Comparison of Two ERCC1 Antibodies as Prognostic and Predictive Biomarkers for Early Non-small Cell Lung Cancer

THOMAS R. MULEY[1,5*], MARIA SIANIDOU[2*], MICHAEL THOMAS[2,5], HELGE BISCHOFF[2], HENDRIK DIENEMANN[3,5], MICHAEL MEISTER[1,5], MARC A. SCHNEIDER[1,5], PHILIPP A. SCHNABEL[4,5] and ARNE WARTH[4]

[1]Translational Research Unit, and Departments of [2]Thoracic Oncology, and [3]Thoracic Surgery, Thorax Clinic, and [4]Institute for Pathology, University Hospital Heidelberg, Heidelberg, Germany; [5]Translational Lung Research Centre Heidelberg (TLRC-H), Member of the German Centre for Lung Research (DZL), Heidelberg, Germany

Abstract. *Aim: Expression of excision repair cross-complementing rodent repair deficiency, complementation group 1 (ERCC1) was suggested to be of predictive value for selecting patients with clinical benefit from platinum-based chemotherapy. Patients and Methods: In order to validate the prognostic and predictive value of ERCC1, we comparatively analyzed 298 patients with non-small cell lung cancer (NSCLC) treated with and without platinum-based adjuvant chemotherapy with two different antibodies against ERCC1 (clones 8F1 and SP68). Results: We found that both antibodies have a different immunoreactivity, with SP68 showing a more distinct, predominantly nuclear staining pattern. There was no prognostic effect for patients with high compared to patients with low ERCC1 expression, regardless of the antibody applied. In contrast, patients with squamous cell carcinoma treated with adjuvant platinum-based chemotherapy who had a low ERCC1 expression had a survival benefit with respect to disease-free and overall survival. This was especially true for expression by the SP68 antibody. Conclusion: Oour data point to a potential predictive value of ERCC1 expression for the selection of adjuvant platinum-based chemotherapy for patients with pulmonary squamous cell carcinomas but not for those with adenocarcinomas. With more specific antibodies in hand, this should be substantiated in subsequent clinical studies.*

*These Authors contributed equally to this study.

Correspondence to: Thomas Muley, Ph.D., Translational Research Unit, Thoraxklinik am Universitätsklinikum Heidelberg, Amalienstr. 5, D-69126 Heidelberg, Germany. Tel: +49 62213961110, Fax: +49 62213961652, e-mail: thomas.muley@med.uni-heidelberg.de

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Despite recent progress in morphological and molecular subtyping of non-small cell lung cancer (NSCLC), patients' prognoses are still poor. Targeted-therapies based on predictive biomarkers, *e.g.* epidermal growth factor receptor (*EGFR*) mutations or anaplastic lymphoma receptor tyrosine kinase (*ALK*) translocations, resulted in improved outcome compared to conventional regimens (1, 2), however, platinum-based chemotherapy remains the mainstay in the adjuvant or palliative setting. Unfortunately, there are no established biomarkers to predict response to platinum-based chemotherapies. Therefore, many patients are affected by severe side-effects without measurable clinical benefit.

Cisplatin compounds interact with DNA double-strands resulting in adduct formation, considered to lead to impaired transcription, cell division, and subsequent cell death. However, evolution has conserved highly effective mechanisms of DNA repair, for example, the nucleotide excision repair system. These protein complexes, with excision repair cross-complementing rodent repair deficiency, complementation group 1 (ERCC1) being a major component, recognize DNA damage and subsequently facilitate DNA excision, synthesis, and ligation of new DNA strands. Therefore, high expression levels of ERCC1 are considered a negative predictor for effective platinum-based therapies. Indeed, several studies indicate that ERCC1 might be used to stratify patients with NSCLC for adjuvant therapies (3-8). However, this was not consistently validated (9) or only found to have minor effects in small subsets of patients (10). Most recently, a large-scale comparative study described a lack of sensitivity of commercially available antibodies to detect the active isoform of ERCC1 (9).

In order to validate the potential prognostic and predictive value of ERCC1 in NSCLC, we immunohistochemically analyzed a matched cohort of 298 patients with and without platinum-based adjuvant chemotherapy using different antibodies. We demonstrate

that ERCC1 expression might be of potential value to stratify patients with squamous cell carcinomas (SQCC) for adjuvant platinum-based chemotherapy.

