

Altered patterns of RB expression define groups of soft tissue sarcoma patients with distinct biological and clinical behavior

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Summary. Background: Function of the retinoblastoma tumor suppressor protein (pRB) may be compromised at a genetic level by gene loss or mutation or at a post-translational level by hyperphosphorylation. In this study, we examined adult soft tissue sarcomas (ASTS) to determine if alterations of pRB were associated with distinct patterns of pRB expression and clinical outcome. Design: We investigated 86 ASTS patients using monoclonal antibodies that distinguish between hyperphosphorylated and underphosphorylated pRB products. We also used microsatellite analysis to investigate the genetic status of the RB locus. We correlated pRB alterations with proliferative activity, and with clinicopathological outcomes. Results: Altered patterns of pRB expression are common in ASTS occurring in 84% of cases, and it is significantly associated with proliferative activity ($p < 0.001$). Patients whose tumors either lack expression of pRB, or express hyperphosphorylated forms of pRB, have poor survivals compared to patients whose tumors exhibit a normal, underphosphorylated pattern of pRB expression ($p = 0.03$). In addition, 63% of cases lacking expression of pRB showed loss-of-heterozygosity at the locus. Conclusions: Inactivation of pRB is common in adult STS, which may be due to either gene loss or post-translational modification, namely hyperphosphorylation. Both mechanisms are associated with tumor cell proliferation and poor survival.

Key words: Retinoblastoma, Sarcoma, Immunohistochemistry, Hyperphosphorylation, Survival

Introduction

Disruption of cell cycle checkpoints is a common finding in human cancer cells. The product encoded by the retinoblastoma gene (pRB) has been identified as a critical cell cycle regulator of the G1/S checkpoint. Progression through this checkpoint is achieved by the activity of complexes formed by cyclins and cyclin-dependent kinases (Cdk) aimed at phosphorylating pRB. pRB phosphorylation results in the liberation of E2F transcription factors, promoting the transcription of genes required for the transition into S phase (Sherr and McCormick, 2002). We previously observed that sarcomas with altered pRB expression, mainly those displaying undetectable pRB levels, were significantly associated with decreased survival (Cance et al., 1990). Further studies from our group and others found that alterations affecting up-stream regulators of pRB, such as cyclin D1 overexpression or p16/INK4A deletions, were common events in soft tissue sarcomas (Orlow et al., 1999; Kim et al., 2001). Thus, we postulated that pRB can be functionally inactivated in the presence of an intact gene by hyperphosphorylation. Furthermore, *in vitro* studies have demonstrated that pRB levels rise during cell cycle progression, and that hyperphosphorylated pRB products accumulate during this process (Xu et al., 1991; Lasorella et al., 2000).

To test the hypothesis that functional inactivation of pRB may be accomplished by two general mechanisms, gene loss or hyperphosphorylation, that these mechanisms will produce two distinct staining patterns by immunohistochemistry, and that both methods of inactivation will have a negative impact on patient survival, we studied a cohort of 86 adult soft tissue sarcoma cases for altered expression of pRB. We used two different anti-pRB monoclonal antibodies: one antibody recognizes both the underphosphorylated and hyperphosphorylated forms of pRB; the other recognizes only the underphosphorylated form. We correlated pRB

expression patterns with proliferative status, RB gene losses, and clinicopathological variables.

Materials and methods

Patient and tissue characteristics

Prospectively acquired frozen tissues from 86 adult patients with soft tissue sarcomas admitted to Memorial Hospital between 1990-1991 were studied. This cohort of specimens has not been previously analyzed. Patient records are part of an ongoing IRB-approved study of adult soft tissue sarcomas. Disease-specific survival data were available for all patients; the median follow up time was 25 months. Diagnoses included in this study were classical well-differentiated (n=21) and dedifferentiated liposarcomas (n=14), leiomyosarcomas (n=22), malignant fibrous histiocytomas (high-grade pleomorphic tumors) (n=20), fibrosarcomas (n=6), rhabdomyosarcomas (n=2) and spindle cell sarcoma (n=1). No cases of myxoid or round cell liposarcomas were included. The cohort contained 58 primary lesions (67%), and 28 recurrent or metastatic lesions (33%). High grade tumors comprised 79% of the cohort (n=68); the remaining 21% were low grade lesions (n=18). This binary grading system takes into account cellularity, differentiation, pleomorphism, necrosis and number of mitoses (Brennan et al., 2001). Specimens were embedded in a cryopreservative solution (ornithine carbonyl transferase compound (OCT) Miles Laboratories, Elkhart, IN), snap frozen in isopentane, and stored at -70 °C until used.

