Nuclear localization of survivin is a positive prognostic factor for survival in advanced non-small-cell lung cancer

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Background: Expression of survivin, a member of the inhibitor of apoptosis protein family, is commonly detected in cancers but not in normal differentiated tissues. Survivin is usually localized in the cytoplasm of cancer cells, but nuclear localization has also been described, and we recently reported that survivin is a nuclear–cytoplasmic shuttling protein.

Patients and methods: Fifty-three tumor specimens from patients with inoperable non-small-cell lung cancer (NSCLC) (55% stage IIIA, 17% stage IIIB and 28% stage IV) who underwent chemotherapy treatment were evaluated with immunohistochemistry for survivin expression and localization. These two sets of data were processed and tested for correlation with major patient characteristics, response to chemotherapy, and overall and relapse-free survival.

Results: Survivin was present only in malignant tissues, and 47/53 (89%) of the specimens were positive. The overall median expression of tumor cells was 40%, and this value was used as a cut-off point for statistical analysis. By dichotomizing the specimens as expressing low or high levels of survivin, a significant association was seen between the expression of survivin and the histology of the tumors (P=0.020), squamous cell carcinoma being the histotype with lower levels of survivin expression. Three patterns of localization were observed: 42% of cases (22/53) showed reactivity confined to the nucleus, 17% (nine of 53) only in the cytoplasm and 30% (16/53) in both the nucleus and the cytoplasm. Interestingly, nuclear survivin levels predicted longer overall and relapse-free survival, in univariate and multivariate analyses. Expression and localization of survivin did not correlate with response to chemotherapy.

Conclusions: Our results indicate that differential localization of survivin may be a prognostic factor for NSCLC. Further studies are warranted.

Key words: inhibitors of apoptosis, non-small-cell lung cancer, nuclear localization, survivin

Introduction

Non-small-cell lung cancer (NSCLC) is the leading cause of cancer death worldwide. Only ~30% of patients diagnosed with NSCLC are able to undergo radical resection. Patients with advanced stages are treated by chemotherapy alone (stage IV) or in combination with a local treatment modality (stage III) [1]. As the benefit of combination chemotherapy in metastatic disease is limited [2], it is important to identify biological markers able to predict response to chemotherapy and survival of patients undergoing treatment.

Chemo- and radiotherapy kill cancer cells mainly by triggering apoptosis or programmed cell death [3]. In human cancer cells and tissues, the expression of four members of the anti-apoptotic inhibitor of apoptosis (IAP) protein family (XIAP, cIAP1, cIAP2 and survivin) has been investigated as a potential factor for chemoresistance, based on their ability to inhibit the key molecules of the apoptotic machinery, the caspases [4]. IAPs are characterized by the presence of one to three copies of a ~70 amino acid baculoviral inhibitory repeat (BIR) motif, which allows interaction with caspases; IAPs interfere with the process of apoptosis, and thus promote tumor progression [5].

The smallest IAP family member, survivin, is a 16.5 kDa protein with a single BIR domain, and a dual activity as both an inhibitor of apoptosis and regulator of cell cycle [6]. Two functionally divergent splice variants of survivin have been identified, survivin-2B and survivin-ΔEx3 [7].

We have recently demonstrated that survivin is a nuclear–cytoplasmic shuttling protein that is actively exported out of the nucleus by the export receptor CRM1 [8]. Localization of its splice variants seems to be differentially regulated: while survivin-2B is a shuttling protein mainly localized in...
the cytoplasm, survivin-ΔEx3 contains a bipartite nuclear localization signal (NLS) that mediates its strong nuclear localization [8].

Survivin expression is not usually detected in normal adult tissues, but is commonly found during fetal development, and in pre-cancerous and cancerous lesions [9]. This finding renders survivin a potential candidate as a tumor marker.

Many reports have indicated a correlation between survivin expression and either unfavorable course of the disease [10–12], lack of response to chemotherapy [13, 14] or radiotherapy [15]. More recently, expression of survivin in the nucleus of tumor cells has been reported to predict a favorable outcome in osteosarcoma [16], bladder [17], gastric [18], and breast [19] carcinomas. These findings suggest that differential nuclear–cytoplasmic localization of survivin may reflect different protein functions and affect patient outcome.

