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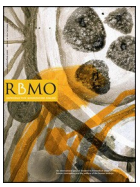

ARTICLE

Raf kinase inhibitor protein expression in smooth muscle tumours of the uterus: a diagnostic marker for leiomyosarcoma?

BIOGRAPHY

Dr Ciarmela is an Associate Professor of Human Anatomy at Università Politecnica delle Marche, Italy. Her research focus is uterine physiology, pathophysiology and the role of growth factors. The aim of her research is to understand the pathogenesis of female diseases and to develop potential therapeutic agents.

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KEY MESSAGE

Raf kinase inhibitor protein (RKIP) was expressed in bizarre leiomyomas and in usual-type leiomyomas. Although it was generally absent in leiomyosarcomas, it could be useful in the diagnosis of leiomyosarcoma. RKIP knockout on a leiomyosarcoma cell line increased the ability of cells to form colonies.

ABSTRACT

Research question: What is the expression pattern of Raf kinase inhibitory protein (RKIP) in different subtypes of leiomyoma (usual type, cellular, apoplectic or haemorrhagic leiomyoma, leiomyoma with bizarre nuclei and lipoleiomyoma) and leiomyosarcoma specimens, and what is its biological role in leiomyosarcoma cells?

Design: Leiomyoma and leiomyosarcoma specimens underwent immunohistochemistry staining. Leiomyosarcoma SK-LMS-1 cell line was RKIP knocked down and RKIP overexpressed, and cell viability, wound healing migration and clonogenicity assays were carried out.

Results: A higher immunohistochemical expression of RKIP was observed in bizarre leiomyomas, than in usual-type leiomyomas. Decreased expression was also found in cellular leiomyoma, with generally absent staining in leiomyosarcomas. Upon RKIP expression manipulation in SK-LMS-1 cell line, no major differences were observed in cell viability and migration capacity over time. RKIP knockout, however, resulted in a significant increase in the cell's ability to form colonies ($P = 0.011$).

Conclusion: RKIP distinct expression pattern among leiomyoma histotype and leiomyosarcoma, and its effect on leiomyosarcoma cells on colony formation, encourages further studies of RKIP in uterine smooth muscle disorders.

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KEYWORDS
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INTRODUCTION

Smooth-muscle tumours are common gynaecologic neoplasms with a high heterogeneity of presentation. Uterine leiomyoma is an extremely frequent benign mesenchymal neoplasm. According to the 2020 World Health Organization, smooth muscle neoplasms can be categorized as leiomyoma, leiomyosarcoma or smooth muscle tumours of uncertain malignant potential (STUMP). The diagnosis of malignant potential is still assessed by histological evaluation, mainly considering cytologic atypia, mitotic index and coagulative tumour cell necrosis (*Hohn et al., 2021*).

Most leiomyomas derive from a transformed somatic stem cell, and genetic, epigenetic factors, sex steroids, growth factors, cytokines, chemokines, inflammatory cells, vascularization and extracellular matrix components seem to promote its development and growth (*Protic et al., 2016*; *Giuliani et al., 2019*; *Zannotti et al., 2021*; *Ciarmela et al., 2022*). Extracellular matrix is an important driver in leiomyoma development, and acts as a reservoir for growth factors, cytokines, chemokines, angiogenic and inflammatory factors, with a predominance of collagen subtypes, fibronectin and proteoglycans (*Ciarmela et al., 2011*; *Islam et al., 2016*; *2018*). Macroscopically benign smooth muscle lesion generally appears as a well-circumscribed round lesion, variable in number and size, and ranging from a few millimetres up to 20 cm in diameter (*Walker and Stewart, 2005*).

Microscopically uterine leiomyoma has a wide range of morphological subtypes. Although the usual-type leiomyoma is the most common, different histological variants are easily recognized: cellular or highly cellular leiomyoma, lipoleiomyoma, apoplectic leiomyoma and bizarre nuclei leiomyoma. Even if the histologic criteria of benign smooth muscle tumours are well established, subtypes can be a diagnostic challenge, especially in the case of bizarre nuclei leiomyoma. In a diagnostic setting, differentiation of bizarre nuclei leiomyoma from leiomyosarcoma can be challenging when histological features overlap, especially by using pathologic morphological analysis alone (*Guo et al., 2022*). Moreover, the diagnosis between leiomyoma and its malignant counterpart can lead to an increase of the questionable category of STUMP. In fact, in 2014 the World Health Organization introduced a category of STUMP that includes all lesions not unequivocally benign or malignant. Smooth muscle tumours of uncertain malignant potential do not fulfill the histologic features for benign leiomyoma or its variants, and its behaviour may be malignant. In 15% of cases, patients with STUMP can relapse with a second localization of STUMP or leiomyosarcoma (*Denschlag et al., 2019*).

Histologically usual-type uterine leiomyoma shows a well-delineated edge and is composed of spindle cells arranged in intersecting fascicles. The cells have eosinophilic cytoplasm and cigar-shaped nuclei with small nucleoli and rare mitosis, usually less than 5 mitotic figures per 10 high power fields, and no significant atypia.

Cellular leiomyoma is characterized by increased cellularity, without atypia and mitotic figures. Lipoleiomyoma shows up as a combination of leiomyoma and mature adipocytes with benign behaviour (*Wang et al., 2006*), whereas apoplectic or haemorrhagic leiomyoma is a combination of stellate zones of recent haemorrhage within nodules of hypercellular smooth muscle, with no nuclear atypia, abnormal mitotic figures or tumour cell necrosis (*Myles and Hart, 1985*). Malignant lesions can be distinguished by increased mitotic rate, atypia and necrosis. Leiomyosarcoma diagnostic hallmarks are coagulative tumour cell necrosis, 15–30 mitotic figures/10 high power fields, and marked pleomorphism. Histopathological diagnosis of STUMP is a rare event in the case of tumours that do not fulfill all the characteristics of leiomyosarcoma, but still have moderate to severe cytological atypia, an increased mitotic activity (6–9 mitoses/10 high power fields) or atypical mitotic figure and ambiguous tumour cell necrosis (*Gupta et al., 2018*; *Gadducci and Zannoni, 2019*). In any case, considering that the diagnostic hallmark of leiomyoma with bizarre nuclei consists of multifocal or diffuse, moderate or severe atypia, it is easy to understand how, in this subtype, benign and malignant characteristics may overlap. For this reason, tumours can be misdiagnosed as STUMP (*Travaglino et al., 2021a*; *2021b*). In this context, immunohistochemistry (IHC) plays a key role in confirming smooth muscle differentiation by smooth muscle-specific actin, desmin and h-Caldesmon; however, the prognostic utility of IHC is still limited. Several IHC stains have been proposed for the detection of lesions at higher risk of malignancy, including p53, p16, KI-67, oestrogen and progesterone receptors. These newly established IHCs can be additional tools for differential diagnosis. *Adams* et al. *(2019)* found that IHC for KI-67 showed different expressions in leiomyosarcoma and leiomyoma. Moreover, an increased level of p53 mRNA is linked to an overexpression of p53 protein in leiomyosarcoma, and it could play an important role in the tumourigenesis of this rare malignant tumour (*Chen and Yang, 2008*; *D'Angelo and Prat, 2010*). All markers used to assess the behaviour of the myometrial lesions, however, are still inconsistent, and histopathological evaluation sometimes remains inconclusive (*Rubisz et al., 2019*).

Raf kinase inhibitory protein (RKIP) is a member of the family of phosphatidylethanolamine-binding proteins It inhibits the MAPK pathway, and thus the RAF-dependent activation of MEK and ERK (*Yeung et al., 1999*). Raf kinase inhibitory protein physically interacts with four kinases (TAK, NIK, IKKa and IKKb) (*Yeung et al., 2001*) by inhibiting their transcriptional activities (*Tang et al., 2010*), which subsequently inhibits the NF-kappaB pathway. If the expression or function of RKIP is dysregulated, pathophysiological change occurs.

Raf kinase inhibitory protein is a multifunctional protein with an emerging role as a regulator of several intracellular signalling pathways, and is associated with an increasing number of diseases (*Gabriela-Freitas et al., 2019*). It is widely expressed in healthy tissues; however, loss of its expression and its correlation with poor prognosis, aggressiveness and treatment resistance has been observed in several malignancies (*Cardoso-Carneiro, 2020*). Indeed, the role of RKIP as a metastasis suppressor protein is well established, having been described first in prostate cancer models (*Yeung et al., 2001*; *Rubisz et al. 2019*).We have recently identified the presence of RKIP in myometrium and leiomyoma, observing the higher expression of RKIP in leiomyoma than in adjacent myometrium (*Janjusevic et al., 2016*).

The aim of the present study, therefore, was to verify the expression of RKIP in leiomyoma and its subtypes, in leiomyosarcoma, especially to determine its biological role in leiomyosarcoma.