Monoclonal antibodies and immunohistochemistry

Two mouse monoclonal antibodies specific for human pRB were used. Clone 3C8 (QED Bioscience, San Diego, CA) recognizes both hyperphosphorylated and underphosphorylated pRB. Specifically, it recognizes an epitope between amino acids 886 and 905. Clone G99-549 (Pharmingen, San Diego, CA) recognizes an epitope between amino acids 514 and 610. Optimal staining conditions for each antibody were determined by trials of different fixatives and titration of antibody concentrations on cytospin preparations of the T24 bladder cancer cell line. The conditions used were those that produced the strongest nuclear intensity without significant cytoplasmic background. Final conditions were as follows: slides stained with 3C8 were fixed in 10% neutral buffered formalin and the antibody concentration was 1.28 µg/ml; those stained with G99-549 were fixed in ice-cold methanol:acetone (1:1 dilution) and the antibody concentration was 5 µg/ml. Proliferative activity was assessed by the use of mouse monoclonal antibody MIB1 (DAKO; dilution 1:1000) directed to the Ki67 antigen. As previously reported, a cut off point of $\geq 20\%$ tumor cells displaying nuclear immunoreactivities was used to define a Ki67 positive proliferative index (Hoos et al., 2001; Hazan et al.,

2002).

Five-micron sections were cut from each tumor block and fixed as described above. The avidin-biotin peroxidase method was used with 0.06% diaminobenzidine as the chromogen. Briefly, sections were washed in phosphate buffered saline followed by quenching of endogenous peroxidases using 0.1% hydrogen peroxide. Sections were blocked with avidin-biotin (Vector Laboratories, Burlingame, CA), followed by 10% normal horse serum (Cappel, West Chester, PA). Tissues were incubated for two hours with primary antibodies, washed extensively, and incubated for 30 minutes with biotinylated horse anti-mouse IgG (Vector Laboratories) at a 1:500 dilution. Sections were washed and incubated for 30 minutes with avidin-biotin peroxidase complexes (Vector Laboratories) at a 1:25 dilution before developing with diaminobenzidine and counterstaining the nuclei with hematoxylin. Cells with nuclear staining were considered positive for pRB expression. Sections were scanned for areas of strongest nuclear immunoreactivity and estimates of the percentage of positive tumor cells were recorded. Cases were considered negative for pRB expression if immunoreactivity was observed in normal cells, but not in tumor cells, indicating that the staining was successful in that section. Cytospins of the T24 bladder cancer cell line were included in all experiments as positive controls. Three investigators (CCC, SM, DP) who were blinded to the clinical and microsatellite data read the cases jointly. A consensus estimate of percent positive cells was reached in all cases.

Immunoprecipitation

10 ml of a lysis buffer prepared after the method of Shew et al (Shew et al., 1990), containing 50mM Tris-HCl (pH 7.4), 0.1% SDS, 150 mM NaCl, 0.5% deoxycholate, 1.0% NP-40, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 mg NaF, and protease inhibitors from one Complete Mini tablet (Boehringer Mannheim) was prepared. 3.9×10^7 logarithmically dividing T24 cells were washed twice in PBS and were incubated in 1 ml of lysis buffer for 30 minutes on ice. The lysed cells were centrifuged at $14,000 \times g$ for 15 minutes at 4°C. The protein concentration was determined by the Bradford method (BioRad) and 3 aliquots of 1.65 mg of protein each were incubated overnight at 4°C with either 10 µg of mAb G99-549, 3 µg of 3C8, or 10 µg of a purified mouse IgG1 (Pharmingen) as a negative control. The concentrations of G99-549 and 3C8 were those recommended by the manufacturers. For the precipitation step, Protein A sepharose beads (CL-4B, Pharmingen) were first incubated with rabbit anti-mouse immunoglobulins (Dako) in a TBS-BSA solution composed of 25 mM Tris-HCl (pH 8.0), 120 mM NaCl, 10% bovine serum albumin (BSA) rocking at 4°C for 30 minutes. The beads were washed three times with TBS-BSA and then added to the cell lysates. This mixture was rocked at 4°C for 45 minutes and centrifuged to collect

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the beads. The beads were washed four times in a wash buffer composed of 10 mM Tris-HCl (pH 7.4), 0.2% NP-40, 1 mM PMSF. Proteins were released from the beads by adding 1X SDS-PAGE buffer (62.5 mM Tris-HCl (pH 6.8), 5% β -mercaptoethanol, 10% glycerol, 2.3% SDS, 0.001% bromophenol blue) and boiling for 5 minutes.

Immunoblotting

100 μ g of T24 cell lysate and the immunoprecipitates described above were fractionated on the same 7.5% polyacrylamide gel and electrophoretically transferred to a nitrocellulose membrane. The membrane was blocked at room temperature for 20 minutes with a solution of 4% non-fat dry milk, 0.02% sodium azide, in TBST (10 mM Tris-HCl (pH 7.4), 0.9% NaCl, 0.05% Tween-20). The blocking solution was changed and the membrane incubated overnight, rocking at 4°C with 1 μ g/ml of 3C8. Next, the membrane was washed three times with TBST and incubated rocking at room temperature for one hour with a 1:3000 dilution of horseradish peroxidase (HRP) -linked sheep anti-mouse Ig (Amersham). Proteins were visualized using the SuperSignal West Pico chemiluminescent system (Pierce).

