Prognostic Impact of Bcl-2 Depends on Tumor Histology and Expression of MALAT-1 lncRNA in Non–Small-Cell Lung Cancer

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Introduction: Apoptosis is a crucial pathway in tumor growth and metastatic development. Apoptotic proteins regulate the underlying molecular cascades and are thought to modulate the tumor response to chemotherapy and radiation. However, the prognostic value of the expression of apoptosis regulators in localized non–small-cell lung cancer (NSCLC) is still unclear.

Methods: We investigated the protein expression of apoptosis regulators Bcl-2, Bcl-xl, Mcl-1, and pp32/PHAPI, and the expression of the lncRNA MALAT-1 in tumor samples from 383 NSCLC patients (median age: 65.6 years; 77.5% male; paraffin-embedded tissue microarrays). For statistical analysis correlation tests, Log rank tests and Cox proportional hazard models were applied.

Results: Tumor histology was significantly associated with the expression of Bcl-2, Bcl-xl and Mcl-1 (all $p < 0.001$). Among the tested apoptotic markers only Bcl-2 demonstrated prognostic impact (hazard ratio = 0.64, $p = 0.012$). For NSCLC patients with non-adenocarcinoma histology, Bcl-2 expression was associated with increased overall survival ($p = 0.036$). Besides tumor histology, prognostic impact of Bcl-2 was also found to depend on MALAT-1 lncRNA expression. Gene expression analysis of A549 adenocarcinoma cells with differential MALAT-1 lncRNA expression demonstrated an influence on the expression of Bcl-2 and its interacting proteins.

Conclusions: Bcl-2 expression was specifically associated with superior prognosis in localized NSCLC. An interaction of Bcl-2 with MALAT-1 lncRNA expression was revealed, which merits further investigation for risk prediction in resectable NSCLC patients.

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Lung cancer is still one of the most lethal and most prevalent cancers in the world.[1] To improve therapy and decrease its high lethality, new molecular markers and targeted therapies are required.[2]

Cancerogenesis and chemotherapy resistance are both driven by deregulation of apoptosis (also referred to as the "programmed cell death").[3] Apoptotic markers (e.g., the Bcl-2 family proteins) regulate the molecular pathways. Bcl-2 itself serves as a prototype for the proapoptotic factors. It was first discovered in low-grade B-cell lymphoma.[4,5] Together with Bcl-xl, another member of this family with structural homology,[6–9] Bcl-2 resides on the mitochondrial outer membrane[10] and interacts with membrane protein channels.[11] By controlling the organelle's permeability and thus the release of caspases inducing proteins (e.g., cytochrome c)[7] both factors function as anti-apoptotic proteins.[7,8] Another family member is Mcl-1 with reported functional impact in human myeloma cells.[12] Moreover, Mcl-1 is a key resistance factor in various cancer cell lines including lung cancer.[13]

The mitochondrial pathway can be influenced by controlling proteins such as the nuclear phosphoprotein pp32/PHAPI, which sensitizes intact cells to caspase activation similar to tumor suppressors.[14,15] Moreover, resistance of cancer cells to the exhaustion of survival factors in vivo might be reduced by pp32/PHAPI.[16]

At present the knowledge about the molecular mechanisms of Bcl-2 family members is well-studied in lymphomas. However, in lung cancer, the prognostic impact of these factors is controversially discussed. Especially, for Bcl-2 there are almost as many studies suggesting a positive effect[17–26] as others suggesting a negative prognostic effect[27–29] (Supplemental Table 1, Supplemental Digital Content, http://links.lww.com/JTO/A677). Only few studies focused on the prognostic impact of Bcl-xl,[27,30–32] Mcl-1, or pp32/PHAPI in lung cancer.

Besides apoptotic factors Guo *et al.* reported cell growth, cell cycle progression, and invasion to depend on the expression of MALAT-1 long noncoding RNA through the regulation of apoptotic gene expression (i.e., caspase-3, -8, Bax, Bcl-2, and Bcl-xl).[33] Although we could demonstrate a functional impact of MALAT-1 on tumor growth, cellular

migration, colony formation, and prognosis in lung cancer,[34] the underlying molecular mechanisms are unclear.

To investigate, whether MALAT-1 regulates lung cancer growth through an interaction with apoptotic factors, we evaluate the prognostic effects of Bcl-2, Bcl-xl, Mcl-1, and pp32/PHAPI in the context of MALAT-1 expression.

METHODS

Study Population

Two collectives of 383 curatively resected non–small-cell lung cancer (NSCLC) patients with complete clinical follow-up information and sufficient tumor material from the Thoracic Departments Ostercappeln, Germany (Niels-Steensen-Klinik, $n = 304$) and Mainz (University Hospital, $n = 79$) were collected. Neoadjuvantly treated patients were excluded. The study was performed compliant to the rules and regulations of the state ethics committee, all subjects gave written consent. Clinicopathological data was obtained by medical chart review, including follow-up data. Clinical tumor, node, metastasis staging (including clinical examination, computed tomography scans, sonography, endoscopy, magnetic resonance imaging, bone scan) was performed according to International Union Against Cancer/American Joint Committee on Cancer recommendations.[35] To determine the definite tumor stage, postsurgical pathological examination was included. The pathological classification was based on the World Health Organization 2004 guideline.[36] The primary pulmonary lesion was classified as 196 squamous cell carcinomas, 135 adenocarcinomas, eight bronchoalveolar carcinomas, 11 adenosquamous carcinomas, and 53 large cell carcinomas. All patients had follow-up visits on a regular basis. Systematic restaging was performed after 3, 6, 12, 18, 24, 36, 48, etc. months or earlier if clinically indicated. Restaging included clinical examination, abdominal ultrasound scan, chest radiograph or computed tomography scans, and blood tests. Survival time was calculated from the date of histological diagnosis to death or last contact, respectively. **Table 1** shows baseline characteristics of the NSCLC study population.

Immunohistochemistry

After lung resection, tumor samples were formalin fixed and paraffin embedded. In this study, tissue microarrays (TMA)[37] were applied for immunohistochemical examination. After paraffin removal, TMA slides were steam heated for 30 minutes in pH 9 buffers and simultaneously stained using a computer controlled autostainer (*DakoCytomation*, Denmark). Unspecific tissue peroxidase was blocked by H_2O_2. The slides were incubated for 60 min with the primary monoclonal antibodies against Bcl-2 (clone 100, epitope: amino acids 41–54; mouse IgG1, 1:100),[38–40] against Bcl-xl (clone H-5, epitope: C-terminus, mouse IgG1, 1:1000),[41,42] and against Mcl-1 (clone RC-13, epitope: amino acids 1–327; mouse IgG1, 1:2000).[43,44] The three antibodies were purchased from Santa Cruz Biotechnology (Heidelberg, Germany). Moreover, a fourth antibody against acidic nuclear phosphoprotein 32A epitope (pp32/PHAPI; clone RJ1, mouse IgG1, 1:2000; Alexis Biochemicals Grünberg, Germany) was applied. After primary antibody incubation, a secondary biotinylated anti-mouse-antibody (Labeled Strept Avidin-Biotin-Methode; *DakoCytomation*) was applied, and the tissue samples were stained with chromogen (RED; Dako REAL detection substrate, *DakoCytomation*), counterstained by hematoxylin solution, and finally covered with Entellan (Merck, Darmstadt, Germany). The stained TMA slides were classified by three investigators (L.H.S., T.S., and R.W.) according to Remmele's Immunoreactive Score (IRS range, 1–12).[45] For immunohistochemical analysis only tumor cells were included. Bcl-2, Bcl-xl, and Mcl-1 were considered as positive if an IRS greater than or equal to 3 was stated. For pp32/PHAPI, a positive staining signal was defined by an IRS≥9. Internal controls were included in all experiments.