MATERIALS AND METHODS

Sample collection

The sample for this study was derived from pathological anatomy, Azienda Ospedaliera Ospedali Riuniti di Ancona, Università Politecnica delle Marche, Ancona and from Department of Woman and Child Health, Fondazione Policlinico Universitario A. Gemelli IRCCS, Rome. All surgical specimens were sent to the histopathology unit and fixed in 4% formalin for 12–18 h, processed with an automatic and fully paraffin-embedded processor and evaluated by an experienced pathologist using well-established histopathologic criteria. The study included five different types of leiomyomas: usual ($n = 7$), cellular ($n = 9$), apoplectic ($n = 4$), lipoleiomyoma ($n = 3$), bizarre nuclei ($n = 4$) and leiomyosarcoma ($n = 12$). All samples were collected between 2003 and 2018. Permission was granted by the Human Investigation Committee of Marche (protocol number 2015 0486, approved on 10 February 2016) was granted.

Cell line culture

The SK-LMS-1 cell line was obtained from ATCC (RRID: CVCL_0628, American Type Culture Collection Manassas, VA, USA). The cell line was maintained in Dulbecco's Modified Eagle's Medium (DMEM) (DMEM 1X, High Glucose; Gibco) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco) (Invitrogen, Carlsbad, CA, USA) and 1% penicillin/streptomycin (Gibco) (Invitrogen, Carlsbad, CA, USA), at 37°C and 5% CO_2.

Haematoxylin and eosin

Tissue sections embedded in paraffin were rehydrated through xylene and a descending graded series of ethyl alcohol. After adding the 50% alcohol, the sections were kept for 5 min in distilled water. They were then stained with haematoxylin (Bio-Optica, Milan, Italy) for 2 min, washed in distilled water, stained with eosin (Bio-Optica, Milan, Italy) for 2 min, washed in distilled water, dehydrated through an ascending graded series of ethyl alcohol and xylene and finally mounted with Eukitt Solution (Orsatec GmbH, Kindler GmbH and Co., Bobingen, Germany).

Immunohistochemistry

Paraffin sections were rehydrated through xylene and a descending graded series of ethyl alcohol. For the RKIP stain, antigenic site retrieval was carried out by immersing the sections in EDTA buffer 1 mM, PH 8 and incubating the samples at 100°C for 20 min in a water bath. To inhibit the endogenous peroxidase activity, the sections were incubated for 1 h with 3% hydrogen peroxide in methanol on a shaker. Subsequently, the sections were washed three times in phosphate buffered saline (PBS), incubated for 20 min at room temperature with normal goat serum (Vector Laboratories, Burlingame, CA, USA), and diluted 1:75. Sections were then incubated overnight at 4°C with RKIP rabbit polyclonal antibody (RRID: AB_11212485) (Merck-Millipore Burlington, MA, USA), and diluted 1:500.

The sections were then washed three times in PBS and incubated for 30 min at room temperature with biotinylated made-in-goat secondary antibody anti-rabbit IgG at a dilution equal to 1:200 (Vector Laboratories, Burlingame, CA, USA). After washing three times in PBS, the ABC method (Vector Laboratories, Newark, CA, USA) was used to localize the antigenic sites by incubating the sections for 1 h at room temperature, followed by washing three times in PBS, and incubation with 3,3'-diaminobenzidine hydrochloride (Sigma-Aldrich, St Louis, MO, USA), used as chromogen. The sections were counterstained in Mayer's haematoxylin (Bio-Optica, Milan, Italy), and subsequently dehydrated through an ascending graded series of ethyl alcohol and xylene, and mounted with Eukitt Solution (Orsatec GmbHm, Bobingen, Germany). Negative controls were prepared using non-immune serum in place of the first antibody.

For the IHC analysis of CD68, the rehydrated slides were not exposed to antigen retrieval. The inhibition of the endogenous peroxidase activity was carried out for 5 min at room temperature with 3% hydrogen peroxide in distilled water (dH2O), the slides were then washed for 5 min in dH2O and incubated with normal horse serum (dilution 2% in PBS homemade, incubation 20 min at room temperature) (Vector Laboratories, Newark, CA, USA). The primary monoclonal mouse antibody raised against CD68 clone KP1 (RRID: AB 2661840 DAKO) (Agilent, Copenhagen, Denmark) was used at a dilution of 1:200 and incubated overnight at 4°C. The secondary biotinylated horse anti-mouse antibody (Vector Laboratories, Burlingame, CA, USA) was used at a dilution of 1:200 and incubated for 30 min at room temperature. For negative controls, immune serum was used in place of the first antibody, or the secondary antibody was omitted.

Assessment of staining

The individual immunostaining intensity of the tissue section was assessed as absent (–), focal expression (+/), diffuse (+), and highly diffuse expression (++). Three independent assessors evaluated the stain.

Raf kinase inhibitory protein stable genetic manipulation in SK-LMS-1 cell line

For the generation of a stable RKIP knockout SK-LMS-1 cell line, the SK-LMS-1 cell line was knocked out using CRISPR/Cas9 technology. For that, the CRISPR/Cas9 knockout kit (CRISPR/Cas9 KO Plasmid - sc-401270-KO-2 and homology-directed repair (HDR) Plasmid – sc-401270-HDR-2) (Santa Cruz Biotechnology, Dallas, TX, USA) was used. For RKIP knockout, cells were transfected with both plasmids. As a control, the cells were also transfected only with homology-directed repair plasmid. For the generation of stable RKIP overexpressing SK-LMS-1 cell line, a pcDNA3 vector (kindly provided by Dr Evan Keller, University of Michigan) containing the full cDNA of RKIP, was used in the SK-LMS-1 cell line. The cells were transfected with the empty vector as control and with RKIP full cDNA containing vector to overexpress it. For both transfections, the cells were plated into six-well plates at a density of 5×10^5 cells per well, allowed to adhere overnight and transfected in serum-free Opti-MEM medium (Gibco) (Invitrogen, Carlsbad, CA, USA) for 24 h. FUGENE HD reagent (Roche, Basel, Switzerland) was used for transfection according to the manufacturer's protocol, with 2 mg of plasmid at a ratio of 6:2 (reagent: plasmid). Then, stable transfectants were selected with 1 mg/ml puromycin (Gibco) (Invitrogen, Carlsbad, CA, USA) for knockout transfection and 1000 mg/ml geneticin (G418) (Santa Cruz Biotechnology, Dallas, TX, USA) for overexpression transfection.

Western blot analysis

The cells (wild-type SK-LMS-1 cells, stable RKIP knockout SK-LMS-1 cells and the corresponding negative controls cells, stable RKIP overexpressing SK-LMS-1 cells and the corresponding negative controls cells) were seeded in six-well plates at a density of 1×10^6 cells per well and allowed to adhere overnight in DMEM (Gibco) (Invitrogen, Carlsbad, CA, USA) 10% FBS

(Gibco) (Invitrogen, Carlsbad, CA, USA) 1% penicillin/streptomycin (Gibco) (Invitrogen, Carlsbad, CA, USA). On the next day, the cells were serum-starved for 2 h before protein isolation. The cells were scraped in cold PBS (Gibco) (Invitrogen, Carlsbad, CA, USA) and lysed in buffer containing 50 mM Tris pH 7.6–8, 150 mM NaCl, 5 mM EDTA, 1 mM Na3VO4, 10 mM NaF, 10 mM NaPyrophosphate, 1% NP-40 and 1/7 of protease cocktail inhibitors (Roche, Basel, Switzerland).

A total of 10% SDS-PAGE gel was used for western blotting. A total of 40 mg protein wsa loaded per lane, with detection by chemiluminescence (Western Bright Sirius HRP substrate) (Advansta, San Jose, CA, USA) using Sapphire Biomolecular Imager (Azure Biosystems) (Dublin, CA, USA). The expression of the analysed proteins was assessed using the antibodies: RKIP (1:1000), vimentin (1:1000), N-Cadherin (1:1000), and phospho-MEK1/2 (1:1000) from Cell Signaling (Danvers, MA, USA), and b-tubulin (1:5000) from Santa Cruz Biotechnology (Dallas, TX, USA).

Cell viability assay

The cells were seeded in 48-well plates in triplicate at a density of 2×10^4 cells per well and allowed to adhere overnight in DMEM (Gibco) (Invitrogen, Carlsbad, CA, USA) 10% FBS (Gibco) (Invitrogen, Carlsbad, USA) 1% penicillin/streptomycin (Gibco) (Invitrogen, Carlsbad, CA, USA). The next day, the cells were submitted to two different medium conditions DMEM (Gibco) (Invitrogen, Carlsbad, CA, USA) 0.5% FBS (Gibco) (Invitrogen, CA, USA) 1% penicillin/streptomycin (Gibco) (Invitrogen, Carlsbad, CA, USA) and DMEM (Gibco) (Invitrogen, Carlsbad, CA, USA) 10% FBS (Gibco) (Invitrogen, Carlsbad, CA, USA) 1% penicillin/ streptomycin (Gibco) (Invitrogen, Carlsbad, CA, USA) and incubated for 24, 48 and 72 h. The viable cells were quantified over time by Sulforhodamine B assay (Sigma-Aldrich, St Louis, MO, USA). The day of condition imposition was considered to be 0 h time point. The results were calibrated to the starting value (time 0 h, considered as 100% of viability) and expressed as the mean § SD. The assays were carried out in triplicate four times.

