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Polymorphisms in the Apoptotic Pathway Gene BCL-2 and Survival in Small Cell Lung Cancer

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Introduction: We investigated the single-nucleotide polymorphism C-938A in the apoptotic gene *BCL-2* to assess the potential impact as a genetic marker for response to chemotherapy and outcome prediction in small cell lung cancer (SCLC) patients. Such a marker might help optimize lung cancer treatment in a tailored approach.

Methods: DNA derived from peripheral blood lymphocytes of 188 Caucasian SCLC patients treated at the Thoraxklinik Heidelberg was genotyped. Chemotherapy response, time to progression (TTP), and overall survival (OS) were evaluated using multivariable regression (unconditional logistic for response and Cox proportional hazard for TTP and OS) with odds ratios and hazard ratios (HRs) and their 95% confidence intervals (CIs) as quantitative outcome measures, respectively.

Results: Small cell lung cancer patients carrying the *BCL-2* -938CC genotype showed significantly worse TTP than patients carrying the *BCL-2* -938AA genotype (HR = 1.86; 95% CI = 1.10–3.13, p = 0.021). The same adverse effect was shown for OS (HR = 2.38; 95% CI = 1.38–4.12, p = 0.002). Also, patients with limited disease (HR = 2.57; 95% CI = 1.18–5.60, p = 0.017) showed worse OS with the *BCL-2* -938CC genotype.

Conclusion: *BCL-2* -938CC genotype shows significantly worse outcome in small cell lung cancer patients. This genetic marker might particularly impact on treatment strategies using BCL-2 antisense approaches.

Key Words: Small cell, BCL-2, Polymorphism, Survival.

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Small cell lung cancer (SCLC) is characterized by a good initial response to chemotherapy. Unfortunately, treatment outcome is often poor because of early relapse. Patients with limited disease are mostly treated with a combination chemotherapy of platinum and etoposide in conjunction with radiotherapy of the chest. Those with extensive disease mostly receive platinum- or anthracycline-containing drug combinations. Median survival times are in the range of 18 to 20 months for limited disease and 9 to 12 months for extensive disease.[1]

One effect of cancer chemotherapeutics is to induce apoptosis via DNA damage. Hence, treatment efficacy not only depends on the amount of DNA damage but also on the capacity of the cell to undergo apoptosis thereafter. A lower ability of apoptosis induction not only increases the risk to develop cancer but also promotes failure to achieve chemotherapy response because of drug resistance.[2] Apoptosis can be induced by an intrinsic mitochondrial-induced and by an extrinsic receptor-mediated pathway. Although DNA damage induced by many chemotherapeutics mainly triggers mitochondrial-induced apoptosis, there is still some crosstalk between the two pathways. One important step in the intrinsic apoptotic pathway is the efflux of cytochrome *c* out of the mitochondria which then further triggers the apoptotic cascade. Proapoptotic and antiapoptotic members of the BCL-2 family regulate this efflux,[3] where BCL-2 acts as an antiapoptotic regulator.

Current approaches targeting BCL-2 attempt to further improve treatment outcome in SCLC. The study by Rudin et al.[4] investigated the effect of the BCL-2 antisense oligonucleotide "oblimersen" in combination with a chemotherapy of etoposide and platinum on toxicity and outcome. No significant effect of "oblimersen" was detected, possibly due to insufficient suppression of BCL-2. It is likely that different

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agents will be tested on their ability to suppress BCL-2 and their influence on survival in SCLC patients.[5,6] It would be interesting to distinguish, before treatment, those patients who have a high chance of benefiting from such an approach or those who do not, on the basis of particular host or tumor characteristics.

Single-nucleotide polymorphisms (SNPs) in BCL-2 could help to characterize those subgroups. SNPs in general account for more than 90% of the genetic variation in the human genome. Many SNPs are known to be involved in pathways such as apoptosis, which are important for cancer development and chemotherapy resistance.[7] The A-allele of the investigated polymorphism C-938A has been associated with increased BCL-2 expression.[8]

The aim of this project was to identify whether the SNP C-938A in the apoptotic gene *BCL-2* could serve as a marker for chemotherapy response and outcome in SCLC patients. Such a marker, if it exists, would allow development of individualized therapy regimens.[9]

Because this functional polymorphism is located in a key apoptotic gene, we postulated that it might result in different levels of apoptosis induced by chemotherapy regimens. Therefore, the aim of this study was to investigate whether this BCL-2 polymorphism has an effect on early response to chemotherapy, overall survival (OS), and time to progression (TTP).

