Expression of X-linked Inhibitor of Apoptosis as a Novel Prognostic Marker in Radically Resected Non-Small Cell Lung Cancer Patients[1]

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ABSTRACT

Purpose: **To assess the pattern of expression and the prognostic value of the inhibitor of apoptosis family member X-linked inhibitor of apoptosis (XIAP; MIHA/ILP-a) in radically resected non-small cell lung cancer patients.**

Experimental Design: **The expression of XIAP and its relationship with overall survival was analyzed by immunohistochemistry on tumors from 144 patients with early-stage non-small cell lung cancer. In addition, the apoptotic and mitotic index, Ki-67, p53, and bcl-2 levels were also assessed.**

Results: **XIAP expression was specific for tumor cells, and the pattern was cytoplasmic. The median expression of XIAP was 20%, and when this value was used as a cutoff point for statistical analyses, 63 of the samples were considered high XIAP-expressing and 81 low XIAP-expressing. Surprisingly, high XIAP-expressing patients had a longer overall survival than the group expressing lower levels (60 *versus* 24 months of median survival; log rank, $P = 0.01$). The positive impact of XIAP expression on survival was confirmed by multivariate analysis ($P = 0.026$). Although no correlation was observed between XIAP expression and the apoptotic index, a significant inverse correlation was observed between XIAP, Ki-67 ($P = 0.006$), and mitotic index ($P = 0.04$).**

Conclusions**: The unexpected inverse correlation of XIAP with proliferation markers and the absence of correlation with apoptotic index, coupled with its role as an independent positive prognostic factor for survival in radically resected NSCLC patients imply a more complex role for XIAP in tumor biology than anticipated by *in vitro* data.**

INTRODUCTION

For patients with NSCLC,[3] resectable early-stage disease is the most powerful prognostic factor. However, even among radically resected NSCLC patients, some have biologically unfavorable cancers, in which resection alone is not likely to lead to a cure (1). The identification of novel biological prognostic markers may help to better assess survival probability in different subgroups of patients and to tailor treatment according to the molecular profile of the tumor.

Tumorigenesis involves a loss of balance between regulators of cell proliferation and apoptosis (2). Among the regulators of apoptosis, an evolutionary conserved gene family of IAP has been identified and implicated in caspase inhibition (3, 4). In humans, four IAPs (XIAP, c-IAP1, c-IAP2, and survivin) (5–7) have been shown to restrain cell death in cancer cells through a mechanism initially thought to involve only inhibition of the effectors caspase-3 and -7 (4). More detailed studies performed on the IAP member XIAP have shown that this molecule is able to inhibit not only the effectors caspase-3 and -7, but also the initiator caspase-9 (8). *In vitro*, XIAP has been shown to be a more potent caspase inhibitor than other IAPs such as survivin (9, 10). Nevertheless, unlike survivin, which has been studied extensively in distinct tumor types (11–14), XIAP expression has not been adequately assessed in patients with solid tumors. We recently observed *in vitro* a constitutional inhibition of caspase-9 in NSCLC cells and postulated that such inhibition might have an impact on the behavior of this type of tumor, especially regarding its relative chemoresistance (15). These observations coupled with the scarcity of data on XIAP in material derived from patients with solid tumors provided the rationale to examine the expression and the prognostic value of XIAP in radically resected NSCLC patients. We analyzed the relationship of the expression of XIAP with overall survival, apoptosis inhibition, tumor cell proliferation, p53, and bcl-2. Here we report that the expression of XIAP in radically resected NSCLC patients did not correlate with the AI, but did inversely correlate with tumor proliferation markers. Unexpectedly, higher XIAP expression translated into a significantly longer overall survival.

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[3] The abbreviations used are: NSCLC, non-small cell lung cancer; IAP, inhibitor of apoptosis; XIAP, X-linked inhibitor of apoptosis; AI, apoptotic index; mAb, monoclonal antibody; MI, mitotic index; PI, fraction of proliferative cells; CI, confidence interval.

