Original Paper

Pathobiology

Pathobiology 2016;83:41–46
DOI: 10.1159/000441227

Received: July 12, 2015
Accepted after revision: September 21, 2015
Published online: February 10, 2016

Raf Kinase Inhibitor Protein Expression and Prognostic Value in Soft Tissue Sarcomas

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Key Words
Raf kinase inhibitor protein · Sarcomas · Prognosis

Abstract
Objective: Soft tissue sarcomas (STSs) are heterogeneous tumors displaying multiple and complex molecular abnormalities with no specific pattern. Despite current therapeutic advances, the patients with STS still have a poor outcome, which makes it necessary to find out new prognostic markers. The Raf kinase inhibitory protein (RKIP) has been associated with prognosis in several human neoplasms; however, its role in STS is unknown. ***Methods:*** In the present study RKIP expression was assessed by immunohistochemistry in a series of 87 STSs, and its expression profile was associated with the patients' pathological parameters. ***Results:*** We found that RKIP is expressed in the cytoplasm of the great majority of cases, and absent in only approximately 18% of cases (16/87). Importantly, we observed that loss of RKIP expression was associated with poor outcome, constituting an independent prognostic marker. ***Conclusion:*** This is the first study assessing RKIP expression levels in STS. We showed that loss of RKIP expression is present in a small subset of cases; however, its absence was associated with poor survival and may be a potential marker for STS prognosis.

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Introduction

Soft tissue sarcomas (STSs) are a heterogeneous group of malignant neoplasms of >50 subtypes of mesenchymal origin and represent <1% of all types of cancer [1]. STS can occur in any soft tissue in the body and may have different etiologies, including external radiation therapy and occupational exposure to certain chemicals such as herbicides [2, 3]. Besides low STS incidence, these neoplasms are aggressive, and the treatment, when possible, is based on traditional chemotherapies, although it is often associated

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1015–2008/16/0831–0041$39.50/0

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Table 1. RKIP expression in STSs and associations with patient's clinicopathological features

Parameter	n	RKIP expression		p
		negative	positive	
Age				
>51 years	47	8 (17)	39 (83)	0.679
≤51 years	39	8 (20.5)	31 (79.5)	
Gender				
Female	34	6 (17.6)	28 (82.4)	0.854
Male	52	10 (19.2)	42 (80.2)	
Ethnicity				
Caucasian	64	12 (18.8)	52 (81.2)	0.953
Not Caucasian	22	4 (18.2)	18 (81.8)	
Localization				
Lower extremities	64	11 (17.2)	53 (82.8)	0.629
Upper extremities	23	5 (21.7)	18 (78.3)	
Classification				
Smooth muscle	17	3 (17.6)	14 (82.4)	0.230
Lipogenic	16	1 (6.2)	15 (93.8)	
Peripheral nerve	7	0 (0)	7 (100)	
Fibroblastic/myofibroblastic	27	5 (18.5)	22 (81.5)	
Miscellaneous	15	5 (33.3)	10 (66.7)	
Pleomorphic cells				
Absent	50	8 (16)	42 (84)	0.747
Present	32	6 (18.8)	26 (81.2)	
Grade				
Low grade (I)	18	2 (11.1)	16 (88.9)	0.447
High grade (II and III)	64	12 (18.8)	52 (81.2)	
Disease progression				
No	27	5 (18.5)	22 (81.5)	0.844
Yes	54	11 (20.4)	43 (79.6)	
Disease recurrence				
Absent	49	8 (16.3)	41 (83.7)	0.492
Present	36	8 (22.2)	28 (77.8)	
Metastasis				
Absent	47	7 (14.9)	40 (85.1)	0.303
Present	38	9 (23.7)	29 (76.3)	

Results are numbers of cases with percentages in parentheses; p values assessed by Pearson χ^2 test.

with resistance [2, 4]. STS cells have been identified to originate from various cell lines, which explains, in part, the variety of phenotype characteristics observed in this tumor [1, 4, 5]. Recent advances in the knowledge of oncogenic mechanisms underlying sarcomagenesis will hopefully translate into more effective molecularly based therapies [2, 3]. Despite some therapeutic improvements, metastasis and death remain a significant problem in half the STS patients, who present with high-risk disease [2]. To a better management of STS progression, the evaluation of prognostic markers is mandatory [6], and the search for more effective biological prognostic factors is desirable.