PATIENTS AND METHODS

Study Population, Response Assessment, and Survival Calculation

A total of 188 patients diagnosed with SCLC and treated with first-line chemotherapy between 1998 and 2007 at the Thoraxklinik Heidelberg were analyzed. Written informed consent was obtained from all participants. The study was approved by the ethics committee of the University of Heidelberg. Response to chemotherapy was assessed by comparison of baseline with follow-up imaging after the second ($n_2 = 174$) or third ($n_3 = 14$) cycle of chemotherapy. Response Evaluating Criteria in Solid Tumors (RECIST 1.0) were applied in a separate retrospective read by an experienced chest radiologist (S.T. or C.P.H.).[10] Patients were categorized into responder (complete response and partial response) and nonresponder (stable disease and progressive disease). Assessment was performed on the basis of pairs of spiral computed tomography (CT) scan for 24 patients. A total of 164 patients were assessed on the basis of paired chest radiographs (two planes, erect).

TTP was calculated from the start of treatment until documentation of the first date of progression or the last date of follow-up. The end point TTP was assessed completely by CT.

OS was calculated from the start of treatment until death or the last date of follow-up.

Limited disease patients were treated with a first-line chemotherapy in combination with thoracic radiation. One patient with limited disease did not receive the planned radiotherapy because of his restricted lung function.

Extensive disease patients were treated by chemotherapy alone, except eight patients, who received chemotherapy and radiotherapy on an individually based treatment decision (younger age; favorable tumor response).

Genotyping

Peripheral blood samples were collected before chemotherapy. DNA was isolated from buffy coat using the QIAamp DNA blood midi kit. Genotyping was performed by polymerase chain reaction followed by fluorescence-based melting curve analysis (LightCycler 480, Roche Diagonstics GmbH, Mannheim, Germany). The newly developed LightCycler method was validated by repeating the genotyping for 200 samples by restriction fragment length polymorphism analysis. In addition, as a quality assurance measure, 10% of the samples were repeated blindly with 100% concordance. A negative control and positive controls were always run as standard controls.

For the polymerase chain reaction followed by melting curve analysis, the following primers and probes were used: forward primer: GCTTCACGCCTCACCAG, reverse primer: TCCAGCAGCTTTTCGGAA, sensor: LCRed640-CCTTCA-TCGTCCCCTCTCC, anchor: CCTGCCTCCGTCCCCGGC-FL (LCRed640: fluorescence dye, FL: fluorescein).

The cycling conditions were as follows: initial denaturation at 95°C for 2 minutes followed by 40 cycles consisting of denaturation at 95°C for 2 seconds, 60°C annealing temperature for 5 seconds, and elongation at 72°C for 6 seconds. Melting curve analyses were performed with an initial denaturation at 95°C for 1 minute, 1 minute at 40°C followed by slow heating of the samples to 80°C and permanent fluorescence detection.

Statistical Analysis

Genotype frequencies were compared in responders and nonresponders. Odds ratios (ORs) and 95% confidence intervals (CIs) for the risk of being a nonresponder were calculated using multivariable unconditional logistic regression analysis for all SCLC patients as well as in the subgroups. The survival time end points in terms of OS and TTP were evaluated analogously using the Cox proportional hazards model with hazard ratio (HR) estimates and 95% CI. For the calculation of ORs and HRs, -938AA was the reference genotype to which we compared the two genotypes CA and CC separately and in combination.

Furthermore, multivariable regressions and by that the outcome measures OR and HR were adjusted in general for clinical stage, chemotherapy regimen, performance status (Eastern Cooperative Oncology Group [ECOG]), and gender; when analyzing the two subgroups with extensive and limited disease, these were adjusted for chemotherapy regimen, performance status (ECOG), and gender only. One-year, 2-year, and 3-year TTP and OS as well as median TTP and median OS rates were calculated from the Kaplan-Meier curves, and the log-rank test was used to assess differences between the Kaplan-Meier curves.