In Situ Hybridization for MALAT-1 RNA Expression Analysis

The full protocol for in situ hybridization for MALAT-1 RNA expression analysis was published before in Schmidt *et al*.[34] In brief, paraffin-embedded TMAs were incubated with Digoxigenin-labeled DNA probes complementary to MALAT-1 RNA. After detection of Digoxigenin-labeled Zyto-Fast chromogenic *in situ* hybridization (CISH) probes and hybridization, incubation with a secondary antibody for visualization was performed. On microscopic examination the NSCLC tissue cores were blindly classified (according to Tanner *et al.*)[46] by two investigators (L.H.S. and T.S.) into either a "weak expression" or a "strong expression" of MALAT-1 RNA transcript.

Next Generation Sequencing

Among several cancer cells lines tested, best transfection rates and strongest downregulation effect of MALAT-1 lncRNA were achieved in A 549 lung cancer cell line, for details please refer Schmidt *et al*.[34] Because of this observation next generation sequencing was performed in A 549 cells as follows: Using Pure Link RNA Mini kit (Life Technologies GmbH, Darmstadt, Germany), total RNA (8 µg) was isolated according to the manufacturer's protocol (for construction details regarding A549 cells please refer Schmidt *et al.*).[34] RNA quality was assessed by the RNA integrity number by Agilent 2100 bioanalyzer using the RNA Nano Chip and quantity was determined by NanoDrop (Peqlab, Erlangen, Germany). Ribo depletion (RiboMinus Eukaryote Kit for RNA-Seq, Life Technologies GmbH) and RNA fragmentation (SOLID Total RNA-Seq Kit, Life Technologies GmbH) were performed. cDNA library was prepared by hybridization and ligation reverse transcription (SOLID Total RNA-Seq Kit, Life Technologies GmbH). After conversion to first-strand cDNA size selection, purification, isolation, and bar coding were performed using SOLID Total RNA-Seq Kit (Life Technologies GmbH).

Mapping of the SOLID sequencing reads against the RefSeq (NCBI Reference Sequence Database) HG19 reference and the expression analysis was performed using LifeScope Genomic Analysis Software 2.5.1.

The transcript expression was quantified by calculating the RPKM values (reads per kilobase of transcript per million mapped reads).[47] RPKM is defined as follows: If the number of reads that map to a given transcript is *Mt* the length of that

TABLE 1. Baseline Characteristics of the Study Population of Resected NSCLC Patients

Variable	Patients (n = 383)	%a
Age[b] (years)	65.6±8.7	
Sex	297 males	77.5
Smoker	301	81.4
Performance status		
ECOG 0	76	20.6
ECOG I	272	73.7
ECOG II	21	5.7
Any adjuvant therapy	76	18.0
Histological tumor type		
Squamous cell carcinoma	176	46.0
Adenocarcinoma	135	35.2
Bronchoalveolar carcinoma	8	2.1
Adenosquamous carcinoma	11	2.9
Large cell carcinoma	53	13.8
Grading		
G1–G2	135	35.9
G3–G4	241	64.1
pT		
pT1	114	30.9
pT 2	220	57.9
pT3+4	34	8.9
pN		
pN 0	239	63.2
pN 1–3	139	36.7
pStage		
I	209	54.6
II	105	27.4
III	62	16.2
IV	7	1.8
Immunohistochemistry		
Bcl-2 positive (IRS ≥ 3)	87	23.4
Bcl-xl positive (IRS ≥ 3)	128	35.5
Mcl-1 positive (IRS ≥ 3)	198	55.2
pp32/PHAPI positive (IRS ≥ 9)	248	67.4
Median survival (days)[c]	1184 (513; 2463)	

[a]% of non-missing values.
[b]Mean ± SD.
[c]Median and quartiles (Q1, Q3).
IRS, immunoreactive score.

transcript is *Lt*, and the total number of mapped reads is *M*, such that $M = Mt$ then RPKM = $(10^9 \times Mt)/(Lt \times M)$. Thus, RPKM normalizes the read count to the length of the transcript and across samples of different coverage, so that direct comparisons of expression level can be performed among transcripts or across experimental conditions. The final results show an expression quantification of previously identified genes and the splice variants that are annotated in the RefSeq database. For each gene, the RPKM value and the total read count are reported. The major transcript with the highest RPKM was chosen to represent the genes with more than one transcript in the report.

Fold change values for strong versus weak MALAT-1 expression were determined as $\log_2$ ($RPKM_{strong}$ MALAT-1 expression/ $RPKM_{weak}$ MALAT-1 expression).

EGFR Protocol and *KRAS* Protocol

DNA extracted from formalin-fixed, paraffin-embedded tumor tissue samples were analyzed using sanger-sequencing technique for *EGFR* analysis and multiplex- polymerase chain reaction (PCR) followed by Snapshot analysis for *KRAS*. The cycling conditions for PCR amplification were done according to the manufacturer's instructions. Primers were removed by adding ExoSAP-IT PCR Product Clean-Up (Affymetrix GmbH, Munich, Germany) to the mix. Next, Snapshot reaction was performed and again unincorporated ddNTP's were removed. Analysis of the reaction was performed using ABI3730 DNA Analyzer (Life Technologies GmbH). Mutations were confirmed by a new PCR/mutation detection reaction. The *EGFR* and *KRAS* mutation status of each patient's tumor was assessed from the individual status of all mutation types and recorded as one of the following: positive (mutation detected for at least one of the mutation types assayed), negative (no mutation detected in any of the mutation types assayed), or undetermined/unknown (a positive or negative result could not be determined as per laboratory assessment (assay fail, insufficient DNA, fail because of assay criteria, or no/insufficient sample).

Statistical Analysis

Standard descriptive statistics such as frequencies, mean, median, and standard deviation were calculated to describe the study population. Clinicopathological correlations and dependencies for antigenic expressions were assessed using χ^2 test or Fisher's exact test, if applicable. The prognostic analysis for overall survival (OS) was carried out by Log rank test and multivariate analyses using Cox proportional hazards utilizing a forward stepwise selection model (inclusion criteria: p value of the Likelihood Ratio test ≤ 0.05). To avoid deviation because of low sample size or extremes, only parameters with complete data (<10 missing values) and with at least 10 cases of interest were included. The following features were regarded as potential explanatory factors (reference category is underlined): sex (male versus female), age (≤70 years versus >70 years), smoking status (nonsmoker versus smoker), pathologic tumor stage (II–IV versus *I*), lymph node status (pN_0 versus $pN_{1–3}$) grading ($G_{1–2}$ versus $G_{3–4}$), and antibody staining using Bcl-2, Bcl-xl, Mcl-1, and pp32/PHAPI (for all: negative expression versus positive expression). All analyses were performed using SPSS 21. The local significance level was set to 0.05. An adjustment to multiplicity was not performed. Therefore, an overall significance level was not determined and cannot be calculated. The presented findings may be used to generate new hypotheses.

Interaction Analysis

Direct interaction partners of Bcl-2 were identified in the IntAct database (https://www.ebi.ac.uk/intact/).[48] The interaction networks were then processed using Cytoscape[49] (Version 3.0.1). The following steps were performed: (1)

import to Cytoscape, (2) removal of interactions without confirmed evidence in humans, (3) removal of nodes with confirmed evidence in other organisms than human, (4) removal of all proteins without any incoming or outgoing edge, (5) integration of expression data (log-fold change) as node attribute, and (6) visualization.

Functional Enrichment

For the functional enrichment analysis of Bcl-2 and its direct interacting partners the BiNGO plugin[50] for Cytoscape was applied. To identify significantly overrepresented geneontology (GO) classes (www.geneontology.org), a hypergeometrical test is used for each GO class against the full GO annotation. *p* values are adjusted according to the Benjamini-Hochberg procedure, which controls the false discovery rate. GO classes with adjusted *p* values ≤ 0.05 are considered as significantly overrepresented in the respective gene set. The effect of MALAT-1 on a distinct GO class is measured by the median log fold change of the genes, which belong to the GO class. The median absolute deviation is used as a measure of variability of the gene cluster.