The Raf kinase inhibitory protein (RKIP; also known as PEBP1, for phosphatidylethanolamine-binding protein 1) is a widely expressed protein in normal human tissues, emphasizing its role in various physiological processes [7, 8]. Functionally, it is an intracellular regulator of important signaling pathways such as RAF/MEK/ERK, G-protein-coupled receptor kinase-2, nuclear factor κB and GSK3β transduction pathways [9–11]. RKIP has been shown to be a multifunctional protein in carcinogenesis, being implicated in the control of cellular growth, motility, epithelial to mesenchymal transition, differentiation, invasion and tumor metastasization [9–11]. Thus, many studies have implicated RKIP in the malignant progression of several human solid tumors, being an important prognostic biomarker for a number of tumors including prostate, colorectal, gastrointestinal stromal tumors, gastric adenocarcinoma of the intestinal subtype, hepatocellular carcinoma, pancreatic ductal adenocarcinoma and also high-grade gliomas [9, 12–15].

To the best of our knowledge, there are no reports of the RKIP expression in STSs. Thus, in this work we aimed to clarify the expression profile and clinical impact of RKIP immunohistochemistry expression in a well-characterized series of STSs [16–19].

Material and Methods

Tissue Samples

Eighty-seven samples of STSs were retrieved from the pathology archives of Barretos Cancer Hospital, Barretos, São Paulo, Brazil, following approval from the local ethical committee (CEP-331/2010). Relevant clinical-pathological data available included the patient's age, gender and race, tumor localization, diagnosis and grade (according to the French Federation of Cancer Centers Sarcoma Group [20]), presence of pleomorphic cells, disease progression, disease recurrence, presence of metastasis and follow-up, as specified in table 1. Not all information was available for all the patients [16–19].

Immunohistochemistry Analysis of RKIP

Representative 3-μm-thick tissue sections were used for immunohistochemical analysis according to the streptavidin-biotin-peroxidase complex system (UltraVision Large Volume Detection System Anti-Polyvalent HRP; LabVision Corporation), as previously described [21–25]. Briefly, deparaffinized and rehydrated slides were submitted to heat-induced antigen retrieval for 20 min at 98°C with 10 mM citrate buffer (pH = 6.0). After incubation with the primary antibody raised against RKIP (dilution 1:600, incuba-