To date, there has been only one report that correlated RNA survivin expression with bad prognosis in NSCLC patients [20] by using reverse transcription (RT)–PCR. Expression of survivin detected by immunostaining was a marker of poor prognosis in a group restricted to patients with small adenocarcinoma of the lung [21]. Investigation on the role of nuclear–cytoplasmic localization of survivin as a tumor marker in NSCLC has been limited to the early stages of the disease [22]. In this study we evaluated the expression and localization of survivin in a set of advanced NSCLC patients, in relation to clinicopathological parameters, survival and response to chemotherapy. Our results indicate that nuclear localization of survivin is a positive prognostic factor for survival in advanced NSCLC.

Materials and methods

Patients and specimens

The study included 53 patients with histologically verified and inoperable NSCLC (55% stage IIIA, 17% stage IIIB and 28% stage IV), treated at the Academic Hospital Vrije Universiteit of Amsterdam within different protocols, between January 1993 and December 1999. The patients, 33 males (62%) and 20 females (38%), ranged in age from 29 to 75 years (mean 56). Thirty-two patients receivedneo-adjuvant chemotherapy followed by surgery or radiotherapy, while 21 patients received palliative chemotherapy. Forty-seven patients received platinum-based chemotherapy and six patients had non-platinum-based chemotherapy. Response to chemotherapy was assessed according to the WHO criteria [23].

The histological features of the surgical specimens were classified according to the WHO, into 23 (43%) adenocarcinomas (including bronchioalveolar carcinomas), 19 (36%) squamous cell carcinomas and 11 (21%) large-cell undifferentiated carcinomas, on the basis of histology and histochemistry (PAS and Alcian Blue stains). Furthermore, 34 tumors (64%) were poorly differentiated, 18 moderately differentiated and only one was well differentiated. The tumor–node–metastasis (TNM) staging system was used according to Mountain [24].

This series of 53 cases was previously used for the analysis of XIAP, cIAP1 and cIAP2 expression in NSCLC [25]. Call charts were updated; the median follow-up was 76 months, and 45 patients were reported dead, 44 patients had relapsed and 43 of them died. Overall survival was calculated from the date of diagnosis to death, whereas survival from chemotherapy was computed from the date of the beginning of chemotherapy to death. Time to progression was defined as the time between diagnosis and the date of documented progression.

Immunohistochemistry

Formalin-fixed paraffin-embedded tissue blocks were freshly cut in 4-μm sections and mounted on poly-L-lysine-coated slides. Following deparaffination and hydration, sections were incubated in a solution with 0.3% H2O2 in absolute methanol for 30 min. Non-specific staining was blocked using normal swine (1:10) serum for 10 min (DAKO, Santa Barbara, CA, USA). The slides were incubated overnight at 4°C with a specific polyclonal primary antibody raised against full-length human survivin (ab6469; Novus Biologicals, Littleton, CO, USA), at 1:2000 dilution. Sections were then rinsed in phosphate-buffered saline (PBS) and incubated for 30 min with biotin-labeled secondary antibody at 1:300 dilution. Following a washing step with PBS, avidin–biotin complex (Strept ABCComplex; DAKO) was applied for 1 h as a reagent at 1:200 dilution. Finally, sections were rinsed in PBS, developed with diaminobenzidine tetrahydrochloride substrate (DAB; Chromogen, Carpinteria, CA, USA) for 3 min, slightly counterstained with hematoxylin, dehydrated and mounted with Depex (BDH Laboratory Supplies, Poole, UK). The validation of the use of the antibody against survivin for immunohistochemistry was carried out in acetone-fixed Jurkat-T leukemia cells and in frozen sections derived from some of the patients included in the series. The omission of the primary antibody in simultaneously incubated sections was used as a negative control. The staining in a set of representative lung tissues from healthy donors obtained from the archive of the Department of Pathology was performed following the same protocol to confirm the specificity of survivin for cancer cells. The percentage of immunoreactive tumor cells was independently assessed by two observers (P.v.d.V. and B.V.) on all sections. Positive samples were analyzed semi-quantitatively in at least five areas at ×400 magnification. Cases were scored positive when ≥5% of the cells reacted with the anti-survivin antibody, as proposed previously [11, 26]. Assessment of all staining results was blinded to knowledge of the clinical outcome of patients.

Statistical analysis

For the analysis of the association between expression of survivin and the major patient and tumor characteristics, we selected its median value of expression as the cut-off point. This methodology precludes strong assumptions about the relationship between marker and risk, while avoiding the bias of searching for ‘optimal cut-off points’ [27]. Correlation of survivin level of expression or localization with clinical features was calculated using the χ²-test. Correlation of survivin expression with expression of cIAP1, cIAP2 and XIAP, which had been previously evaluated in the same series [25], was tested by the Spearman’s correlation coefficient (rho). Cumulative survival curves were estimated using the Kaplan–Meier method and compared using the log-rank test. Multivariate survival analysis was performed according to the Cox regression model.