Patients and Methods

Patients. We retrospectively screened our archives for cohorts of surgically resected early NSCLC (stage I-IIIb). All tumors were resected between 2003 and 2008 at the the Thorax Clinic Heidelberg at Heidelberg University, Germany, and were classified according to the criteria of the current (2004) WHO classification for lung cancer (16). Usage of the tissue was approved by the local Ethics Committee (no. 206/2005). For further details including age, sex, ECOG status, histology, tumor stage, TNM (6th edition) (17), grading, and adjuvant treatment see Table I. Histologies other than SQCC and adenocarcinomas (ADC) were combined as 'other NSCLC' (*i.e.* large cell carcinoma, adenosquamous carcinoma). Overall survival (OS) and disease-free survival (DFS) were recorded; for DFS, an event was defined as any definite clinical or pathological evidence of local or distant recurrence or death for any reason.

Immunohistochemistry. Immunohistochemical stains were performed after pre-treatment of formalin-fixed paraffin-embedded tissue sections (citrate buffer pH 6, 20 minutes, 98˚C) using an autostainer (AS480; medac Diagnostika, Wedel, Germany). The following antibodies were applied: i) monoclonal mouse anti-ERCC1, clone 8F1 (dilution 1:100; Thermo Fisher Scientific, Runcorn, UK); ii) monoclonal rabbit anti-ERCC1, clone SP68 (dilution 1:1000; Spring Bioscience, Pleasanton, USA). The streptavidin-biotin-peroxidase detection system (UltraVision; Thermo Fisher Scientific) was used with 3,3'-diaminobenzidine as a chromogen. All reagents were provided by medac Diagnostica (Wedel, Germany). Evaluation was carried out according to a previously established semiquantitative scoring system considering the proportion of positively stained cells, as well as the staining intensity (4). Lymphocytes were used as an internal positive control for strong immunoreactivity. Tonsillar tissues were used as positive controls; for negative controls the primary antibodies were omitted. All evaluations were performed by one experienced pathologist (AW) blinded to all clinical data and treatment.

Statistics. Statistical analysis was carried out using SPSS 20.0 (IBM, Ehningen, Germany). Uni- and multivariate survival analyses were performed using log-rank test and Cox regression analyses, respectively. Survival was calculated from start of therapy (=date of surgery) until last observation or death. The median follow-up was calculated using an 'inverse' survival curve (death=censored, alive=event).

A *p*-values less than 0.05 were considered significant. Survival curves were constructed according to Kaplan and Meier (17). Comparisons of the staining results were performed with Spearman rank correlation and Cohen's Kappa coefficient. Comparison of clinical parameters between groups with and without adjuvant chemotherapy was made with Mann–Whitney *U*-test.

Results

Overall, comparative evaluation of the staining results of both antibodies demonstrated a more distinct nuclear staining pattern of the SP68 clone, while the 8F1 clone had both nuclear and cytoplasmic immunoreactivity. Since they are present in almost all tumors, lymphocytes as an internal source of strong ERCC1 expression were proven a helpful parameter in assessing the staining intensity (Figure 1). Direct comparison of the staining results per case underscored the different staining pattern of both clones, with there being only mild-to-moderate agreement (Spearman rank correlation r=0.531; Cohen's Kappa coefficient=0.34). The same was true when the whole cohort was analyzed for different histologies, where the 8F1 clone in particular showed a much higher heterogeneity (Figure 2).

Table I. *Patient characteristics for the 'surgery alone' cohort and the cohort of surgery followed by adjuvant platinum-based chemotherapy.*

	Surgery alone			Surgery + adjuvant chemotherapy			
		N	%		N	%	*p*-Value
Mean age (years)	65.9	136	100	58.5	162	100	<0.001
Gender							
Male		105	77.2		119	73.5	0.456
Female		31	22.8		43	26.5	
ECOG PS							
0		87	64		107	66.0	0.708
1		43	31.6		51	31.5	
2		5	3.7		4	2.5	
3		1	0.7		0	0	
Histology							
SQCC		59	43.4		51	31.5	0.037
ADC		59	43.4		78	48.1	
Other NSCLC		18	13.2		33	20.4	
p-Stage							
I		55	40.4		46	28.4	0.139
II		40	29.4		58	35.8	
IIIA		18	13.2		37	22.8	
IIIB		23	16.9		21	13.0	
Grading							
1		2	1.5		2	1.2	0.009
2		49	36.0		36	22.2	
3		85	62.5		124	76.5	
Surgery							
Wedge resection		4	2.9		0	0	0.32
Lobectomy		95	69.9		113	69.8	
Bilobectomy		7	5.1		8	4.9	
Pneumonectomy		30	22.1		41	13.8	
Adjuvant chemotherapy							
Carboplatin-based		--	--		59	36.4	--
Cisplatin-based		--	--		103	63.6	
Status							
Alive		78	57.4		104	64.2	0.07
Tumor-dependent death		36	26.5		50	30.9	
Tumor-independent death		22	16.2		8	4.9	

ECOG PS: eastern cooperative oncology group performance status; SQCC: squamous cell carcinoma; ADC: adenocarcinoma; NSCLC: non-small cell lung cancer.

