Histol Histopathol (2014) 29: 1325-1334

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Expression of Raf Kinase Inhibitor Protein (RKIP) is a predictor of uveal melanoma metastasis

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Summary. Melanoma arising from melanocytes within the choroid is the most frequent primary intraocular neoplasm in adults. It is biologically distinct from cutaneous melanoma by a very strong propensity to metastasize the liver. Raf kinase inhibitor protein is a member of an evolutionarily conserved group of proteins called phosphatidylethanolamine-binding proteins. It is an interacting partner of Raf-1 and a negative regulator of the mitogen-activated protein kinase cascade initiated by Raf-1. Raf kinase inhibitor protein expression is low in many human cancers and represents an indicator of poor prognosis and/or induction of metastasis. In the present study, we examined the immunohistochemical expression levels of Raf kinase inhibitor protein and phosphorylated Raf kinase inhibitor protein in primary uveal melanoma with and without metastasis, and evaluated their association with other high risk characteristics for metastasis in order to assess whether Raf kinase inhibitor protein and phosphorylated Raf kinase inhibitor protein can be used to predict metastasis. A significant low expression of Raf kinase inhibitor protein was seen in patients with metastasis but not in patients without metastasis. The latter more frequently had a high expression of Raf kinase inhibitor protein. No significant difference was seen in phosphorylated Raf kinase inhibitor protein expression between patients with and without metastasis. Raf kinase inhibitor protein expression is a suitable and easily determinable marker in the primary tumour that could predict the risk of uveal melanoma to metastasize, and hence guide strategies for monitoring and therapy.

Key words: RKIP, pRKIP, Uveal melanoma, Immunohistochemistry

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Introduction

Melanoma arising from melanocytes within the choroid is the most frequent primary intraocular neoplasm in adults. This tumour predominately occurs in the blue-eyed, blond population of Northern European ancestry; approximately 1 percent of malignant melanomas occur in African-Americans (Margo and McLean, 1984). Most studies find a higher incidence of uveal melanoma in men than women. Sun exposure is not a risk factor for choroidal and ciliary body melanoma (Shah et al., 2005). It has been suggested that most of them arise from preexisting benign nevi, congenital melanosis oculi and oculo-dermal melanocytosis (nevus of Ota) (Spagnolo et al., 2012). Malignant melanoma may be located at any point in the uveal tract, with the choroid and ciliary body being more frequent locations than the iris. It is biologically distinct from cutaneous melanoma by a very strong propensity to metastasize the liver. Indeed, up to 50% of patients with primary uveal melanoma develop distant metastasis, the liver being involved in up to 90% of individuals and the median survival being 4-5 months. Patients' survival has not been improved despite new diagnostic and therapeutic modalities, which have led to a better eye preservation rate only (Singh and Topham, 2003). Uveal

melanoma also differs from cutaneous melanoma in the presence of chromosomal aberrations, with frequent loss of chromosome 3 (Prescher et al., 1996), which is also a prognostic indicator. Currently, malignant melanoma of the uvea are divided into three cell types: 1) spindle cell; 2) epithelioid cell; and 3) mixed cell type (McLean et al., 1983).

RKIP (Raf kinase inhibitor protein) is a member of an evolutionarily conserved group of proteins called PEBP1 (phosphatidylethanolamine-binding protein 1). It is an interacting partner of Raf-1 and a negative regulator of the mitogen-activated protein kinase (MAPK) cascade initiated by Raf-1 (Yeung et al., 1999). Subsequently, RKIP was shown also to suppress the activation of the nuclear factor kappa B (NFκB) transcription factor by blocking the inactivation of the inhibitor of NFκB, IκB (Yeung et al., 2001). Both pathways play an important role in cancer and invasion (Reddy et al., 2003; Greten and Karin, 2004). In mammals, RKIP is also a negative regulator of G-protein coupled receptors (GPCRs) by inhibiting G-protein coupled receptor kinase (GRK-2) (Lorenz et al., 2003). Actually, RKIP has been implicated in various intracellular signalling pathways that control cell growth (Akaishi et al., 2006), motility (Al-Mulla et al., 2010), epithelial to mesenchymal transition (EMT) (Baritaki et al., 2009) and differentiation (Hellmann et al., 2010). The RKIP is a conserved cytosolic protein with wide tissue expression and does not share significant homology with other kinase inhibitors (Serre et al., 2001). Aberrant RKIP expression may play a critical role in the malignant process considering that RKIP is a metastasis suppressor gene product. Studies have shown that overexpression of RKIP inhibited metastasis and down-regulation of RKIP increases the metastatic potential. In mice, RKIP that was exogenously expressed led to a decreased level of metastasis and invasion in transformed metastatic cells (Fu et al., 2003).

