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Expression and prognostic implications of apoptosis-related proteins in locally unresectable non-small cell lung cancers

Eelco Fokkema^a, Wim Timens^b, Elisabeth G.E. de Vries^c, Steven de Jong^c, Vaclav Fidler^d, Coby Meijer^c, Harry J.M. Groen^{a,*}

^a Department of Pulmonary Diseases, University Medical Center Groningen, The Netherlands

^b Department of Pathology, University Medical Center Groningen, The Netherlands

^c Department of Medical Oncology, University Medical Center Groningen, The Netherlands

^d Department of Biostatistics and Bioinformatics, University Medical Center Groningen, The Netherlands

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Summary

Background: Apoptosis related proteins in early staged NSCLC seem to have prognostic value. We studied the value of a combination of eight of those proteins in advanced NSCLC.

Patients and methods: Bronchoscopically procured tumor biopsies of NSCLC patients were stained immunohistochemically and rated for expression of eight different cellular proteins. Patients were treated with 60 Gy radiotherapy with or without carboplatin as radiosensitizer.

Results: Apoptotic proteins in tumors that showed positive staining were the highest for Bax (99%), Fas (92%), FasL (87%), Rb (87%), p21(WAF1) (73%), and p53 (70%), and the lowest for c-myc (58%) and Bcl-2 (58%). In the Cox regression analysis Bcl-2 positivity (RR=0.61, 95% CI, 0.37–0.98, $p=0.04$) was predictive for overall survival. Only Bcl-2 staining percentage (RR₁₀ (RR associated with an increase in stained cells of 10%)=0.93, 95% CI, 0.89–0.99), p53 (RR₁₀=0.94, 95% CI, 0.89–0.99) and FasL (RR₁₀=0.92, 95% CI, 0.86–0.99) were predictive for a longer progression-free survival. No specific constellation of apoptotic proteins was associated with tumor response.

Conclusion: Bcl-2 expression in tumor tissue of patients with unresectable NSCLC predicts a better overall survival, while Bcl-2, p53, and FasL expressions predict for a longer progression-free survival.

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1. Introduction

Most important prognostic factors in non-small cell lung cancer (NSCLC) patients are clinical parameters such as disease

* Corresponding author. University Medical Center Groningen, P.O. Box 30.001, 9700 RB Groningen, The Netherlands
Tel.: +31 50 3616161; fax: +31 50 3619320.
E-mail address: h.j.m.groen@int.umcg.nl (H.J.M. Groen).

stage, performance status, and weight loss. Prognosis of these patients is still poor. One and 5 years survival for patients with locally unresectable NSCLC is about 27% and 6%, respectively [1]. Treatment of these patients mainly consists of concurrent radiation and chemotherapy. The impact of such therapy on prognosis depends on the tumor volume and tumor response to treatment. For instance, large variations in cellular radiosensitivity, which correlate with radioresponsiveness in human tumors [2,3] have been found in tumor cells [4] and also in normal cells [5] taken from different individuals. Identifying biological factors that determine tumor resistance towards radiation or chemotherapy may be helpful for optimizing therapy. Especially, variability in the tumor cell's ability to go into apoptosis may be crucial. In vitro studies in small cell lung cancer cell lines show that chemoradiotherapy induces morphological changes in cell size and cell size heterogeneity which are more pronounced in the sensitive GLC4 than in GLC4-CDDP tumors. An increase in p21 in GLC4 cell line after radiation may facilitate apoptosis. The increase in number of Bcl-2 positive cells after combined treatment and the consistently negative p21 status after any treatment in the resistant GLC4-CDDP cell line may protect these tumor cells from apoptosis as a part of their resistance mechanism to cisplatin [6].

Nowadays, several pathways leading to apoptosis have been revealed. For instance, upon radiation DNA damage, the tumor suppressor gene p53 induces Bax protein expression leading to apoptosis [7]. Overexpression of the oncogene c-myc can also activate apoptosis in a p53-dependent manner [8]. Furthermore, active p53 can also induce cell cycle arrest via p21 (WAF1) inhibiting Rb phosphorylation. Rb protein hypophosphorylation suppresses the transition from G1 to S phase. One observation in an interleukin-3 dependent cell line suggests that the absolute level of p21 can regulate whether a cell will arrest in G1 or goes into apoptosis [9]. Apoptosis can also be induced by death receptors, such as Fas or DR4/5. Other mechanisms, such as Bcl-2 protein members binding to Bax as heterodimers, prevent apoptosis.