RESULTS

Immunohistochemical Expression of Regulators of Apoptosis

The characteristics of $n = 383$ NSCLC patients are summarized in Table 1; all of them had sufficient tissue material for the immunohistochemical analysis. Positive protein expression, as defined by an IRS greater than or equal to three, was found in 87 cases (23.4%) for Bcl-2, in 128 cases (35.5%) for Bcl-xl, and in 198 cases (55.2%) for Mcl-1. Because of a high frequency of positive staining results for pp32/PHAPI (≥80% of tumor samples), an IRS greater than or equal to nine was set to indicate positive antigen expression. Strong expression was found for $n = 248$ NSCLC tissue samples (67.4%; Table 1). pp32/PHAPI was present predominantly in the nuclei (Fig. 1*D*), whereas Bcl-2, Bcl-xl, and Mcl-1 displayed a cytoplasmic expression pattern (Fig. 1*A*-*C*).

Expression of Apoptosis Regulators Correlates with Tumor Histology

Squamous cell carcinoma histology was associated with positive expression for Bcl-2, Bcl-xl, and Mcl-1 (all $p < 0.05$). Of interest, in adenocarcinomas Bcl-2 expression was lower compared with non-adenocarcinoma patients, whereas Bcl-xl and Mcl-1 expression was more prominent in adenocarcinomas than in non-adenocarcinomas. Moreover, performance status (Eastern Cooperative Oncology Group [ECOG] 0 versus ECOG ≥ 1; $p = 0.002$) and smoking status (never smokers versus smokers; $p < 0.001$) were significantly associated with Mcl-1. For pp32/PHAPI, no positive association was found (Table 2).

Bcl-2 Influences Prognosis in Non-Adenocarcinoma NSCLC

Univariate Kaplan-Meier estimates did not demonstrate any significant effect on OS for the tested antigens. The

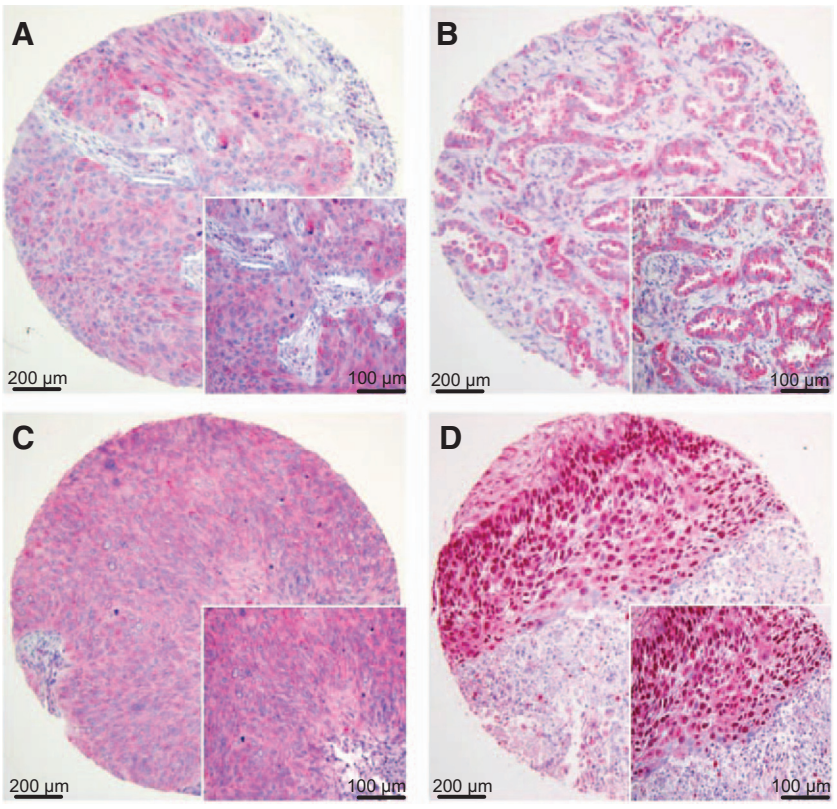

FIGURE 1. Immunohistochemical staining results for Bcl-xl, Bcl-2, Mcl-1, and pp32/PHAPI. All tested Bcl-2 family proteins Bcl-2 (A), Bcl-xl (B), and Mcl-1 (C) displayed a cytoplasmatic expression pattern, pp32/PHAPI (D) was also found in the nuclei. All images at x20, inlay x40.

TABLE 2. Clinicopathological dependencies regarding immunohistochemistry.

	positive antigen expression (%)			
Variables	**Bcl-2**	**Bcl-xl**	**Mcl-1**	**pp32/PHAPI**
Sex	p = 0.077	p = 0.086	p = 0.898	p = 0.787
Male	25.6%	33.1%	55.0%	67.8%
female	15.7%	43.8%	55.8%	65.8%
Age	p = 0.897	p = 0.816	p = 0.571	p = 0.122
<70 years	23.3%	35.8%	54.4%	64.8%
≥70 years	24.0%	34.5%	57.8%	73.1%
Performance Status	p = 0.123	p = 0.491	p = 0.002	p = 0.671
ECOG 0	16.0%	37.3%	39.7%	70.4%
ECOG ≥ 1	25.0%	32.6%	60.4%	67.0%
Smokers	p = 0.335	p = 1.000	p < 0.001	p = 0.193
never-smokers	17.9%	34.9%	79%	74.6%
smokers	24.3%	34.4%	50.5%	65.4%
NSCLC Histology	p = 0.037	p < 0.001	p = 0.034	p = 0.222
Non-squamous cell ca.	19.0%	44.7%	66.3%	70.3%
Squamous cell carcinoma	28.5%	25.6%	49.1%	64.2%
pStage	p = 0.805	p = 0.32	p = 0.522	p = 0.797
I	22.6%	37.8%	53.1%	66.5%
II - IV	24.0%	32.5%	57.0%	68.7%
Grading	p = 0.365	p = 0.726	p = 0.736	p = 0.242
G1-G2	20.3%	33.3%	57.0%	72.0%
G3-G4	24.9%	35.5%	55.0%	65.5%
Lymph node status	p = 0.443	p = 0.254	p = 0.322	p = 0.486
pN0	21.7%	37.7%	53.8%	66.1%
pN1-3	25.5%	31.6%	59.4%	70.0%
MALAT-1 expression (ISH)	p = 1.000	p = 0.342	p = 0.345	p = 0.789
weak	36.1%	33.7%	32.6%	33.7%
strong	35.2%	40.2%	38.7%	36.1%

All p-values according to Fisher's exact test

p values of the Log rank test are displayed in Figure 2 in the corresponding charts (Fig. 2*A*: *p* value for Bcl-2 = 0.218; Fig. 2*B*: *p* value for Bcl-xl = 0. 590; Fig. 2*C*: *p* value for Mcl-1 = 0.270; Fig. 2*D*: *p* value for pp32/PHAPI = 0.385).

For the tested apoptotic markers, subgroup analyses with regard to tumor histology (adenocarcinoma versus non-adenocarcinoma), therapy status (no adjuvant therapy *vs*. adjuvant therapy), sex (female versus male) and MALAT-1 expression (weak MALAT-1 expression versus strong MALAT-1 expression) were performed. Depending on histology a prognostic effect was shown for Bcl-2 in non-adenocarcinoma (Fig 3*A*; $p = 0.036$). Only a low number of adenocarcinoma tumors exhibited Bcl-2 expression without any correlation (Fig 3*B*; $p = 0.993$).