Wound healing migration assay

The cells were seeded in six-well plates, allowed to adhere overnight, and cultured at 95% of confluence in DMEM (Gibco) (Invitrogen, Carlsbad, CA, USA) 10% FBS (Gibco) (Invitrogen, Carlsbad, CA, USA) 1% penicillin/streptomycin (Gibco) (Invitrogen, Carlsbad, CA, USA). Monolayer cells were washed with PBS (Gibco) (Invitrogen, Carlsbad, CA, USA), scraped with a plastic 1000 ml pipette tip, and then incubated with fresh DMEM (Gibco) (Invitrogen, Carlsbad, CA, USA) medium 1% penicillin/streptomycin (Gibco) (Invitrogen, Carlsbad, CA, USA) with either 0.5% FBS (Gibco) (Invitrogen, Carlsbad, CA, USA) or 10% FBS (Gibco) (Invitrogen, Carlsbad, CA, USA). The 'wounded' areas were photographed by phase contrast microscopy at specific time points: 0, 12, 24, 36, 48 and 72 h. The relative migration distance was calculated by the following formula: percentage of wound closure (%) = 100 (A-B)/A, where A is the width of the cell wounds before incubation (0 h) and B is the width of the cell wounds after incubation. The results are expressed as the mean § SD. The assay was carried out in quadruplicate three times.

Clonogenicity assay

The cells were seeded in 12-well plates at a density of 700 cells per well and they were allowed to adhere overnight in DMEM (Gibco) (Invitrogen, Carlsbad, CA, USA) 10% FBS (Gibco) (Invitrogen, Carlsbad, CA, USA) 1% penicillin/ streptomycin (Gibco) (Invitrogen, Carlsbad, CA, USA). Then, they were left to grow for 9–11 days DMEM (Gibco) (Invitrogen, Carlsbad, CA, USA) 10% FBS (Gibco) (Invitrogen, Carlsbad, CA, USA) 1% penicillin/streptomycin (Gibco) (Invitrogen, Carlsbad, CA, USA), with medium renewal after 3 days. The colonies were stained with 0.5% Crystal Violet (Sigma-Aldrich, St Louis, MO, USA) for 30 min and manually counted. Results were expressed as the mean colonies § SD. The assays were carried out in triplicate at least twice.

Statistical analysis

For in-vitro assays, Student's test was used for single comparisons between the different conditions studied, and differences between groups were tested using two-way analysis of variance. GraphPad Prism 9 version was used for statistical analysis. The level of significance in all the statistical analyses was set at $P < 0.05$.

RESULTS

Expression of Raf kinase inhibitory protein in different histotypes of leiomyoma and in leiomyosarcoma

Immunohistochemical staining was carried out to detect the RKIP expression in usual leiomyomas, cellular leiomyomas, apoplectic leiomyomas, lipoleiomyomas, bizarre leiomyomas and leiomyosarcomas. The immunostaining intensity of RKIP of all tissues analysed is presented in TABLE 1.

The presence of RKIP in endothelial cells was generally observed. Furthermore, the presence of RKIP in smooth muscle cells was observed, in usual-type leiomyomas (FIGURE 1B) ($n = 7$), and in different patients with apoplectic leiomyoma (FIGURE 2B) ($n = 4$). Haematoxylin and eosin staining was reported for usual leiomyoma (FIGURE 1A, panel A) and for apoplectic leiomyoma (FIGURE 2A, panel A); negative control for IHC staining was reported respectively (FIGURE 1A, panel B, and FIGURE 2A, panel B).

In lipoleiomyoma, RKIP was present in adipose cells and in the inflammatory component ($n = 3$) (FIGURE 3B, panels a and b), confirmed with immunostaining for CD68 (FIGURE 3B, panel C). Haematoxylin and eosin staining (FIGURE 3A panel A) and negative control for IHC staining (FIGURE 3A, panel B) were reported.

In cellular leiomyoma, the expression of RKIP was confined to endothelial and a few smooth cells ($n = 9$) (FIGURE 4B). Haematoxylin and eosin staining (FIGURE 4A, panel a) and negative control for IHC staining (FIGURE 4A, panel b) were reported.

Bizarre nuclei leiomyoma showed a diffuse stain of RKIP, located in pleomorphic cells ($n = 4$) (FIGURE5B).

In leiomyosarcoma tissue, RKIP expression was generally absent (TABLE 1) ($n = 12$). It was possible to observe negative RKIP immunoreactivity in case 4737-9 (FIGURE 6B, panel a). Positivity of RKIP in leiomyosarcoma specimens has been observed in some low-grade cancer or to be present with focal expression. Indeed, in FIGURE 6 (panel b), the staining of a patient (2067) with RKIP positivity restricted in the area close to the myometrium is reported, but the central part of the tumour is negative. Haematoxylin and eosin staining (FIGURE 5A, panel a), bizzarre leiomyoma, leiomyosarcoma (FIGURE 6A, panel a) and negative control for IHC staining (FIGURE 5A,

TABLE 1 EXPRESSION OF RAF KINASE INHIBITORY PROTEIN IN DIFFERENT HISTOLOGICAL TYPES OF LEIOMYOMA TISSUES ACCORDING TO IMMUNOHISTOCHEMICAL EVALUATION

Type of leiomyoma	Sample ID	Staining intensity	Cancer grade
Usual leiomyoma	16B24597	+	
	16B24330	+	
	16B24839	+/-	
	16B24705	+	
	16B28287	+	
	13-306-9	+	
	5892	+	
Apoplectic leiomyoma	16B18221	+/-	
	15B29363	++	
	15B4415	+	
	16B10567	–	
Lipoleiomyoma	16B23397	++	
	16B28597	++	
	16B17161	+	
Cellular leiomyoma	16B6611	+/-	
	15B282837	+/-	
	15B24965	–	
	15B19918	–	
	16B23869	–	
	16B09589	–	
	15B27194	+/-	
	12493	+/-	
	4724	+/-	
Bizzarre leiomyoma		+	
	1	+	
	5B18704	++	
	15B18248	+/-	
	16B18874	–	High grade
	16B18802	–	Low grade
Leiomyosarcoma	6375-1	–	Medium grade
	21707-	+	Epithelioid low grade
	4737-9	+/-	Low grade
	3285	+/-	High grade
	4188-D	–	High grade
	2067-H	–	Epithelioid low grade
	2497-4	–	Low grade
	5945-6	–	High grade
	24067-5	–	High grade
	8736-5	–	Low grade
	8096-15		
	25095-8		

panel b, and FIGURE 6A, panel b) were reported.

Effect of Raf kinase inhibitory protein on leiomyosarcoma biological behavior in vitro

To explore the biological role of RKIP in leiomyosarcoma cells, in-vitro knockout and overexpression of RKIP were carried out in the SK-LMS-1 cell line. As shown in FIGURE 7, RKIP protein expression was efficiently impaired by RKIP knockout, in SK-LMS-1 cells (knockout cells compared with the control SK-LMS-1 cells, transfected only with the HDR plasmid (calcitonin receptor cells [CTR]) ($P < 0.001$). In addition, RKIP protein expression was efficiently increased by RKIP overexpression, in SK-LMS-1 cells (RKIP+ cells), compared with the control SK-LMS-1 cells transfected with the empty vector (calcitonin receptor cells) ($P < 0.001$).

Concerning the biological assays, first, the effects of RKIP knockout and RKIP overexpression on leiomyosarcoma cell viability over time were studied (FIGURE 8). No statistically significant differences were found in cell viability of knockout cells compared with CTR at 0.5% FBS growth condition and 10% FBS growth condition (FIGURE 8A) ($P = 0.928$ and $P = 0.969$). No statistically significant differences were found in the cell viability of RKIP+ cells compared with empty cells neither at 0.5% FBS growth condition and 10% FBS growth condition (FIGURE 8B) ($P = 0.811$ and $P = 0.157$).

To study the effects of RKIP knockout and RKIP overexpression on leiomyosarcoma cellular migration, a wound-healing migration assay was conducted (FIGURE 9). When considering the assay as a whole, no statistically significant differences were found over time in the migration of knockout cells and RKIP+ cells compared with calcitonin receptor cells neither at 0.5% (FIGURE 9A) ($P = 0.273$) (FIGURE 9C) ($P = 0.294$) and 10% FBS growth conditions (FIGURE 9B) ($P = 0.168$) (FIGURE 9D) ($P = 0.085$). It is apparent that the curve separates at 48 h in knockout cells with 0.5% FBS ($P = 0.0208$) (FIGURE 9A) and RKIP+ cells with 10% FBS ($P = 0.0201$) (FIGURE 9D). The same, however, was not observed at 72 h, indicating that the differences at 48 h are a mere statistical artifact without biological meaning. Additionally, the effects of RKIP knockout and RKIP overexpression on the ability of leiomyosarcoma cells to form colonies were evaluated by a clonogenicity assay (FIGURE 10). It was found that knockout cells formed a significantly ($P = 0.011$) higher number of colonies than CTR cells (FIGURE 10A), whereas the number of colonies formed by RKIP+ cells was not statistically significant different from CTR cells (FIGURE 10B) ($P = 0.164$). Finally, to confirm whether the differences were caused by RKIP-driven alterations in the expression of mesenchymal markers or MAPK pathway, a western blot was conducted in the genetically manipulated cell lines (FIGURE 10C and FIGURE 10D). As can be observed, no major differences were found in MAPK activation, nor vimentin expression, but a decrease in N-Cadherin expression was found in RKIP+ cells compared with the respective CTR (FIGURE 10D) ($P = 0.033$), which was not corroborated with the RKIP knockout cells (FIGURE 10C) ($P = 0.387$). As the expression levels of N-Cadherin are similar between RKIP KO and RKIP+ cells, it is not clear whether the RKIP+ cells are losing N-Cadherin or whether it is the empty CTR cells that are overexpressing it.