Induction of apoptosis by chemotherapy or radiation depends on the function of these proteins. It has been shown that these proteins can be mutated, absent, up or down regulated in tumor cells. Moreover, the differential expression of some of these proteins may elicit a specific signal pathway reflecting at least in part the heterogeneity in tumor response and metastatic rate. Also effects downstream in the apoptotic pathways can be hampered. In NSCLC for instance, not only the Fas receptor but also a defective caspase-3 relocalization can influence the ability of a tumor cell to go into apoptosis [10]. The effect of treatment may therefore be related to the functionality and expression of apoptotic proteins. In the light of these interactions the question raises whether expression of these proteins has prognostic implications. Until now, most studies concerning prognostic implications of expression of these proteins in tumors from NSCLC patients have been performed in tissue obtained from patients with early stage NSCLC by surgical procedures. In the present prospective study we obtained tumor tissue from locally unresectable stage IIIA/B NSCLC patients treated with either radiation alone or radiation combined with carboplatin as radiosensitizer. The results of the parental clinical study have been published previously

[11]. We investigated whether single or combined expression of p53, p21(WAF1), Rb, c-myc, Bcl-2, Bax, and Fas and FasL in tumor biopsies, obtained from these patients, had prognostic implications.

2. Patients and methods

2.1. Patients and biopsies

In a randomized study, patients with locally advanced and inoperable NSCLC were treated with radiotherapy (60 Gy) administered as 2 Gy per day for 5 days a week during 6 weeks with or without continuous intravenous carboplatin (total dose: 860 mg/m²/6 weeks) as radiosensitizer. The study was approved by the local medical ethics committees of all hospitals and all patients gave written informed consent. Tumor responses were measured according to WHO criteria. Patients were followed every 3 months by history, physical examination, chest X-ray and additional imaging tests when there was suspicion for metastases. Overall survival was calculated from the time of randomization until death, loss of follow-up, or end of study. Time to progression was calculated from the time of randomization until time of local tumor progression or the occurrence of metastases.

Tumor biopsies prior to treatment were obtained by bronchoscopy. All biopsies were immediately fixed in 10% formalin and embedded in paraffin according to routine procedures. From all embedded biopsies 3 μ m sections were cut and placed on 3-aminopropyltriethoxysilan (APES) coated slides.

2.2. Chemicals

Phosphate buffered saline (PBS, 150 mM NaCl, 7.6 mM Na₂HPO₄·2H₂O, 1.6 mM KH₂PO₄, pH 7.35) was freshly made in our laboratory. Antigen retrieval (AR) solution consisted of 2% blocking reagent (Roche Biochemicals, Germany) for nucleic acid hybridization and detection with 0.2% SDS in maleic acid buffer (pH 6.0). Tris buffer was 0.1 M Tris-HCl at pH 9.0. Bovine serum albumine (BSA) was obtained from Serva (Heidelberg, Germany), human AB serum was obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). Imidazol was purchased from Merck (Darmstadt, Germany), di-ammine benzidine tetrahydrochloride (DAB) from ICN Biomedicals (Zoetermeer, The Netherlands). Mounting medium was purchased from International Medical (Zutphen, The Netherlands).

2.3. Tissue staining

Prior to immunohistochemistry slides were deparaffinized and air dried. One slide per tumor stained with standard haematoxylin/eosin was revised by an independent pathologist to establish whether it contained NSCLC tumor tissue. For all immunohistochemistry purposes except for c-myc staining, epitope demasking was performed by preheating. Slides were covered with antigen retrieval (AR) solution and incubated in a pressure cooker three times for 10 min at 115°C with 5 min cooling at room temperature in between. For c-myc, AR consisted of incubation

overnight at 80 °C in Tris buffer. After AR, slides were washed with PBS, incubated for 30 min with 0.3% H₂O₂ in PBS to block endogenous peroxidase activity and washed again with PBS. Subsequently, slides were incubated with the primary antibody diluted in PBS/1% BSA for 1 h. Primary antibodies used were: mouse monoclonal anti-p53, clone BP53-12-1 from Biogenex (San Ramona, CA)(1:800); mouse monoclonal anti-p21, clone EA10 (WAF 1) from Calbiochem (Cambridge, Massachusetts)(1:50); mouse monoclonal anti-Rb, NCL-Rb from Novocastra Laboratories (Newcastle upon Tyne, UK)(1:50); mouse monoclonal anti-c-myc, clone 9E10.3 from Neomarkers, (Hoersholm, Denmark)(1:150); mouse monoclonal anti-Bcl-2, clone 124 from Dako (Glostrup, Denmark)(1:50); rabbit anti-Bax, N-20 from Santa Cruz (Heidelberg, Germany)(1:600); mouse monoclonal anti-Fas, clone CH-11 from Upstate Biotechnology (Lake Placid, NY)(1:100); mouse monoclonal anti-FasL from Transduction Laboratories (Lexington, KY)(1:160).