The genotype frequencies were tested for deviation from the Hardy-Weinberg equilibrium using the χ^2 goodness-of-fit test. All statistical analyses were performed with SAS software version 9.1.3.

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RESULTS

Of all 188 patients analyzed, 101 showed limited disease and 87 extensive disease (Table 1). There was 100% concordance between the newly developed fluorescence-based melting curve analysis and the restriction fragment length polymorphism analysis for the 200 samples genotyped with both methods for the purpose of validation. The *BCL-2* -938A-allele was found to have a frequency of 0.53, and genotypes were in Hardy-Weinberg equilibrium.

Association of Genotypes with Chemotherapy Response

For the chemotherapeutics used and for further patients' characteristics, see Table 1.

The median time interval between baseline scan and treatment start was 6 days (range, 0–35 days) and between end of treatment and first restaging was 18 days (range, 5–44 days).

Among all 188 patients, 148 were responders and 40 were nonresponders to first-line chemotherapy. No statistically significant association of *BCL-2* C-938A with chemotherapy response was found (Table 2).

TABLE 1. Patients' Characteristics

	Patients with Small Cell Lung Cancer	
	Responder (CR + PR) (N = 148), n (%)	**Nonresponder (SD + PD) (N = 40), n (%)**
Gender		
Male	108 (73)	33 (83)
Female	40 (27)	7 (18)
Age		
≤60 yr	77 (52)	18 (45)
>60 yr	71 (49)	22 (55)
Stage		
Limited disease	83 (56)	18 (45)
Extensive disease	65 (44)	22 (55)
Performance status		
ECOG 0–1	130 (88)	36 (90)
ECOG 2–3	18 (12)	4 (10)
Chemotherapy regimen		
Extensive disease patients		
Etoposide and platinum-based chemotherapy	46 (31)	9 (23)
Etoposide, ifosfamide, and platinum-based chemotherapy	1 (0.7)	0 (0)
Other combinations	18 (12)	13 (33)
Limited disease patients		
Etoposide and platinum-based chemotherapy	59 (40)	11 (28)
Etoposide, ifosfamide, and platinum-based chemotherapy	11 (7)	3 (8)
Other combinations	13 (9)	4 (10)

Values are given as n (%).

ECOG, Eastern Cooperative Oncology Group; CR, complete remission; PR, partial remission; SD, stable disease; PD, progressive disease.

Association of Genotypes with TTP and OS

The median TTP was 8.9 months (95% CI = 8.1–10.3) (responders: 9.5 [95% CI = 8.2–10.7], nonresponders 7.8 [95% CI = 4.8–9.1]). The median OS was 13.3 months (95% CI = 11.5–15.9) (responders: 14.7 [95% CI = 11.5–18.5], nonresponders 11.8 [95% CI = 8.8–13.8]).

BCL-2 -938CC had significantly worse TTP compared with *BCL-2* -938AA in all SCLC patients (HR = 1.86; 95% CI = 1.10–3.13, p = 0.021, CC: median 7.8 months, 95% CI = 5.3–9.8 versus AA: median 10.4 months, 95% CI = 7.8–12.4).

For extensive disease patients and for limited disease patients, similar but not statistically significant effects were observed (compare Table 3). The corresponding Kaplan-Meier curves are shown in Figures 1*A–C*.

Evaluation of OS showed results similar to TTP. In all patients with SCLC, *BCL-2* -938CC was associated with worse survival (HR = 2.38; 95% CI = 1.38–4.12, p = 0.002, CC: median 11.2 months, 95% CI = 8.5–14.6 versus AA: median 14.4 months, 95% CI = 12.0–21.9) (Table 4).

This impact could also be shown in patients with limited disease (HR = 2.57; 95% CI = 1.18–5.60, p = 0.017, CC: median 14.6 months, 95% CI = 11.2–18.3 versus AA: median 21.2 months, 95% CI = 14.1–39.3). In patients with extensive disease, a similar but statistically not significant effect was observed (compare Table 4).

