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BAX and p16^{INK4A} are independent positive prognostic markers for advanced tumour stage of nonsmall cell lung cancer

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BAX and p16^{INK4A} are independent positive prognostic markers for advanced tumour stage of nonsmall cell lung cancer. C. Gessner, U. Liebers, H. Kuhn, P. Stiehl, C. Witt J. Schauer, G. Wolff. ©ERS Journals Ltd 2002.

ABSTRACT: Clinical studies suggest prognostic relevance of p16^{INK4A} in nonsmall cell lung cancer (NSCLC) while conflicting results for p53 have been published. However, the importance of the apoptosis regulating gene BAX, a downstream regulator of p53, on the prognosis of NSCLC is unknown. The present study investigated the prognostic relevance of BAX with respect to the status of p53 and p16^{INK4A} in 61 patients with advanced NSCLC.

Protein expression of BAX, p53 and p16^{INK4A} was investigated retrospectively by immunohistochemistry. Tumour deoxyribonucleic acid (DNA) was screened for p53 mutations by single strand-conformation polymorphism polymerase chain reaction (PCR) and BAX frameshift mutations by fragment length analysis.

Patients with positive BAX protein expression had a significantly longer median survival (14 months) than those patients without BAX expression (6 months, p=0.0004). In contrast, p53 status did not influence prognosis. Patients with p16^{INK4A} negative tumours had a significantly shorter survival (4 months) than those with p16^{INK4A} protein expression (15 months, p=0.0001). Furthermore, the loss of p16^{INK4A} protein expression correlated strongly with the pressure of distant and advanced lymph-node metastases. The best survival was seen in a subgroup of 20 patients with positive p16^{INK4A} expression and intact BAX (p=0.0002).

The results of the present study suggest that the loss of BAX and p16^{INK4A} expression are independent markers for poor prognosis in nonsmall cell lung cancer. The study suggests that multimarker analysis of genes involved in apoptosis may be useful for determining individual therapy and for identifying targets for gene-replacement therapy. This should be assessed in a prospective study with a larger cohort of patients.

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Lung cancer is the most prevalent and lethal cancer in the Western world with increasing incidence attributable mainly to cigarette smoking [1, 2]. Despite the development of new chemotherapeutic drugs and multimodal treatment strategies, the survival rate of nonsmall cell lung cancer (NSCLC) remains unchanged and poor. Identification of new prognostic markers for the characterization of lung-cancer biology may be helpful, as they could serve as a basis for predicting response to radiation and chemotherapy at a molecular level [3].

Genes regulating apoptosis and cell-cycle progression are candidates for prognostic markers, *e.g.* p16 and p53, which have already been targets for investigation. p16 is a gene regulating cell-cycle progression. A tumour suppressive property was first described for p16^{INK4A} by OKAMOTO *et al.* [4], and its prognostic relevance has been shown for various cancers. Inactivation of p16^{INK4A} correlated with bad prognosis in pancreatic adenocarcinoma [5], malignant melanoma [6], and resected NSCLC [7]. Experimental data have also shown cooperation of p16^{INK4A} and p53 in induction of apoptosis [8, 9]. Studies investigating the prognostic relevance of p53 have shown conflicting results [10]. NSCLC with p53 mutations show resistance to the effects of radiation and cisplatin [11] due to the lack of the proapoptotic function of intact p53.

BAX is a proapoptotic gene, which is regulated by p53, and is therefore a likely candidate for a marker of prognosis. The gene product is a downstream effector of the tumour suppressor gene p53 and is upregulated by transcriptional activation. Furthermore, there is strong evidence for a prognostic relevance of BAX in malignancy. STURM *et al.* [12] found that low BAX expression was a negative prognostic factor in patients with resected liver metastases from colorectal cancer. Also, there is evidence that genetic damage to BAX or p16^{INK4A} genes causes resistance towards apoptosis as in tumorigenesis, but also resistance to anti-tumour therapy. In patients with breast cancer, a reduced expression of BAX was correlated with a poor response to chemotherapy and shorter overall survival [13]. Restoration of BAX expression in breast cancer

cell lines increased sensitivity to cytotoxic drug therapy [14] and also suppressed tumourigenesis [15].