Depending on the primary antibody, rabbit anti-mouse immunoglobulin conjugated to peroxidase, goat anti-rabbit antibody conjugated to peroxidase (both 1:50) or a biotinylated rabbit anti-mouse antibody (1:300)(all from Dako) for Fas and FasL, were used for the secondary step (diluted in PBS/1% BSA/1% AB serum). Depending on the secondary antibody, goat anti-rabbit antibody conjugated to peroxidase, rabbit anti-goat antibody conjugated to peroxidase (1:50) or streptavidin conjugated peroxidase (1:300)(all from Dako) were used for the tertiary step (diluted in PBS/1% BSA/1% AB serum).

After each step the slides were washed with PBS for 5 min. To visualize the bound antibodies, slides were incubated in DAB-medium (25 mg DAB in 50 mL PBS with 50 mg imidazole and 50 µL 30% H₂O₂) for 7 min. Slides were counterstained with haematoxylin for 2 min and rinsed with H₂O. After dehydration slides were covered using mounting medium. As positive controls for p53 and Rb, NSCLC tumor tissue that showed positive staining in an earlier staining procedure was used. For p21 we used a positive breast carcinoma, for c-myc a positive human ovarian tumor, for Fas human liver tissue, for FasL human testis, and for Bcl-2 and Bax human tonsil and infiltrating lymphocytes in the tumor tissue as positive controls. As a negative control for the staining procedure, the primary antibody was omitted. Furthermore, normal tissue next to tumor tissue was used as negative control for p53, p21(WAF1), and c-myc.

2.4. Staining analysis

All tumor biopsies were reviewed by an independent pathologist for presence of tumor tissue. Stained biopsies were considered evaluable if they contained at least 100 tumor cells. The number of stained tumor cells were counted in representative parts of the slides. All stained tumor biopsies were evaluated in a semiquantitative way for intensity of staining on a 5-point scale (0 = negative, 4 = strongly positive), and localization of staining (nucleus, cytoplasmic or membrane). All evaluations were performed without knowledge of prior treatment. Another classification, the positivity-intensity index, combined staining intensity and percentage of positive cells. For this positivity-intensity index, we choose two arbitrary categories: tumors that

showed ≤10% positive cells, or showed a staining intensity of zero or 1+ were categorized as 0 and others were categorized as 1.

2.5. Statistics

Cox regression analysis was used with the overall survival time and progression-free survival as the outcome variables and the eight immunohistochemical factors and patient characteristics including treatment as covariates. The association of these covariates and the tumor response was evaluated by logistic regression and two-sided Fisher's exact test. The nominal level of statistical significance used was 5%.

3. Results

3.1. Patient characteristics and tumor biopsies

Bronchoscopic evaluation prior to treatment revealed that 117 patients had central endobronchial tumors out of 160 patients who entered the randomized study assessing the radiosensitizing effect of carboplatin in stage III NSCLC. From 95 patients tumor blocks were available for further studies. Characteristics of the patients from whom tumor biopsies were taken are shown in Table 1. These patients were equally distributed over treatment with radiotherapy alone and treatment with radiotherapy combined with carboplatin (Table 1).

3.2. Immunohistochemistry

Seventy-five to 89 patients out of the 95 pretreatment patients with tumor blocks had enough evaluable tissue for subsequent immunohistochemical stainings. Positive tumor stainings were highest for Bax (99%), Fas (92%), FasL (87%), Rb (87%), p21(WAF1) (73%), and p53 (70%), and lowest for c-myc (58%) and Bcl-2 (58%). Table 2 shows the staining characteristics of the apoptotic proteins. Rb, p53, and p21(WAF1) showed mainly nuclear staining as expected (Fig. 1). Bcl-2 was more cytoplasmatic while Bax had both nuclear and

Table 1 Patient characteristics

	Number of patients
Patients with evaluable biopsies	95
Male/female	86/9
Stage IIIA/IIIB	45/50
Performance status WHO 0/1	58/37
Histology	
Squamous cell carcinoma	58
Adenocarcinoma	23
Large cell carcinoma	14
Mean weight loss in last 3 months (% of body weight)	2.2%
Radiotherapy with/without carboplatin	49/46
Tumor response/no response	43/52

Table 2 Immunohistochemical staining results from endobronchial biopsies of stage III NSCLC patients