The corresponding Kaplan-Meier curves are shown in Figures 1*D–F*.

DISCUSSION

To our knowledge, this is the first report in patients with SCLC, analyzing the association between the *BCL-2* C-938A polymorphism and chemotherapy response and outcome.

In our study, the *BCL-2* -938 CC-genotype was found to be associated with an adverse influence on TTP and OS, which means that *BCL-2* -938AA was shown to be a marker of improved TTP and OS. These results are somewhat unexpected for two reasons. First, the *BCL-2* -938A-allele has been associated with increased BCL-2 expression.[8] An increase of the antiapoptotic BCL-2 should lead to a less favorable chemotherapy response and outcome because of a reduced ability of tumor cells to undergo apoptosis. Second, BCL-2 overexpression has been shown to be a marker of chemotherapy resistance regardless of small and non-small cell histology.[11,12] This resulted in the development of new promising treatment options for SCLC[13] by blocking *BCL-2* expression by antisense oligonucleotides.[4,14–16]

This notion cannot explain our findings in SCLC patients of a beneficial effect of the *BCL-2* AA-genotype. However, in keeping with our results, BCL-2 overexpression was previously shown to have a positive influence on survival in non-small cell lung cancer.[17] Conflicting results on the influence of *BCL-2* overexpression on survival in SCLC have previously been published.[18–21] In concordance with our findings on *BCL-2* -938AA and its positive influence on TTP and OS, the A-allele was also associated with a longer survival in patients with breast cancer.[22] After radical prostatectomy, the *BCL-2* CC-genotype leads to earlier recur-

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TABLE 2. Chemotherapy Response According to BCL-2 C-938A Polymorphism

	Responder (CR + PR) (N = 148), n (%)	Nonresponder (SD + PD) (N = 40), n (%)	OR	95% CI	p
All patients with small cell lung cancer					
AA (reference)	37 (25)	10 (25)	1		
CA	86 (58)	21 (53)	0.78[b]	0.33–1.87	0.575
CC	25 (17)	9 (23)	1.08[b]	0.36–3.24	0.887
CA + CC[a]	111 (75)	30 (75)	0.84[b]	0.36–1.95	0.690
Patients with extensive disease					
AA (reference)	14 (10)	5 (13)	1		
CA	40 (27)	10 (25)	0.57[c]	0.15–2.16	0.411
CC	11 (7)	7 (18)	1.56[c]	0.33–7.27	0.573
CA + CC[a]	51 (35)	17 (43)	0.75[c]	0.22–2.65	0.660
Patients with limited disease					
AA (reference)	23 (16)	5 (13)	1		
CA	46 (31)	11 (28)	1.06[c]	0.3–3.49	0.918
CC	14 (10)	2 (5)	0.75[c]	0.12–4.70	0.756
CA + CC[a]	60 (41)	13 (33)	1.00[c]	0.32–3.20	0.995

[a] CA + CC = C-allele carriers.
[b] Adjusted for clinical stage, chemotherapy regimen, performance status (ECOG), and gender.
[c] Adjusted for chemotherapy regimen, performance status (ECOG), and gender.
ECOG, Eastern Cooperative Oncology Group; CR, complete remission; PR, partial remission; SD, stable disease; PD, progressive disease; OR, odds ratio; CI, confidence interval.