The prognostic relevance of p16^{INK4A} status and of BAX status with respect to p53 in advanced NSCLC was investigated. Based on the data about the prognostic relevance in other cancers and the cooperation of p16^{INK4A} and p53 in inducing apoptosis, a possible prognostic relevance of BAX and p16^{INK4A} protein expression in advanced tumour stage of NSCLC was hypothesized.

Patients and methods

Patients

Representative tumour samples (two) were obtained from 61 consecutive patients with NSCLC at autopsy from primary tumours (47 male, 14 female, median age 65 yrs ranging between 46–83 yrs) and analysed retrospectively. All patients were treated between 1992–1996 at the University of Leipzig Medical centre and associated academic hospitals. The histological tumour classification was performed by two pathologists according to World Health Organization guidelines [16]. The tumour stage was determined at the time of diagnosis according to the revised tumour, node, metastasis (TNM) staging system of lung cancer [17]. Survival times and clinical data were assessed retrospectively with the help of the patients' physicians (table 1).

Table 1. – Tumour parameters

Tumour parameters	n (%)
Subjects n	61
T-stage of primary tumour	
T1	9 (15)
T2	23 (38)
T3	13 (21)
T4	16 (26)
Lymph node metastases	
N0	19 (31)
N1	13 (21)
N2	14 (23)
N3	15 (25)
Distant metastasis	
M0	28 (46)
M1	33 (54)
Tumour stage groups	
IA	5 (8)
IB	8 (13)
IIA	1 (2)
IIB	6 (10)
IIIA	5 (8)
IIIB	3 (5)
IV	33 (54)
Histological classification	
Adenocarcinoma	18 (29)
Bronchioloalveolar carcinoma	3 (5)
Squamous cell carcinoma	33 (54)
Large cell carcinoma	7 (12)

Immunohistochemical analysis for BAX, p53, and p16^{INK4A}

Protein expression of p53, p16^{INK4A}, and BAX was analysed by immunohistochemistry using 2 μm formalin-fixed paraffin-embedded tissue slices. The slices were boiled after deparaffination for 10 min in citrate buffer (0.01 mol·L^{-1}, pH 6.0) for antigen demasking. After blocking with fish serum (SEA BLOCK^{TM}, Pierce, Rockford, IL, USA), slices were incubated for 60 min with the primary antibodies anti-p53 antibody (DO-1, PharMingen, Hamburg, Germany; dilution 1:50), anti-BAX monoclonal antibody (YTH-2D2, Trevigen, Gaithersburg, MD, USA; dilution 1:500), and anti-p16^{INK4A} antibody (G175-405, PharMingen, Hamburg, Germany; dilution 1:50). The bound primary antibodies were visualized by biotin-conjugated secondary antibodies and peroxidase-conjugated streptavidin (Jackson Immuno Research Laboratories, West Grove, PA, USA), using metal-enhanced i.e. 3,3′-diamino (no gap between comma and 3) 3′-diaminobenzidine (DAB) as a substrate (Pierce, Rockford, IL, USA). Slices were counterstained with haematoxylin.

Slices were analysed in a blinded fashion by two observers who were unaware of clinical and histological data. Four high power fields (400×) were evaluated for localization and percentage of positive cells (0–100% in 5% steps). Samples were considered as positive, if >10% of tumour cells showed moderate or intense nuclear staining.

Mutation analysis for p53

Deoxyribonucleic acid (DNA) was extracted from 30-μm slices of paraffin embedded tissue. Genomic DNA was isolated after deparaffination and rehydration using the InViSorb^{TM} Genomic DNA Kit II (InViTek, Berlin, Germany).

For detection of mutations in the DNA binding region of p53 single-strand conformation polymorphism polymerase chain reaction (PCR) analysis was performed. Template DNA was subjected to PCR using oligonucleotide primer pairs for amplification of exons 5–8. The amplified fragments were analysed on a 10% nondenaturing polyacrylamide gel with silver staining using a DNA silver staining kit (Amersham Pharmacia Biotech, Freiburg, Germany) according to the manufacturers specifications.