In all cases, a P value ≤0.05 was considered to indicate statistical significance. Statistical analysis was performed using the SPSS software program 9.0 (SPSS Inc., Chicago, IL, USA).

Results

Immunohistochemical analysis of survivin expression and localization in advanced NSCLC

Survivin expression was detected in 47/53 (89%) tumor specimens. The intensity of the staining was usually homogeneous within each sample tested, and the proportion of
survivin-positive tumor cells ranged from 0% to 100% (median value of expression 40%). Positive immunoreactivity for survivin was present only in tumor cells and not in the neighboring normal lung epithelial cells. Survivin specificity for tumor cells was confirmed when specimens from normal lung of healthy donors were analyzed (not shown). In these samples, only macrophages showed reactivity to survivin, limited to the cytoplasm, with neither bronchial nor alveolar epithelium immunoreactivity.

The present study on survivin extends the analysis of IAPs expression in a set of NSCLC tissues [25] previously analyzed for expression of cIAP1, cIAP2 and XIAP. A significant association was observed between expression of survivin and cIAP2 (rho 0.387; \( P = 0.004 \)), and survivin and cIAP1

Figure 1. Differential localization of survivin in non-small-cell lung cancer cells. (A) Strong cytoplasmic immunoreactivity in squamous cell carcinoma cells. Cell nuclei are negative (counterstained with hematoxylin) (B) Strong nuclear staining in adenocarcinoma cells. Some survivin-negative nuclei are visible. (C) Strong nuclear and cytoplasmic overlapping staining in squamous cell carcinoma. All the cells stained positive for survivin expression. In some cases, strong nuclear positivity is visible. Scale bar, 100 \( \mu \)m. (D–F) Enlarged examples of, respectively, cytoplasmic, nuclear, and nuclear and cytoplasmic staining.

Figure 2. Different patterns of survivin expression in tissues from advanced non-small-cell lung cancer. (A) Sample of squamous cell carcinoma negative for survivin expression. (B) Cytoplasmic staining in squamous cell carcinoma. Stroma and nuclei are counterstained with hematoxylin. (C) Exclusive survivin nuclear staining in squamous cell carcinoma. (D) Adenocarcinoma with some strongly stained cells for survivin in the nucleus, and weakly stained cells in the cytoplasm. Scale bar, 100 \( \mu \)m.
However, no significant correlation was noted between the expression of survivin and XIAP (rho = 0.072; \( P = 0.607 \)).

The localization of survivin within a cell was usually either cytoplasmic (Figure 1A and D) or nuclear (Figure 1B and E). Cells with survivin expression in both the nucleus and the cytoplasm (Figure 1C and F) were rarely observed. Nine of the 53 tumors (17\%) contained only cells with cytoplasmic survivin, 22 samples (42\%) contained only cells with nuclear survivin, and 16 samples (30\%) contained a mixed population of positive cells, some with nuclear survivin and others with cytoplasmic survivin (Figure 2).

**Table 1.** Characteristics of 53 patients and univariate significance for survival of selected parameters

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>No. of patients</th>
<th>%</th>
<th>( P ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Overall survival</td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \leq 56 )</td>
<td>25</td>
<td>47</td>
<td>0.4996</td>
</tr>
<tr>
<td>( &gt;56 )</td>
<td>28</td>
<td>53</td>
<td>0.1306</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>33</td>
<td>62</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>20</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>Histology</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>19</td>
<td>36</td>
<td>0.6685</td>
</tr>
<tr>
<td>Non-squamous cell carcinoma</td>
<td>34</td>
<td>64</td>
<td>0.0014</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
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<tr>
<td>III</td>
<td>38</td>
<td>72</td>
<td>0.0178</td>
</tr>
<tr>
<td>IV</td>
<td>15</td>
<td>28</td>
<td>0.6000</td>
</tr>
<tr>
<td>Differentiation grade</td>
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<td></td>
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<tr>
<td>Well + moderately differentiated</td>
<td>19</td>
<td>36</td>
<td>0.6000</td>
</tr>
<tr>
<td>Poorly differentiated</td>
<td>34</td>
<td>64</td>
<td>0.0631</td>
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<td>Type of chemotherapy</td>
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<tr>
<td>Neo-adjuvant</td>
<td>32</td>
<td>60</td>
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</tr>
<tr>
<td>Palliative</td>
<td>21</td>
<td>40</td>
<td>0.0631</td>
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<tr>
<td>Survivin expression</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>23</td>
<td>43</td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>30</td>
<td>57</td>
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<tr>
<td>Nuclear survivin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>38</td>
<td>72</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
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<td>28</td>
<td></td>
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<tr>
<td>Cytoplasmic survivin</td>
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<tr>
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<tr>
<td>Negative</td>
<td>28</td>
<td>53</td>
<td>0.8054</td>
</tr>
</tbody>
</table>