DNA extraction and microsatellite analysis

Matched pairs of frozen normal and tumor tissue samples were available from 34 of the 86 patients. Tumor specimens contained at least 80% tumor cells as determined by light microscopy of cryostat cut sections stained with hematoxylin and eosin. DNA was extracted from these samples using the QIAamp Tissue Kit (Qiagen). The RB 1.20 locus was amplified by PCR using fluorescently tagged (6-FAM) primers (PE Biosystems), and the products analyzed on an ABI 310 genetic analyzer. The primer sequences were: 5' AAGTAAGAAAATCAAGCACTT 3' and 5' AATTAACAAGGTGTGGTGGT 3'. The cycling conditions were 95°C x 5', (95°C x 30", 62°C x 30", 72°C x 1') x 35 cycles, 72°C x 5', 4°C. The amplification reaction consisted of 10 pmol of each primer, 0.75 U Taq polymerase (Boehringer), 2.0 mM MgCl₂, and 1X reaction buffer (Boehringer). 0.3 microliters of the 20 μ l reaction were analyzed. Cases were considered informative if two peaks were observed in the normal specimen. R-values were calculated by first determining the ratio between the heights of the two peaks of the normal and tumor specimens respectively. The ratio of the tumor peak heights was divided by the ratio of the normal peak heights to yield the R-value. An R-value of $1.5 < R < 0.5$ was considered to represent loss of one allele (LOH) (Mora et al., 2000).

Statistical analysis

Fisher's exact test was used to assess the association between altered patterns of pRB expression and

clinicopathologic factors, as well as the separate correlations between pRB status, Ki-67 expression, and LOH. The Kaplan-Meier method was used to estimate the survival functions. Proportional hazards analysis was used to obtain maximum likelihood estimates of relative risks.

Results

Analysis of antibody specificities

We confirmed the specificities and utilities of these antibodies for immunohistochemistry using the T24 cell line. This cell line has been extensively characterized with respect to its pRB status. In these cells, the p16^{INK4A} promoter is methylated, and no p16 protein is produced. As a result, much of the pRB in these cells is hyperphosphorylated (Bender et al., 1998). A cell lysate of exponentially growing T24 cells was analyzed by immunoprecipitation and immunoblotting using both mAbs G99-549 and 3C8. G99-549 recognizes only underphosphorylated pRB; 3C8 recognizes both under- and hyperphosphorylated pRB. Western blotting using 3C8 antibody revealed multiple bands with distinct electrophoretic mobilities, corresponding to pRB proteins with different degrees of phosphorylation (Fig. 1a). However, the combined immunoprecipitation and Western blotting utilizing the G99-549 antibody identified a single, fastest migrating, underphosphorylated form of pRB.

Cytospin preparations of same T24 cells were subjected to immunohistochemical analysis (see Figure 1b). We observed a strong nuclear staining in the majority of cells when using antibody 3C8. In addition, we found cytosolic immunoreactivities in cells undergoing mitosis, since nuclear membranes are no longer present. The pattern of staining was very different when using antibody G99-549. Staining was less intense, and fewer cells stained positive. Mitotic figures did not stain with this antibody. The fact that mitotic cells, which contain hyperphosphorylated pRB, did not react with mAb G99-549 provides further evidence that this antibody recognizes underphosphorylated pRB in immunohistochemical assays. Other studies have also confirmed the utility of these reagents for immunohistochemistry (Juan et al., 1998; Chatterjee et al., 2004).

Expression patterns of pRB in adult soft tissue sarcomas using mAb 3C8

The staining patterns were divided into three groups based on the percentage of positively staining tumor cells. Representative samples of these patterns are shown in Figure 2. A frequency distribution plot of the number of positively staining cases versus the percentage of positive nuclei is shown in Figure 3. Together, these data suggested the presence of three populations of tumors. Group 1 (n=14) were tumors with a weak, heterogeneous

staining pattern, displaying nuclear reactivity in 1-10% of tumor cell nuclei. These cases were considered to have wild-type levels of pRB expression, as this is the same pattern observed in normal mesenchymal cells from which these tumors derive (Cordon-Cardo and Richon, 1994; Shi et al., 1996). Tumors with undetectable staining were considered to have no expression of pRB and were designated Group 2 (n=21). In these cases, vascular endothelial cells in the tumor were positive and served as an internal control. Group 3 (n=51) were cases with a more intense, homogeneous pattern of staining in greater than 10% of tumor cell nuclei. These cases were considered to accumulate pRB. This group exhibited an almost normal distribution of staining intensities varying from 20%-80% positive cells. The large majority of these cases had greater than

40% of tumor cells with positive nuclear staining (Fig. 3). Considering the cases from groups 2 and 3 as a single group characterized by "altered" expression of pRB, we observed altered pRB phenotypes in 72 of 86 (84%) cases.