Detection of BAX frameshift mutations

A 94 bp fragment of the BAX exon 3 encompassing the G(8) tract was amplified by PCR using primer sequences and cycling conditions as described previously [12, 18], with the reverse primer labelled with the fluorescence dye HEX (Applied Biosystems, Foster City, CA, USA). PCR fragment length was analysed using an ABI 310 Sequencer (Perkin Elmer, Weiterstadt, Germany). The human colon carcinoma cell line LoVo carrying mutations in both BAX alleles (one insertion (G(9)) and one deletion (G(7)) was used

as a positive control, while the human colon carcinoma line SW620 served as wild type control. In dilution experiments, the sensitivity (cut-off: 10% mutated cells) and in blinded experiments the specificity (100%) of the fragment length analysis were confirmed.

Statistical analysis

Overall survival was estimated by the Kaplan-Meier product-limit method. Statistical comparison was by means of the Logrank Mantel-Cox Test. Most biological and pathological variables were used as dichotomized (categorical) variables. Patients were stratified as positive ($p53_{pos}$, BAX_{pos}, and $p16^{INK4A}{}_{pos}$) *versus* negative ($p53_{neg}$, BAX_{neg}, and $p16^{INK4A}{}_{neg}$). p53 genetic status was subdivided into p53 wild type (wt) *versus* p53 mutated (mut). The cut-off value for immunohistochemical data of BAX, p53, and $p16^{INK4A}$ was set at 10% positive stained cells, *i.e.* $\geqslant$10% stained cells in a tumour was considered positive, <10% stained cells was considered negative [12].

For correlation of BAX, p53, and $p16^{INK4A}$ with tumour classification as well as clinical parameters, Pearson's Chi-squared test was applied.

For survival analysis, the equal distribution of patients with different therapies in the positive and negative subgroups for BAX, p53 and $p16^{INK4A}$ was considered using Fisher's Exact test.

Results

Follow-up

At the time of diagnosis most of the 61 patients had stage IV tumours (33 of 61), while only 28 (46%) were stages I to III. Sixteen cases received best supportive care only due to their advanced stage, 23 were treated by surgical resection, 18 by chemotherapy and 17 by radiation therapy. Five patients underwent surgery and adjuvant chemotherapy, four underwent surgery followed by radiotherapy and four received chemotherapy and radiotherapy. These patients were equally distributed in the positive and negative subgroups for BAX (Fisher's Exact test $p>0.15$), p53 (Fisher's Exact test $p>0.99$), and $p16^{INK4A}$ (Fisher's Exact test $p>0.99$) as well as in p53 mutation status subgroups (Fisher's Exact test $p>0.39$).

Median overall survival was 9 months. The overall 1-, and 2-yr survival rates (including the 30-day perioperative mortality of patients treated by surgery) were 38%, and 10%, respectively.

Immunohistochemical analysis of p53 expression

Overexpression of p53 protein was found in 31 cases with a mean number of 63±4% (mean±SEM) of p53 expressing cells. Twenty-three tumours showed 0%, one sample 5%, and six samples 10% of cells positively stained for p53.

To analyse the clinical relevance of p53, the protein expression status was correlated with tumour and clinical parameters. Neither tumour parameters (size, lymph node metastasis status, distant metastasis status, tumour stage, grading, and histological classification) nor clinical parameters (age, gender, and therapy status) showed a significant correlation with p53 expression.

Immunohistochemical analysis of BAX expression

In 31 patients BAX expressing cells were not found. In the remaining 30 cases, BAX expression was observed in 65±4% of all cells evaluated. There was a significant correlation of distant metastasis and grading type with BAX expression. Tumours with distant metastasis were BAX negative in 64% while tumours without distant metastasis were BAX negative in only 36% ($p<0.03$).

Loss of BAX expression was found in grading type G2 in 37%, and in grading type G3 in 66% ($p<0.03$) indicating that the absence of BAX expression could be associated with less histological differentiation of tumour tissue. Grading type G1 and G4 were not represented in the tumour samples.

Immunohistochemical analysis of $p16^{INK4A}$ expression

$p16^{INK4A}$ expressing cells were found in 35 cases with mean expression of 54±4% in the cells that were evaluated, 25 samples had no expression and one had 5% of positively stained cells.

Neither histological classification nor clinical parameters showed a significant correlation with $p16^{INK4A}$ expression. Nor was there a correlation between tumour size or stage and $p16^{INK4A}$ expression.

