The Retinoblastoma Family Member pRb2/p130 Is an Independent Predictor of Survival in Human Soft Tissue Sarcomas

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Abstract

Purpose: pRb2/p130, a member of the Retinoblastoma gene family, has been shown to be a powerful prognostic factor in several malignancies. We sought to evaluate pRb2/p130 protein expression and its clinical effect in patients affected with soft tissue sarcomas (STS).

Experimental Design: Expression of pRb2/p130 was evaluated by immunohistochemistry on formalin-fixed, paraffin-embedded sections in 41 STSs. Results obtained were correlated with clinical-pathologic variables and disease-free and overall survival (OS) in univariate and multivariate analysis.

Results: Expression of pRb2/p130 was diminished in 25 (61%) tumors, whereas the remaining ones (39%) were classified as high expressors. No correlation between pRb2/p130 expression and clinical-pathologic variables was observed. However, a direct relationship between pRb2/p130 expression and clinical outcome of the patients was found in the subgroup of nonmetastatic tumors (n = 31). In univariate analysis, reduced pRb2/p130 expression was a negative prognostic factor and correlated with shorter disease-free survival (P = 0.021) and OS (P = 0.017) survival. In multivariate analysis, reduced pRb2/p130 expression was confirmed to be an independent predictor of shorter OS when considered together with tumor stage and grading (risk ratio, 7.893; confidence interval, 1.618-38.509; P = 0.011).

Conclusions: This study shows for the first time the potential prognostic value of pRb2/130 expression evaluated on formalin-fixed, paraffin-embedded sections in STSs patients. pRb2/p130 immunoreactivity can be used to predict OS in patients with nonmetastatic STSs and, therefore, may represent a new prognostic marker.

Soft tissue sarcomas (STS) are uncommon tumors (1% of all cancers) originating from mesenchymal tissue, and can arise in various sites (1, 2). The majority of STS is located in the limb or limb girdle, whereas retroperitoneal, visceral, and gynecologic sarcomas are less common. The WHO in 2002 has recognized ∼50 tumor subtypes related to STSs, which, in general, are named according to the tissue they most closely resemble (3). The prognosis of STSs is still unsatisfactory. Studies on large cohorts of patients have identified as important determinants of survival the following prognostic factors: volume of the disease, depth, grade, and presence of metastasis (1, 3). Five-year survival rate, following American Joint Committee on Cancer and Unione Internationale Contro Cancrum (Italian) staging, strictly correlates with the stage: 5-year survival is 90% for stage I, 70% for stage II, 50% for stage III, and 0% to 15% for stage IV. Prognosis can be modified by the histologic type, the location, and the level of surgical resection involving wide or radical margins (4, 5). Surgery, supported by adjuvant radiotherapy, is often curative for localized disease (stage I and II; ref. 1). Adjuvant or neoadjuvant chemotherapies do not significantly affect the natural history of localized STSs (1). In metastatic disease, anthracycline-based chemotherapy has a palliative role, with a median time to progression of 4 months and an overall survival (OS) of 11 months.

To better define the natural history of STSs, as well as to identify hypothetical targets for new kind of therapies, many researches have focused their attention on different prognostic markers. Those could be useful to improve the results of the therapeutic decision-making process because the three well-known prognostic variables (grade, depth, and volume) cannot explain all the biological differences in aggressiveness between STSs.

The Retinoblastoma gene family (RB/p105, p107, and Rb2/p130) regulates cell cycle progression through the G1 phase of the cell cycle. Retinoblastoma family members are nuclear proteins, known also as pocket proteins for their unique structure, which are phosphorylated in a cell cycle–dependent...
manner and exhibit growth suppressive properties in a cell type–dependent manner. Whereas pRb/p105 is found in both cycling and quiescent cells, Rb2/p130 and p107 act exclusively in a cell cycle–dependent fashion to regulate several cellular transcription factors such as E2Fs (6).

The Rb2/p130 gene maps to human chromosome 16q12.2, an area in which deletions or loss of heterozygosity have been found in several human neoplasms, including breast, hepatic, prostatic, and ovarian cancer (7). Loss of pRb2/p130 expression has been observed and reported to correlate with clinical aggressiveness in lung (8), endometrial cancer (9), and choroidal melanoma (10).