PATIENTS AND METHODS

Patients. Patients ($n = 144$) with early-stage (I to IIIA) NSCLC were enrolled in the present study. Patients underwent radical surgery (lobectomy or pneumectomy) of primary tumor and lymph nodes between January 1988 and December 1995 at the Academic Hospital Vrije Universiteit of Amsterdam and received no other treatment before or after surgery. The histopathological features of the surgical specimens were classified according to WHO criteria. The TNM staging system was updated according to the new staging system (16). Data regarding age, stage, sex, smoking, histology, differential grade, p53, and bcl-2 of 116 of the 144 patients were collected previously and reported (17). The follow-up of these patients was updated, and 28 new patients were included in the present series.

Antibodies. The mAb anti-XIAP (MIHA/ILP-a) clone 2F1 (MBL, Nagoya, Japan) was used in this study. This mAb recognizes the COOH-terminal region of the XIAP molecule (amino acids 352–449), and was used diluted 1:75 in PBS-1% BSA. The mouse mAb against Ki-67 (MIB-1; Immunotech, Marseilles, France) was used at a dilution of 1:40. The monoclonals against p53 (PAb 1801; Oncogene Science, Uniondale, NY) and against bcl-2 (clone 100, provided by Dr. Francesco Pezzella) were used diluted 1:200 and 1:25, respectively. The use of the mAb anti-XIAP for immunohistochemistry was validated in acetone-fixed NSCLC cell lines (NCI-H460, A549, and SW1573) and in frozen sections derived from some of the patients included in the series.

Immunohistochemistry. Formalin-fixed paraffin-embedded tissue was cut in 4-μm sections and mounted on poly-L-lysine-coated slides. After deparaffinization and rehydration, sections were incubated in a solution with 0.3% H_2O_2 in absolute methanol for 30 min to block endogenous peroxidase. Antigen retrieval for XIAP and Ki-67 was performed by heating the slides for 1 h in a pressure cooker in 10 mM citrate buffer (pH 6.0), cooling at room temperature for at least 30 min, and washing with PBS. Nonspecific staining was blocked using normal rabbit serum at a 1:50 dilution for 10 min (DAKO, Santa Barbara, CA). Subsequently, the slides were incubated overnight at 4°C with the specific primary antibodies against XIAP and Ki-67. Sections were then rinsed in PBS and incubated for 30 min with biotin-labeled $F(ab')_2$ fragments of the secondary antibody diluted at 1:500 (rabbit antimouse). Avidin-biotin complex (Strept ABComplex; Dako) was applied for 1 h as a reagent diluted at 1:200. Finally, sections were rinsed in PBS, developed with diaminobenzidine tetrahydrochloride substrate (DAB; Chromogen, Carpinteria, CA) for 3 min, slightly counterstained with hematoxylin, dehydrated, and mounted with Depex (BDH Laboratory Supplies, Poole, United Kingdom). Immunohistochemistry for p53 and bcl-2 was performed as described previously (17).

The XIAP-expressing Jurkat-T leukemia cells and tonsil tissue were used as positive controls for XIAP and Ki-67 expression, respectively. Incubation with an isotype-matched antibody was used as a negative control for XIAP, whereas the omission of the primary antibody in simultaneously incubated sections was used as a negative control for all of the other antibodies (Ki-67, p53, and bcl-2).

The percentage of immunoreactive tumor cells was assessed by two observers (P. v. d. V. and C. G. F.) on all sections. Positive cells were analyzed semiquantitatively. The observers assessing all staining results were blinded to the clinical outcome of patients.

Counting of Apoptotic and Mitotic Cells. Apoptotic cells were counted on H&E-stained tissue sections basically as described previously (18). Briefly, with a standard light microscope at ×400 magnification (×40 objective; field diameter, 450 μm), the total number of apoptotic cells was counted in 4–10 fields of vision (~1.59 mm^2), systematically spread over the tumor, making sure that in each section, ~2500 tumor cells were counted. The number of fields counted was dependent on the growth pattern and size of the cells. In the case of a solid growth pattern and relatively small tumor cells, 4 fields were counted, whereas in the sections presenting extensive lumen formation and small and widely separated tumor fields, the counting of up to 10 fields was required to reach ~2500 cells. Mitosis was analyzed basically as described previously (18, 19), and mitotic figures were counted in the same fields described above. Finally, the numbers of apoptotic and mitotic figures were divided by the total number of tumor cells analyzed and taken as the AI or MI, respectively. The PI was defined as the percentage of Ki-67-positive cells and was estimated semiquantitatively.