Moreover in non-adenocarcinoma survival rates increased for lymph node negative patients (Fig. 3*C*; $p = 0.049$) in contrast to lymph node positive patients (Fig. 3*D*; $p = 0.151$). However, further stage depending subdivision (i.e., stage I versus II versus III versus IV) regarding all tested immunohistochemical markers and regarding the three non-adenocarcinoma subtypes (squamous cell carcinoma, large cell carcinoma, and adenosquamous cell carcinoma) did not yield in any additional clinical relevant results (data not shown).

For MALAT-1 univariate Kaplan-Meier estimates did not reveal a prognostic effect neither for weak MALAT-1 expression ($p = 0.136$; Fig. 4*A*) nor for strong MALAT-1 expression ($p = 0.734$; Fig. 4*B*).

Prognostic Value of Bcl-2 Depends on MALAT-1 lncRNA Expression

To determine the prognostic value of the tested apoptotic markers Bcl-2, Bcl-xl, Mcl-1, and PP32/PHAPI, Cox proportional hazards model for comparison with established prognostic factors was applied. Besides Bcl-2 expression (hazard ratio (HR) 0.64, 95% CI 0.452–0.905, $p = 0.012$), tumor stage (HR 1.917, 95% CI 1.449–2.537, $p < 0.001$), sex (HR 1.72, 95% CI 1.173–2.522, $p = 0.005$) and age (HR 1.576, 95%-CI 1.177–2.110, $p = 0.002$) were relevant prognostic parameters in the multivariate analysis (Table 3A: whole study collective; $n = 304$ patients).

Moreover, MALAT-1 expression was found to influence multivariate analysis. In case of weak MALAT-1 expression the same parameters as mentioned above were prognostically significant (Table 3B: patients with weak MALAT-1 expression; $n = 155$ patients), whereas in case of strong MALAT-1 expression only lymph node stage (HR 0.372, 95% CI 0.162–0.850, $p = 0.019$) and tumor stage (HR 5.415, 95% CI 2.308–12.702, $p < 0.001$) were prognostically significant, but not Bcl-2 (Table 3C: patients with strong MALAT-1 expression; $n = 87$ patients).

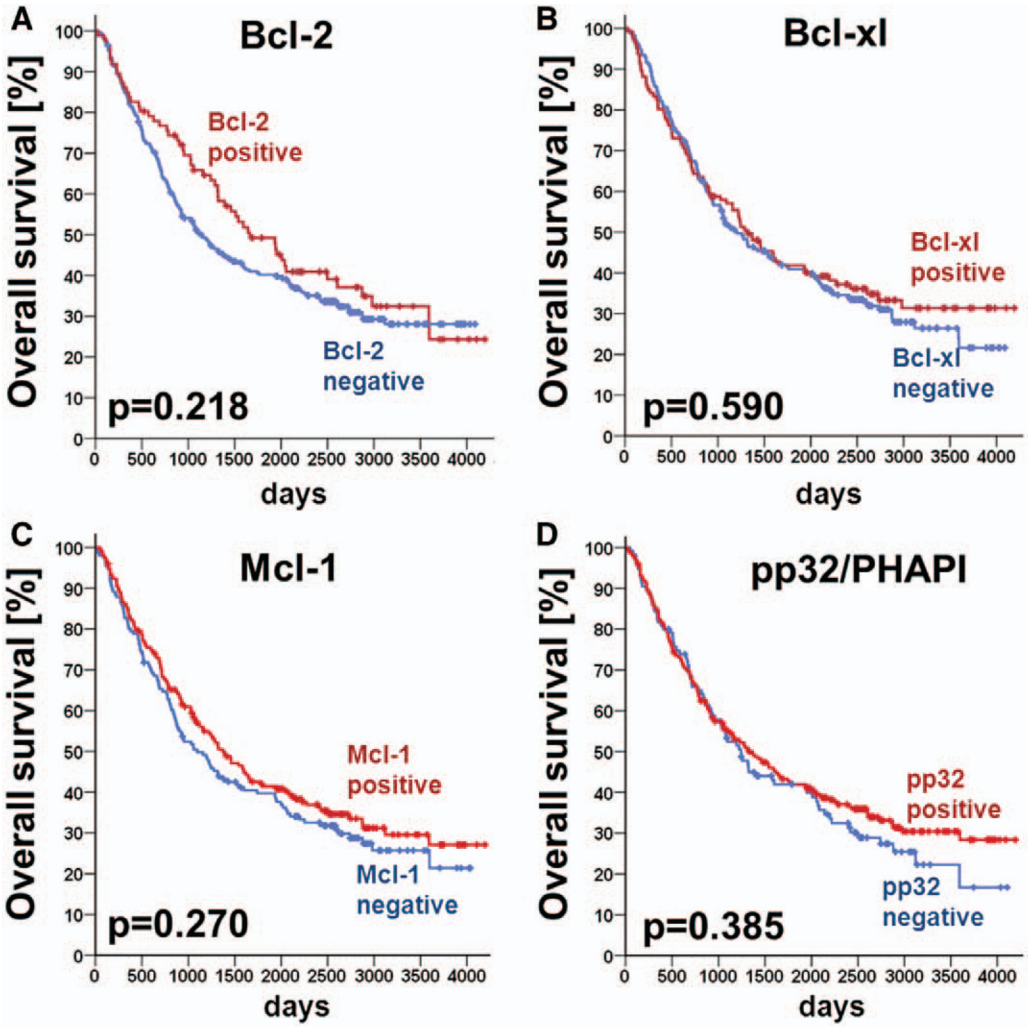

FIGURE 2. Kaplan-Meier survival analysis for all tested apoptotic factors (n = 383). The blue line indicates negative antigenic expression, and the red line stands for a positive immunohistochemical signal. The p values of the Log rank test are displayed in the corresponding charts. In the total study collective no prognostic effect was found for Bcl-2 (A; p = 0.218, log rank test), for Bcl-xl (B; p = 0.590, log rank test), for Mcl-1 (C; p = 0.270, log rank test) nor for pp32/PHAPI (D; p = 0.385, log rank test).

Interaction Analysis and Functional Enrichment

Bcl-2 interaction network (Fig, 5) displays the direct interaction partners of Bcl-2 and their log-fold change values in dependency of MALAT-1. Among the upregulated factors, we found Bcl-2, Bcl2l1, BID, and BAX, whereas BIK, Bcl2l11, TP53, and MAPK1 were downregulated by MALAT-1 (Fig. 5 and Table S2). Moreover, interactions among the Bcl-2 interacting proteins were revealed (e.g., Bcl-2l1 interacts with TP53, Bcl2l11, BAD, MAPK8, and BAX; Fig. 5). The BiNGO analysis of significantly overrepresented GO-terms confirmed the annotated functions and compartmental localizations for the selected gene set (data not shown).

Within the functional enrichment analyses with regard to MALAT-1 expression (up/downregulated genes), biological function was of interest. Among the downregulated genes in the BiNGO analysis, focus was put on the gene sets that were mostly affected by MALAT-1. The gene set TP53, NLRP1 displayed the second lowest median log FC (-0.79, mad = 0.36) and was associated with "activation of caspase activity," suggesting that MALAT-1 inhibits caspase activation. Likewise, another gene set containing MAPK1, BNIP3L, TP53, NR4A1, BIK, BAD, BCL2L11, and NLRP1 (median log FC = −0.62, mad = 0.41) shows a negative effect of MALAT-1 on both induction of apoptosis and on regulation of caspase activity. For the complete list, please refer to the Supplemental Digital Content (http://links.lww.com/JTO/A677).