In the present study, to investigate a possible role for the Rb2/p130 protein in the pathogenesis of STSs, we did immunohistochemistry on a series of 41 STSs specimens. pRb2 expression was lost or decreased in 60% of STSs samples and significantly correlated with recurrence of disease and poor survival in the subset of nonmetastatic tumors.

These results support a role for pRb2/p130 as a tumor suppressor gene in STSs and suggest that loss of pRb2/p130 expression could represent a novel prognostic marker for STS patients.

Materials and Methods

Patients and clinicopathologic data. Forty-one patients with histologically verified STSs were enrolled in the present study. Sections from paraffin-embedded STSs were obtained from patients who underwent surgical resection as a treatment for sarcoma in the Department of Surgery at the Regina Margherita Hospital and S. Giovanni Battista Hospital, Turin, Italy. Tumor tissues were submitted for routine histopathologic examination. Follow-up data were available for all 41 patients. The patients’ age ranged from 19 to 80 yr (mean, 53 yr; median, 56 yr). The tumors were staged according to Enzinger and Weiss classification (11). All of the primary tumors (n = 41) were untreated before primary tumor resection. None of the patients with primary tumor (n = 41) had received either chemotherapy or radiotherapy before surgery. Those patients who were irradiated received a dose ≥60 Gy postoperatively. The chemotherapy regimen included Adryamicin or Epirubicin (75 mg/m²) in association with Ifosfamide (12 or 16 mg/m²), every 21 d, for 6 cycles.

The grade of STSs differentiation was estimated according to the Coindre-Trojani classification. Of the tumor samples, 4 (10%) were grade 1, 13 (32%) were grade 2, and 24 (58%) were grade 3. Representative clinical data of the STS patients are summarized in Table 1.

Immunohistochemistry. A total of 41 formalin-fixed and paraffin-embedded STS specimens were processed. Sections of each specimen were cut at 3 μm, mounted on glass, and dried overnight at 37°C. All sections were dewaxed, rehydrated, quenched in 0.5% hydrogen peroxide, and microwave pretreated in Retrieval Citra Plus Buffer (pH 7.2; Biogenex). After blocking with normal serum for 1 h at room temperature, a rabbit polyclonal antibody against pRb2/p130 (ADL-1; refs. 12, 13) was incubated with tissue sections at a 1:100 dilution at 4°C overnight. Negative control slides were also processed in parallel using a nonspecific immunoglobulin IgG (Sigma Chemical Co.) at the same concentration as the primary antibody. The positive immunostaining of infiltrating lymphocytes represented an internal-positive control for preservation of antigenicity in the sections examined. All slides were processed using the peroxidase-antiperoxidase method (Dako). Diaminobenzidine was used as the final chromogen, and Gill’s hematoxylin was used for counterstaining. Additionally, to assess overall tumor morphology, tissue biopsies were deparaffinized as described above, stained with Gill’s hematoxylin, and counterstained with eosin.