Characterization of altered expression of pRB

To better define the significance of these altered patterns of pRB expression, sections were stained with the proliferation marker MIB-1. This antibody recognizes the Ki-67 antigen, which is expressed throughout the cell cycle but not in resting cells. A statistically significant correlation ($p=0.0008$) was observed between "altered" pRB expression (Groups 2 and 3) and a positive proliferative index (Table 1). These

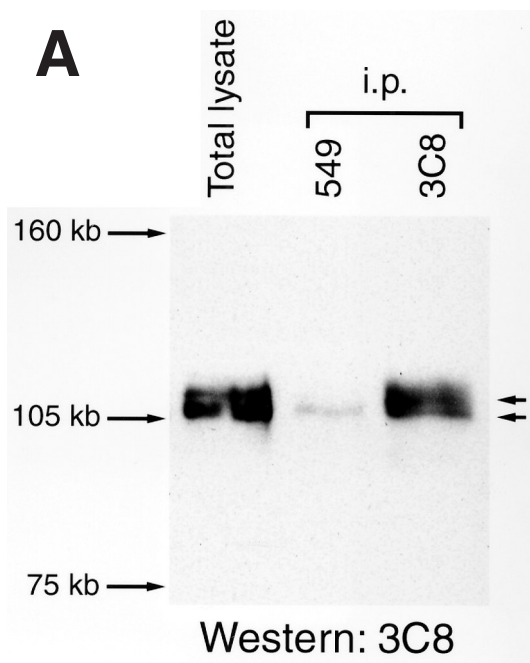
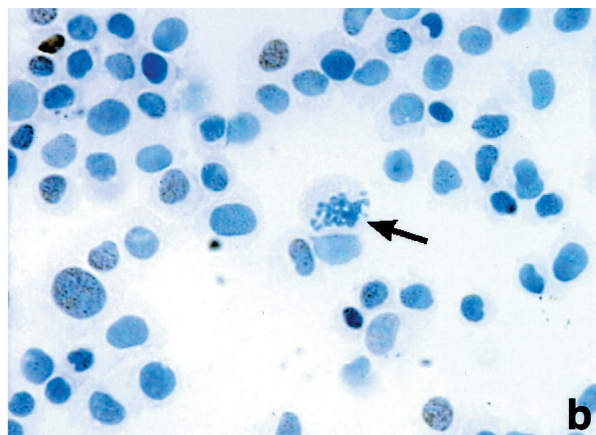
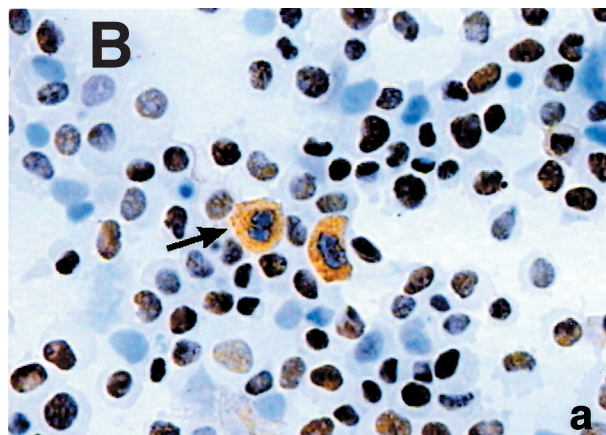


Fig. 1. Antibody characterization. **A.** Immunoprecipitation/immunoblot analysis. Protein lysates from exponentially growing T24 cells were immunoprecipitated with monoclonal antibodies that recognize either both hyperphosphorylated and underphosphorylated pRB (3C8), or only underphosphorylated pRB (549). The immunoprecipitates (middle and right lanes) as well as a whole cell lysate (left lane) were subjected to immunoblotting analysis with the 3C8 antibody. Multiple pRB species are present in the total lysate and in the 3C8 immunoprecipitate. A single, lower molecular weight species, corresponding to underphosphorylated pRB is present in the 549 lane. No bands were observed using a negative control antibody for the immunoprecipitation (data not shown). **B.** Immunohistochemical staining of mitotic figures. Cytospins of the bladder carcinoma cell line T24 were stained with either 3C8 (left), or G99-549 (right). The arrows indicate mitotic figures. Note the cytoplasmic staining of the mitotic figures with 3C8, but not with G99-549, which recognizes only the underphosphorylated form of pRB. Nuclear staining of other cells is evident with both antibodies. Original magnification, x 400