TABLE 3. Time to Progression According to BCL-2 C-938A Polymorphism

	n	HR	95% CI (from)	p	1-yr TTP (%)	95% CI	2-yr TTP (%)	95% CI	3-yr TTP (%)	95% CI	Median TTP (mo)	95% CI
All patients with small cell lung cancer												
AA (reference)	47	1			35	20–50	16	6–31	6	1–22	10.4	7.8–12.4
CA	107	0.96[a]	0.64–1.46	0.860	31	21–41	9	4–17	9	4–17	8.9	7.6–10.8
CC	34	**1.86[a]**	**1.10–3.13**	**0.021**	17	6–33	0	—	0	—	7.8	5.3–9.8
CA + CC[b]	141	1.10[a]	0.74–1.63	0.644	27	19–36	7	3–13	7	3–13	8.8	7.6–9.8
Patients with extensive disease												
AA (reference)	19	1			13	2–34	0	—	0	—	6.2	4.8–8.9
CA	40	0.65[c]	0.34–1.22	0.179	20	9–35	3	0.3–15	0	—	6.9	5.5–8.7
CC	18	1.49[c]	0.69–3.21	0.316	0	—	0	—	0	—	5.0	3.1–7.8
CA + CC[b]	68	0.77[c]	0.42–1.42	0.400	15	6–27	2	0.2–11	0	—	6.5	5.2–7.8
Patients with limited disease												
AA (reference)	28	1			49	27–68	28	10–48	10	1–34	11.6	10.2–21.3
CA	57	1.42[c]	0.81–2.52	0.225	39	26–52	14	6–25	14	6–25	10.9	9.1–12.4
CC	16	2.01[c]	0.96–4.20	0.063	32	11–56	0	—	0	—	13.3	6.8–12.1
CA + CC[b]	73	1.53[c]	0.88–2.65	0.130	37	26–49	11	5–20	11	5–20	10.7	9.4–11.9

Values in bold indicate HR estimates with $p < 0.05$.
[a] Adjusted for clinical stage, chemotherapy regimen, ECOG, and gender.
[b] CA + CC = C-allele carriers.
[c] Adjusted for chemotherapy regimen, ECOG, and gender.
CI, confidence interval; TTP, time to progression; ECOG, Eastern Cooperative Oncology Group.

rence,[23] and it is also associated with poor prognosis and lower survival in renal cancer,[24] whereas in leukemia patients, the *BCL-2* -938A-allele was associated with worse survival.[8] Interestingly, an increase in BCL-2 copy number in SCLC cell lines and tumors leading to higher BCL-2 expression has previously been associated with higher sensitivity to treatment with BCL-2 antisense oligonucleotides in SCLC cell lines.[25]

Taken together, our findings indicate a negative prognostic impact of the *BCL-2* -938CC genotype in SCLC, where progression and survival of patients with SCLC were strongly associated with this polymorphism.

Because of the fact that this is a retrospective study of patients who were not part of a uniform clinical trial, the cohort shows a variety in initial treatment regimes as well as methods to follow-up chemotherapy response. This is because the retro-