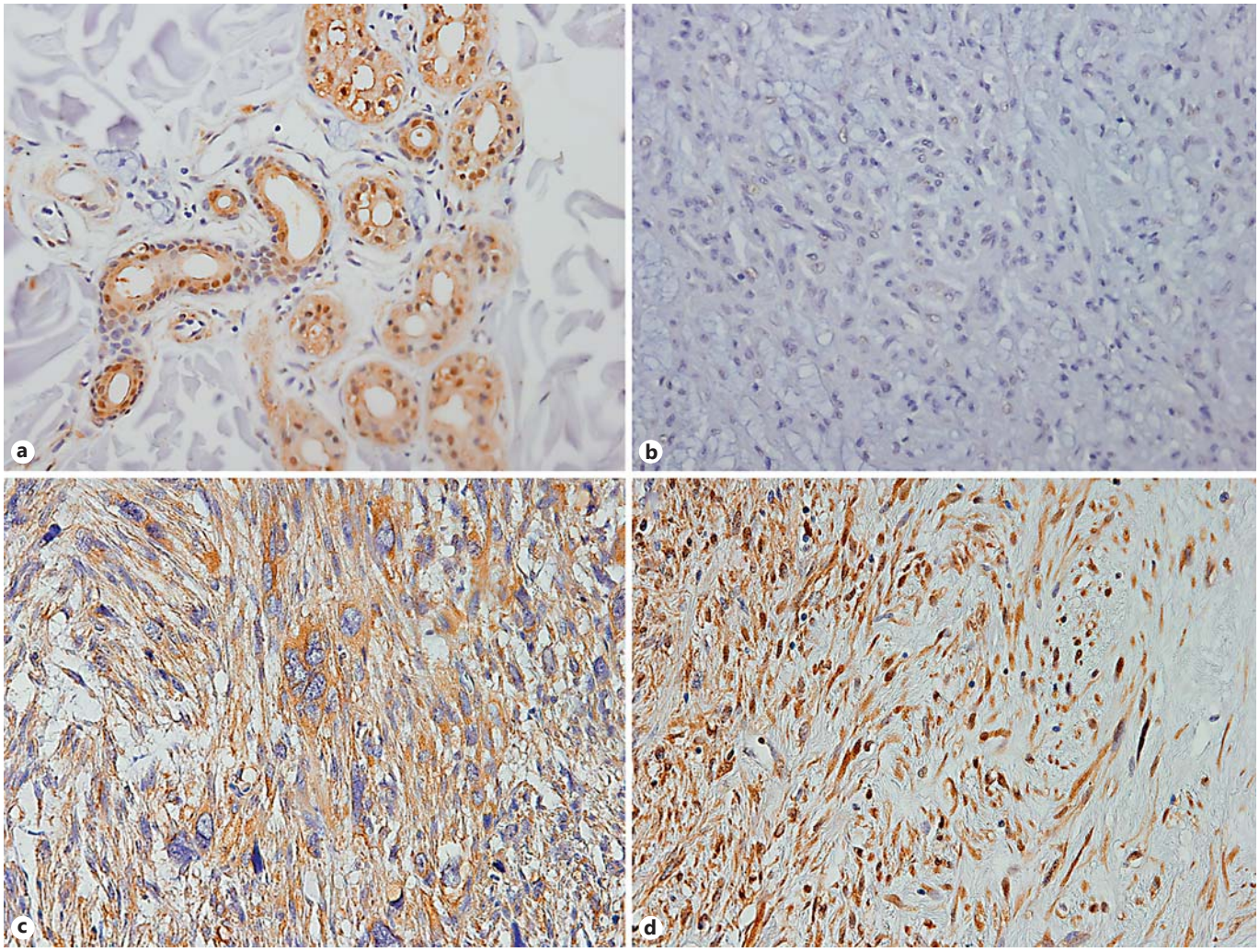

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Fig. 1. Immunohistochemistry analysis of RKIP in STS. Fibroblastic/myofibroblastic tumor sample with positive staining in the normal adjacent (**a**) and negative expression in the tumor counterpart (**b**). ×200. **c** Leiomyosarcoma of the smooth muscle depicting positive staining. ×200. **d** A tumor with miscellaneous differentiation showing positive expression. ×200.

tion 1 h at room temperature; Upstate Biotechnology), the secondary biotinylated goat antipolyvalent antibody was applied for 10 min followed by incubation with the streptavidin-peroxidase complex. The immune reaction was visualized by 3,3′-diaminobenzidine as a chromogen. All sections were counterstained with Gill-2 hematoxylin. For negative controls, primary antibodies were omitted and also replaced by a universal negative control antibody (CEA, rabbit anti-human, DAKO Corporation). A prostate carcinoma was used as positive control.

Sections were scored double-blind for cytoplasmic expression following a semiquantitative criterion. The score used was the sum of the percentage of positive cells (0, negative; 1, less than 25% positive cells; 2, 26–50% positive cells; 3, more than 50% positive cells) and the staining intensity (0, negative; 1, weak; 2, moderate; 3, strong). Scores between 0 and 2 were classified as negative, 3 and 4 as moderately positive, and 5 and 6 as strongly positive [21–25].

Correlations between RKIP expression and clinical data of the patients were performed using the χ^2 test.

Statistical Analysis

The statistical analysis was performed using SPSS software for Windows, version 17.0.