More recently, sorafenib, (Buzzacco et al., 2012) a multi-kinase inhibitor, targeting Raf kinase and VEGFR, has proven to have therapeutic a effect in uveal melanoma, thus heralding a new era of molecular targeting therapy and reinforcing the utility of blocking Ras/MAPK signals in the treatment of this tumor. The inhibitory activity of RKIP on the Raf-1/MEK/ERK pathway is, at least in part, regulated by protein kinase C (PKC) induced phosphorylation of RKIP at serine 153 (Corbit et al., 2003). Mutant RKIP that has serine 153 substituted with valine failed to associate with Raf-1 and was not phosphorylated following PKC stimulation. It has also been reported that phosphorylated Raf kinase inhibitor protein (pRKIP) binds to GRK-2 and, thus, inhibits GRK-2-mediated phosphorylation of G protein coupled receptors (GPCRs) resulting in the inhibition of receptor internalization and cell signalling integrity (Lorenz et al., 2003). The resultant inhibition of receptor internalization has been predicted to promote cell growth and survival by maintaining appropriate extracellular signaling stimulation (Lorenz et al., 2003).

In the present study, we examined the expression levels of RKIP and pRKIP in primary uveal melanoma with and without metastasis, and evaluated their association with other high risk characteristics for metastasis in order to assess if RKIP and pRKIP can be used to predict metastasis.

Materials and methods

The authors performed a retrospective analysis of clinical records and formalin-fixed, paraffin-embedded (FFPE) tissue specimens of all cases of primary choroidal and/or ciliary body melanoma treated by primary enucleation at the Eye Clinic, University of Catania, Catania, Italy during the eight years up to October 2012. Enucleations were performed in the case of tumours not suitable for radiotherapy procedures, such as plaque brachytherapy or proton beam radiotherapy. Cases were excluded if the paraffin blocks containing the tumour could not be located for the preparation of additional slides for the immunohistochemical staining study, representative tumour tissue was not present in the paraffin blocks, if the tumour was completely necrotic, or the tumour had been treated previously by a method such as plaque radiotherapy or proton beam radiotherapy, which might have altered the histopathologic features and immunoreactivity of the tumours. Formalin-fixed and paraffin-embedded tissue specimens were obtained from the surgical pathology files at the Anatomic Pathology, Department G.F. Ingrassia, University of Catania, Catania, Italy. From formalin-fixed and paraffin-embedded tissue specimens multiple sections (at least 5) were obtained. Due to the retrospective nature of the study, no written informed consent from patients was obtained. The research protocols were approved by the Local Medical Ethical Committee (University of Catania) and conformed to the ethical guidelines of the Declaration of Helsinki. In order to use uniform criteria, all histological slides were evaluated by two pathologists (RC and LP). However, there is no general consensus among ophthalmic pathologists regarding the number of spindle and epithelioid cells required for the diagnosis of mixed cell type melanoma. Tumors with a few epithelioid cells (3 to 5 percent) were classified as mixed. The study consisted of 32 uveal melanomas without metastasis and 12 uveal melanomas with metastasis. From clinical charts the following data were collected: size and location of the tumour evaluated through ophthalmoscopy and A and B scan ultrasonography The presence of metastasis was assessed using standard modalities, including physical examination, liver ultrasound and total body computed tomography.