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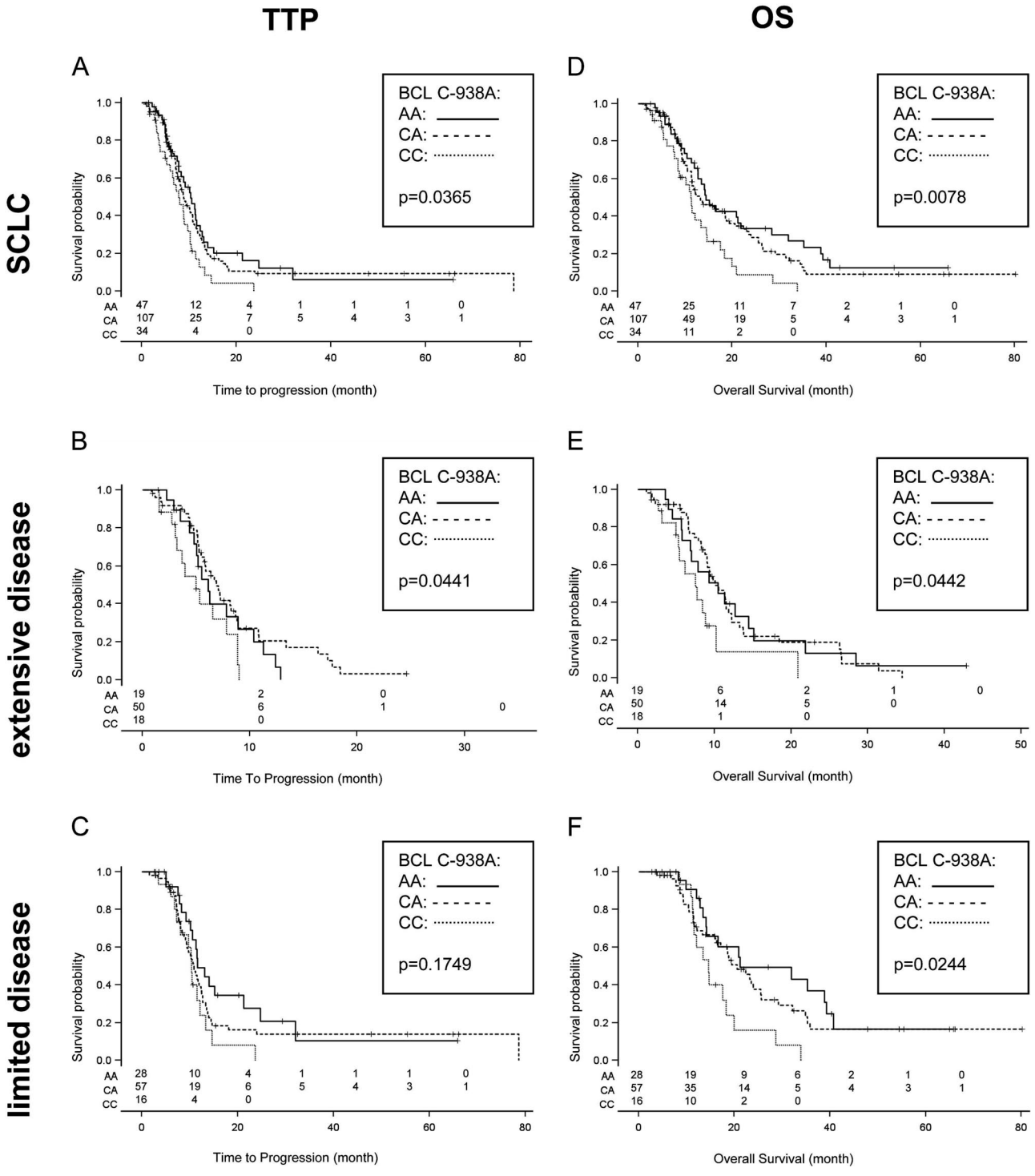

FIGURE 1. Time to progression (TTP) and overall survival (OS) for BCL-2 C-938A with number of subjects at risk. TTP in all patients with small cell lung cancer (*A*), in patients with extensive disease (*B*), and in patients with limited disease (*C*). OS in all patients with small cell lung cancer (*D*), in patients with extensive disease (*E*), and in patients with limited disease (*F*).

spective study design did not allow the possibility of influencing the given clinical process. On the one hand, the cohort therefore is not an artificial construct and reflects the heterogeneity in the population of patients with SCLC. On the other hand, this variety could lead to imbalances that might bias the results of the study. We tried to avoid bias by adjusting for clinical stage, chemotherapy regimen, performance status (ECOG), and gender in the statistical analysis.

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TABLE 4. Overall Survival According to BCL-2 C-938A Polymorphism

	n	HR	95% CI (from)	*p*	1-yr OS (%)	95% CI	2-yr OS (%)	95% CI	3-yr OS (%)	95% CI	Median OS (mo)	95% CI
All small cell lung cancer patients												
AA (reference)	47	1			68	52–80	33	19–49	23	11–39	14.4	12.0–21.9
CA	107	1.14[a]	0.74–1.76	0.543	54	44–63	29	19–39	9	4–18	13.3	11.4–18.5
CC	34	**2.38[a]**	**1.38–4.12**	**0.002**	42	24–59	9	2–24	0	—	11.2	8.5–14.6
CA + CC[b]	141	1.30[a]	0.85–1.97	0.223	51	42–60	24	17–33	7	3–14	12.2	11.3–15.9
Patients with extensive disease												
AA (reference)	19	1			39	18–60	13	2–34	7	0.4–25	10.5	5.8–14.5
CA	40	0.89[c]	0.48–1.65	0.710	34	21–48	19	9–32	0	—	10.1	9.0–11.8
CC	18	1.95[c]	0.89–4.30	0.097	14	1–42	0	—	0	—	7.5	5.0–10.2
CA + CC[b]	68	1.01[c]	0.56–1.84	0.968	29	18–42	15	7–26	0	—	9.4	8.3–11.3
Patients with limited disease												
AA (reference)	28	1			91	68–98	49	26–69	37	16–58	21.2	14.1–39.3
CA	57	1.32[c]	0.72–2.44	0.372	71	56–81	38	23–52	16	7–30	20.6	15.9–25.5
CC	16	**2.57[c]**	**1.18–5.60**	**0.017**	67	38–85	16	3–39	0	—	14.6	11.2–18.3
CA + CC[b]	73	1.51[c]	0.84–2.72	0.167	70	57–79	33	21–45	12	5–23	18.5	14.6–23.2