Results

Characterization of RKIP Expression in STSs

In the present study, 87 STSs were studied for RKIP immunohistochemical expression. RKIP positivity was found in the cytoplasm of the great majority of samples,

Table 2. Prognostic factors in STSs

Parameter	n	Univariate analysis		Multivariate analysis	
		mean months ± SD	p	HR	p
Pleomorphic cells					
Absent	49	62.9±4.9		1	
Present	31	37.9±5.0	0.002	3.0 (1.4–6.6)	0.006
Metastasis					
Absent	46	68.7±4.8		1	
Present	38	40.5±4.3	0.002	1.9 (0.8–4.5)	0.157
Disease progression					
No	27	73.1±5.9		1	
Yes	54	48.0±4.5	0.013	3.4 (1.0–12.0)	0.053
RKIP expression					
Positive	68	50.9±4.6		1	
Negative	16	30.2±5.1	0.007	3.0 (1.3–6.9)	0.009

SD = Standard deviation; HR = hazard ratio; figures in parentheses are 95% confidence intervals.

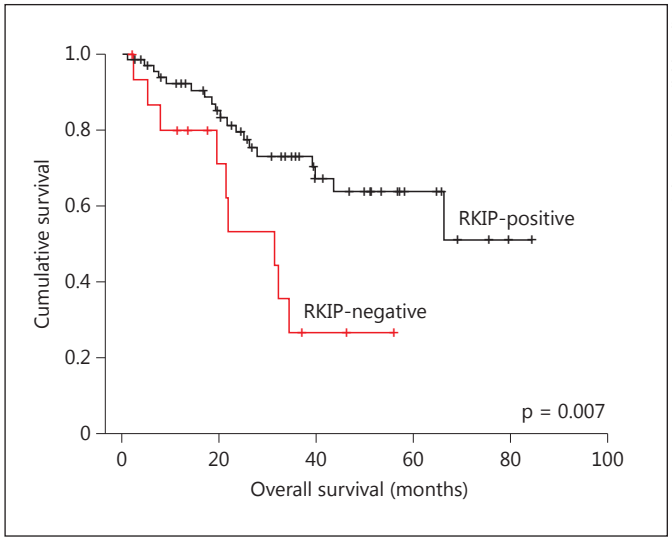

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Fig. 2. Disease-specific survival according to RKIP expression in STS (n = 84). Cumulative survival is significantly lower in cases with RKIP loss of expression (p = 0.007).

but also in the nucleus of some normal and tumoral tissues (fig. 1). When the normal adjacent tissue was available, RKIP expression was found to be highly positive (fig. 1a). Overall, considering only the cytoplasmic expression, RKIP was found to be highly expressed in the tumor tissues, its expression being negative in only 18.4% (16/87) of the cases (fig. 1b). No statistical differences were observed between RKIP expression and the tumors' histological subtypes (table 1); however, the lowest RKIP expression was found in the miscellaneous tumors (33% of the samples have absence of the protein). Specifically, by subgrouping the cases in 5 different classifications, RKIP positivity was found in 100% (7/7) of the peripheral nerve tumors, in 93.8% (15/16) of the lipogenic tumors, in 82.4% (14/17) of the smooth muscle tumors (fig. 1c), in 81.5% (22/27) of fibroblastic/myofibroblastic tumors, and in 66.7% (10/15) of the miscellaneous tumors (fig. 1d; table 1). Furthermore, no significant associations were found between RKIP expression and the other clinical-pathological parameters available (table 1).

Correlations with Patient Survival

Regarding the follow-up information, it was available for 84 patients, and overall survival was defined as the time between the date of first consultation and the date of last information or patient death (table 2). For univariate analysis we determined the cumulative survival probabilities, calculated using the Kaplan-Meier method, and the differences between survival rates were tested using the log-rank test. We found a significant association (p = 0.007) between absence of RKIP expression and poor prognosis in STSs (table 2; fig. 2). As it was expected, we also observed a significant association of other clinicopathological features of tumors, such as presence of pleomorphic cells, the presence of metastasis and disease progression with patient prognosis by univariate analysis

(table 2). Multivariate analysis was done using the Cox proportional hazards model, where we observed that only the absence of RKIP expression, the presence of pleomorphic cells and disease progression were independent prognostic markers in this series of STSs.