DISCUSSION

In the present study, we observed that the RKIP was expressed in all types of leiomyomas, but with different degrees of

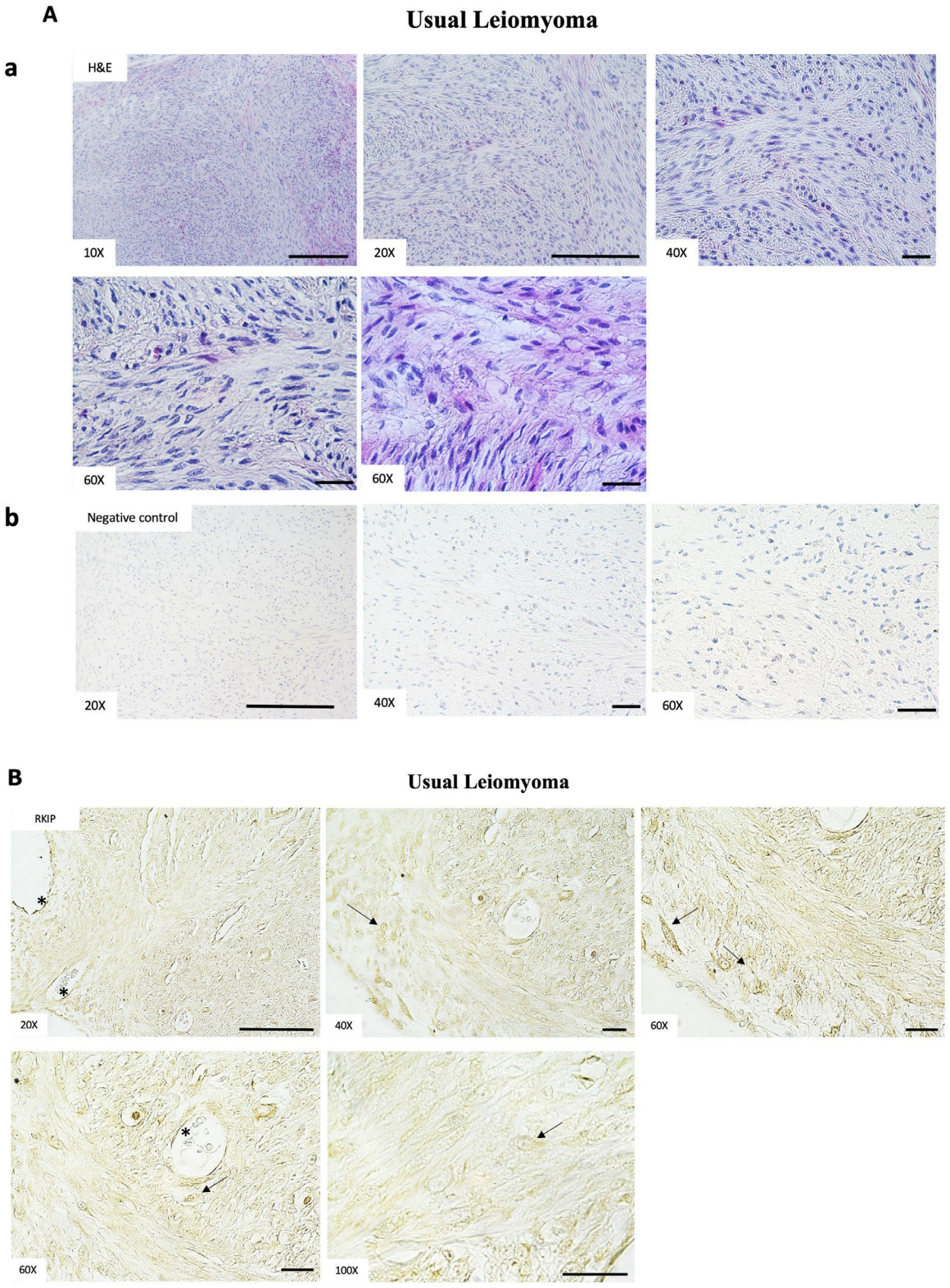

FIGURE 1 Expression of Raf kinase inhibitory protein (RKIP) in usual leiomyoma tissues (case 16B24330). (A) (panel a) with magnifications 10X, 20X, 40X and 60X shows haematoxylin and eosin (H&E) of the usual leiomyoma tissues; (panel b) with magnifications 20X, 40X and 60X shows immunohistochemical negative control (non-immune serum in place of the first antibody); (B) with magnifications 20X, 40X, 60X and 100X shows immunohistochemical staining for RKIP in usual leiomyomas. Arrows: smooth muscle cells). Representative images show that RKIP expression is also present in endothelial cells (*). Bar: 100 ym in micrographs with magnifications 10X and 20X, 10 ym in micrographs with magnifications 40X, 60X and 100X.

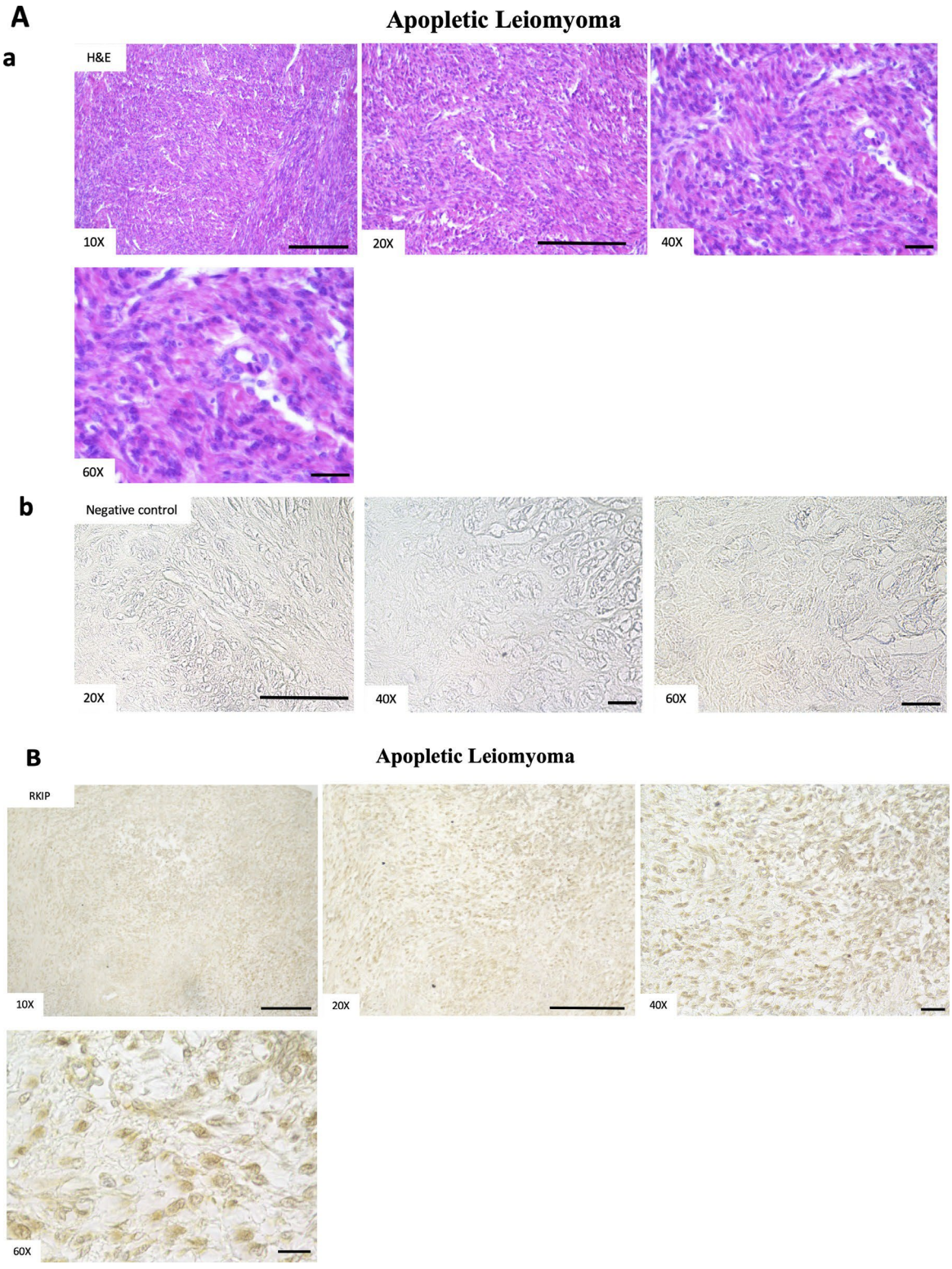

FIGURE 2 Expression of Raf kinase inhibitory protein (RKIP) in apoplectic leiomyoma (case 15B29363). (A) (panel a) with magnifications 10X, 20X, 40X and 60X shows the haematoxylin & eosin (H&E) of apoplectic leiomyomas tissues; (panel b) with magnifications 20X, 40X and 60X shows immunohistochemical negative control (non-immune serum in place of the first antibody); (B) with magnifications 10X, 20X, 40X and 60X shows the immunohistochemical staining for RKIP in apoplectic leiomyomas. RKIP is expressed in apoplectic cells. Bar: 100 ym in micrographs with magnifications 10X and 20X, 10 ym in micrographs with magnifications 40X, 60X and 100X.