(rho = 0.311; \( P = 0.023 \)). However, no significant correlation was noted between the expression of survivin and XIAP (rho = 0.072; \( P = 0.607 \)).

The localization of survivin within a cell was usually either cytoplasmic (Figure 1A and D) or nuclear (Figure 1B and E). Cells with survivin expression in both the nucleus and the cytoplasm (Figure 1C and F) were rarely observed. Nine of the 53 tumors (17\%) contained only cells with cytoplasmic survivin, 22 samples (42\%) contained only cells with nuclear survivin, and 16 samples (30\%) contained a mixed population of positive cells, some with nuclear survivin and others with cytoplasmic survivin (Figure 2).

**Prognostic implications of survivin expression and localization**

By dichotomizing the specimens as expressing low or high levels of survivin, we observed no significant difference in overall survival, relapse-free survival and survival from chemotherapy (Table 1). The correlations between patients expressing survivin at different levels and their clinicopathological characteristics are presented in Table 2. The expression of survivin correlated with histology (\( P = 0.020 \)), squamous cell carcinoma being the histotype with lower levels of expression.

For the analysis of the localization of survivin, we divided the patients into two groups based on the presence or absence of nuclear survivin staining: the first group (nuclear survivin-positive) of 38 patients (72\%) included tumors with nuclear survivin, or tumors with a mixed population of cells with nuclear or cytoplasmic survivin. The second group (nuclear survivin-negative) of 15 patients (28\%) included tumors with no survivin expression, or only cytoplasmic survivin expression. By univariate analysis the nuclear survivin-positive patients had significantly longer overall (23 versus 14 months; \( P = 0.0446 \)) and disease-free (16 versus
9 months; $P = 0.0417$) survival compared with nuclear survivin-negative patients (Figure 3A and B).

Nuclear localization of survivin did not show any statistically significant correlation with the patients’ clinicopathological parameters (data not shown).

In addition to nuclear survivin, stage III and neo-adjuvant type of chemotherapy were also found to have prognostic significance for longer overall and relapse-free survival by univariate analysis (Table 1).

A multivariate analysis was performed according to the Cox regression model, for both overall and disease-free survival, including as covariates nuclear survivin localization, stage and histology. These last two factors were chosen based on the results of the univariate analysis, and because they are known to influence survival of lung cancer. Nuclear survivin expression was found to be an independent favorable prognostic indicator for overall and relapse-free survival, together with stage (Table 3).

To investigate the possible prognostic relevance of cytoplasmic localization of survivin, patient samples were then divided into cytoplasmic survivin-positive and cytoplasmic survivin-negative groups. The first group included 25 patients (47%) with tumors containing cells with cytoplasmic survivin staining, or a mixed population of cells with nuclear or cytoplasmic survivin staining. The cytoplasmic survivin-negative group included 28 patients (53%) with tumors that expressed no survivin or expressed the protein only in the nucleus of tumor cells. By univariate analysis no correlation between cytoplasmic accumulation of survivin and survival or clinicopathological parameters was found (Table 1; data not shown).

Neither survivin expression nor localization correlated with the survival time from chemotherapy by univariate analysis (Table 1).
Table 3. Multivariate analysis for overall and relapse-free survival

<table>
<thead>
<tr>
<th>Variable</th>
<th>Overall survival</th>
<th>Relapse-free survival</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hazard ratio</td>
<td>P value</td>
</tr>
<tr>
<td>Histology&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.9778</td>
<td>0.9461</td>
</tr>
<tr>
<td>Stage&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.4115</td>
<td>0.0081</td>
</tr>
<tr>
<td>Nuclear survivin&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.4407</td>
<td>0.0241</td>
</tr>
</tbody>
</table>

<sup>a</sup>Squamous cell versus non-squamous cell carcinoma.
<sup>b</sup>Stage III versus stage IV carcinoma.
<sup>c</sup>Nuclear survivin-positive versus nuclear survivin-negative patients.