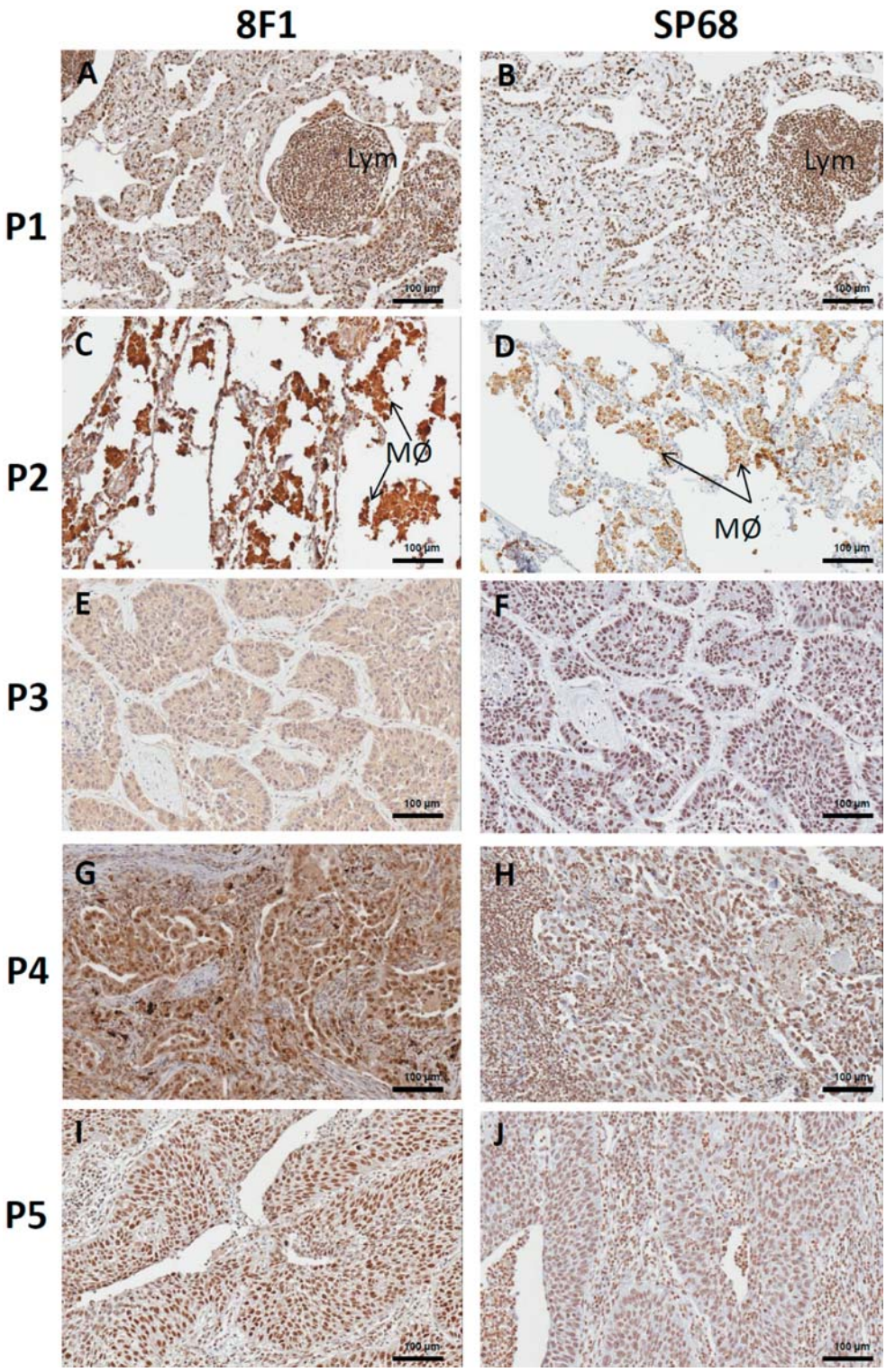

Figure 1. *Representative staining results of ERCC1 antibodies (clones 8F1 and SP68) in lymphocytes (Lym; A, B) and normal lung (C, D). Note the strong immunoreactivity of lymphocytes but also that of alveolar macrophages (MØ). Comparative immunoreactivity of ERCC1 using the SP68 and the 8F1 clones in the same tumors (E-J). Overall the 8F1 clone (E, G, I) has more pronounced cytoplasmic immunoreactivity and somewhat less distinct nuclear staining compared to the SP68 clone (F, H, J). In some cases, the cytoplasmic immunoreactivity dominates the overall staining pattern and makes it hard to clearly separate cytoplasmic from nuclear immunoreactivity (G). P1-P5, Selected patients with lung cancer (P1 and P2 normal lung tissue, P3 ADC; P4 ADC; P5 SQCC), (original magnification ×20).*