Statistical Analysis. Statistical analysis was performed using the SPSS software program, version 9.0 (SPSS Inc., Chicago, IL). The median values for expression were used as cutoff points for statistical analyses. For the analysis of the association between XIAP expression and either the major patient and tumor characteristics (age at surgery, sex, pT, pN, stage, histology, tumor grade) or biological markers (Ki-67, AI, MI, p53, and bcl-2), XIAP was dichotomized at its median value (20%). Associations were tested by means of χ^2 tests. Overall survival (defined as the time between the date of surgery and date of death) was estimated by the Kaplan-Meier method, and survival curves were compared by the log-rank test. To adjust for potential confounders, Cox proportional hazards survival analysis was applied.

RESULTS

Expression of XIAP and Clinical Features. The expression of XIAP was cytoplasmic and rather diffuse (Fig. 1*A*), sometimes with a more distinct granular staining pattern, particularly in adenocarcinoma sections (Fig. 1*B*). The pattern of granular staining for XIAP observed on some paraffin sections was confirmed when immunohistochemistry was performed on frozen sections derived from matched cases, excluding the possibility of staining artifacts and altered subcellular localization of the signal attributable to antigen retrieval (data not shown). XIAP staining was specific for tumor tissue compared with the neighboring normal lung epithelial cells (Fig. 1*C*), including bronchial and alveolar compartments. Alveolar macrophages were the only normal cells that consistently stained positive for XIAP in some sections (Fig. 1*C*). The intensity of XIAP staining was usually homogeneous within a case tested, but the number of tumor cells stained positive by the anti-XIAP antibody ranged from 0 to 100%, depending on the case examined (mean, 30%;