Values in bold indicate HR estimates with $p < 0.05$.
[a] Adjusted for clinical stage, chemotherapy regimen, ECOG and gender.
[b] CA + CC = C-allele carriers.
[c] Adjusted for chemotherapy regimen, ECOG, and gender.
CI, confidence interval; OS, overall survival; HR, hazard ratio.

However, because of the retrospective character of the study, the results can merely be hypothesis generating. The results need to be confirmed in a prospective trial using a multivariate analysis to verify *BCL-2* C-938A as a marker of survival and TTP in patients with SCLC. Particularly, it would be interesting to evaluate the association of *BCL-2* C-938A genotypes with the effect of *BCL-2* antisense treatment in SCLC patients.

We did not find significant results for chemotherapy response despite the fact that there were significant effects for TTP and OS. This might be due to the fact that tumor progression and death of a group of patients are harder end points than response to chemotherapy, e.g., because it is difficult to distinguish tumor from necrosis or atelectasis by CT.[26] In addition, only 24 pairs of CT scans were available to follow-up chemotherapy response. The rest of the patients were followed up by chest radiograph. This makes the data on chemotherapy response more heterogeneous and less reliable. Another factor could be that response as categorical outcome has less statistical power than the quantitative end points TTP and OS. Alternatively, the results could reflect a limited association of the early response outcome determined in this study and the two survival end points.

The observed negative effects were only seen for homozygous carriers of the C-allele and not for all C-allele carriers. This may suggest a recessive effect of the C-allele. The results in the subgroup of patients with extensive disease and in the subgroup of patients with limited disease reflect the results in the main group of all patients with SCLC. The results point in the same direction as in the main group but significance is not always reached. This could be due to the lower case numbers in these subgroups. Another factor that needs to be critically assessed is the fact that the number of CC-allele carriers was not evenly distributed among the two stage groups. In the group of patients with extensive disease stage, 18 patients carried the CC genotype. This reflects 21% of all patients with extensive disease stage. In the group of patients with limited disease stage, 16 patients carried the CC genotype. This reflects 16% of all patients with limited disease stage. Because extensive disease stage patients have a known worse outcome, the imbalance of the genotypes by stage could contribute to the observed worse outcome for the CC genotype in the group as a whole.

One advantage of our study is that we investigated a genetic polymorphism as a host factor in DNA derived from peripheral blood lymphocytes. This can be easily and noninvasively obtained and analyzed within a short time frame, making it ideal for routine clinical use. However, we are aware that the measured host factor does not necessarily reflect the situation in a given tumor, where clonal transformation might have occurred. One hundred forty of 188 patients were treated with a standard regimen of platinum-based therapy in combination with etoposide. Thus, the remaining treatment group, receiving different drug combinations, mostly adriamycin/cyclophosphamide/vincristine, is too small to differentiate in both subgroups a predictive impact of the polymorphism on outcome. However, all patients received chemotherapy and mostly a combination of platinum-based treatment and etoposide. In the overall group receiving chemotherapy, *BCL-2* -938CC indicates inferior outcome in terms of TTP and OS. A prospective study design with different therapy regimen subgroups would provide an

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opportunity to elucidate the predictive value of the investigated polymorphisms.

In conclusion, this is the first study to our knowledge that revealed statistically significant associations for *BCL-2* C-938A, and TTP as well as OS in SCLC patients, indicating a negative prognostic impact of the *BCL-2* -938CC genotype.

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