In contrast to BAX and p53 analysis, loss of $p16^{INK4A}$ expression was more frequent in patients with lymph-node metastases. In N2 stage 79% and in N3 stage 53% of the samples were negative for $p16^{INK4A}$ protein, while in N0 and N1 stages absence of $p16^{INK4A}$ expression was found in 16% and 31% of the cases, respectively ($p<0.002$). Furthermore, a loss of $p16^{INK4A}$ expression was observed in 58% of tumours from patients with distant metastasis, but in only 25% of patients without distant metastasis ($p<0.01$).

Loss of $p16^{INK4A}$ expression was found in grade 2 (G2) in 28%, and in grade 3 (G3) in 59% of the cases ($p<0.016$) which was similar to findings shown for BAX expression.

Analysis of p53 and BAX mutations

In 32 primary tumours (53%) mutations in the p53 gene were found. Altogether 37 mutations were detected: seven in exon 5, five in exon 6, 17 in exon 7, and eight in exon 8. Five primary tumours showed mutations in two exons. Tumours with mutated p53 did not have significantly increased protein expression. For 53% of cases with p53 mutations *versus* 50%

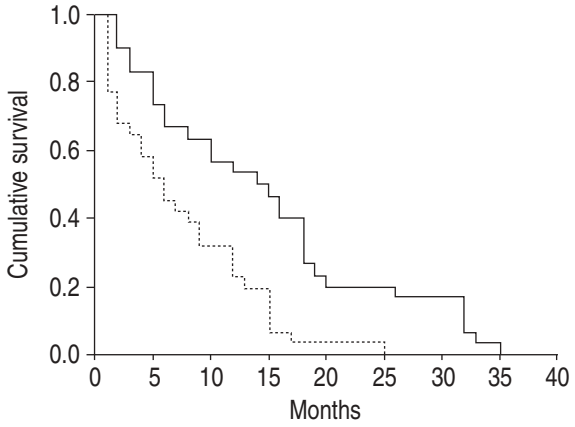

Fig. 1. – Survival analysis for BAX expression. Kaplan-Meier-Analysis of overall survival in the BAX positive group (solid line; n=30) and in the BAX negative group (dotted line; n=31; Logrank-Mantel-Cox p=0.0004).

of cases without p53 mutation a positive stain for p53 was detected.

BAX was expressed in 37±7% of tumours with p53 mutations *versus* 26%±6 in tumours with p53 wild type (Fisher′s Exact test p>0.99), suggesting that p53 is not the only determinant of BAX protein expression in these samples.

None of the 61 tumours showed a frameshift mutation in the BAX gene.

Correlation of p53, BAX, p16^{INK4A} with survival

To determine the prognostic impact of p53 protein expression and mutation status (exons 5–8), as well as expression of BAX and p16^{INK4A}, univariate survival was analysed.

There was no prognostic impact of p53 protein expression ($p53_{pos}$: median survival 7 months; $p53_{neg}$: median survival 9 months; p=0.79) or p53 mutational status (p53wt: median survival 10 months; p53mut: median survival 6 months; p=0.64).

Patients with BAX expression in tumours showed increased survival times (median survival 14 months) when compared with patients without expression (median survival 6 months; p=0.0004; fig. 1). Furthermore, dilutional effects due to heterogeneous groups of patients were excluded by comparing survival times and BAX expression for subgroups, such as stages I–III, stage IV, and therapeutic intervention (table 2).

Based on the data of a previous study on metastatic colorectal cancer [12], where inclusion of the p53 mutational status in the survival analysis corroborated the power of BAX as a prognostic marker, a further survival analysis was carried out for the p53wt/p53mut and $p53_{pos}$/$p53_{neg}$ subgroups and BAX expression, also listed in table 2. The best outcomes were seen in the p53wt/BAX_{pos} group (median survival 18 months), and in the $p53_{pos}$/BAX_{pos} group (median survival 16 months).