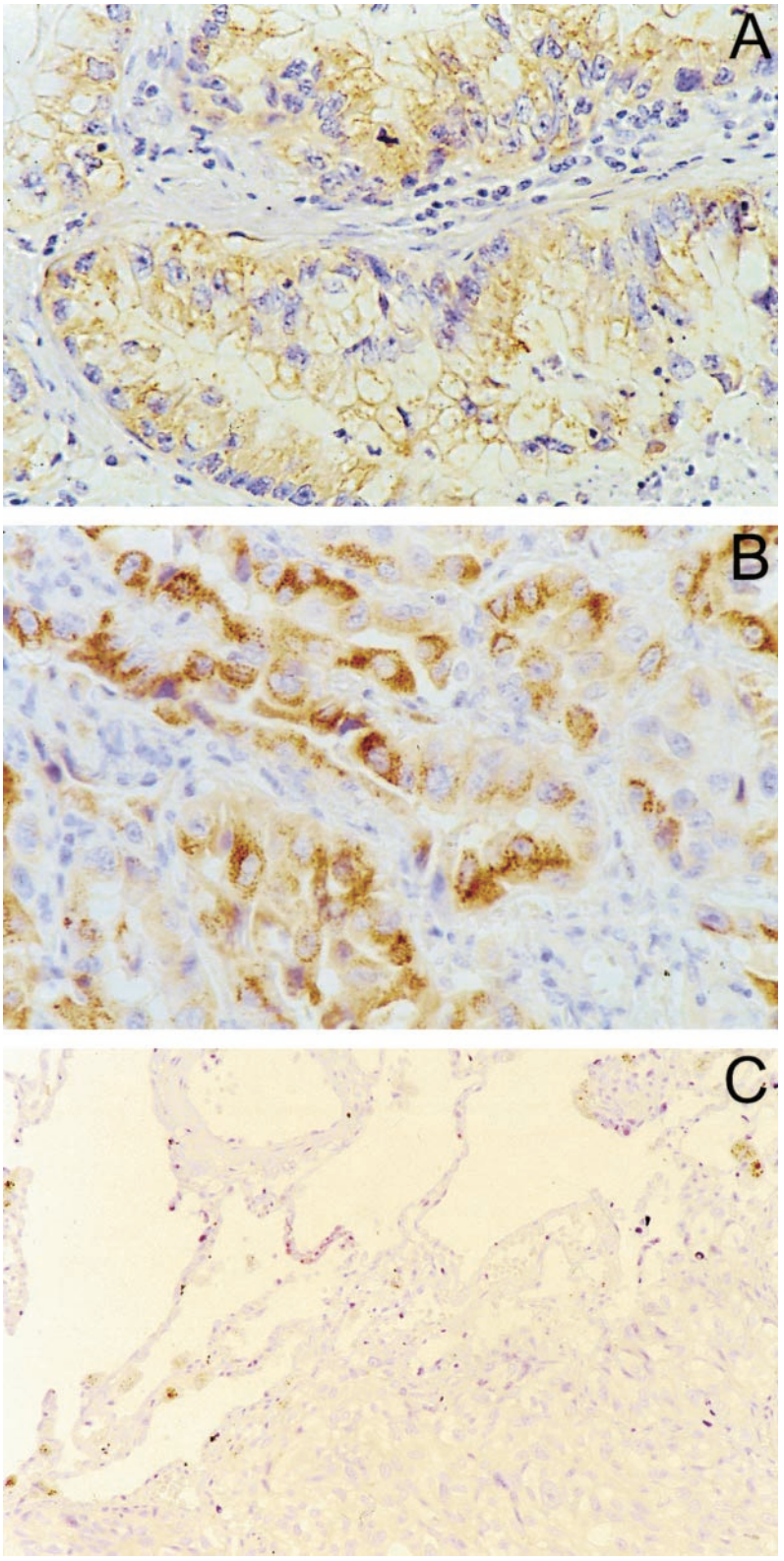

Fig. 1 Staining of tumor samples with mAb against XIAP. *A*, homogeneous cytoplasmic staining. *B*, distinct granular staining pattern observed particularly in adenocarcinoma sections (magnification, ×40). *C*, section representing cases with low expression of XIAP in tumor cells. The absence of XIAP staining in normal lung epithelial cells and the occasional positive staining of alveolar macrophages are also demonstrated (magnification, ×20).

Table 1 XIAP expression and clinicopathological characteristics

	Low XIAP[a]	High XIAP	All	χ^2
Age				
≤65 years	31	41	72	
>65 years	50	22	72	0.001
Sex				
Male	69	51	120	
Female	12	12	24	0.499
Histology[b]				
Squamous	45	29	74	
Adenocarcinoma	18	22	40	
Large cell	18	12	30	0.240
Differentiation grade				
Well differentiated	2	2	4	
Moderately differentiated	20	21	41	
Poorly differentiated	59	40	99	0.486
Tumor status				
T_1	16	22	38	
T_2	59	36	95	
T_3	6	5	1	0.109
Nodal status				
N_0	58	50	108	
N_1	17	11	25	
N_2	6	2	8	0.437
Stage[c]				
IA	10	20	30	
IB	43	25	68	
II	20	16	36	
IIIA	8	2	10	0.018

[a] Cutoff point at the median level, 20%.
[b] Large cell tumors are included as poorly differentiated tumors.
[c] TNM staging according to Mountain (16).

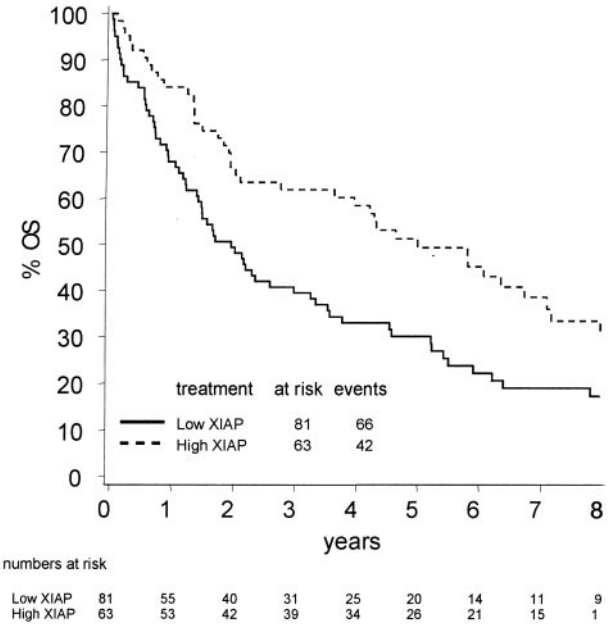

Fig. 2 Kaplan-Meier curve of the overall survival (*OS*) according to XIAP expression (log rank, $P = 0.01$).