CI, confidence interval.

Expression and localization of survivin does not predict response to chemotherapy

As reported previously for the other IAPs [25], no significant correlation was observed between response to chemotherapy and expression or localization of survivin in the present study (Table 2; data not shown). Furthermore, no correlation was found between modality of treatment (neo-adjuvant versus palliative) and scheme of chemotherapy (platinum-based versus platinum-free).

Discussion

Survivin is overexpressed in several different tumor types at both protein and mRNA levels, including in NSCLC [9]. In our study the majority of tumor samples had positive survivin staining, and absence of staining in surrounding normal tissues. This result therefore supports the view that survivin has a specific function in malignant transformation. In addition, we found that expression of survivin correlated with the expression of cIAP1 and cIAP2 (but not XIAP) determined previously [25] in the same series of samples. Thus, deregulation of different members of the IAP family occurs simultaneously in a subset of NSCLC.

In contrast to previous studies in NSCLC [20, 21] and other tumor types [28], survivin expression did not correlate with worse prognosis in our series. This finding can be explained by the different patient populations analyzed, or by methodological differences, such as the technique used to probe survivin expression (immunohistochemistry versus PCR), or the antibody reacting against a specific survivin epitope. In this regard, it has to be noted that different antibodies may recognize different pools of survivin [29]. In addition, the conditions of the statistical analysis, such as the level of expression chosen to score positive cases for survivin activation, may also have influenced the results.

In this group of advanced NSCLC, expression levels of survivin did not predict response to chemotherapy. This result is consistent with our previous finding with IAPs [25]. On the other hand, in radically resected NSCLC patients, we have described XIAP as an independent positive prognostic factor for survival [30]. This suggests that far advanced disease may be a more difficult area in which to find strong biological prognosticators than early disease.

On the other hand, there is recent evidence that subcellular localization of survivin may also correlate with prognosis. Although initially described as an exclusively cytoplasmic protein when overexpressed in tumor cells [9], the existence of two different nuclear–cytoplasmic pools of survivin is now recognized [29]. The expression of survivin in the nucleus appears to positively affect overall and relapse-free survival in gastric [18] and breast [19] carcinomas. Consistent with these findings, in our series of NSCLC tumors, localization of survivin in the nucleus correlated with a better prognosis. However, in early-stage NSCLC, nuclear survivin, detected with the same antibody we used, was not related to any clinicopathological parameters or survival [22]. Only in esophageal squamous cell carcinoma did expression of survivin in the nucleus predict shorter survival [26]. The biological basis for the correlation between survivin localization and prognosis remains to be established. A likely possibility is that the differential localization might reflect different functions of survivin, in respect of inhibition of apoptosis or regulation of the cell cycle. Our results are promising, and encourage further studies to understand the influence of nuclear–cytoplasmic localization on prognosis and to reconcile conflicting data, not only within different tumor types, but also within same tumors at different stages.

We have recently investigated possible mechanisms responsible for the differential nuclear–cytoplasmic localization of survivin, and we have reported that survivin is a CRM1-dependent nuclear shuttling protein, able to translocate continuously in and out of the nucleus [8]. However, the molecular mechanism underlying the differential localization in tumor cells is still unclear. Furthermore, the existence of splice variants of survivin [7] adds further complexity to this issue. In fact, the variant ΔEx3 contains a NLS that localizes it constitutively into the nucleus [8]. Thus, the expression of ΔEx3 variant may be partially responsible for the nuclear levels of survivin detected in this study. It needs to be noted, in this regard, that the polyclonal antibody used for this study recognized the three variants by immunofluorescence of cells transfected with expression plasmids encoding each of the isoforms (data not shown).

Survivin is increasingly investigated as a potential target for anticancer therapy. It has been reported that suppression of survivin expression using antisense oligonucleotides induces
tumor cell apoptosis in vitro and in vivo [31]. Further analysis on the importance of survivin localization is needed in order to define better the target of therapy.

In conclusion, in this study we have identified nuclear survivin expression as an important prognostic factor in patients with advanced NSCLC who undergo chemotherapy treatment, independent of the therapy. The nuclear–cytoplasmic localization in tumor tissues will be explored with the generation of specific antibodies against different spliced forms of survivin.

Acknowledgements

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