Variable	p53	p21	Rb	Bcl-2	Bax	c-myc	Fas	FasL
Number of patients ^a	89	85	86	84	80	76	80	75
Number of positive staining	62 (70%)	62 (73%)	75 (87%)	49 (58%)	79 (99%)	44 (58%)	74 (92%)	65 (87%)
Localization staining								
Nuclear	58	51	62	9	26	0	7	0
Nuclear and cytoplasmatic	4	11	12	9	48	4	14	0
Cytoplasmatic	0	0	1	26	5	40	53	61
Membraneous	—	—	—	5	—	—	0	4
Staining intensity								
1+	8	13	17	20	3	17	6	12
2+	16	46	38	18	39	23	43	23
3+	15	3	16	11	35	4	25	24
4+	23	0	4	0	2	0	0	6
Histology								
Squamous cell								
Positive staining	42	43	50	33	51	29	48	43
Negative staining	14	13	3	21	1	19	4	5
Adenocarcinoma								
Positive staining	13	12	15	11	17	8	16	13
Negative staining	7	5	5	8	0	9	2	3
Large cell								
Positive staining	7	7	10	5	11	7	10	9
Negative staining	6	5	3	6	0	4	0	2

^a Patients with evaluable biopsies that contained at least 100 tumor cells per immuno-histochemically stained slide. Biopsy tissue containing too much necrosis or if no proper tissue could be defined were considered non-evaluable. The number of specimen are different to the total of 95 biopsies due to availability of sufficient tissue for all stainings.

cytoplasmatic localization. Fas and FasL were mainly cytoplasmatic (Fig. 2). Squamous cell carcinomas showed higher percentage of positive staining for p53, p21(WAF1), Rb, and Bcl-2 than adenocarcinoma or large cell carcinoma. No asso-

ciation was observed between loss of p21(WAF1) staining and Rb protein ($n = 69$, $p = 0.67$), nor between p53 and Bcl-2 protein staining ($n = 83$, $p = 1.00$). Loss of p53 staining was also not associated with the presence of Fas staining. The

Fig. 1 Typical immunohistochemical staining of tumor biopsies of p53, p21, c-myc, and Rb (immunoperoxidase staining, original magnification 100×).

Fig. 2 Typical immunohistochemical staining of tumor biopsies of Bcl-2, Bax, Fas, and FasL. Bax staining shows predominantly nuclear staining (immunoperoxidase staining, original magnification 100 $\times$).

positivity-intensity index did not add additional information over the positive/negative staining classification.

3.3. Relation of tumor markers with prognosis and tumor response

The Cox regression analysis with all the covariates but the immunohistochemical factors revealed an overall survival effect for stage (stage 3B versus stage 3A, RR=0.63, 95% CI, 0.43–0.92, $p=0.015$) and for mediastinal lymph nodes (N2 versus N3, RR=1.69, 95% CI, 1.02–2.8, $p=0.043$). Subsequently these two variables plus the eight immunohistochemical factors were entered into the regression model. Only Bcl-2 positivity was predictive for a better survival (RR=0.61, 95% CI, 0.37–0.98, $p=0.04$). Using progression-free survival as outcome variable, Bcl-2 staining percentage (RR₁₀ (RR associated with an increase in stained cells of 10%)=0.93, 95% CI, 0.89–0.99, $p=0.012$), p53 staining percentage (RR₁₀=0.94, 95% CI, 0.89–0.99, $p=0.027$) and FasL staining percentage (RR₁₀=0.92, 95% CI, 0.86–0.99, $p=0.021$) were predictive for a longer progression-free survival.

Tumor response was not associated with a single or combination of (mechanistically related) apoptotic proteins. Loss of Rb staining however was associated with unresponsiveness to radiotherapy, although the number of nonresponding patients was small ($n=6$).

4. Discussion

Tumor suppressor proteins such as p53 and Rb are overexpressed in tumor tissue of the majority of locally advanced NSCLC patients. In our study, 70% of these tumors showed p53 overexpression, which was not related with tumor responses after radiation. Also overall survival time was

not associated with p53 expression. Two small studies in stage III NSCLC patients treated with radiation contrasted with our results. Failure to stain for p53 correlated with both response and better survival in one study [12] and the presence of a mutated p53 gene predicted resistance towards radiotherapy in the other study [13]. However, most clinical pathological studies are performed in early-staged NSCLC, where clinical risk factor associations are made on resected specimen. In more advanced NSCLC such studies are sparse. Larger studies in resected early staged NSCLC patients revealed no association with p53 expression [14,15]. Meta-analyses showed that p53 protein overexpression studied in resected NSCLC tumors is a poor prognostic factor in NSCLC patients [16], especially in adenocarcinoma, with a 5-year survival difference of 9.1% (95% CI, 2.3–16.0) between patients with and without p53 protein overexpression [17].