Survival time analysis for p16^{INK4A} protein expression including subgroups is also shown in table 2. Here patients with p16^{INK4A} expression in their tumours showed the best outcome compared with those without p16^{INK4A} expression (p=0.0001; fig. 2). Further, survival was analysed with respect to the combination of p16^{INK4A} status with p53wt/p53mut, $p53_{pos}$/$p53_{neg}$ and BAX_{pos}/BAX_{neg}, listed in table 2. The worst outcome was observed for the p16$^{INK4A}{}_{neg}$/BAX_{neg} group of patients (median survival 2 months),

Table 2. – Survival analysis for different subgroups

Groups	Median survival (months)		
	Expression	No expression	p-value (Logrank-Mantel-Cox-Test)
BAX	14 (30)	6 (31)	<0.0004
stages I–III group	18 (18)	12 (10)	<0.037
stage IV group	6 (12)	3 (21)	<0.017
therapy group	16 (25)	8 (20)	<0.005
best supportive care	5 (5)	2 (11)	NS
p53wt and	18 (13)	8 (16)	
p53mut	12 (17)	3 (15)	<0.0003
$p53_{pos}$ and	16 (16)	4 (14)	
$p53_{neg}$	12 (13)	9 (17)	<0.0005
p16^{INK4A}	15 (35)	4 (26)	<0.0001
stages I–III group	18 (18)	12 (10)	<0.037
stage IV group	9 (14)	2 (19)	<0.028
therapy group	15 (26)	6 (19)	<0.0004
best supportive care	5 (9)	2 (7)	<0.018
p53wt and	15 (19)	5 (10)	
p53mut	13 (16)	3 (16)	<0.0006
$p53_{pos}$ and	16 (17)	5 (14)	
$p53_{neg}$	12 (18)	3 (12)	<0.0001
BAX_{pos} and	16 (20)	6 (10)	
BAX_{neg}	12 (15)	2 (16)	<0.0002

Data are presented as months of median survival (number of patients). wt: wild type; mut: mutated; $_{pos}$: positive; $_{neg}$: negative; NS: nonsignificant.

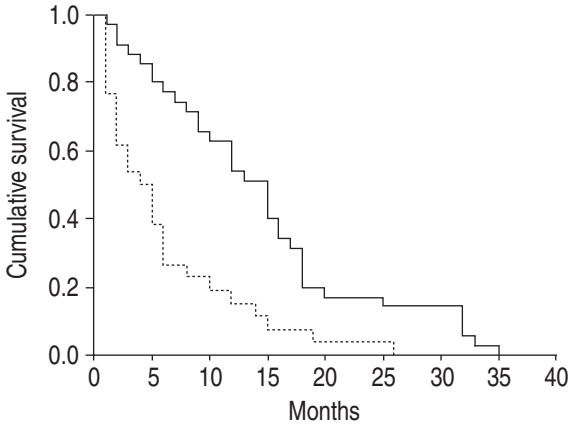

Fig. 2. – Survival analysis for $p16^{INK4A}$ expression. Kaplan-Meier-Analysis of overall survival in the $p16^{INK4A}$ negative group (dotted line; n=26) and in the $p16^{INK4A}$ positive group (solid line; n=35; Logrank-Mantel-Cox p=0.0001).

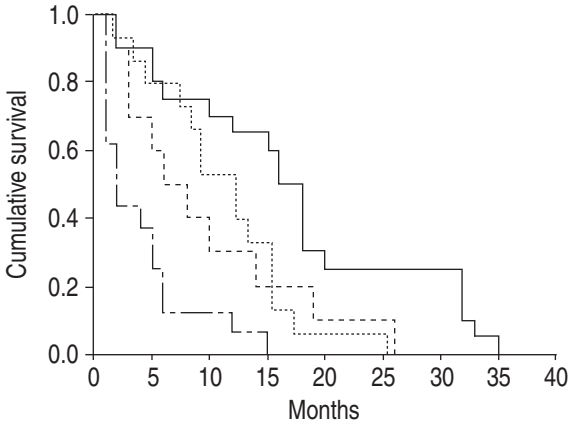

Fig. 3. – Combination of survival analysis for $p16^{INK4A}$ expression and BAX expression. Subgroup analysis for four groups: $p16^{INK4A}{}_{pos}/BAX_{pos}$ (n=20; —), $p16^{INK4A}{}_{pos}/BAX_{neg}$ (n=15; ····), $p16^{INK4A}{}_{neg}/BAX_{pos}$ (n=10; - - -), and $p16^{INK4A}{}_{neg}/BAX_{neg}$ (n=16; – - –) (Logrank-Mantel-Cox p=0.0002). pos: positive group; neg: negative group.

the best for the $p16^{INK4A}{}_{pos}/BAX_{pos}$ patient group (median survival 16 months, fig. 3).