Immunohistochemistry

Sections were processed as previously described (Leonardi et al., 2012). Briefly, they were incubated for 30 min in 0.3% H_2O_2/methanol to quench endogenous

peroxidase activity then rinsed for 20 min with phosphate-buffered saline (PBS; Bio-Optica, Milan, Italy). The sections were heated (5 min × 3) in capped polypropylene slide-holders with citrate buffer (10 mM citric acid, 0.05% Tween 20, pH 6.0; Bio-Optica, Milan, Italy), using a microwave oven (750 W) to unmask antigenic sites. The blocking step performed before application of the primary antibody with goat serum (Vector Laboratories, Burlingame, CA, USA), 1:20 work dilution in PBS-T, 1 h in a moist chamber. Then, the sections were incubated overnight at 4 °C with rabbit polyclonal antibodies RKIP and pRKIP (FL-187: sc-28837; and hSer 153: sc-32626 for RKIP and pRKIP, respectively, Santa Cruz Biotechnology, Milan, Italy) were used at a dilution 1:200. The secondary antibody, biotinylated anti-mouse antibody was applied for 30 min at room temperature, followed by the avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA, USA) for a further 30 min at room temperature. The immunoreaction was visualized by incubating the sections for 4 min in a 0.1% 3,3'-diaminobenzidine (DAB) and 0.02% hydrogen peroxide solution (DAB substrate kit, Vector Laboratories, CA, USA). The sections were lightly counterstained with Mayer's hematoxylin (Histolab Products AB, Göteborg, Sweden) mounted in GVA mountant (Zymed Laboratories, San Francisco, CA, USA) and observed with a Zeiss Axioplan light microscope (Carl Zeiss, Oberkochen, Germany).

Evaluation of immunohistochemistry

Immunostained slides were separately evaluated by two pathologists (RC and LP), who were blinded to patient identity, clinical status and group identification, using a light microscope.

The RKIP and pRKIP-staining status was identified as either negative or positive. Immunohistochemistry positive staining was defined as the presence of brown chromogen detection on the edge of the hematoxylin-stained cell nucleus, distributed within the cytoplasm or in the membrane. Stain intensity and the proportion of immunopositive cells were assessed by light microscopy. Intensity of staining (IS) was graded on a scale of 0-3, according to the following assessment: no detectable staining = 0, weak staining = 1, moderate staining = 2, strong staining = 3, as described previously (Allegra et al., 2013). The percentage of RKIP and pRKIP immunopositive cells (Extent Score (ES)) was independently evaluated by two investigators and scored as a percentage of the final number of 100 cells in five categories: <5% (0); 5-30% (+); 31-50% (++); 51-75% (+++), and >75% (++++). Counting was performed at 200×magnification. The staining intensity was multiplied by the percentage of positive cells to obtain the intensity reactivity score (IRS). Positive and negative controls were performed to test the specific reaction of primary antibodies used in this study at a protein level. Positive controls consisted of sections of rat liver. For negative controls, testing sections of uveal melanoma were treated with normal rabbit serum instead of the specific antibodies.

Statistical analysis

Non parametric comparison of the median values of all parameters in patients without metastasis and with metastasis was performed by Kolmogorov-Smirnov test or chi-square test. Agreement among observers was tested by Cohen K. Considering the median of the values of RKIP and pRKIP detected in all patients, each case has been classified into two categories (high and low) that expressed RKIP and pRKIP higher/equal or lower than the median value. Univariate and multivariate analysis were based on a Cox proportional hazards regression model (time free from metastasis as outcome); this model included gender, age, melanoma location (choroid or ciliary body), temporal or nasal location, cell type (epithelioid, spindle cells or mixed), echographic parameters (height, greatest and minor diameters, internal reflectivity), and value of RKIP and pRKIP expression. All predictors that had a P value <0.15 (cut off) in the univariate analysis were included in the multivariate analysis. Survival analysis according to RKIP and pRKIP expression levels was performed by Kaplan-Meyer test; survival rates were compared by log-rank (Mantel-Cox) test. P values <0.05 were considered as statistically significant.