SD, 30%). The overall median expression of XIAP was 20%, and when we used this value as the cutoff point for statistical analyses, 63 patients were considered high XIAP-expressing and 81 low XIAP-expressing. A description of the clinicopathological features of the cases according to XIAP expression is provided in Table 1.

Survival Analysis. The median follow-up was 104 months. Survival, analyzed according to the expression of the XIAP protein, is shown in Fig. 2. The patients with high XIAP-expressing tumors had a significantly longer survival than the low XIAP-expressing patients (log rank, $P = 0.01$). The median overall survival was 60 months (95% CI, 33–85 months) in the high XIAP-expressing cases and 24 months (95% CI, 17–39 months) in patients with lower XIAP expression. The 5-year survival rates for the high and low XIAP-expressing groups were 50 and 30%, respectively. An advantage in survival was also observed when the group showing the highest expression (XIAP ≥ 90%) was compared with the group presenting the lowest expression (XIAP ≤ 10%; 52 *versus* 26 months of median survival; $P = 0.03$).

Relationship between XIAP Expression, Apoptosis, and Proliferation. Cells with apoptotic features on H&E slides were detected in 139 cases with material assessable for analysis. The mean AI was 0.65% (SD, 0.41%; 95% CI, 0.58–0.72%). The mean AI in high XIAP-expressing sections (0.60%; SD, 0.42%; 95% CI, 0.49–0.71%) was not significantly different from low XIAP-expressing tumors (0.69%; SD, 0.39%; 95% CI, 0.6–0.78%; $P = 0.2$). Moreover, when the patients were dichotomized according to AI, no significant difference in overall survival was observed (data not shown).

IAPs, including XIAP, have been suggested to be involved in cellular processes other than apoptosis inhibition, such as proliferation (4, 20–22). We explored the fraction of proliferative tumor cells on the sections and related to XIAP expression. Initially, the percentage of Ki-67-positive cells was analyzed as the PI. The mean PI calculated for all 144 patients was 49.3% (SD, 25.1%; 95% CI, 44.8–53.6%). When analyzed according to XIAP expression, the mean PI was significantly reduced in patients expressing higher (41%; SD, 27%; 95% CI, 34–48%) than lower XIAP levels (53%; SD, 22%; 95% CI, 48–58%; $P = 0.006$). Tumor proliferation was also assessed by the MI, and the mean MI calculated for the whole series was 0.76% (SD, 0.46%; 95% CI, 0.68–0.84%). When we used the dichotomized variable, the mean MI in high XIAP-expressing sections was also lower than in low XIAP-expressing patients (0.67 *versus* 0.83; $P = 0.04$), corroborating the findings described for PI. Taken together, these results strongly suggest a relationship of XIAP with tumor proliferation in radically resected NSCLC patients.

Because of the relationship between XIAP and proliferation markers, the prognostic significance of PI and MI was also explored, but no significant effect on overall survival was observed (data not shown). The relationship between the dichotomized values of XIAP and all other variables analyzed is summarized in Table 2.

Correlation between XIAP, p53, and bcl-2 Expression. We previously reported the characteristics and prognostic influence of p53 and bcl-2 for the initial 116 patients of this series (17). Here, we updated the follow-up and included additional patients. The protocol and criteria used to score the slides were the same, and the sections were analyzed by the same pathologist (P. v. d. V.).