Table 1. Rb2 expression and clinicopathologic variables in a series of 41 STSs

<table>
<thead>
<tr>
<th>Age (yr)</th>
<th>Total (%)</th>
<th>pRb2 expression High (%)</th>
<th>pRb2 expression Low (%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;56</td>
<td>19 (46)</td>
<td>7 (37)</td>
<td>12 (63)</td>
<td>0.7</td>
</tr>
<tr>
<td>≤56</td>
<td>22 (54)</td>
<td>9 (41)</td>
<td>13 (59)</td>
<td></td>
</tr>
<tr>
<td>Tumor grade</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>4 (10)</td>
<td>2 (50)</td>
<td>2 (50)</td>
<td></td>
</tr>
<tr>
<td>G2</td>
<td>12 (22)</td>
<td>4 (31)</td>
<td>8 (69)</td>
<td></td>
</tr>
<tr>
<td>G3</td>
<td>24 (58)</td>
<td>10 (42)</td>
<td>14 (58)</td>
<td>1.0</td>
</tr>
<tr>
<td>Tumor stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>II/III (4/27</td>
<td>31 (76)</td>
<td>12 (39)</td>
<td>19 (61)</td>
<td>0.7</td>
</tr>
<tr>
<td>IV</td>
<td>10 (24)</td>
<td>4 (44)</td>
<td>6 (56)</td>
<td></td>
</tr>
<tr>
<td>Tumor site</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extremities</td>
<td>28</td>
<td>12 (43)</td>
<td>16 (57)</td>
<td></td>
</tr>
<tr>
<td>Abdomen</td>
<td>11</td>
<td>3 (27)</td>
<td>8 (73)</td>
<td></td>
</tr>
<tr>
<td>Torax</td>
<td>2</td>
<td>1 (50)</td>
<td>1 (50)</td>
<td>0.37</td>
</tr>
<tr>
<td>Histotype</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MFH</td>
<td>8</td>
<td>2 (25)</td>
<td>6 (75)</td>
<td></td>
</tr>
<tr>
<td>LMS</td>
<td>6</td>
<td>3 (50)</td>
<td>3 (50)</td>
<td></td>
</tr>
<tr>
<td>LPS</td>
<td>13</td>
<td>3 (23)</td>
<td>10 (77)</td>
<td></td>
</tr>
<tr>
<td>RMS</td>
<td>3</td>
<td>2 (67)</td>
<td>1 (33)</td>
<td></td>
</tr>
<tr>
<td>SCHW</td>
<td>7</td>
<td>5 (71)</td>
<td>2 (29)</td>
<td></td>
</tr>
<tr>
<td>SINOV</td>
<td>4</td>
<td>1 (25)</td>
<td>3 (75)</td>
<td>0.2</td>
</tr>
</tbody>
</table>

NOTE: Statistical analyses were done by the Fisher χ² test. A P value of <0.05 is considered significant.

Abbreviations: MFS, malignant fibrous histiocytoma; LMS, leiomyosarcoma; LPS, liposarcoma; RMS, rhabdomyosarcoma; SCHW, Schwannoma; SINOV, sinoviosarcoma.

*pOnly stages II and III were included in the analysis (see Materials and Methods for details).

pRb2/p130 staining evaluation. Immunohistochemical staining in sample sections was categorized as weakly positive (low nuclear expression) or strongly positive (high nuclear expression). A strongly positive result was recorded when >14% of the cells (corresponding to the median value of the percentage of positive cells) exhibited strong nuclear staining. The sections were initially scanned at low power to determine the areas that were evenly labeled. The cases were evaluated independently by two different pathologists (E.B. and A.G.), and discrepancies in estimation were reconciled by concurrent review using a multihheaded microscope. At least 10 high-power fields were chosen randomly, and 2,000 cells were counted.

Statistical analysis. The association between pRb2 expression and other molecular and clinicopathologic variables were calculated using contingency table methods and tested for significance using the Fisher χ² test.

Disease-free (DFS) and OS curves were calculated using the Kaplan-Meier method, and the log-rank test was used to compare survival curves. Time to recurrence was calculated from surgery to the first documented recurrence. In the absence of recurrence, follow-up time was calculated from surgery to the date of the last documented follow-up. Data were stratified for the variables of interest to detect association with the survival rate. Univariate and multivariate relative risks were calculated using the Cox proportional hazards regression. All calculations were done using the STATA statistical software package (Stata Corporation), and the results were considered statistically significant when the P value was <0.05.
Results

Expression levels of pRb2/p130 in STSs specimens. To investigate the role of pRb2/p130 in human STSs, the expression of pRb2/p130 protein was evaluated by immunostaining in a series of 41 primary human STSs. Only cells with a clear nuclear staining were scored as positive (Fig. 1A). As shown in Table 1, the series included different histotypes and tumors with different stage and grade.