Results

Clinicopathological characteristics of uveal melanomas

This study included a total of 44 patients, 22 men and 22 women, with uveal melanoma. At the time of diagnosis, median age was 64 years (range 29 to 85 years). The clinicopathological parameters are listed in tables 1, 2 and 3. Of the tumours, 31 (70.4%) were located in the choroid and 13 (29.6%) tumours involved the choroid and ciliary body. There was only one case with extrascleral extension that was evaluated visually during surgery, with ultrasonography and tomography and confirmed histologically. Histologically, 26 (59.1%) tumours were classified as mixed; 12 (27.3%), as spindle cells; 6 (13.6%), as epithelioid cells, respectively. Pathological T stage was T1a (pT1a) for 1 (2.3%) tumour, 22 (50%) were pT2a, 10 (22.7%) were pT2b, 1 (2.3%) was pT2d, 7 (15.9%) were pT3a, and 3 (6.8%) were pT3b. Liver involvement was detected in 12 cases (27.3%). The median follow-up period was 41 months (range: 0-112 months). The group of patients (n=32) affected by uveal melanoma without metastasis included 17 males and 15 females, with an age ranging between 29 and 85 years (median 63 yrs). The group of patients (n=12) affected by metastatic uveal melanoma included 5 males and 7 females, with an age ranging between 48 and 76 years (median 67 yrs). During the follow-up period, 6 of them died due to disease progression.

Between the two groups (patients with or without metastasis), a significant difference was seen in cell type (p=0.025), disease free survival (p=0.006), and RKIP (p<0.001). Median follow-up was greater in the group without metastasis (55 vs 38 months) (table 3).

Correlations between RKIP and pRKIP expression and clinicopathological factors in uveal melanomas

In the whole group (n=44) median RKIP value was 8 and median pRKIP value was 5. RKIP expression was low in 12 (27.2%) tumours (Fig. 1a), and high in 32 (72.7%) tumours (Fig. 1b). pRKIP expression was low in 22 (50%) tumours (Fig. 2a), and high in 22 (50%) tumours (Fig. 2b). No expression of pRKIP was identified for in non-tumoral ocular tissue. Interobserver agreement measured as kappa coefficient was 0.938 (excellent).

A significantly low expression of RKIP was seen in patients with metastasis (10/12, 83.3%), but not in patients without metastasis (2/32, 6.3%) (chi-square, p<0.001); this latter more frequently had a high expression of RKIP (30/32, 93.7%). No significant difference was seen in pRKIP expression between patients with and without metastasis (Table 4).

Factors related to the presence of metastasis at univariate analysis on a Cox proportional hazards regression model were: tumour greater diameter (p=0.061), pT stage (p=0.091), cell type (p=0.008), and RKIP level (p<0.001); at multivariate analysis only RKIP level (p<0.001) and pT stage (p=0.014) were significant. No correlation was found between histological type and both RKIP and pRKIP expression (Spearman).

Figure 3 shows the results of Kaplan-Meier survival analyses in patients with uveal melanomas with low and high RKIP and pRKIP expression. The survival times free from metastasis (SE, with 95% CI) estimated were respectively: 21.9 (6.9) (CI: 8.4 to 35.3) and 105.3 (4.5) (CI: 96.5 to 114.2) for RKIP; and 80.2 (9.7) (CI: 61.1 to 99.3) and 58.2 (5.8) (CI: 47 to 69.5) for pRKIP

The log-rank test showed a significant difference

Table 1. Demographics, tumour parameters, disease free time, follow-up and RKIP and pRKIP expression in primary uveal melanoma without metastasis.