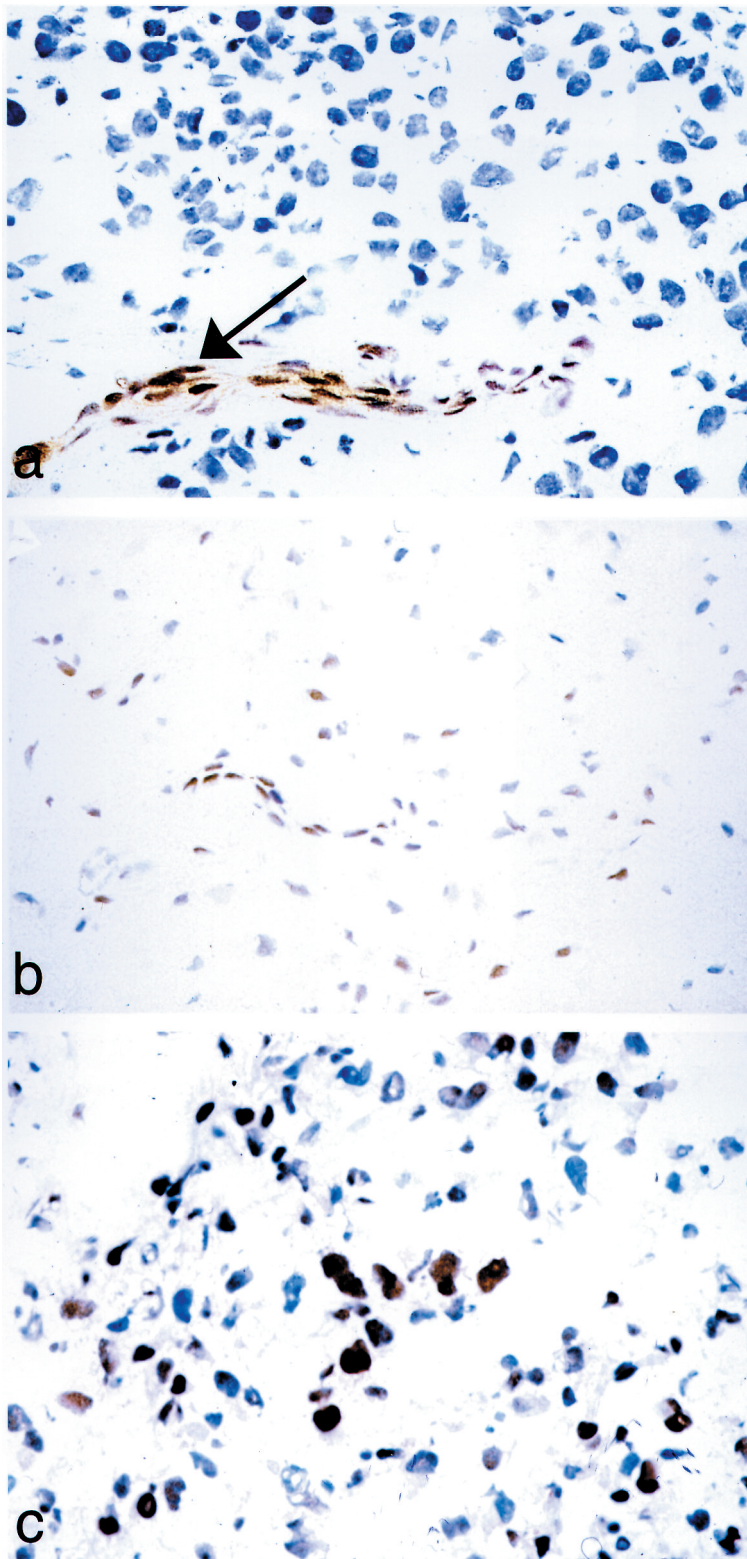


Fig. 2. Patterns of pRB staining in STS using mAb 3C8. **(A)** Sarcoma demonstrating negative pRB staining in tumor cells. The arrow indicates intense staining in vascular endothelial cells; **(B)** another tumor demonstrating wild-type pRB staining, and **(C)** a third tumor demonstrating accumulation of pRB. Original magnification: A, C, x 400; B x 200

data suggest that altered pRB expression is associated with a loss of cell cycle control due to the lack of a functional pRB.

To characterize the biological basis for these altered expression patterns, we stained all cases with mAb G99-549, specific for the underphosphorylated form of pRB (see above and Fig. 1). Cases in Group 3 that demonstrated intense, homogeneous staining in a substantial proportion of tumor nuclei using mAb 3C8 showed undetectable or markedly reduced staining using mAb G99-549 (Fig. 4, compare left and right panels). A comparison between the staining patterns using this antibody and 3C8 is described graphically for Group 3 cases in Figure 5. Cases in Group 1 (wild-type staining using 3C8) still had heterogeneous staining with G99-549, although some cases were negative.

In Group 2 (cases lacking pRB expression), we investigated the potential association between undetectable pRB by immunohistochemistry and genetic losses. We used the microsatellite marker RB 1.20 to assess loss-of-heterozygosity at the RB locus. This intronic polymorphism resides near the 3' end of exon

20 and has been shown to be 78% to 94% informative (Yandell and Dryja, 1989; Henson et al., 1994). We analyzed 34 matched pairs of normal and tumor tissue available for our cohort of 86 patients, and 30 of 34 cases were informative for a rate of 88%. In one case from Group 3 the RB1.20 locus could not be amplified from tumor DNA, resulting in 29 usable cases. Overall, LOH was observed in 10/29 (34%) cases. A representative electropherogram demonstrating loss of one allele is shown in Figure 6. We observed an excellent correlation between the wild-type protein

Table 1. Correlation between altered pRB expression and proliferative index as determined by Ki-67 immunoreactivity.

	Ki67		Total*
	-	+	
pRB Wild-type	10	3	13
pRB Altered	18	51	69
Total	28	54	82

p: 0.0008; *: 4/86 cases stained for pRB expression did not have available tissue sections for MIB-1 staining.