The median percentage of positive cells was 14 (range, 0-80; mean, 18), and this value was used to classify tumors as displaying a low (≤14%) or a high (>14%) pRb2/p130 expression. Overall, low staining for pRb2/p130 was detectable in 25 (61%) tumors, whereas the remaining ones (39%) were classified as high expressors (Fig. 1B). Low staining (Fig. 1C) was observed in 2 (50%) of the 4 low grade (G1), in 9 (69%) of the 13 G2, and in 14 (58%) of the 24 G3 tumors, but these differences were not statistically significant. No correlation was also observed with either age at diagnosis, histotype, tumor stage, and tumor site (Table 1).

pRb2/p130 expression and clinical outcome. To analyze a more homogeneous group of patients, the relationship between pRb2/p130 expression levels and clinical outcome was analyzed within the subset of patients who did not show metastases at diagnosis (stage II and III; n = 31). During the follow-up period, recurrence and death of disease were observed in 18 and 17 patients, respectively. When these tumors were stratified according to pRb2/p130 expression, 14 (74%) of the 19 low expressor tumors recurred during the period of follow-up (mean, 32 months; range, 2-71 months), whereas only 4 (33%) occurred among the remaining 12 cases, and this difference was significant (P = 0.04). Moreover, median DFS of patients whose tumors expressed low levels of pRb2 (13 months; mean, 26.2 ± 21.9 months) was shorter compared with high expressor patients (46 months; mean, 44.2 ± 17.9 months), and this difference was significant (P = 0.02). In univariate setting, patients with tumors displaying low staining for pRb2 were more likely to recur compared with patients who had tumor positive for pRb2 expression, as confirmed by the Kaplan-Meier curves of DFS, which displayed a significant separation between the two groups of patients (P = 0.02 by log-rank test; Fig. 2A).

Similarly, in the same subset of patients, 14 (74%) of the 19 patients with low expressor tumors and only 3 (25%) of the 12 high expressors died of disease during the period of follow-up (mean, 49 months; range, 9-84 months). This difference was significant (P = 0.02). Moreover, median survival of patients whose tumors expressed low levels of pRb2 (40 months; mean, 43.8 ± 23.4) was significantly shorter compared with high expressor patients pRb2 (60 months; mean, 59.8 ± 12.4 months; P = 0.037). Thus, patients with tumors displaying low staining for pRb2 were more likely to die for the disease compared with patients who had tumors expressing high levels of the protein as confirmed by the Kaplan-Meier curves, which displayed a significant separation between the two groups of patients (P = 0.017 by log-rank test; Fig. 2B). Hence, recurrence and death occurred more frequently when STS in our series displayed low staining for pRb2.

In univariate analysis, none of the available clinicopathologic variables displayed a relationship with DFS (data not shown). On the other hand, higher tumor grade (P = 0.048 by log-rank test) and stage (P = 0.045) were also associated with a shorter OS in the same subset of STS patients (data not shown).

We then did a multivariate analysis by building a Cox hazards model that included tumor grade, tumor stage, and pRb2 staining and evaluated their relationship with OS. As shown in Table 2, reduced pRb2 staining was confirmed to be the strongest independent prognostic factor (P = 0.011; confidence interval, 1.618-38.509; relative risk, 7.893) in terms of OS.

It is noteworthy that when stage IV tumors were included in the model, only stage was confirmed to be an independent prognostic factor, but reduced pRb2 staining was still associated with an increased risk of death for the disease (relative risk, 2.26; confidence interval, 0.865-5.934), although it was no longer significant (P = 0.1).

Discussion

Despite various efforts to improve treatment for human STSs, this disease remains at high risk for recurrence and death. The main reason for this is the heterogeneity of STS as reflected by variable biological behavior. Clinical prognostic factors such as histologic grade, size, depth, and status of surgical resection margins have been identified to better define the risk of patients to die of disease. Nevertheless, because the natural course of STS cannot be reliably predicted, it remains a clinical dilemma whether some or all of these patients should receive adjuvant therapy. In recent years, many different biological prognostic factors have been studied in STSs; unfortunately, none of them seems to play a definitive role because of methodologic limitations of retrospective studies and because of contrasting results.