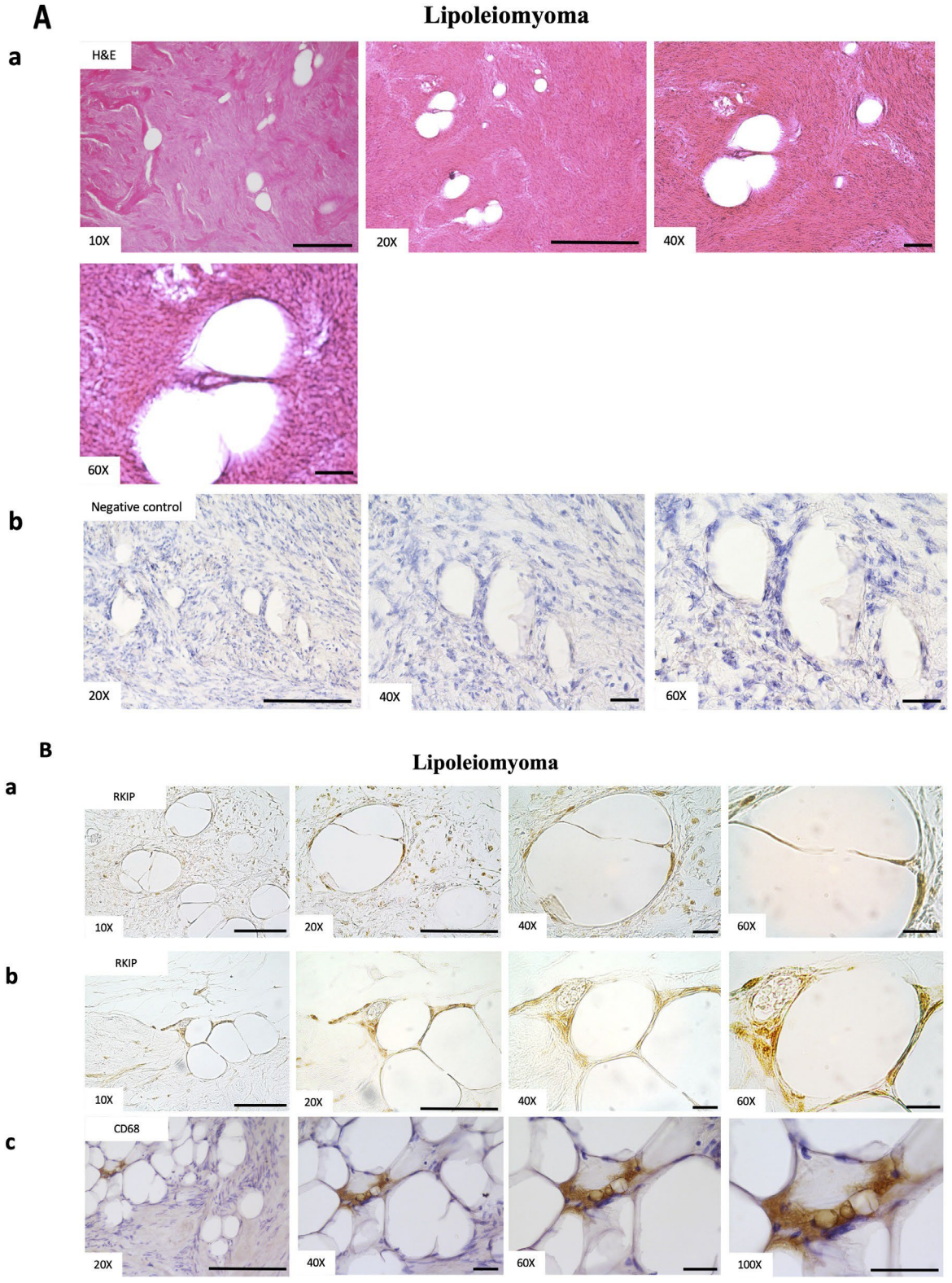

FIGURE 3 Expression of Raf kinase inhibitory protein (RKIP) in lipoleiomyoma tissues (16B23397). (A) (panel a) with magnifications 10X, 20X, 40X and 60X shows the haematoxylin and eosin (H&E) of lipoleiomyoma tissues; (panel b) with magnifications 20X, 40X and 60X shows immunohistochemical negative control (non-immune serum in place of the first antibody); (B) (panel a) with magnification 10X, 20X,40X and 60X shows the immunohistochemical staining for RKIP in lipoleiomyoma tissue, in particular, the expression of RKIP is present in adipocytes cells; (panel b) with a magnification 10X, 20X, 40X and 60X shows a high expression of RKIP in adipose cells with inflammation; (panel c) the inflammation was confirmed with the immunohistochemical staining for CD68, in with 20X, 40X, 60X and 100X magnifications, showing the high expression of CD68 and shows the 'crown-like structures' typical structures of adipose cells with inflammation. Bar: 100 ym in micrographs with magnifications 10X and 20X, 10 ym in micrographs with magnifications 40X, 60X and 100X.

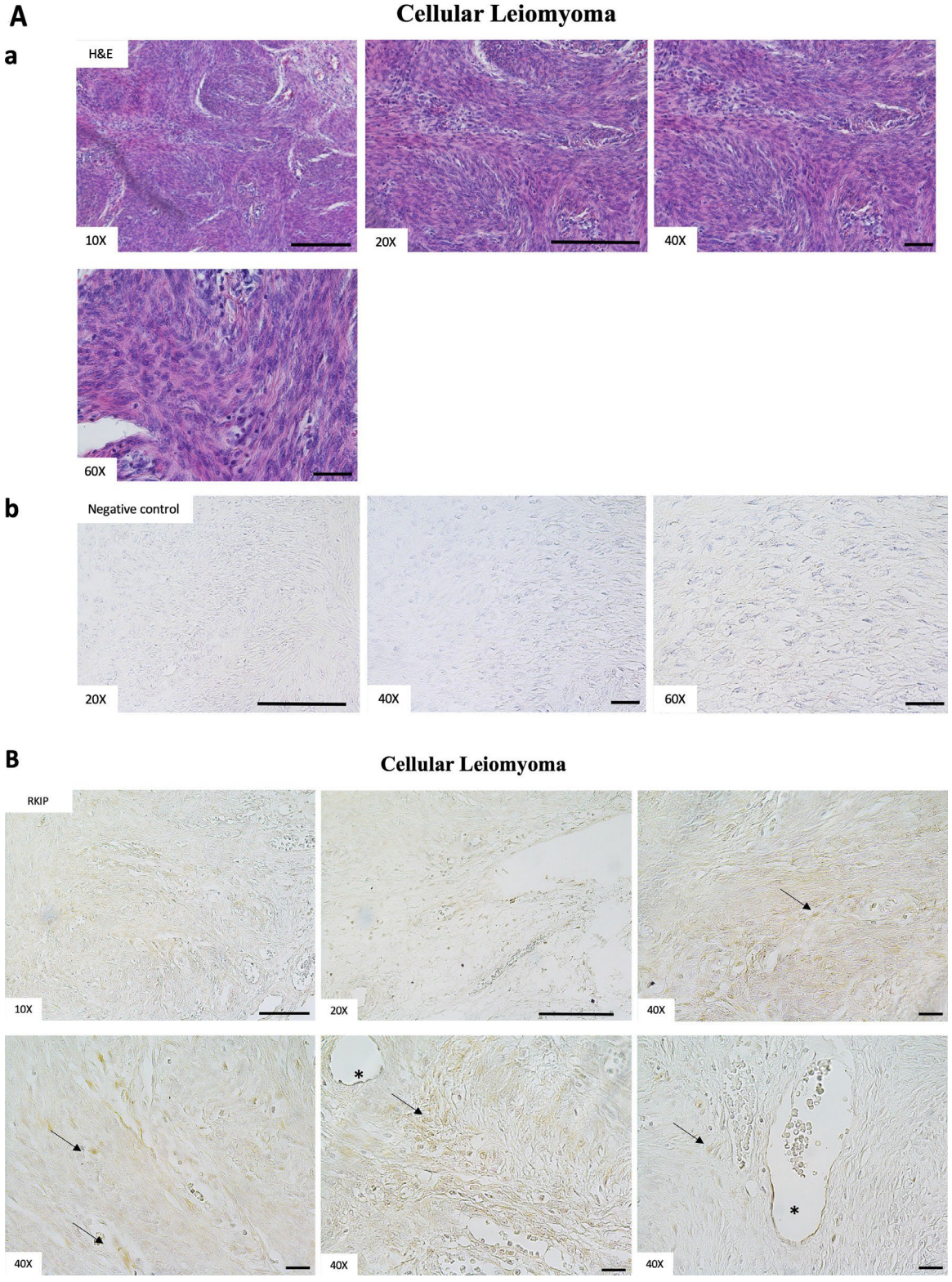

FIGURE 4 Expression of Raf kinase inhibitory protein (RKIP) in cellular leiomyoma tissues (case 16B6611). (A) (panel a) with magnifications 10X, 20X, 40X and 60X shows haematoxylin and eosin (H&E) of cellular leiomyomas; (panel b) with magnifications 20X, 40X and 60X shows immunohistochemical negative control (non-immune serum in place of the first antibody); (B) with magnifications 10X, 20X and 40X immunohistochemical staining for RKIP in cellular leiomyomas, shows low RKIP expression in cellular leiomyomas, whereas the expression of RKIP is present in endothelial cells (arrows: smooth muscle cells; asterix: endothelial cells). Bar: 100 ym in micrographs with magnifications 10X and 20X, 10 ym in micrographs with magnifications 40X, 60X and 100X.