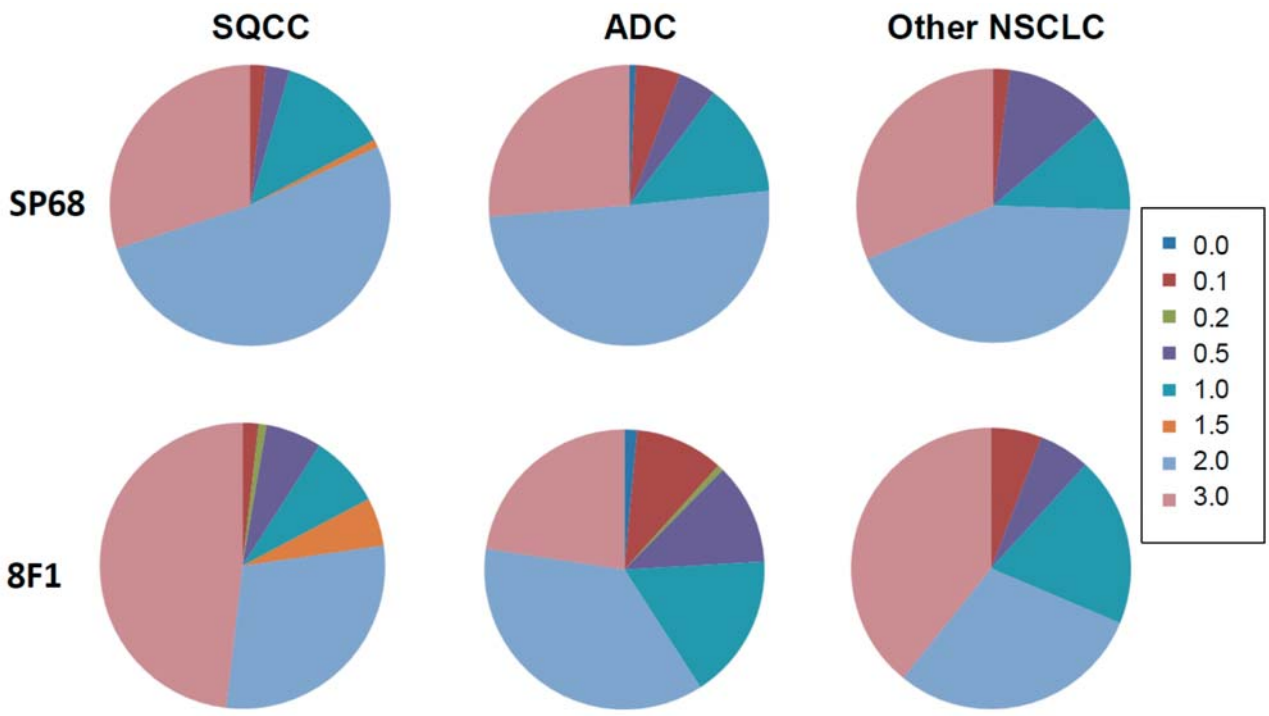

Figure 2. *Distribution of immunoreactivity scores for ERCC1 stratified according to histology using the SP68 and the 8F1 clones. ADC, Adenocarcinoma; SQCC, squamous cell carcinoma; other NSCLC.*

Impact of ERCC1 expression on DFS and OS. In order to analyze the potential prognostic and predictive value of ERCC1 expression with respect to adjuvant chemotherapy, we performed Kaplan–Meier survival analyses. Patients were stratified according to low- or high-ERCC1 expression using the median immunohistochemical score (=2) as a cut-off. The cohorts with and without adjuvant chemotherapy were analyzed separately. The median follow-up was 63.7 months for patients without and 48.8 months for patients with adjuvant chemotherapy.

There was no significant survival benefit in patients treated with surgery-alone with respect to ERCC1 expression neither in DFS nor in OS, irrespective of histology.

In patients treated with adjuvant chemotherapy, there was a statistically significant survival benefit in regard to DFS in patients with SQCC and low ERCC1 expression (score ≤2). This result was irrespective of the antibody applied. No difference in DFS was observed in ADC (Figure 3). The survival benefit in DFS translated into a significant benefit in OS only in the group of patients with SQCC with low ERCC1 expression detected by the SP68 antibody (Figure 4).