Mutational Analysis

To understand the relationship of the tested apoptotic factors and the mutation status of *EGFR* and *KRAS*, we reevaluated the mutational status of a randomly assigned study subgroup of our study collective. Mutation status was analyzed for a subcohort of n = 146 patients with comparable clinical characteristics (p = 0.128, Mann-Whitney-U test for

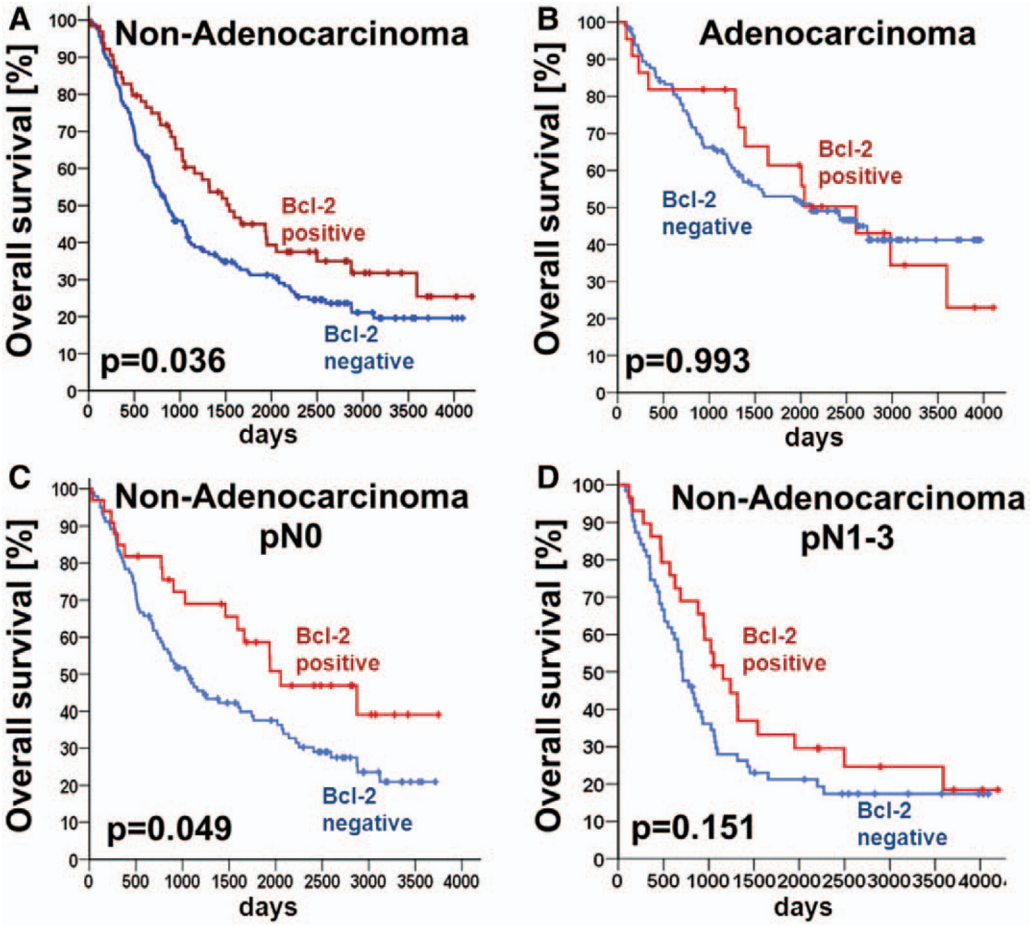

FIGURE 3. The prognostic effect of Bcl-2 depends on tumor histology and lymph node stage (Kaplan-Meier survival analysis, $n = 383$). The blue line indicates negative antigenic expression, and the red line stands for a positive immunohistochemical signal. The p values of the Log rank test are displayed in the corresponding charts. Depending on histology a prognostic effect was shown for Bcl-2 in non-adenocarcinoma (A; $p = 0.036$) but not for adenocarcinoma (B; $p = 0.993$). Moreover, in non-adenocarcinoma survival rates increased for lymph node negative patients (C; $p = 0.049$) in contrast to lymph node positive patients (D; $p = 0.151$).

age; $p = 0.956$ and $p = 0.202$, χ^2 test for sex and tumor stage, respectively).

Among *EGFR* mutated patients (six of 146 (4%) positive, either at position 18, 19, or 21) Bcl-2 was not expressed. For *KRAS* (14 of 146, 10% positive) only in 1 case Bcl-2 was expressed. Because of the low number of Bcl-2 in mutated patients, no comparative survival analysis was calculated for Bcl-2. Likewise, we could not show any relevant association or prognostic effect for other apoptotic factors (i.e., Bcl-xl, Mcl-1, and pp32/PHAPI) and mutation status ($p > 0.05$, all comparisons, for both *EGFR* and *KRAS*, log rank test).

DISCUSSION

Apoptosis is a central pathway with strong impact on lung cancer pathogenesis. Apoptotic regulators such as the Bcl-2 family proteins are involved in the cellular response to various stresses, including oncogene activation, growth factor depletion, and DNA damage. Bcl-2 is well characterized in indolent B-cell lymphoma.[4,5] Functionally, Bcl-2 prevents apoptotic cell death as induced through the so-called mitochondrial or intrinsic pathway of caspase activation.[17,19,20,22,23,25,26] In lung cancer, the functional role of Bcl-2 and its family members is less clear. Published studies of Bcl-2 expression in lung cancers suggest a positive prognostic effect,[17–26] whereas others demonstrate a negative prognostic effect[27–29] (Supplemental Table 1, Supplemental Digital Content, http://links.lww.com/JTO/A677).

Because previous studies are based on heterogeneous NSCLC patient populations, the prognostic effect of Bcl-2 might be influenced by various confounders. To reduce heterogeneity, only curatively resected patients without neoadjuvant treatment and with a definite NSCLC histology were included into the analysis. With regard to the discovered subgroup-specific effects of Bcl-2, we suggest to reevaluate the published studies.

Of the four tested markers a nonsignificant correlation with improved survival was found for patients with NSCLC expressing Bcl-2 or Mcl-1. To investigate whether these effects are random or systematic, exploratory subgroup analyses for the main clinical parameters (i.e., tumor histology, lymph node status, and tumor stage) were performed.

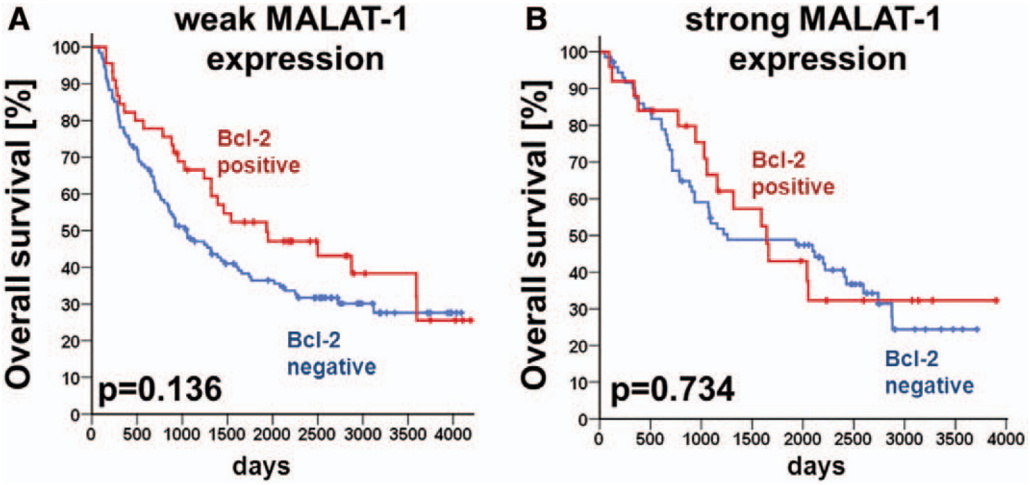

FIGURE 4. Univariate prognostic analysis for Bcl-2 and MALAT-1. The blue line in the Kaplan-Meier charts for overall survival (n = 383) indicates negative antigenic expression, and the red line stands for a positive immunohistochemical signal. The p values of the Log rank test are displayed in the corresponding charts. In univariate analysis, no prognostic effect was found neither for weak MALAT-1 expression (A; p = 0.136) nor for strong MALAT-1 expression (B; p = 0.734).