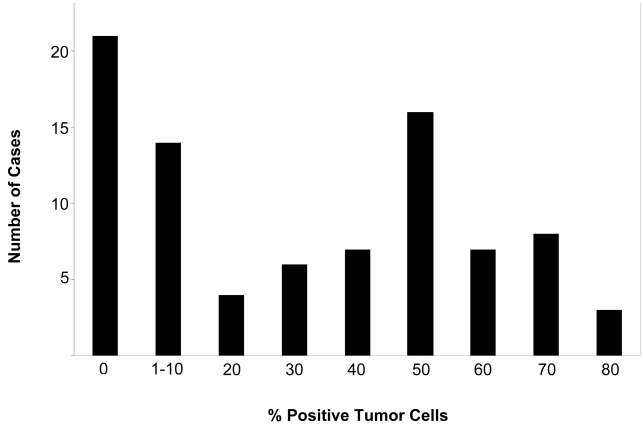


Fig. 3. Frequency distribution of 3C8-positive sarcomas. Histogram plot of the number of 3C8-positive cases by percentage positive tumor cells. Cases exhibiting between 20% and 80% positive tumor cells are distributed in a near Gaussian fashion, suggesting they belong to a single population. Cases that demonstrate less than 10% positive cells or 0% positive cells do not fall into this distribution. They appear to define different populations of tumors.

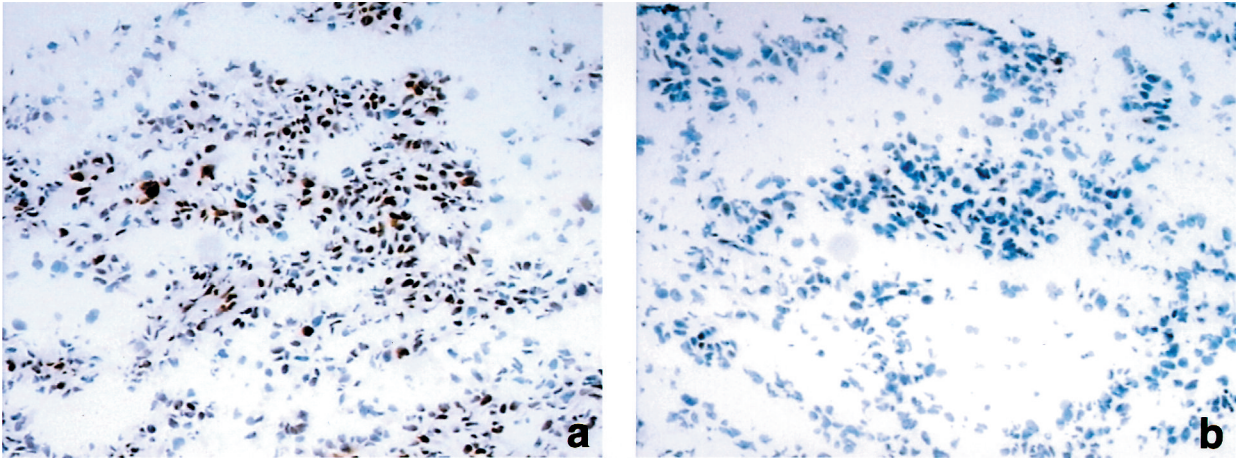


Fig. 4. Comparative immunohistochemical staining of a soft tissue sarcoma using mAbs 3C8 and G99-549. A soft tissue sarcoma from group 3 is stained with mAb 3C8 (a), that recognizes both the underphosphorylated and hyperphosphorylated forms of pRB, and G99-549 (b), that recognizes only the underphosphorylated form of pRB. Note the markedly decreased staining using the monoclonal antibody specific to the underphosphorylated form of pRB. Original magnification, x 200

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expression pattern (Group 1) and lack of LOH. Specifically, 9 of these 10 (90%) cases retained both alleles. In cases with no staining by IHC (Group 2) 5 of 8 (63%) tumors had evidence of LOH using this marker. The difference between these groups was statistically significant (Group 1 versus Group 2, $p=0.04$). In cases with evidence of pRB accumulation (Group 3), 7 of 11 (64%) cases retained both alleles.

Clinicopathologic correlations and survival analyses

To determine the clinical significance of pRB phenotypes, we correlated pRB staining profiles with clinicopathological parameters. Analysis of the entire cohort demonstrated a significant association between altered pRB staining, defined as either absent pRB expression (Group 2) or accumulation of pRB (Group 3), and tumor grade ($p \leq 0.001$). Specifically, 95% of patients in Group 2, and 85% of patients in Group 3 had high grade tumors. In contrast, only 36% of patients in Group 1 had high grade tumors. The survival curves of the

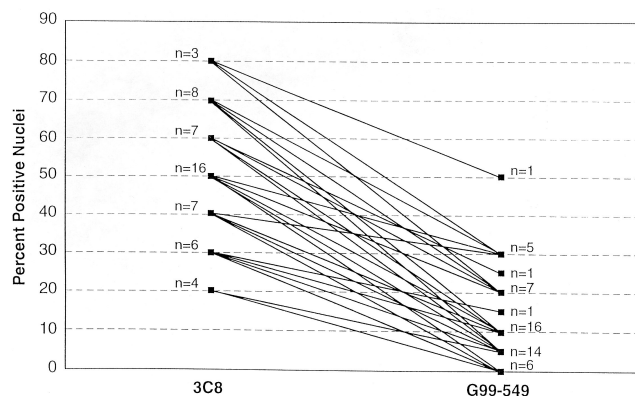


Fig. 5. Comparison of staining patterns in Group 3, pRB overexpressing cases using mAbs 3C8 and G99-549. The percentage of cells staining, plotted on the vertical axis, is compared between mAbs 3C8 (left) and G99-549 (right). See text for full discussion.