Discussion

We analyzed the new SP68 monoclonal antibody against ERCC1 in comparison to the well-known 8F1 clone with regard to their prognostic and predictive impact on adjuvant platinum-based chemotherapy in a large matched cohort of surgically-resected patients with NSCLC. We demonstrated that these antibodies have a different immunoreactivity, with SP68 showing a more distinct staining pattern. In the group without adjuvant treatment, there was no survival benefit in DFS or OS in patients with high ERCC1 expression compared to patients with low expression, regardless of the antibody applied, which is consistent with recently reported results from a large well-characterized ADC cohort (15). In contrast, patients with adjuvant platinum-based chemotherapy for SQCC and a low ERCC1 expression had a significant survival benefit with respect to DFS and OS, which was especially true for staining by the SP68 antibody, while the 8F1 antibody failed to show a prognostic impact for OS. Although some studies clearly indicate a predictive value of ERCC1 expression for platinum-based chemotherapy, others were unable to validate this (for review see (11)).

One possible explanation for these discrepancies is the usage of different antibodies, as well as different staining procedures or scoring systems, but also a lack of specificity of commercially-available antibodies. Recently Ma *et al.* provided evidence that the 8F1 clone cross-reacts with an unrelated nuclear protein, namely phosphocholine-cytidylyltransferase 1-alpha (PCYT1A) (12). PCYT1A is a protein involved in phosphatidylcholine biosynthesis and