Whereas no further prognostic effect for Mcl-1 was found, Bcl-2 showed a strong impact on survival in our study collective. On the one hand, we could confirm the reported association of Bcl-2 with squamous cell carcinomas.[20,22,23,29] On the other hand, Bcl-2 expression in patients with non-adenocarcinomas was associated with increased OS (p = 0.036). The median OS was longer for Bcl-2-positive patients with non-adenocarcinoma histology without lymphatic spread (p = 0.049). The observation of an improved survival for Bcl-2 positive patients was also confirmed by multivariate analysis. Bcl-2 positive NSCLC patients showed decreased hazard ratios (i.e., their lethality is reduced by a factor of 0.64) compared with Bcl-2-negative patients. Besides Bcl-2, age, sex, and tumor stage were also identified as independent risk factors.

A recent study described an interaction with MALAT-1 lncRNA in cervical cancer cells. Here, downregulation of MALAT-1 induced the expression of Caspase-3, Caspase-8, Bax, and suppressed the expression of Bcl-2 and Bcl-xl[33]. To evaluate this hypothesis, we performed RNASeq experiments in human lung cancer cell lines with differential MALAT-1 expression. Therefore, downregulation of MALAT-1 was tested in several cancer cell lines. Best transfection rates and strongest downregulation of MALAT-1 lncRNA was achieved in A 549 lung cancer cell line.[34] Because of this observation and also because of cost effectiveness, next generation sequencing was exclusively performed in A 549 cells.

We found Bcl-2 expression to depend on MALAT-1 expression status in a positively related manner. i.e., strong MALAT-1 expression results in elevated Bcl-2 expression values (log-fold change = 0.46), confirming the hypothesis of Guo *et al*.[33]

Furthermore, among Bcl-2 family proteins Bcl-2 showed the highest upregulation depending on MALAT-1. Whether this is because of direct or indirect regulation should be evaluated in further studies. A functional enrichment analysis with regard to cellular localization, biological processes, and molecular functions of Bcl-2 and its interacting partners was performed. The analysis confirms the typical characteristics of Bcl-2 and its interacting partners, e.g., mitochondric localization. A further analysis separated for MALAT-1 expression did not generate new insights into the effect of MALAT-1 onto apoptotic regulation.

To test whether Bcl-2 is an independent risk factor dependent on MALAT-1 expression in vivo, we combined our expression profiles for the apoptotic markers with MALAT-1 lncRNA expression data and performed a multivariate Cox proportional hazard analysis stratified by MALAT-1 expression status (i.e., patients with weak versus patients with strong MALAT-1 expression). For NSCLC patients with strong MALAT-1 expression, Bcl-2 is no longer an independent risk factor, which may be explained by the positive regulation of MALAT-1 on Bcl-2 expression. In patients with strong MALAT-1 expression, Bcl-2 and corresponding Bcl-2 family members might be elevated. Tumor stage remains the strongest risk factor in both MALAT-1 groups. Of note, the prognostic impact of stage might be dependent of MALAT-1 expression, because the HR for stage II versus stage I increases from HR = 1.750 to HR = 5.002 and the HR for stage III and IV versus I rises from HR = 3.065 to HR = 6.472, respectively.

Despite the fact that we analyzed a large, clinically well characterized study cohort of localized and locally advanced NSCLC without neoadjuvant treatment, we cannot provide deeper insights into the molecular pathways. Whether the Bcl-2 related prognostic effect is independent on the EGFR and K-ras, somatic mutation status cannot be answered conclusively because of the small number of observed mutations. Moreover, the influence of the assumed anti-apoptotic effect of Bcl-2 in advanced stages of NSCLC on chemo-resistance remains unclear. Further studies should specifically address this question in a homogeneous collective of patients receiving adjuvant chemotherapy

In summary, three of the four markers tested (Bcl-xl, Mcl-1, and pp32/PHAPI) did not reveal any prognostic effect,

TABLE 3. Overall Survival: Explanatory Prognostic Factors in a Cox Proportional Hazards Model

Prognostic Factor	*p* value	HR[a] (95% CI)[b]
A) Total study cohort		
Tumor stage	<0.001	
(II vs. *I*)		1.544 (1.115–2.139)
(III and IV vs. *I*)		2.824 (1.974–4.039)
Age (≤70 years vs. ≥70 years)	0.002	1.598 (1.193–2.141)
Sex (male vs. female)	0.005	0.595 (0.405–0.873)
Bcl-2 expression (negative vs. positive expression)	0.014	0.657 (0.465–0.930)
Lymph node status (pN0 vs. pN1-3)	0.537	
Smoking status (nonsmoker vs. smoker)	0.378	
Bcl-xl expression (negative vs. positive expression)	0.790	
Mcl-1 expression (negative vs. positive expression)	0.348	
pp32/PHAPI expression (negative vs. positive expression)	0.353	
Grading (G1–2 vs. G3–4)	0.190	
B) Weak MALAT-1 expression		
Tumor stage	<0.001	
(II vs. I)		1.750 (1.103–2.777)
(III and IV vs. I)		3.065 (1.873–5.014)
Age (≤70 years vs. ≥70 years)	0.003	1.879 (1.250–2.826)
Sex (male vs. female)	0.018	0.556 (0.333–0.930)
Bcl-2 expression (negative vs. positive expression)	0.017	0.579 (0.363–0.923)
Lymph node status (pN0 vs. pN1-3)	0.588	
Smoking status (nonsmoker vs. smoker)	0.513	
Bcl-xl expression (negative vs. positive expression)	0.985	
Mcl-1 expression (negative vs. positive expression)	0.437	
pp32/PHAPI expression (negative vs. positive expression)	0.773	
Grading (G1–2 vs. G3–4)	0.262	
C) Strong MALAT-1 expression		
Tumor stage	0.002	
(II vs. I)		5.002 (2.057–12.162)
(III and IV vs. I)		6.472 (2.426–17.268)
Lymph node status (pN0 vs. pN1-3)	0.028	0.364 (0.159–0.835)
Age (≤70 years vs. ≥70 years)	0.311	
Sex (male vs. female)	0.636	
Bcl-2 expression (negative vs. positive expression)	0.818	
Smoking status (nonsmoker vs. smoker)	0.300	
Bcl-xl-2 expression (negative vs. positive expression)	0.219	
Mcl-1 expression (negative vs. positive expression)	0.685	
pp32/PHAPI expression (negative vs. positive expression)	0.173	
Grading (G1–2 vs. G3–4)	0.397	

[a]Hazard ratio < 1 indicates improved survival.
[b]Confidence interval.

whereas Bcl-2 could be identified as a prognostic factor, especially in non-adenocarcinoma lung cancer patients. Furthermore, Bcl-2 expression was positively related to MALAT-1 lncRNA expression status. With respect to weak MALAT-1 expression, Bcl-2-negative patients with resected lung cancer demonstrated an adverse prognosis. Non-adenocarcinoma patients with a negative expression of Bcl-2 might benefit from intensified postoperative care and treatment regimens. However, prospective studies are required to confirm this hypothesis.

Moreover, our data neither provides biochemical associations between MALAT-1 and Bcl-2 in cancer cells, nor a causal relationship between MALAT-1 and Bcl-2 because of the multivariate analysis including MALAT-1 as potential independent risk factor does not result in a significant *p* value (data not shown). However, our analysis provides a strong basis for further functional studies of interactions between MALAT-1 and molecules of the apoptotic network using analytical cell models.