Discussion

The analysis showed prognostic values for BAX and for $p16^{INK4A}$. p53 overexpression or p53 mutations alone showed no significant influence on survival. Although many studies have investigated the implication of p53 in NSCLC carcinogenesis it appears that the prognostic significance of p53 alterations in lung cancer is rather weak [10, 19]. In contrast to p53, patients with BAX expression had a significantly better prognosis in the analysis of overall survival (8 months longer survival). These data concur with the results of a previous study, in this study reduced expression of BAX was a negative prognostic factor in patients with colorectal adenocarcinoma [12]. A positive prognostic impact of high BAX expression has also been demonstrated for ovarian carcinoma [20], breast cancer [21, 22], and pancreatic cancer [23]. In contrast, BAX expression was not associated with better outcome in another study, which used, however, a protein expression level of 50% of tumour cells as a cut-off value for classifying BAX expression as "positive" or "negative" [24]. In the present study the cut-off value was set at 10% positively stained cells for "positive" as previously reported in STURM *et al.* [12].

One possible reason for the lack of BAX expression is a frameshift mutation in the BAX gene, which is frequently found in colorectal cancers [12, 18] and which leads to a truncated protein that is not expressed. In concordance with findings of other groups [25, 26] there were no frameshift mutations of the BAX gene in the cohort of the present study, which indicates that somatic frameshift mutation in the BAX gene is not an important mechanism of BAX inactivation in lung carcinogenesis.

When information about the p53 mutation status, p53 protein expression, and BAX protein expression are added, the dichotomy of the positive prognostic relevance of BAX becomes even more striking. This result is in line with findings in colorectal cancer [12]. Loss of BAX has the consequence that BAX cannot work as an effector of p53 in inducing apoptosis, leading to a growth advantage of tumour cells despite intact p53. Interestingly, BAX expression was detectable in some patients carrying mutated p53. However, patients with BAX expression and p53 overexpression showed better outcome than those without p53 overexpression. A possible reason for this unexpected finding could be a missing correlation of p53 mutation status and p53 overexpression.

The present study also included the investigation of an effect of $p16^{INK4A}$ with respect to the prognosis in NSCLC. Immunohistochemistry was used to analyse $p16^{INK4A}$ inactivation because genetic (homozygous and heterozygous deletions, and point mutations or deletions in exons one and two) and epigenetic (hypermethylation of the CpG island in the promoter region) alterations result in nonfunctional proteins or partial or complete loss of $p16^{INK4A}$ expression [27, 28]. In 40% of the tumours no $p16^{INK4A}$ in protein expression was found, this is similar to results of others [7, 29]. Two representative samples from primary tumours were investigated, which were comparable in each case. No probes from lymph-node metastases or distant metastases were analysed, but MARCHETTI *et al.* [29] have shown concordance of $p16^{INK4A}$ status in primary tumour and node metastasis for node positive NSCLC.

Tumour stage, tumour size, lymph node metastasis status, and status of distant metastasis are conventional prognostic markers. No association between a lack of $p16^{INK4A}$ protein expression and tumour stage was observed in this study in accordance with the results of TAGA *et al.* [30] and HUANG *et al.* [31]. However, other groups did find such a correlation [7, 29, 32]. Furthermore, in line with KAWABUCHI *et al.* [32] a correlation between tumour grading and loss of $p16^{INK4A}$ protein expression was observed, which is in contrast to the results of TAGA *et al.* [30], MARCHETTI

et al. [29], and HUANG *et al.* [31]. A further correlation was shown for tumour grading and loss of BAX protein expression. In some analyses p16^{INK4A} inactivation was found to be strongly correlated with lymph node metastasis in early stage NSCLC [29, 32] whereas this was not the case in other reports [30, 31]. In this study, a correlation was found between p16^{INK4A} protein expression and lymph-node metastasis status. Status of distant metastasis correlated with BAX as well as with p16^{INK4A} protein expression. While most authors investigated earlier tumour stages, in the present report mainly advanced tumours were studied which might have contributed to the conflicting results described earlier.