Discussion

STSs have a poor prognosis since they are often diagnosed in advanced stages, frequently with metastasis [2, 3]. Therefore, it is important to identify reliable biomarkers for STS outcome.

Loss of RKIP expression is pathologically linked to cancer development [9, 12–15], and initial studies have characterized it as a metastasis suppressor [14]. RKIP is a *bona fide* inhibitor of RAF-MEK-1 by preventing activation of the RAF/MEK/ERK signal transduction [26]. Importantly, some alterations in the MAPK pathway were already described in STS. RAF1 and MEK-1/2 mRNA were detected in STS cell lines that demonstrated dose- and time-dependent inhibition of cell growth when treated with a MEK inhibitor [27]. Similar observations have been reported with sorafenib that inhibited the MKK pathway and induced apoptosis [28]. In a xenograft model, MKK signaling was necessary for tumor growth and vascularization, suggesting that MKK had a predominantly proangiogenic effect in this model [29]. Thus, we hypothesized that the constitutive activation of this pathway due to the absence of its endogenous regulator RKIP can be involved in STS poor prognosis markers. However, to date, no large case series have evaluated the expression of RKIP in STSs.

In the present study we showed that RKIP immunohistochemistry loss is an independent prognostic factor in a well-characterized series of 87 STSs [16–19].

According to the literature, RKIP expression is absent in metastatic tumors with high expression in primary tumors [9, 30, 31]. Our results are in accordance with this observation, since we found that the majority of STSs express RKIP; yet, it was observed that among the RKIP-negative tumors, there were 9 that presented metastasis and 7 with no evidence of metastasization (table 1). In our analysis no correlations between the presence of RKIP expression and histological type of tumor or other clinical-pathological parameter were found (table 1). On the other hand, our results showed a statistical correlation with the absence of RKIP molecule expression and poor prognosis of STSs (table 2). These data are in accordance with other papers from our group, evaluating the RKIP protein expression profile in different types of tumors such as gastrointestinal stromal tumors, bladder cancer, gastric cancer and gliomas [12, 15, 21–25, 30]. Thus, in the present study we showed that RKIP immunohistochemistry loss is an independent prognostic factor in a well-characterized series of 87 STSs [16–18].

We are aware that our cohort comprises a wide variety of tumor types with divergent biological behavior, histological grade and differentiation, and such heterogeneities could at least in part interfere with our results. However, we have already described associations with prognosis of other proteins in this same series of cases [16–19], and we found that the presence of metastasis, a well-known prognostic factor in STS, is also associated in our series with poor prognosis, validating the reliability of our data and the series that we are studying.

Despite the increasing importance of RKIP as a metastasis and prognostic marker in human cancer, the mechanisms of RKIP downregulation remain to be elucidated [32]. Some studies have investigated the methylation status of the RKIP promoter in colorectal cancer as a possible mechanism; however, the results are discrepant [32–34]. Other studies have suggested that RKIP is under the regulation of the transcription repressor Snail [35], the tumor suppressor gene *TP53* [36], or a double-negative feedback loop by the transcription factor BACH1 [37]. Further studies are needed to understand the mechanisms associated with RKIP downregulation in STS.

The understanding of the mechanisms regulating RKIP expression in STSs would be important to explore the potential mechanism of RKIP reactivation in order to revert the negative effects of its absence. Another issue that deserves further studies is the role of RKIP in STS therapy response, since this protein has also been involved in the modulation of therapy response [38, 39].

In conclusion, we report for the first time the expression profile of RKIP in a heterogeneous group of STS. We showed that only a small subset of cases exhibited RKIP loss, yet its loss is an independent biomarker of prognosis.

Acknowledgments

This project was partially supported by a CNPq Universal Grant (476192/2013-7) to Rui Manuel Reis. Olga Martinho is recipient of a Post-Doc fellowship (SFRH/BPD/108351/2015) from Fundação para a Ciência e Tecnologia (FCT), Portugal.

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