The retinoblastoma susceptibility gene, RB, is a tumor suppressor gene, which when deleted is associated with the development of retinoblastoma. Children with heritable retinoblastoma
frequently develop second malignancies, principally sarcomas, in which pRb expression is frequently lost (14). To date, no studies have been reported on the role of the other two Retinoblastoma family members, pRb2/p130 and p107, in STSs.

In the present study, we investigated for the first time the prognostic role of pRb2/p130 in primary STSs. Our findings indicate that loss of pRb2 expression in a subset of 31 patients who had not metastatic disease at diagnosis (stage II-III) was associated with increased risk of recurrence and death during the period of follow-up. These findings are consistent with most of the studies analyzing the prognostic role of pRb2 expression in several cancer types. In particular, loss of pRb2/p130 expression significantly increases the risk of recurrence and death from disease in choroidal melanoma, endometrial, lung (8–10), and hepatocellular carcinomas (15). In our study, loss of Rb2/p130 expression retained an independent negative prognostic role for OS also in a multivariate analysis that included tumor grade and stage (Table 2). We could not perform a multivariate analysis for DFS because no other prognostic marker, but pRb2, correlated with DFS in univariate setting. This finding further supports the prognostic role of pRb2 in STSs patients, although it needs to be confirmed on a larger cohort of STSs.

Our finding of a relatively frequent decreased expression of pRb2 protein in STSs raises questions concerning the mechanism responsible for its loss in these tumors. It is likely that inactivating mutations, as observed previously in human primary nasopharyngeal carcinomas (16), lung tumors (17), and Burkitt’s lymphomas, (18) are involved. Recent studies (7, 19) seem to rule out promoter hypermethylation as the mechanism involved in controlling pRb2 expression. However, we cannot exclude that posttranscriptional mechanisms, such as proteasome-mediated degradation (20), might also play a role in the regulation of pRb2 in STS cells. Studies are ongoing to identify which means is involved in Rb2 down-regulation in STSs.

pRb2/p130 was the last member of the Rb family to be identified. Like pRb and p107, it has well-characterized cell growth suppressive properties similar to, yet distinct from, the other family members. Using a tetracycline-regulated gene expression system to control the expression of pRb2/p130 in a JC virus–induced hamster brain tumor cell line, we showed that forced expression of pRb2/p130 reduces the tumor mass in nude mice (21). In another study in nude mice, we showed that ectopic expression of pRb2/p130 suppresses the tumorigenicity of the SKOV3 ovarian cancer cell line overexpressing erbB-2 (22). Finally, in vivo retroviral transduction of pRb2/p130 in established tumors, derived from injection of the lung adenocarcinoma cell line H23 grown in nude mice, reduced the mass 12-fold with respect to the control viruses (17). The result of our study additionally supports a role for pRb2/p130 as a bona fide tumor suppressor gene in human cancer.

In conclusion, we present evidence for a role of pRb2/p130 as an independent prognostic variable of clinical outcome in STS patients. Patients with STs that show loss of pRb2/p130 are at higher risk of recurrence and death of disease and may eventually benefit from more aggressive adjuvant therapy. However, the reliability of pRb2/p130 as a potential marker in the clinical routine assessment and management of patients with STS deserves to be further evaluated in long-term follow-up studies on a larger number of cases.

Table 2. Contribution of various potential prognostic factors to OS by Cox regression analysis in STS patients

<table>
<thead>
<tr>
<th>Variable</th>
<th>Risk ratio</th>
<th>95% confidence interval</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor grade*</td>
<td>10.135</td>
<td>1.290-79.634</td>
<td>0.028</td>
</tr>
<tr>
<td>Tumor stage</td>
<td>0.969</td>
<td>0.120-7.803</td>
<td>0.977</td>
</tr>
<tr>
<td>pRb2/p130</td>
<td>7.893</td>
<td>1.618-38.509</td>
<td>0.011</td>
</tr>
</tbody>
</table>

*The risk ratio is given as higher versus lower grade tumors.
† The risk ratio is given as higher (III) versus lower stage (II) tumors.
‡ The risk ratio is given as low expressing versus high expressing tumors.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.
Role of pRb2/p130 in Human Soft Tissue Sarcoma

References

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