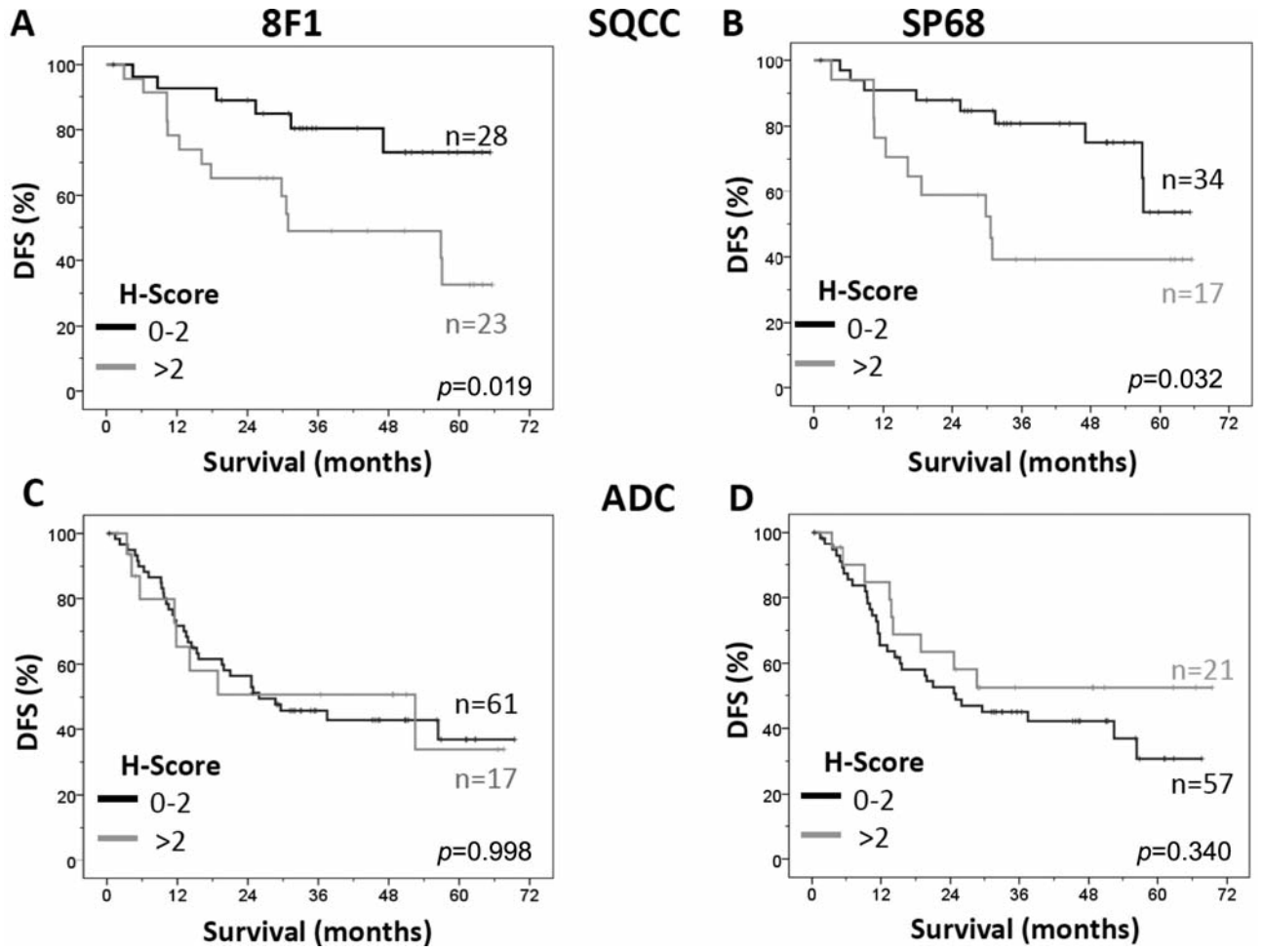

Figure 3. *Prognostic impact of ERCC1 staining with 8F1 (A, C) and SP68 (B, D) on disease-free survival in patients treated by surgery followed by platinum-based adjuvant chemotherapy stratified by squamous cell carcinomas (SQCC; A, B) and adenocarcinomas (ADC; C, D).*

nuclear membrane expansion, with rapid translocation between an inactive cytoplasmic and an active nuclear membrane-associated form. This crossreactivity might explain the strong cytoplasmic staining by the 8F1 clone observed herein, which is likely not exclusively related to ERCC1 but clearly hampers the interpretation of the nuclear immunosignal. Based on this finding, most of the results on ERCC1 expression using the 8F1 clone have to be questioned. Furthermore, the original study by Olaussen *et al*. (4) was not validated most recently in a large comparative study with commercially available antibodies using the identical study cohort (9), which was attributed to a lack of specific ERCC1 antibodies. Only a portion of ERCC1 is present as an active isoform and currently no antibody seems to be available which specifically detects this isoform (9). Different immunoreactivities of commercially available antibodies to ERCC have also been described (13). Therefore, the usefulness of ERCC1 protein expression to stratify patients with respect to adjuvant therapies is currently considered to be of limited value.

Nevertheless, although currently available antibodies are likely not specific for the active form of ERCC1, the finding that patients with SQCC with high ERCC1 expression have a significantly worse outcome under platinum-based therapy compared to those with low expression (14), which was also demonstrated in this study, merits further investigations. Furthermore, the SP68 clone used here has not yet been tested in terms of specific recognition of the active isoform of ERCC1. Finally, we are aware of the limitations of our retrospective cohorts, which differed in some of the clinical characteristics and especially in the median follow-up time. Therefore, statistics were performed separately for the cohorts with and without adjuvant chemotherapy. Further analyses might