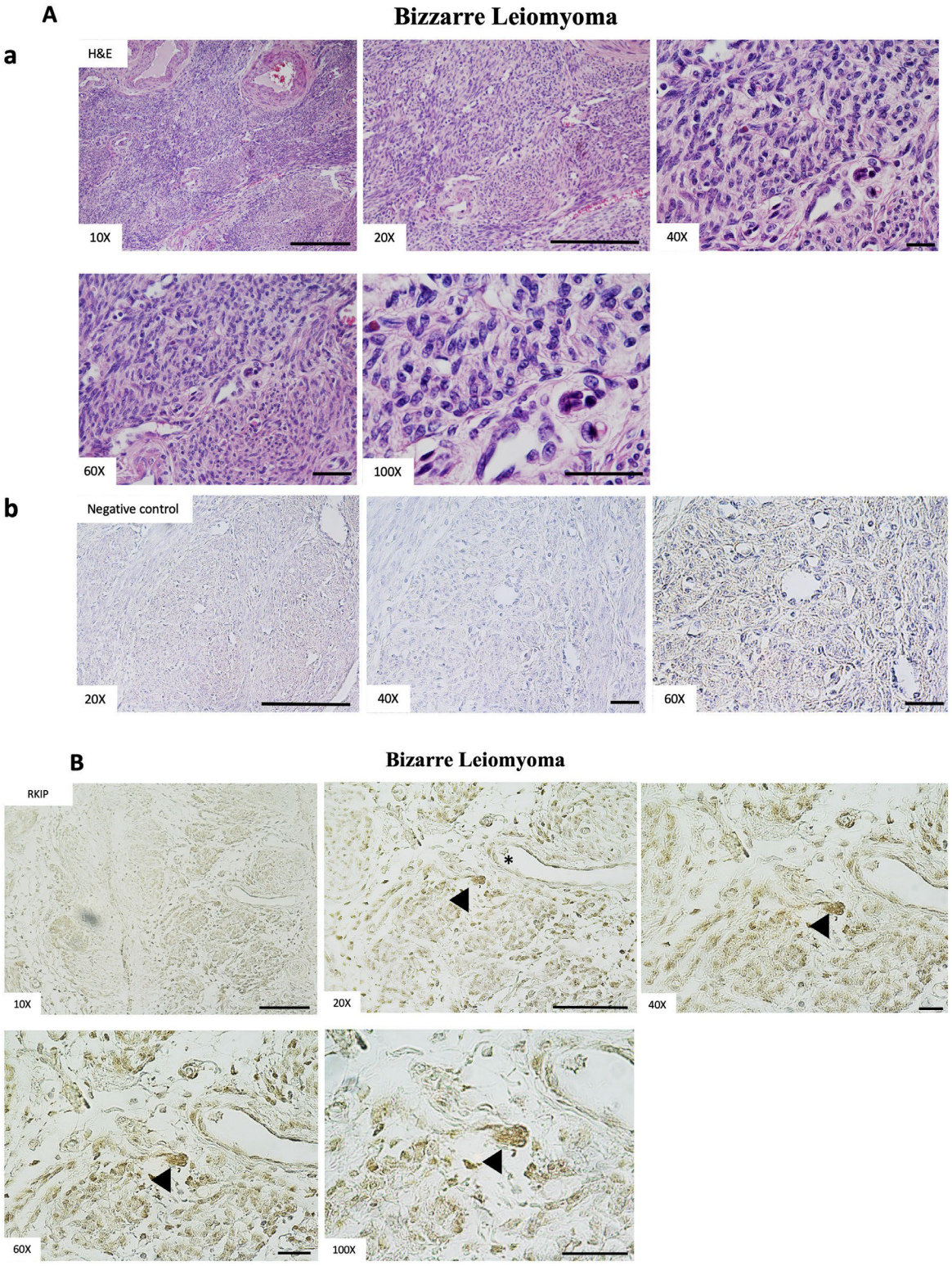

FIGURE 5 Expression of Raf kinase inhibitory protein (RKIP) on bizarre leiomyoma tissues (case 15B18704). (A) (panel a) with magnifications 10X, 20X, 40X, 60X and 100X shows the haematoxylin and eosin (H&E) of bizarre leiomyomas (panel b) with magnifications 20X, 40X and 60X show immunohistochemical negative control (non-immune serum in place of the first antibody) (B) with magnifications 10X, 20X, 40X, 60X and 100X shows immunohistochemical staining for RKIP in bizarre leiomyomas, shows high RKIP expression, especially staining is found to be positive in the 'bizarre cells' (arrowheads) (asterix: endothelial cells). Bar: 100 ym in micrographs with magnifications 10X and 20X, 10 ym in micrographs with magnifications 40X, 60X and 100X.

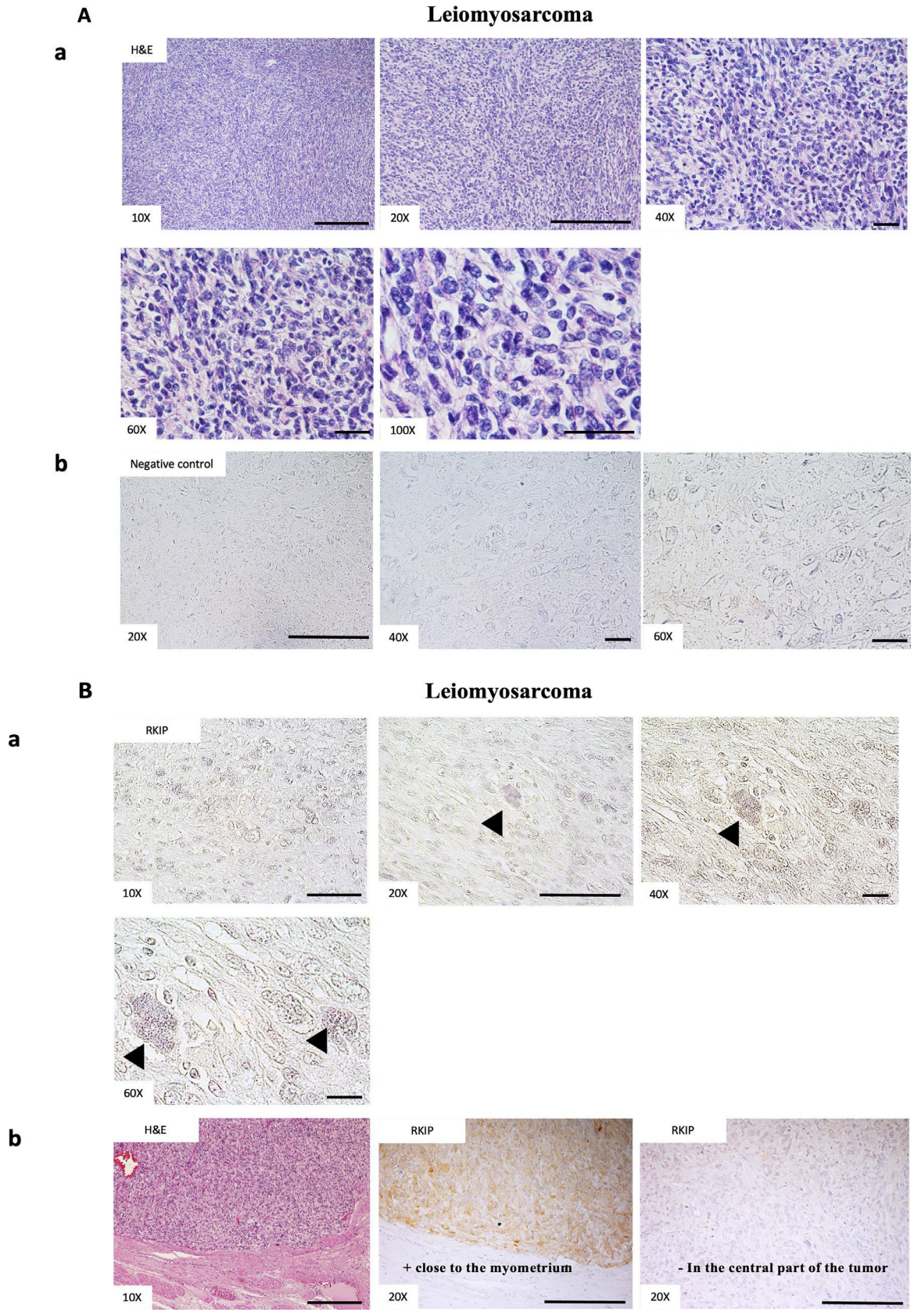

FIGURE 6 Expression of Raf kinase inhibitory protein (RKIP) in leiomyosarcoma tissues. Representative images from two different patients. (A) (panel a) with magnifications 10X, 20X, 40X, 60X and 100X shows the haematoxylin and eosin (H&E) of leiomyosarcoma tissues; (A) (panel b) with magnifications 20X, 40X and 60X shows immunohistochemical negative control (non-immune serum in place of the first antibody); (B) (panel a) shows the