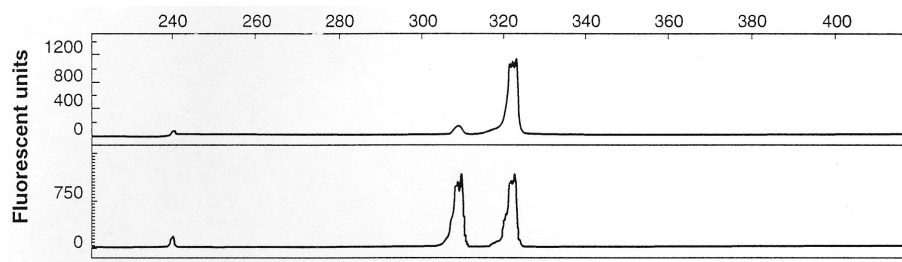


Fig. 6. Microsatellite Analysis. A representative electrophoretic profile of RB1.20 products from tumor DNA (top) and the corresponding normal DNA sample (bottom) is shown. In the tumor sample note the markedly decreased height of the lower molecular weight peak indicating loss of that allele in the tumor specimen.

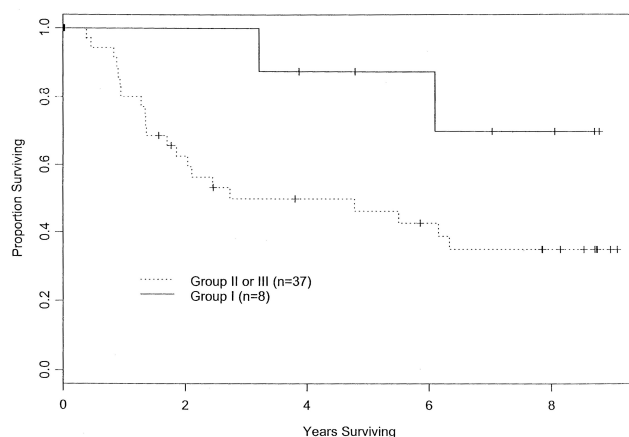
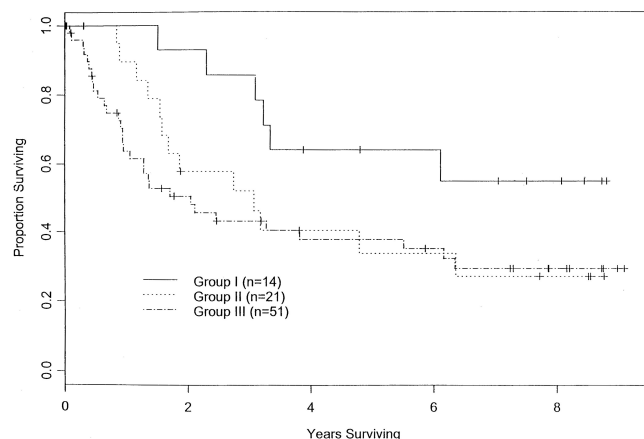


Fig. 7. Survival analysis. The Kaplan-Meier method was used to estimate survival and the log-rank method was used to compare the curves. **Left.** Analysis of the entire cohort demonstrates that the survival of patients in groups 2, no pRB, and group 3, accumulated pRB, were not statistically different. These groups had a markedly decreased survival when compared with group 1, wild-type pRB expression ($p=0.03$). **Right.** Survival analysis of patients with completely resected primary STS. The survival of patients in groups 2, no pRB, and group 3, overexpressed pRB, were not statistically different. These groups had a markedly decreased survival when compared with group 1, wild-type pRB expression ($p=0.09$).

Table 2. Patients with completely resected soft tissue sarcomas stratified by pRB expression (n=45).

Strata	N	# DOD	Univariate Analysis Cox Model		Multivariate Analysis Cox Model	
			Rel. Risks	p-value	Rel. Risks	p-value
Grade						
High	36	21	3.9	0.07	3.3	0.12
Low	9	2				
Size						
≥ 10 cm	18	5	3	0.03	2.9	0.05
> 10 cm	26	17				
Site						
Extremity	28	12	0.6	0.16	0.8	0.57
Other	17	11				
pRB Grouping						
pRB=0% or pRB>10%	37	21	3.7	0.08	2.3	0.28
pRB=1-10%	8	2				

three groups are shown in Figure 7A. We observed that patients whose tumors either lacked pRB (Group 2), or accumulated pRB (Group 3) had similarly poor outcomes in comparison to patients with wild-type expression (Group 1). This association was significant with $p=0.03$. Although this finding is not surprising given the strong correlation of these patterns with high tumor grade, it demonstrates that similar biologic behaviors of these tumors may be associated with two different mechanisms that disable RB function.