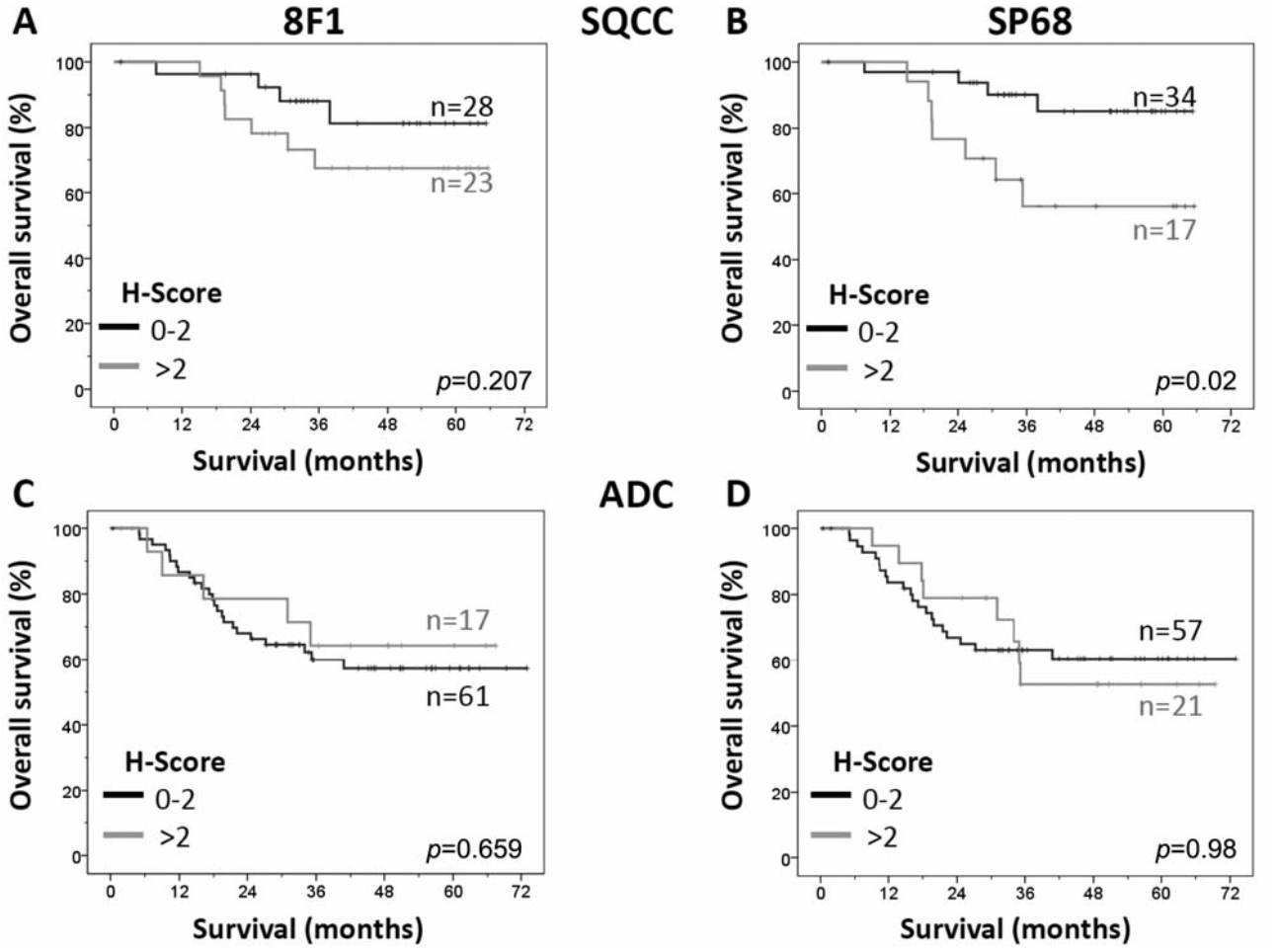

Figure 4. *Prognostic impact of ERCC1 staining with 8F1 (A, C) and SP68 (B, D) on overall survival in patients treated by surgery followed by platinum-based adjuvant chemotherapy stratified by squamous cell carcinomas (SQCC; A, B) and adenocarcinomas (ADC; C, D).*

better be performed in randomized prospective cohorts, which might be the only way to guarantee balance between potential prognostic factors.

In conclusion, we demonstrate that commercially-available antibodies against ERCC1 show different immunoreactivities in NSCLC. The SP68 clone has a rather distinct nuclear staining pattern and retrospective survival analyses point to a potential predictive value of ERCC1 expression for the selection of adjuvant platinum-based chemotherapies for pulmonary SQCC but likely not for ADC. With the availability of more specific antibodies, this should be substantiated in subsequent studies.

Conflicts of Interest

Parts of this study were supported by a research grant from medac Diagnostika (Wedel, Germany). No other conflicts of interest in regard to the presented work are declared.

References

1 Mok TS, Wu YL, Thongprasert S, Yang CH, Chu DT, Saijo N, Sunpaweravong P, Han B, Margono B, Ichinose Y, Nishiwaki Y, Ohe Y, Yang JJ, Chewaskulyong B, Jiang H, Duffield EL, Watkins CL, Armour AA and Fukuoka M: Gefitinib or carboplatin-paclitaxel in pulmonary adenocarcinoma. N Engl J Med *361*: 947-957, 2009.

2 Kwak EL, Bang YJ, Camidge DR, Shaw AT, Solomon B, Maki RG, Ou SH, Dezube BJ, Janne PA, Costa DB, Varella-Garcia M, Kim WH, Lynch TJ, Fidias P, Stubbs H, Engelman JA, Sequist LV, Tan W, Gandhi L, Mino-Kenudson M, Wei GC, Shreeve SM, Ratain MJ, Settleman J, Christensen JG, Haber DA, Wilner K, Salgia R, Shapiro GI, Clark JW and Iafrate AJ: Anaplastic lymphoma kinase inhibition in non-small-cell lung cancer. N Engl J Med *363*: 1693-1703, 2010.

3 Olaussen KA, Mountzios G and Soria JC: ERCC1 as a risk stratifier in platinum-based chemotherapy for nonsmall-cell lung cancer. Curr Opin Pulm Med *13*: 284-289, 2007.