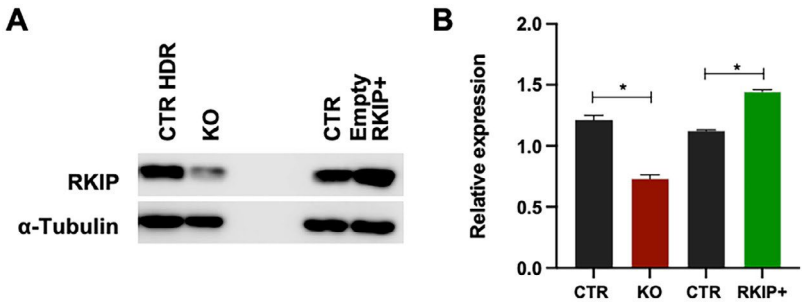

FIGURE 7 In-vitro knockout of Raf kinase inhibitory protein (RKIP) and in-vitro overexpression of RKIP in the SK-LMS-1 cell line. Western blot analysis confirms the success of the transfection. Stable RKIP knockout SK-LMS-1 cells (knockout cells) and stable RKIP overexpressing SK-LMS-1 cells (RKIP + cells) were generated. In the knockout cells, RKIP protein expression was efficiently impaired compared with control cells ($P < 0.001$), transfected only with the homology-directed repair (HDR) plasmid (calcitonin receptor cells [CTR]). In the RKIP+ cells, RKIP protein expression was efficiently increased compared with control cells ($P < 0.001$), transfected with the empty pcDNA3 vector (calcitonin receptor cells). a-Tubulin was used as a loading control; (B) band densitometry analysis with Image J software was used to quantify western blot results. Relative protein expression results for RKIP are shown as the ratio between the RKIP and a-tubulin. Results are expressed as mean SD, and statistically significant differences ($P < 0.05$) were found using Student t-test.

intensity and types of expression. In cellular leiomyoma, RKIP stain seemed to be lower compared with usual-type leiomyoma. In contrast to leiomyoma, leiomyosarcoma was generally negative for RKIP. In the smooth muscle cell cytoplasm, RKIP was present and was also generally observed in the vascular endothelium. It was interesting to note that RKIP tends to be absent in leiomyosarcoma. This observation was in line with numerous studies that have shown RKIP to be present and expressed in healthy tissues, whereas it was often downregulated in tumours (*Karamitopoulou et al., 2013*; *Shvartsur et al., 2017*). Furthermore, in our previous study (*Rhim 2003*; *Janjusevic et al., 2016*), we showed that RKIP expression is significantly increased in leiomyoma compared with adjacent myometrial tissue, confirming that the concept that leiomyoma is, in most cases, a fibrotic lesion. The expression pattern of RKIP seems to be inversely related to increased cell proliferation, as in cellular leiomyoma, and to metastatic behaviour, as in leiomyosarcoma. We have recently identified a factor that presents opposite behaviour. The hypusinated eIF5A, in fact, was present in myometrium, leiomyoma and leiomyosarcoma with increasing expression, respectively. In addition, the inhibition of eIF5A hypusination resulted in decreased cell proliferation and decreased expression of fibronectin in leiomyoma and leiomyosarcoma cells (*Greco et al., 2023*). Therefore, both factors, eIF5A and RKIP, could be added to IHC markers currently used in the diagnostic setting of uterine smooth muscle tumours, such as Ki-67, p53 and the steroid receptors (*Chen and Yang, 2008*).

In addition to the intensity of RKIP staining, it is worth noting that the discriminating expression of RKIP in bizarre cells present in leiomyoma is not found in the same type of cells present in leiomyosarcoma specimens.

The results of the present study suggested that RKIP could be used as a marker in the distinction between bizarre nuclei leiomyoma and leiomyosarcoma; therefore, RKIP could be added to evaluate and distinguish STUMP clinical behaviour. A limitation of the present study was the low number of cases available to test RKIP expression. The differentiation between benign and malignant myometrial lesions continues to be a considerable challenge for pathologists, and sometimes the final diagnosis remains inconclusive.

Lipoleiomyoma had a high expression of RKIP in adipose cells with inflammation, as confirmed by IHC for CD68 showing the presence of 'crown like structures', typical of fat cells with inflammation (*Cinti et al., 2005*). This finding is in line with the role of RKIP in immune cells mediating inflammatory diseases (*Touboul et al., 2021*).

To understand the biological role of the loss and overexpression of RKIP in malign leiomyosarcoma, we also conducted in-vitro studies with the SK-LMS-1 leiomyosarcoma cell line. Although we did not observe any RKIP effect on the cell viability over time and migration of the leiomyosarcoma cells, we observed a statistically significant increase in the ability of cells after RKIP knockout to form colonies. Moreover, in the present study, it was also noticed that cells overexpressing RKIP seemed to show a trend towards a diminished ability to form colonies, probably through N-Cadherin expression modulation, although this was not statistically significant (*Cessna et al., 2022*). A lack of an effect of RKIP on cell proliferation and angiogenesis has previously been reported; however, an effect was more evident *in vivo*, with RKIP expression significantly decreasing with malignant progression of endometrial cancer (*Martinho et al., 2012*). The non-significant trend observed for the colony formation assay in the present study is in line with what was expected based on a previous study that showed RKIP-inhibition resulted in higher colony formation (*Martinho et al., 2013*). The clonogenicity cell survival test assesses the ability of single cells to survive and reproduce to form colonies (*Franken et al. 2006*). The formation of clones is interpreted as a trait of cancer cells with tumour-initiating capabilities. In this way, the clonogenicity assay is a standard tool in cancer research to evaluate cellular growth, and it may be fundamental to study the malignancy of tumours as well as the mechanisms underlying the onset of the metastasis, which is a distinctive feature of the leiomyosarcoma (*Leitao et al., 2004*; *Wu et al., 2006*). Hence, the findings on the clonogenicity of leiomyosarcoma cells after RKIP knockout suggest that RKIP plays a biological role in leiomyosarcoma cells; however, further studies will be needed in the future, mainly in-vivo assays, to validate the findings.

The present study has some weaknesses, owing mainly to the low number of cases available and to the use of only one cell

immunohistochemical staining for RKIP in leiomyosarcoma, RKIP expression appears completely absent in panels at magnifications 10X, 20X, 40 and 60X (arrowheads: bizarre cells; (case 4737-9); (B) (panel b) shows that RKIP expression (case 2067) was high in the tumour close to the myometrium and completely absent in the central part of the sarcoma, with magnifications 10X and 20X. Bar: 100 ym in micrographs with magnifications 10X and 20X, 10 ym in micrographs with magnifications 40X, 60X and 100X.

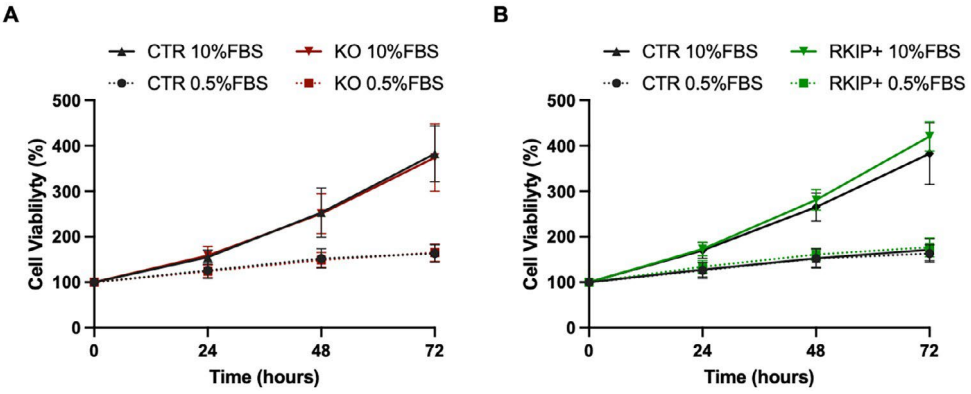

FIGURE 8 In-vitro role of Raf kinase inhibitory protein (RKIP) knockout and RKIP overexpression in SK-LMS-1 cell biological behaviour and cell viability over time. Cell viability in two different growth conditions (0.5% fetal bovine serum [FBS] and 10% FBS) was measured at 24, 48, and 72 h by sulforhodamine B assay. (A) Cell viability of knockout cells was not statistically significant different over time compared with calcitonin receptor cells [CTR] cells for the 0.5% FBS ($P = 0.928$) growth condition and the 10% FBS growth condition ($P = 0.969$); (B) cell viability of RKIP+ cells were not statistically significant different over time compared with empty (empty pcDNA3 vector) cells at the 0.5% FBS growth condition ($P = 0.811$) and the 10% FBS growth condition ($P = 0.157$). The experiments were conducted in triplicate four times. Results are expressed as mean SD, and statistically significant differences (with a $P < 0.05$) were not found on two-way analysis of variance.