It was also shown that patients with NSCLC expressing p16^{INK4A} in tumour tissue had a significantly better prognosis than those with p16^{INK4A} negative tumours (fig. 2). The results of the present study support data from other groups describing loss of p16^{INK4A} protein expression as a negative prognostic marker in early stage of NSCLC [29–32]. In contrast, HOMMURA *et al.* [33] found no prognostic benefit with p16^{INK4A} protein expression in early stage NSCLC. However, when NSCLC with tumour stage IV were included in the present investigations, the positive prognostic impact of p16^{INK4A} protein expression for survival became more obvious.

To evaluate a possible dilution effect in the statistical analysis by heterogeneous patient groups, subgroups reflecting stage and therapy status were analysed. Here the independent prognostic impact of BAX and p16^{INK4A} could also be shown. However, BAX status in the patient group receiving best supportive care showed no prognostic influence. While these results do not exclude any influence of other parameters, the differences in survival time of the subgroups suggest a combined effect of different prognostic parameters, such as therapy status (surgery, radiotherapy, and chemotherapy) and protein expression. Moreover, combination of protein expression analysis of p53, BAX, and p16^{INK4A} expression in the present study seems to identify subgroups of patients with very good prognosis.

Taking into account the relatively small number of cases in this work, further investigations and a larger study are necessary to evaluate subgroups of patients for new or modified therapeutic strategies, such as more aggressive adjuvant therapy perhaps in combination with surgery, or combined therapy including gene therapy to replace the lost gene.

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References

1. Parker SL, Tong T, Bolden S, Wingo PA. Cancer statistics, 1997. *CA Cancer J Clin* 1997; 47: 5–27. [published erratum appears in *CA Cancer J Clin* 1997; 47: 68].
2. Denissenko MF, Pao A, Tang M, Pfeifer GP. Preferential formation of benzo[a]pyrene adducts at lung cancer mutational hotspots in P53. *Science* 1996; 274: 430–432.
3. Salgia R, Skarin AT. Molecular abnormalities in lung cancer. *J Clin Oncol* 1998; 16: 1207–1217.
4. Okamoto A, Demetrick DJ, Spillare EA, *et al.* Mutations and altered expression of p16^{INK4A} in human cancer. *Proc Natl Acad Sci USA* 1994; 91: 11045–11049.
5. Hu YX, Watanabe H, Ohtsubo K, *et al.* Frequent loss of p16 expression and its correlation with clinicopathological parameters in pancreatic carcinoma. *Clin Cancer Res* 1997; 3: 1473–1477.
6. Reed JA, Loganzo F Jr, Shea CR, *et al.* Loss of expression of the p16/cyclin-dependent kinase inhibitor 2 tumor suppressor gene in melanocytic lesions correlates with invasive stage of tumor progression. *Cancer Res* 1995; 55: 2713–2718.
7. Kratzke RA, Greatens TM, Rubins JB, *et al.* Rb and p16^{INK4A} expression in resected non-small cell lung tumors. *Cancer Res* 1996; 56: 3415–3420.
8. Sandig V, Brand K, Herwig S, Lukas J, Bartek J, Strauss M. Adenovirally transferred p16^{INK4A}/CDKN2 and p53 genes cooperate to induce apoptotic tumor cell death. *Nat Med* 1997; 3: 313–319.
9. Gorgoulis VG, Zacharatos P, Kotsinas A, *et al.* Alterations of the p16-pRb pathway and the chromosome locus 9p21-22 in non-small-cell lung carcinomas: relationship with p53 and MDM2 protein expression. *Am J Pathol* 1998; 153: 1749–1765.
10. Greatens TM, Niehans GA, Rubins JB, *et al.* Do molecular markers predict survival in non-small-cell lung cancer? *Am J Respir Crit Care Med* 1998; 157: 1093–1097.
11. Lowe SW, Bodis S, McClatchey A, *et al.* p53 status and the efficacy of cancer therapy *in vivo*. *Science* 1994; 266: 807–810.
12. Sturm I, Kohne CH, Wolff G, *et al.* Analysis of the p53/BAX pathway in colorectal cancer: low BAX is a negative prognostic factor in patients with resected liver metastases. *J Clin Oncol* 1999; 17: 1364–1374.
13. Krajewski S, Blomqvist C, Franssila K, *et al.* Reduced expression of proapoptotic gene BAX is associated with poor response rates to combination chemotherapy and shorter survival in women with metastatic breast adenocarcinoma. *Cancer Res* 1995; 55: 4471–4478.
14. Wagener C, Bargou RC, Daniel PT, *et al.* Induction of the death-promoting gene bax-alpha sensitizes cultured breast-cancer cells to drug-induced apoptosis. *Int J Cancer* 1996; 67: 138–141.
15. Yin C, Knudson CM, Korsmeyer SJ, Van Dyke T. Bax suppresses tumorigenesis and stimulates apoptosis *in vivo*. *Nature* 1997; 385: 637–640.
16. The World Health Organization histological typing of lung tumours. Second edition. *Am J Clin Pathol* 1982; 77: 123–136.
17. Mountain CF. Revisions in the International System for Staging Lung Cancer [see comments]. *Chest* 1997; 111: 1710–1717.
18. Rampino N, Yamamoto H, Ionov Y, *et al.* Somatic frameshift mutations in the BAX gene in colon cancers of the microsatellite mutator phenotype. *Science* 1997; 275: 967–969.
19. Kalemkerian GP. Biology of lung cancer. *Curr Opin Oncol* 1994; 6: 147–155.