As the cohort contained primary, recurrent, and metastatic lesions, we sought to confirm the importance of altered pRB immunoreactivity in a more homogeneous subset of our patients. For this purpose, we analyzed the correlation of altered pRB expression with the survival of patients with primary, completely resected sarcomas (n=45). As illustrated in Figure 7B altered pRB phenotypes were still associated with decreased survival. However, the association did not reach statistical significance ($p=0.09$). To further explore the importance of altered pRB expression with regard to the pathologic factors of grade and size, known predictors or poor outcomes in these patients (Brennan et al., 2001), we performed univariate and multivariate analyses on this subset. As summarized in Table 2, high tumor grade conferred a high risk of death in univariate and multivariate analyses, $RR=3.9$, ($p=0.07$) and $RR=3.3$, ($p=0.12$), respectively, although size greater than 10 centimeters was the only variable to achieve statistical significance in this subset. Altered pRB expression conferred a high risk of death in univariate analysis $RR=3.7$; however, it failed to reach statistical significance in this subset ($p=0.08$).

Discussion

Soft tissue sarcomas have been classified histogenetically according to their morphological resemblance to normal cognate tissues. Currently, the best predictors of outcome for patients with soft tissue

sarcomas are the assessment of tumor grade and size, which are characteristics of poor differentiation and uncontrolled growth (Cormier and Pollock, 2004). The use of immunohistochemical procedures aimed at identifying differentiation antigens has led to the proper diagnosis of difficult sarcoma lesions. More recently, immunohistochemical and genetic markers have been utilized to further define growth deregulation in sarcomas. Previous studies from our group centered on correlating pRB immunostaining to clinical outcomes, without directly linking these expression patterns to the underlying genetic or epigenetic alterations affecting RB function (Cance et al., 1990; Karpel et al., 1995). As part of these analyses, we reported the significant association between high tumor grade and high proliferative Ki67 index (Hoos et al., 2001).

In recent years, the function of pRB in the cell cycle has been better delineated (reviewed in (Weinberg, 1995; Sherr and McCormick 2002)). pRB is a component of the G1/S checkpoint, acting as a transcriptional repressor when bound to proteins from the E2F family. Hyperphosphorylation of pRB causes it to dissociate from E2F proteins allowing transcription of many genes, including those involved in DNA synthesis, the hallmark of S phase. pRB phosphorylation during G1 is tightly controlled by cyclin-dependent-kinases (Cdk's), mainly Cdk4, their activating partners the D-type cyclins, and their inactivating regulators, the cyclin-dependent kinase inhibitors (Cki's), mainly p16^{INK4A}. In transformed cells, overexpression of cyclin D1 (Weinstat-Saslow et al., 1995), activating mutations of CDK4 (Wolfel et al., 1995), and inactivation of p16^{INK4A} (Kamb et al., 1994; Gruis et al., 1995; Orlow et al., 1999) have been observed. Each of these events could contribute to hyperphosphorylation of pRB and disrupt the G1/S checkpoint in a manner analogous to that when RB is altered at a genetic level. Our observation that accumulation of hyperphosphorylated pRB products was associated with increased proliferation is consistent with *in vitro* studies demonstrating increasing levels of pRB

as cells progress from G0/G1 to G2/M (Xu et al., 1991; Lasorella et al., 2000). Recently, we have shown that RB pathway defects are also associated with overexpression of the mitotic checkpoint protein Mad2, contributing to aneuploidy, a characteristic of advanced cancers (Hernando et al., 2004).

Building on our previous results, the current study provides evidence in human soft tissue sarcomas that loss of expression of pRB, or accumulation of pRB – including hyperphosphorylated products – is associated with loss of growth control, as evidenced by a high proliferative index. Accumulation of pRB was a more frequent alteration than absence of pRB expression in this study, and both events were associated with high tumor grade and decreased survival. These findings suggest that alteration of pRB, whether by genetic events or by post-translational modification, is one of the biologic determinants of a high proliferative index, high tumor grade and poor outcome in adult soft tissue sarcomas.

Previous studies in primary sarcomas from our group and others, have reported alterations of genes and proteins involved in the regulation of pRB phosphorylation. Specifically, we found that cyclin D1 overexpression was a frequent event in adult soft tissue sarcomas, and that it was associated with high grade tumors and poor survival (Kim et al., 2001). Amplification and overexpression of CDK4, mapping to 12q13-14 a region frequently amplified in human tumors, was reported to be a common event in osteosarcoma and to be associated with metastatic potential (Wei et al., 1999). Others and we have also reported the common inactivation of the cyclin-dependent kinase inhibitor p16/INK4A in soft tissue sarcomas, a phenomenon associated with an aggressive biologic behavior and poor outcome (Orlow et al., 1999).

Similar findings regarding pRB expression have been described in colon and bladder cancers. Yamamoto et al. (1999) described an association between increased pRB expression and malignancy in colon cancer. Using immunohistochemistry, they noted only weak expression of pRB in normal mucosa, and increased expression in adenomatous polyps and frank carcinomas. In bladder cancer, comparable patterns of altered pRB expression have been described. Similar to the findings presented herein, both the lack of pRB expression and the strong expression of pRB were associated with a poor prognosis (Cote et al., 1998). More recent work has shown that the accumulated pRB in bladder tumors contained hyperphosphorylated forms (Chatterjee et al., 2004).

In sum, data from this study provide evidence that inactivation of pRB is frequent in adult soft tissue sarcomas, that hyperphosphorylation is a common means by which pRB is inactivated, and that both genetic and post-translational mechanisms are associated with tumor cell proliferation and poor survival.

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