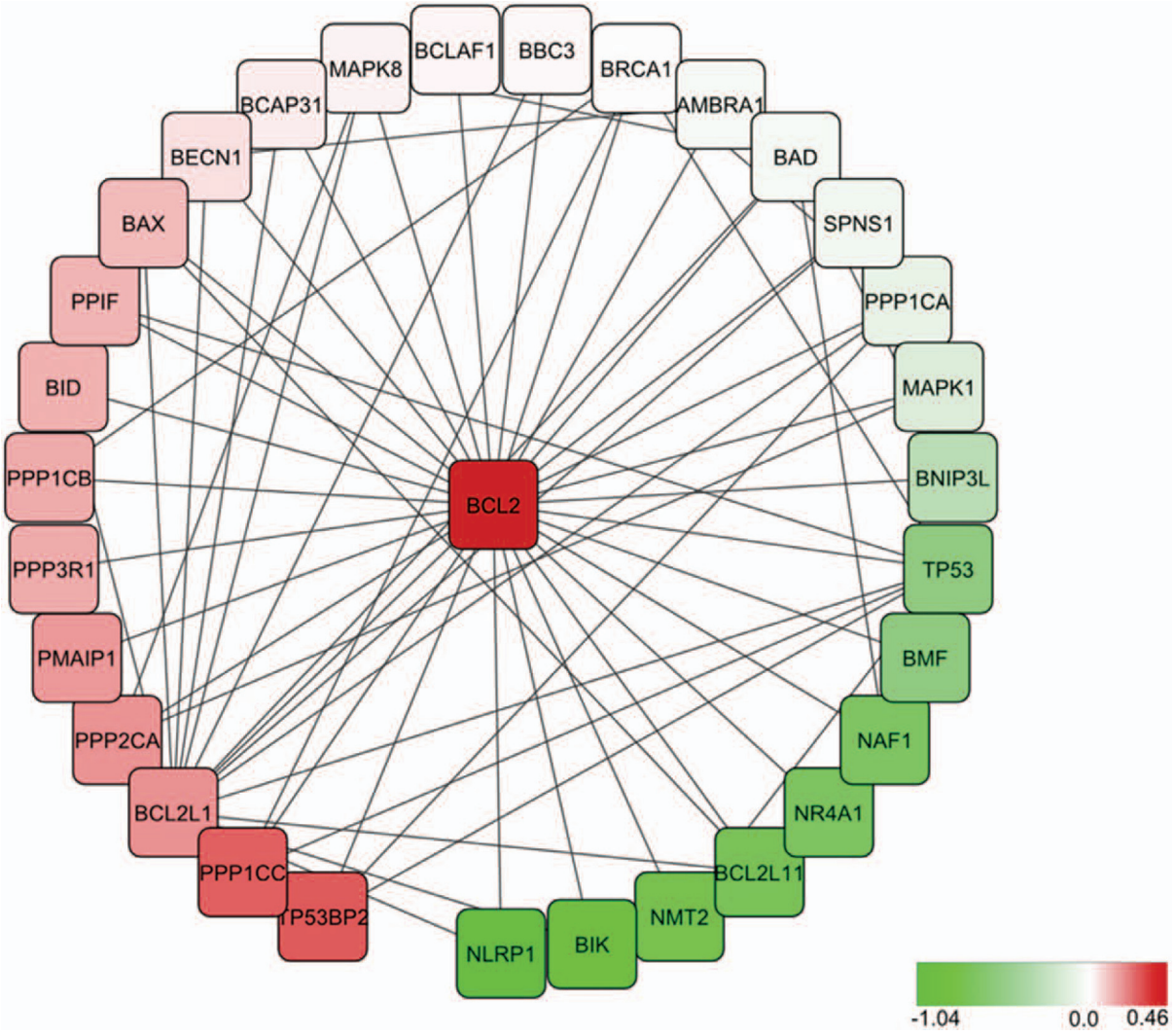

FIGURE 5. Bcl-2 and its direct interaction partners on protein level. Protein-protein interaction network of Bcl-2 according to the IntAct database. Edges indicate direct protein-protein interactions. The effect of MALAT1 (fold change [logFC] of strong MALAT-1 versus weak MALAT-1) on the expression of the interaction partners of Bcl-2 is color coded. Green nodes indicate downregulated genes, red nodes upregulated genes. Bcl-2 shows the strongest change in expression (logFC = 0.46), whereas NLRP1 displays the lowest logFC among the downregulated genes (logFC = –1.04). Among the upregulated factors, we found Bcl-2, Bcl2l1, BID, and BAX whereas BIK, Bcl2l11, TP53, and MAPK1 were downregulated by MALAT-1. Moreover, interactions among the Bcl-2 interacting proteins are demonstrated by crosslinks.

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REFERENCES

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA Cancer J Clin* 2011;61:69–90.
2. Brambilla E, Gazdar A. Pathogenesis of lung cancer signalling pathways: roadmap for therapies. *Eur Respir J* 2009;33:1485–1497.
3. Zhu CQ, Shih W, Ling CH, Tsao MS. Immunohistochemical markers of prognosis in non-small cell lung cancer: a review and proposal for a multiphase approach to marker evaluation. *J Clin Pathol* 2006;59:790–800.
4. Tsujimoto Y, Finger LR, Yunis J, Nowell PC, Croce CM. Cloning of the chromosome breakpoint of neoplastic B cells with the t(14;18) chromosome translocation. *Science* 1984;226:1097–1099.
5. Tsujimoto Y, Cossman J, Jaffe E, Croce CM. Involvement of the bcl-2 gene in human follicular lymphoma. *Science* 1985;228:1440–1443.
6. Boise LH, González-García M, Postema CE, et al. bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. *Cell* 1993;74:597–608.
7. Gross A, McDonnell JM, Korsmeyer SJ. BCL-2 family members and the mitochondria in apoptosis. *Genes Dev* 1999;13:1899–1911.
8. Kelekar A, Thompson CB. Bcl-2-family proteins: the role of the BH3 domain in apoptosis. *Trends Cell Biol* 1998;8:324–330.
9. Muchmore SW, Sattler M, Liang H, et al. X-ray and NMR structure of human Bcl-xL, an inhibitor of programmed cell death. *Nature* 1996;381:335–341.