20. Tai YT, Lee S, Niloff E, Weisman C, Strobel T, Cannistra SA. BAX protein expression and clinical outcome in epithelial ovarian cancer. *J Clin Oncol* 1998; 16: 2583–2590. [published erratum appears in *J Clin Oncol* 1998; 16: 3211].
21. Bargou RC, Daniel PT, Mapara MY, *et al.* Expression of the bcl-2 gene family in normal and malignant breast tissue: low bax-alpha expression in tumor cells correlates with resistance towards apoptosis. *Int J Cancer* 1995; 60: 854–859.
22. Bargou RC, Wagener C, Bommert K, *et al.* Overexpression of the death-promoting gene bax-alpha which is downregulated in breast cancer restores sensitivity to different apoptotic stimuli and reduces tumor growth in SCID mice. *J Clin Invest* 1996; 97: 2651–2659.
23. 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.
24. Apolinario RM, van der Valk P, de Jong JS, *et al.* Prognostic value of the expression of p53, bcl-2, and bax oncoproteins, and neovascularization in patients with radically resected non-small-cell lung cancer. *J Clin Oncol* 1997; 15: 2456–2466.
25. Gotoh K, Yatabe Y, Sugiura T, *et al.* Frameshift mutations in TGFbetaRII, IGFIIR, BAX, hMSH3 and hMSH6 are absent in lung cancers. *Carcinogenesis* 1999; 20: 499–502.
26. Caligo MA, Ghimenti C, Marchetti A, *et al.* Microsatellite alterations and p53, TGFbetaRII, IGFIIR and BAX mutations in sporadic non-small-cell lung cancer. *Int J Cancer* 1998; 78: 606–609.
27. Shapiro GI, Park JE, Edwards CD, *et al.* Multiple mechanisms of $p16^{INK4A}$ inactivation in non-small cell lung cancer cell lines. *Cancer Res* 1995; 55: 6200–6209.
28. Gazzeri S, Gouyer V, Vour′ch C, Brambilla C, Brambilla E. Mechanisms of $p16^{INK4A}$ inactivation in non small-cell lung cancers. *Oncogene* 1998; 16: 497–504.
29. Marchetti A, Buttitta F, Pellegrini S, *et al.* Alterations of P16 (MTS1) in node-positive non-small cell lung carcinomas. *J Pathol* 1997; 181: 178–182.
30. Taga S, Osaki T, Ohgami A, *et al.* Prognostic value of the immunohistochemical detection of $p16^{INK4A}$ expression in nonsmall cell lung carcinoma. *Cancer* 1997; 80: 389–395.
31. Huang CI, Taki T, Higashiyama M, Kohno N, Miyake M. p16 protein expression is associated with a poor prognosis in squamous cell carcinoma of the lung. *Br J Cancer* 2000; 82: 374–380.
32. Kawabuchi B, Moriyama S, Hironaka M, *et al.* p16 inactivation in small-sized lung adenocarcinoma: its association with poor prognosis. *Int J Cancer* 1999; 84: 49–53.
33. Hommura F, Dosaka-Akita H, Kinoshita I, *et al.* Predictive value of expression of $p16^{INK4A}$, retinoblastoma and p53 proteins for the prognosis of non-small-cell lung cancers. *Br J Cancer* 1999; 81: 696–701.