4 Olaussen KA, Dunant A, Fouret P, Brambilla E, Andre F, Haddad V, Taranchon E, Filipits M, Pirker R, Popper HH, Stahel R, Sabatier L, Pignon JP, Tursz T, Le Chevalier T and Soria JC. DNA repair by ERCC1 in non-small cell lung cancer and cisplatin-based adjuvant chemotherapy. N Engl J Med *355*: 983-991, 2006.
5 Vilmar AC, Santoni-Rugiu E and Sorensen JB: ERCC1 and histopathology in advanced NSCLC patients randomized in a large multicenter phase III trial. Ann Oncol *21*: 1817-1824, 2010.
6 Vilmar A and Sorensen JB: Excision repair cross-complementation group 1 (ERCC1) in platinum-based treatment of non-small cell lung cancer with special emphasis on carboplatin: a review of current literature. Lung Cancer *64*: 131-139, 2009.
7 Besse B, Olaussen KA and Soria JC: ERCC1 and RRM1: ready for prime time? J Clin Oncol *31*: 1050-1060, 2013.
8 Ceppi P, Volante M, Novello S, Rapa I, Danenberg KD, Danenberg PV, Cambieri A, Selvaggi G, Saviozzi S, Calogero R, Papotti M and Scagliotti GV: ERCC1 and RRM1 gene expressions but not EGFR are predictive of shorter survival in advanced non-small-cell lung cancer treated with cisplatin and gemcitabine. Ann Oncol *17*: 1818-1825, 2006.
9 Friboulet L, Olaussen KA, Pignon JP, Shepherd FA, Tsao MS, Graziano S, Kratzke R, Douillard JY, Seymour L, Pirker R, Filipits M, Andre F, Solary E, Ponsonnailles F, Robin A, Stoclin A, Dorvault N, Commo F, Adam J, Vanhecke E, Saulnier P, Thomale J, Le Chevalier T, Dunant A, Rousseau V, Le Teuff G, Brambilla E and Soria JC: ERCC1 isoform expression and DNA repair in non-small-cell lung cancer. N Engl J Med *368*: 1101-1110, 2013.
10 Bepler G, Williams C, Schell MJ, Chen W, Zheng Z, Simon G, Gadgeel S, Zhao X, Schreiber F, Brahmer J, Chiappori A, Tanvetyanon T, Pinder-Schenck M, Gray J, Haura E, Antonia S and Fischer JR: Randomized international phase III trial of ERCC1 and RRM1 expression-based chemotherapy versus gemcitabine/carboplatin in advanced non-small cell lung cancer. J Clin Oncol *31*: 2404-2412, 2013.
11 Allingham-Hawkins D, Lea A and Levine S. ERCC1 expression analysis to guide therapy in non-small cell lung cancer. PLoS Curr 2: RRN1202, 2010.
12 Ma D, Baruch D, Shu Y, Yuan K, Sun Z, Ma K, Hoang T, Fu W, Min L, Lan ZS, Wang F, Mull L and He WW: Using protein microarray technology to screen anti-ERCC1 monoclonal antibodies for specificity and applications in pathology. BMC Biotechnol *12*: 88, 2012.
13 Arbogast S, Behnke S, Opitz I, Stahel RA, Seifert B, Weder W, Moch H and Soltermann A: Automated ERCC1 immunohistochemistry in non-small cell lung cancer: comparison of anti-ERCC1 antibodies 8F1, D-10, and FL-297. Appl Immunohistochem Mol Morphol *19*: 99-105, 2011.
14 Pierceall WE, Olaussen KA, Rousseau V, Brambilla E, Sprott KM, Andre F, Pignon JP, Le Chevalier T, Pirker R, Jiang C, Filipits M, Chen Y, Kutok JL, Weaver DT, Ward BE and Soria JC: Cisplatin benefit is predicted by immunohistochemical analysis of DNA repair proteins in squamous cell carcinoma but not adenocarcinoma: theranostic modeling by NSCLC constituent histological subclasses. Ann Oncol *23*: 2245-2252 2012.
15 Warth A, Penzel R, Lindenmaier H, Brandt R, Stenzinger A, Herpel E, Goeppert B, Thomas M, Herth FJ, Dienemann H, Schnabel PA, Schirmacher P, Hoffmann H, Muley T and Weichert W: EGFR, KRAS, BRAF and ALK Gene alterations in lung adenocarcinomas: patient outcome, interplay with morphology and immunophenotype. Eur Respir J *43*: 872-883, 2014.
16 Travis WD, Brambilla E, Muller-Hermelink HK and Harris CC (eds.): World Health Organization Classification of Tumors. Pathology and genetics of tumours of the lung, pleura, thymus and heart. IARC Press: Lyon 2004.
17 Sobin LH and Wittekind CH (eds.): TNM classification of malignant tumours sixth edidion, Wiley-Liss Inc. 2002.
18 Kaplan E and Meier P: Non-parametric estimation from incomplete observations. J Am Stat Assoc *53*: 457-458, 1958.

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