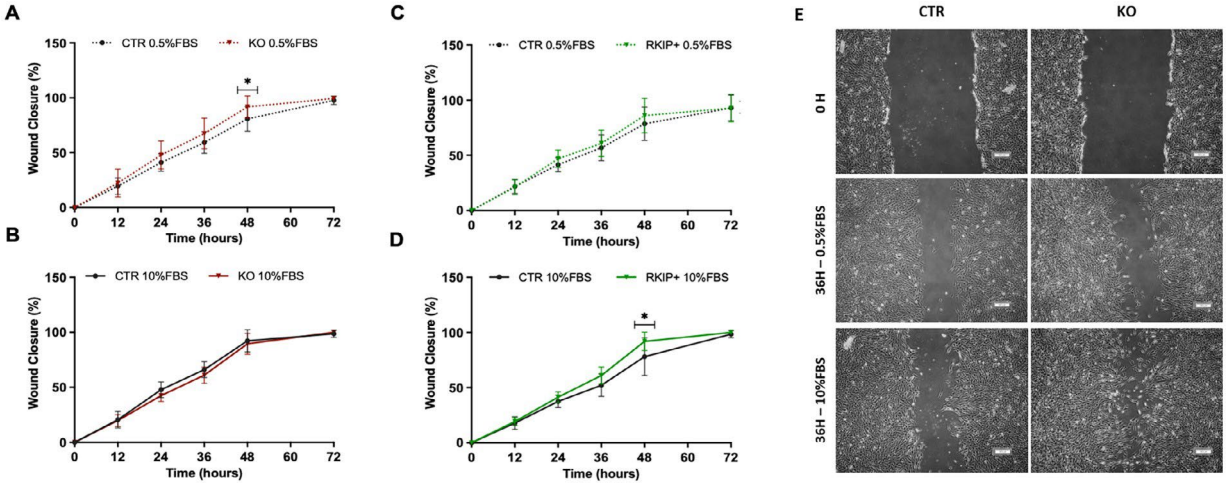

FIGURE 9 In-vitro role of Raf kinase inhibitory protein (RKIP) knockout and RKIP overexpression in SK-LMS-1 cells biological behaviour and cellular migration over time. Cellular migration in two different growth conditions (0.5% fetal bovine serum [FBS] and 10% FBS) was assessed by wound healing migration assay. A standardized scratch (wound) was applied to monolayer cells and digital images were taken at several time points (0, 12, 24, 36, 48 and 72 h). (A) Cell migration of knockout cells over time was not statistically significantly different compared with calcitonin receptor cells (CTR) at the 0.5% FBS growth condition ($P = 0.273$) nor at the 10% FBS growth condition ($P = 0.168$); (B) cellular migration of RKIP+ cells was not statistically significantly different over time compared with empty (empty pcDNA3 vector) cells at (C) the 0.5% FBS growth condition ($P = 0.294$) and (D) 10% FBS growth condition ($P = 0.085$). The experiments were conducted three times in quadruplicate. Results are expressed as mean SD, and statistically significant differences (with a $P < 0.05$) were not found on the two-way analysis of variance over time. Student's t-test for each time point showed an apparent curve separation at 48 h, which was significant (*) at (A) ($P = 0.0208$) and (D) ($P = 0.0201$). (A) P-values were as follows: $P = 0.496$ at 12 h; $P = 0.129$ at 24 h; $P = 0.121$ at 36 h; $P = 0.0208$ at 48 h; $P = 0.131$ at 72 h; (B) the P-values were as follows: $P = 0.797$ at 12 h; $P = 0.068$ at 24 h; $P = 0.110$ at 36 h; $P = 0.487$ at 48 h; $P = 0.174$ at 72 h; (C) P-values were as follows: $P = 0.845$ at 12 h; $P = 0.089$ at 24 h; $P = 0.422$ at 36 h; $P = 0.254$ at 48 h; $P = 0.966$ at 72 h; (D) the P-values were: $P = 0.481$ at 12 h; $P = 0.083$ at 24 h; $P = 0.069$ at 36 h; $P = 0.0201$ at 48 h; $P = 0.125$ at 72 h; (E) representative images at 0 and 36 h; 100x magnification. KO, knockout, Bar: 100mm

line. Considering the low incidence of leiomyosarcoma and leiomyoma variants, however, the results obtained represent important preliminary data on critical pathologies. Further research is needed to understand if RKIP can be a suitable marker for this tumour diagnosis and its role as a possible key player in the tumourigenesis process of leiomyosarcoma. Subsequently, it would be important to carry out a more in-depth study with a greater number of participants and a correlation with clinical data, mainly in malignant specimens, to assess the role of RKIP as a diagnosis marker and as a prognostic marker. In future evaluations of the biological role of RKIP in leiomyosarcoma, it is crucial to evaluate RKIP effect in more cell lines, and in other processes and assays such as invasion and spheroid formation assays. Ultimately, in-vivo assays are needed to validate these findings.

In conclusion, the difference in RKIP expression in different cases and histological types underscores the

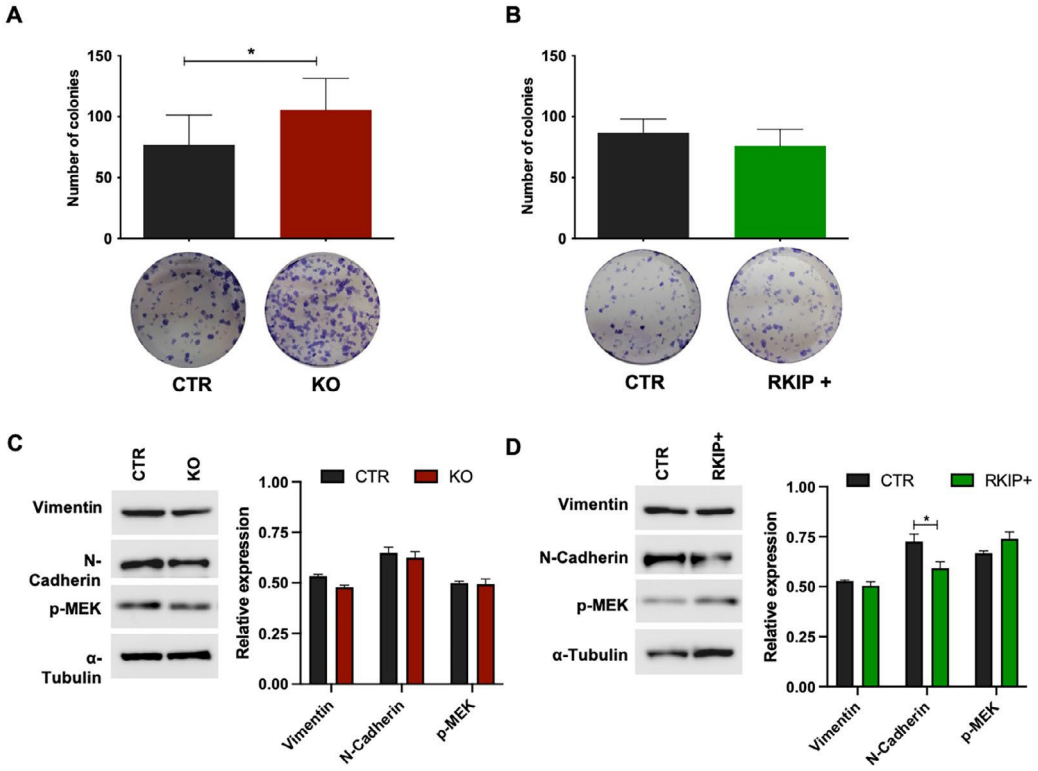

FIGURE 10 In-vitro role of Raf kinase inhibitory protein (RKIP) knockout and RKIP overexpression in SK-LMS-1 cells biological behaviour and the ability to form colonies. The ability to form colonies was assessed by clonogenicity assay, at 10% fetal bovine serum conditions, counting the stained colonies with crystal violet (representative images, 1x magnification, are shown below the graphs). (A) Knockout (KO) cells formed a significantly greater ($P = 0.011$) number of colonies compared with calcitonin receptor cells [CTR] cells; (B) the number of colonies formed by RKIP+ cells was not significantly different compared with CTR cells ($P = 0.164$); (C) western blot analysis for mesenchymal (vimentin: $P = 0.281$ and N-Cadherin: $P = 0.387$) and MAPK pathway (phospho-MEK: $P = 0.977$) in RKIP knockout cells; (D) western blot analysis for mesenchymal (vimentin: $P = 0.624$ and N-Cadherin: $P = 0.033$) and MAPK pathway (phospho-MEK: $P = 0.093$) for RKIP+ cells. a-Tubulin was used as a loading control. Band densitometry analysis with Image J software Quantification of western blot was used to quantify the results. Relative protein expression results are shown as the ratio between the proteins and a-tubulin. The experiments were conducted in triplicate at least twice. Results are expressed as the mean ± S.D., and differences with a $P < 0.05$ on Student's t-test were considered statistically significant (indicated as *).

importance of continuing to study RKIP as a possible marker candidate in myometrial histopathological studies. The biological role of RKIP in leiomyosarcoma was studied for the first time, and its downregulation seems to favour the clonogenicity in the leiomyosarcoma.

DATA AVAILABILITY

Data will be made available on request.

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