Table 2 Relationship between the dichotomized expression of XIAP and AI, PI, MI, p53, and bcl-2

	XIAP, %		
	Low	High	*P*
AI	0.69 (0.6–0.78)	0.60 (0.49–0.71)	0.2
PI	53 (48–58)	41 (34–48)	0.006
MI	0.83 (0.72–0.92)	0.67 (0.52–0.76)	0.04
p53	35 (27–42)	23 (18–32)	0.04
bcl-2	27 (19–36)	34 (26–42)	0.09

The addition of new cases led to no major change in the previous results reported for bcl-2 (17). In total, 143 cases were assessable, and the range of expression was 0–100%, with a median expression <0.5%. No significant difference in the mean expression of bcl-2 between high and low XIAP-expressing patients was observed (34 *versus* 27%; $P = 0.09$). The results of the p53 staining also did not significantly change when the new cases were added and the initial 116 patients were updated (17). In total, p53 was assessable in 142 patients, and nuclear accumulation of p53 was observed in 94 cases (66%), whereas in 19 cases, cytoplasmic staining in scattered cells was observed. All analyses were based on nuclear staining only. The range of expression was 0–100%, and the median expression was 5%. When analyzed according to XIAP, the mean expression of p53 was significantly higher in low XIAP-expressing cases (35 *versus* 23%; $P = 0.04$). Because the inhibition of apoptosis induced by XIAP has been reported to depend on p53 status (23), we analyzed the correlation between XIAP and AI in relation to high and low p53 expression. In line with our previous observations, no correlation was observed between XIAP and AI in this context (data not shown). The same type of analysis was done for bcl-2, and once more no significant correlation was observed. Furthermore, no significant difference in survival was observed when the survival analysis was performed according to the dichotomized values of p53 and bcl-2 (data not shown).

Multivariate Analysis. The results presented showed that the expression of XIAP protein was inversely related to stage (Table 1), a known prognostic factors for NSCLC patients. Multivariate analysis including age at surgery, sex, histology, and differentiation concluded that XIAP is an independent predictor of the outcome of radically resected NSCLC patients (Table 3). Stage replaced by pT and pN did not essentially modify the model.

Table 3 Multivariate analysis

Factor	Hazards ratio	*P*	95% CI
Age at surgery	1.017	0.14	0.99–1.04
Poorly differentiated	0.86	0.53	0.53–1.38
Histology			
Adenocarcinoma	1.39	0.15	0.88–2.19
Large cell	1.41	0.19	0.83–2.40
Stage			
IB	0.85	0.55	0.50–1.44
II	1.36	0.29	0.76–2.42
IIIA	3.08	0.007	1.36–6.95
XIAP[a]	0.63	0.026	0.42–0.95

[a] XIAP >20%.

DISCUSSION

In this study, the pattern of expression and biological relevance of XIAP expression was analyzed for the first time in NSCLC patients. Relevant features of XIAP expression in this series include its selective expression in neoplastic cells in relation to normal lung epithelium and a characteristic granular staining pattern displayed in adenocarcinomas sections, which might suggest a typical subcellular distribution of XIAP in this NSCLC subtype. Furthermore, the prevalence of XIAP expression in patients with a lower stage of cancer suggests an association of XIAP expression with a less aggressive phenotype of NSCLC.

In fact, this possible association of XIAP with predictors of favorable prognosis is in line with the main observation of this study: the patients who expressed higher levels of XIAP achieved a significantly longer overall survival. This finding was unexpected based on *in vitro* data that indicate an antiapoptotic role for XIAP. Moreover, our data are at odds with a recent report showing that the expression of XIAP in a series of 79 acute myelogenous leukemia patients was correlated with a shorter survival (24). The discrepancy between this study and our series may suggest a different role for XIAP, depending on the type of cancer. In addition, our results are at striking contrast to studies involving the IAP molecule survivin, for which a relationship between expression, low levels of apoptosis, and a worse prognosis has been described for different tumor types, including NSCLC (11–14). In that study in NSCLC, an association between the expression of survivin, analyzed by semiquantitative PCR, and a poorer survival was reported (14). The divergence between the outcome of the expression of XIAP or survivin expression in NSCLC suggests that distinct IAP family members might have different functions and impact on the prognosis within the same tumor type.

Our data are supported by fact that the analysis of XIAP protein levels was based on the standard approach of selecting the median value of expression as a cutoff point. This methodology precludes strong assumptions about the relationship between marker and risk, while avoiding the bias of searching for "optimal" cutoff points (25). However, some loss of information may occur with the use of this type of cutoff point, thus increasing the probability of failing to detect a real association (25). Furthermore, the positive impact of XIAP expression on the overall survival of NSCLC patients in the present study was confirmed by multivariate analysis. In addition, the fact that our observations are based on protein levels gives weight to these results because evidence for translational control of XIAP (26) and dissociation between mRNA and protein levels has been reported for XIAP (24).