10. Hsu YT, Wolter KG, Youle RJ. Cytosol-to-membrane redistribution of Bax and Bcl-X(L) during apoptosis. *Proc Natl Acad Sci U S A* 1997;94:3668–3672.
11. Sharpe JC, Arnoult D, Youle RJ. Control of mitochondrial permeability by Bcl-2 family members. *Biochim Biophys Acta* 2004;1644:107–113.
12. Derenne S, Monia B, Dean NM, et al. Antisense strategy shows that Mcl-1 rather than Bcl-2 or Bcl-x(L) is an essential survival protein of human myeloma cells. *Blood* 2002;100:194–199.
13. Zhang H, Guttikonda S, Roberts L, et al. Mcl-1 is critical for survival in a subgroup of non-small-cell lung cancer cell lines. *Oncogene* 2011;30:1963–1968.
14. Bai J, Brody JR, Kadkol SS, Pasternack GR. Tumor suppression and potentiation by manipulation of pp32 expression. *Oncogene* 2001;20:2153–2160.
15. Brody JR, Kadkol SS, Hauer MC, Rajaii F, Lee J, Pasternack GR. pp32 reduction induces differentiation of TSU-Pr1 cells. *Am J Pathol* 2004;164:273–283.
16. Hoffarth S, Zitzer A, Wiewrodt R, et al. pp32/PHAPI determines the apoptosis response of non-small-cell lung cancer. *Cell Death Differ* 2008;15:161–170.
17. Cox G, Louise Jones J, Andi A, Abrams KR, O'Byrne KJ. Bcl-2 is an independent prognostic factor and adds to a biological model for predicting outcome in operable non-small cell lung cancer. *Lung Cancer* 2001;34:417–426.
18. Fokkema E, Timens W, de Vries EG, et al. Expression and prognostic implications of apoptosis-related proteins in locally unresectable non-small cell lung cancers. *Lung Cancer* 2006;52:241–247.
19. Fontanini G, Boldrini L, Vignati S, et al. Bcl2 and p53 regulate vascular endothelial growth factor (VEGF)-mediated angiogenesis in non-small cell lung carcinoma. *Eur J Cancer* 1998;34:718–723.
20. Higashiyama M, Doi O, Kodama K, Yokouchi H, Nakamori S, Tateishi R. bcl-2 oncoprotein in surgically resected non-small cell lung cancer: possibly favorable prognostic factor in association with low incidence of distant metastasis. *J Surg Oncol* 1997;64:48–54.
21. Jeong SH, Jung JH, Han JH, et al. Expression of Bcl-2 predicts outcome in locally advanced non-small cell lung cancer patients treated with cisplatin-based concurrent chemoradiotherapy. *Lung Cancer* 2010; 68:288–294.
22. Laudanski J, Chyczewski L, Niklińska WE, et al. Expression of bcl-2 protein in non-small cell lung cancer: correlation with clinicopathology and patient survival. *Neoplasma* 1999;46:25–30.
23. Ohsaki Y, Toyoshima E, Fujiuchi S, et al. bcl-2 and p53 protein expression in non-small cell lung cancers: correlation with survival time. *Clin Cancer Res* 1996;2:915–920.
24. Renouf DJ, Wood-Baker R, Ionescu DN, et al. BCL-2 expression is prognostic for improved survival in non-small cell lung cancer. *J Thorac Oncol* 2009;4:486–491.
25. Silvestrini R, Costa A, Lequaglie C, et al. Bcl-2 protein and prognosis in patients with potentially curable non-small-cell lung cancer. *Virchows Arch* 1998;432:441–444.
26. Yoo J, Jung JH, Lee MA, et al. Immunohistochemical analysis of non-small cell lung cancer: correlation with clinical parameters and prognosis. *J Korean Med Sci* 2007;22:318–325.
27. Groeger AM, Esposito V, De Luca A, et al. Prognostic value of immunohistochemical expression of p53, bax, Bcl-2 and Bcl-xL in resected non-small-cell lung cancers. *Histopathology* 2004;44:54–63.
28. Kim YC, Park KO, Kern JA, et al. The interactive effect of Ras, HER2, P53 and Bcl-2 expression in predicting the survival of non-small cell lung cancer patients. *Lung Cancer* 1998;22:181–190.
29. Poleri C, Morero JL, Nieva B, et al. Risk of recurrence in patients with surgically resected stage I non-small cell lung carcinoma: histopathologic and immunohistochemical analysis. *Chest* 2003;123:1858–1867.
30. Berrieman HK, Smith L, O'Kane SL, Campbell A, Lind MJ, Cawkwell L. The expression of Bcl-2 family proteins differs between nonsmall cell lung carcinoma subtypes. *Cancer* 2005;103:1415–1419.
31. Karczmarek-Borowska B, Filip A, Wojcierowski J, et al. Estimation of prognostic value of Bcl-xL gene expression in non-small cell lung cancer. *Lung Cancer* 2006;51:61–69.
32. Sánchez-Ceja SG, Reyes-Maldonado E, Vázquez-Manríquez ME, López-Luna JJ, Belmont A, Gutiérrez-Castellanos S. Differential expression of STAT5 and Bcl-xL, and high expression of Neu and STAT3 in non-small-cell lung carcinoma. *Lung Cancer* 2006;54:163–168.
33. Guo F, Li Y, Liu Y, Wang J, Li Y, Li G. Inhibition of metastasis-associated lung adenocarcinoma transcript 1 in CaSki human cervical cancer cells suppresses cell proliferation and invasion. *Acta Biochim Biophys Sin (Shanghai)* 2010;42:224–229.
34. Schmidt LH, Spieker T, Koschmieder S, et al. The long noncoding MALAT-1 RNA indicates a poor prognosis in non-small cell lung cancer and induces migration and tumor growth. *J Thorac Oncol* 2011;6:1984–1992.
35. Mountain CF. Revisions in the International System for Staging Lung Cancer. *Chest* 1997;111:1710–1717.
36. Travis WD, Colby TV, Corrin B, et al. Histological Typing of Lung and Pleural Tumours. 3 ed. Berlin, Heidelberg, New York: Springer Verlag Berlin Heidelberg; 1999:1–70.
37. Schmidt LH, Biesterfeld S, Kümmel A, et al. Tissue microarrays are reliable tools for the clinicopathological characterization of lung cancer tissue. *Anticancer Res* 2009;29:201–209.
38. Friess H, Lu Z, Graber HU, et al. bax, but not bcl-2, influences the prognosis of human pancreatic cancer. *Gut* 1998;43:414–421.
39. Fu YC, Chi CS, Yin SC, Hwang B, Chiu YT, Hsu SL. Norepinephrine induces apoptosis in neonatal rat endothelial cells via down-regulation of Bcl-2 and activation of beta-adrenergic and caspase-2 pathways. *Cardiovasc Res* 2004;61:143–151.
40. Ghosh A, Sarkar SN, Rowe TM, Sen GC. A specific isozyme of 2'-5' oligoadenylate synthetase is a dual function proapoptotic protein of the Bcl-2 family. *J Biol Chem* 2001;276:25447–25455.
41. Hartmann K, Artuc M, Baldus SE, et al. Expression of Bcl-2 and Bcl-xL in cutaneous and bone marrow lesions of mastocytosis. *Am J Pathol* 2003;163:819–826.
42. Roth KA, Kuan C, Haydar TF, et al. Epistatic and independent functions of caspase-3 and Bcl-X(L) in developmental programmed cell death. *Proc Natl Acad Sci U S A* 2000;97:466–471.
43. Romani AA, Desenzani S, Morganti MM, Baroni MC, Borghetti AF, Soliani P. The BH3-mimetic ABT-737 targets the apoptotic machinery in cholangiocarcinoma cell lines resulting in synergistic interactions with zoledronic acid. *Cancer Chemother Pharmacol* 2011;67:557–567.
44. Opferman JT, Iwasaki H, Ong CC, et al. Obligate role of anti-apoptotic MCL-1 in the survival of hematopoietic stem cells. *Science* 2005;307: 1101–1104.
45. Remmele W, Stegner HE. [Recommendation for uniform definition of an immunoreactive score (IRS) for immunohistochemical estrogen receptor detection (ER-ICA) in breast cancer tissue]. *Pathologe* 1987;8:138–140.
46. Tanner M, Gancberg D, Di Leo A, et al. Chromogenic in situ hybridization: a practical alternative for fluorescence in situ hybridization to detect HER-2/neu oncogene amplification in archival breast cancer samples. *Am J Pathol* 2000;157:1467–1472.
47. Mortazavi A, Williams BA, McCue K, Schaeffer L, Wold B. Mapping and quantifying mammalian transcriptomes by RNA-Seq. *Nat Methods* 2008;5:621–628.
48. Kerrien S, Aranda B, Breuza L, et al. The IntAct molecular interaction database in 2012. *Nucleic Acids Res* 2012;40(Database issue):D841–D846.
49. Cline MS, Smoot M, Cerami E, et al. Integration of biological networks and gene expression data using Cytoscape. *Nat Protoc* 2007;2:2366–2382.
50. Maere S, Heymans K, Kuiper M. BiNGO: a Cytoscape plugin to assess overrepresentation of gene ontology categories in biological networks. *Bioinformatics* 2005;21:3448–3449.