In the present study the percentage of patients with Bcl-2 expression was somewhat higher (58%) as compared with other studies (about 40%). We found a survival advantage after radiotherapy. No association between tumor response after radiation and Bcl-2 expression was observed, although positive Bcl-2 expression was related to a prolonged progression-free survival. This is a surprising effect since Bcl-2 expression is normally associated with decreased apoptosis, implying worse survival of patients with Bcl-2 expression. However, contradictory results involving prognostic impact of Bcl-2 expression have been found. In several studies, Bcl-2 expression in NSCLC implied a better prognosis after treatment [18–26]. In other studies, Bcl-2 expression was reported as being related with worse survival [27,28], or reported as not related with survival [29–32].

Almost all patients expressed Bax protein in the present study. As a single factor, its expression did not affect survival in this, nor in another study [24].

In our study, Rb was undetectable by immunohistochemistry in 13% of stage III NSCLC patients. Although the number of Rb negative patients was small in this study, all these patients did not respond to radiotherapy as measured with CT scans. Also in another study patients with Rb negative NSCLC tumors showed a tendency to do worse, especially in adenocarcinomas [33]. Furthermore, significantly more stage III and IV NSCLC tumors had altered Rb protein expression than stage I and II [34]. This suggests that loss of Rb expression is an indicator for worse prognosis. However, Kwiatkowski [19] showed abnormal Rb protein expression in 79 of 242 NSCLC patients and found no prognostic impact.

The cyclin dependent kinase (CDK) inhibitor p21(WAF1) was present in 73% of biopsies and although CDK inhibitors are described as disruptors of the Rb pathway we found no association between the failure to express p21 and Rb expression. P21 did not predict survival or progression-free survival in our study. Prognostic impact of p21 is not as intensively investigated as p53 in NSCLC tumors. Studies in breast, gastric, endometrial and head and neck cancer showed contradicting results. High as well as low expression of p21 was correlated with a short patient survival [35]. In NSCLC positive expression of p21 predicted a favorable prognosis in a study by Shoji et al. [36]. Other CDK inhibitors such as p16 show the same contradicting results with respect to prognostic significance [37]. While loss of p16 or Rb expression was associated with increased proliferative activity in p53 positive tumors, loss of p16 protein alone did not result in shorter patients survival.

In the present study the dominant oncogene protein c-myc was overexpressed in 42% of patients which is in line with other studies where about half of patients had a detectable c-myc protein [38,39].

In our study Fas and FasL proteins were expressed in most NSCLC tumors. In a Japanese study [40], Fas protein overexpression occurred in 37% of resected stage III NSCLC. Fas mRNA expression and high levels of Fas protein were associated with p53 wild-type status alone [41]. We could not confirm such association at the protein level, as was also found by Esposito et al. [42]. In contrast to these results Fas was an independent factor in another study, predicting a better survival [43]. We found that FasL expression and not Fas was associated with a longer progression-free survival. One possible explanation for this phenomenon is that the ligand should first associate with the Fas receptor before apoptosis is induced. After radiation FasL may be the limiting factor in the interaction of both proteins and therefore can be the determining factor in the Fas/FasL system to be associated with time to tumor progression.

At this moment, it appears that clinical parameters are still better predictors for clinical outcome than expression of single apoptosis related proteins or a combination of expression of these proteins. However, limitations of immunohistochemistry in estimating biological properties may account partially for this lack of effect. For instance, immunohistochemical staining misses about one third of p53 mutations (mostly splicing and nonsense) and occasionally yields false positive results [44]. It is known that wild-type p53 can be stabilized or induced by c-myc and thus can be detected by immunohistochemistry. Perhaps such mechanisms are also true for other apoptotic proteins. Other problems in immunohistochemical studies are the different sensitivities

of antibodies, recognition of different epitopes by different antibodies used in those studies, and the different cut-off points from where a tumor is defined as positive or negative. However, the major advantage of immunohistochemistry is that it shows which cells in the tumor are stained and the localization of the cellular staining, providing information about cellular heterogeneity in tumor tissue, which is quite extensive in NSCLC.

This study also shows high expression of apoptotic proteins in tumor tissue. These proteins may be used as new treatment targets [45]. Downregulation of Bcl-2 using antisense technology or relocalisation of Fas and FasL to the cell membrane followed by heteromerization may be examples of such approach.

In conclusion, in the present study Bcl-2 was the most obvious individual cellular protein with prognostic implication out of eight apoptosis related proteins in patients with locally unresectable NSCLC.

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