To gain further insight into the role of XIAP in NSCLC patients and its relationship with a better prognosis, we analyzed the AI and correlated it with XIAP expression. Here, the H&E method was used to detect apoptotic cells instead of the terminal deoxynucleotidyl transferase-mediated nick end labeling technique because of its higher reproducibility and reliability, and the possibility to simultaneously analyze mitotic figures on the

same section (18). Furthermore, the methodology for scoring apoptotic cells by the terminal deoxynucleotidyl transferase-mediated nick end labeling technique still requires adequate validation (18). As one of the most interesting aspects of this study, high XIAP expression was not associated with a low AI, contradicting *in vitro* data that link this molecule to apoptosis inhibition (6, 8). Conflicting data have been accumulated on the correlation between apoptosis and prognosis in several tumor types, including NSCLC (27–29). As suggested by Tanaka *et al.* (28), a possible explanation of these findings is that most of the studies focused only on apoptosis and neglected the fact that tumor growth is a net effect of cell death and proliferation, processes that occur simultaneously and are likely related at the molecular level. Several molecules have in fact been shown to act at the interface between cell death and proliferation, including IAPs (20–22). We analyzed tumor proliferation by PI and MI and compared it with the expression of XIAP. Surprisingly, higher XIAP levels correlated with lower tumor proliferation as assessed by both PI and MI. In reality, XIAP has been implicated in a regulatory system that balances cell response to environmental stimuli through a positive regulation of the nuclear factor κB pathway (21). However, to the best of our knowledge, the present report is the first to suggest a possible negative effect of the expression of XIAP, or any other IAP, on cell proliferation. One should be cautious to interpret these results, however, because the pathways leading to the loss of balance between cell proliferation/death in tumorigenesis are complex and may sometimes be divergent. Here, proliferation on its own did not fully explain the longer overall survival observed in the high XIAP-expressing cases in our study. In fact, conflicting data have been generated on the relationship between proliferation markers and prognosis in NSCLC (30).

Although weak ($P = 0.04$), the relationship between lower p53 expression and higher levels of XIAP was another intriguing finding of the present study. This is not unprecedented because a possible involvement of p53 in the transcriptional regulation of XIAP expression has been suggested (23). Our results; however, contrast with the study on survivin in gastric carcinomas, in which a direct association between this IAP molecule and accumulated p53 was found (13). These discrepancies highlight the need for additional studies to unravel the molecular implications, if any, of the interaction between p53 and IAPs in cancer cells. An additional difference from our data in relation to the studies focusing on survivin is the strong association between XIAP and bcl-2 reported in different studies (12, 13) but not observed in our series on XIAP.

In conclusion, two major observation in this study of NSCLC may have biological and clinical implications. The first observation was that higher levels of XIAP expression in radically resected NSCLC patients do not correlate with either lower AI or the antiapoptotic protein bcl-2, suggesting a dissociation between XIAP and the apoptotic process in this context. This is consistent with our *in vitro* data showing no significant impact of XIAP overexpression on apoptosis of NSCLC cells (15). In contrast, the higher expression of XIAP in the present study was associated with a lower PI and MI, implying a relationship between XIAP and the negative control of tumor proliferation. Taken together, these results suggest that XIAP probably has a more complex role in tumor biology than initially suggested. This is in line with recent reports suggesting that IAPs, including XIAP, would play not only a simultaneous role in apoptosis and proliferation, but also interact with regulatory molecules such as the recently described Smac/DIABLO (31, 32) and XIAP-associated factor 1, an antagonist of the antiapoptotic abilities of XIAP (33). Hence, additional studies are warranted to further delineate the interactions and biological functions of XIAP. The second observation was that XIAP expression is a novel independent prognostic factor for better outcome of a representative population of radically resected NSCLC patients. This finding may contribute to a new framework to classify these patients according to the biological aggressiveness of their tumors. To better establish the clinical relevance of XIAP, it would be interesting to see whether our findings can be observed in other solid tumor types.

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