Sex	Age (yrs)	Location	Thickness (mm)	Largest diameter (mm)	Cell type	Extrascleral extension	Pathological T stage	DFS (months)	Follow-up (months)	RKIP				pRKIP			
										IS	ES	IRS		IS	ES	IRS	
M	52	choroid	7.9	13.3	spindle	N	pT2a	95	95	2	4	8	H	0	0	0	L
F	29	choroid	14.2	16.2	mixed	N	pT2a	90	90	3	3	9	H	0	0	0	L
M	54	choroid	9.2	17	spindle	N	pT2a	112	112	3	4	12	H	0	0	0	L
F	30	choroid/cil.body	12.05	9.2	spindle	N	pT2b	72	72	3	4	12	H	0	0	0	L
F	83	choroid/cil.body	6.84	14.2	mixed	N	pT2b	74	74	2	4	8	H	0	0	0	L
F	55	choroid	9.8	13.9	spindle	N	pT2a	73	73	3	3	9	H	2	4	8	H
M	74	choroid/cil.body	10.04	16.1	spindle	N	pT2b	72	72	2	4	8	H	3	3	9	H
M	80	choroid	6.04	10.5	mixed	N	pT1a	68	68	3	3	9	H	3	4	12	H
M	68	choroid	12.8	20.1	mixed	N	pT2a	68	68	2	4	8	H	2	2	4	L
F	59	choroid	8.4	14.7	mixed	N	pT2a	59	59	3	4	12	H	3	3	9	H
F	55	choroid	10.5	16.2	mixed	N	pT2a	58	58	2	3	6	L	2	3	6	H
F	54	choroid/cil.body	9.76	9.5	mixed	N	pT2b	58	58	3	4	12	H	0	0	0	L
M	84	choroid/cil.body	11.9	14.8	mixed	N	pT2b	56	56	2	4	8	H	2	4	8	H
M	73	choroid	9.7	11.3	mixed	N	pT2a	55	55	3	3	9	H	0	0	0	L
M	76	choroid/cil.body	8.9	13.3	mixed	N	pT2b	55	55	2	4	8	H	0	0	0	L
F	63	choroid/cil.body	10.3	17.2	spindle	N	pT2b	54	54	2	4	8	H	1	3	3	L
F	45	choroid	13.7	10.2	mixed	N	pT2a	50	50	2	4	8	H	3	4	12	H
M	58	choroid	13.1	15.3	spindle	N	pT2a	49	49	3	3	9	H	2	3	6	H
M	75	choroid	6.1	11.7	mixed	N	pT2a	41	41	2	4	8	H	3	4	12	H
M	81	choroid	9.2	14.3	spindle	N	pT2a	39	39	2	4	8	H	2	4	8	H
F	84	choroid	11.7	17.4	mixed	N	pT3a	30	30	3	4	12	H	2	3	6	H
M	75	choroid	8.8	11.3	mixed	N	pT2a	73	73	3	3	9	H	1	3	3	L
M	49	choroid/cil.body	13.79	16.6	mixed	N	pT3b	25	25	2	4	8	H	1	3	3	L
F	51	choroid	9.42	16	mixed	N	pT3a	24	24	3	4	12	H	2	4	8	H
M	73	choroid	9.24	17.7	epithelioid	N	pT2a	23	23	2	4	8	H	1	3	3	L
M	62	choroid	13.68	14.7	mixed	N	pT3a	23	23	2	4	8	H	0	0	0	L
F	85	choroid/cil.body	7.3	12.5	spindle	Y	pT2d	22	22	3	3	9	H	1	3	3	L
F	66	choroid/cil.body	8.95	15.4	mixed	N	pT2b	18	18	2	3	6	L	3	4	12	H
M	72	choroid	7.42	10.5	mixed	N	pT3b	12	12	3	3	9	H	3	4	12	H
F	61	choroid	12.05	12.4	spindle	N	pT2a	12	12	3	3	9	H	2	3	6	H
M	63	choroid	8.6	10.5	mixed	N	pT3a	11	11	2	4	8	H	2	4	8	H
F	52	choroid/cil.body	9.5	13.2	mixed	N	pT2b	9	9	3	4	12	H	2	4	8	H

DFS, disease free survival; RKIP, Raf kinase inhibitor protein; pRKIP, phosphorylated Raf kinase inhibitor protein; cil.body, ciliary body.

between the two groups in RKIP ($p<0.001$), but not in pRKIP expression.

Discussion

Approximately 50 percent of patients diagnosed with an uveal melanoma develop metastasis within 10 to 15 years after enucleation (Collaborative Ocular Melanoma Study Group, 2001). Once metastasis is diagnosed uveal melanoma is typically fatal within 1 year because there are currently no effective treatments. The variables that are best predictive of poor patient survival are epitheliod

Table 2. Demographics, tumour parameters, disease free time, follow-up and RKIP and pRKIP expression in primary uveal melanoma with metastasis.

Sex	Age (yrs)	Location	Thickness (mm)	Largest diameter (mm)	Cell type	Extrascleral extension	Pathological T stage	DFS (months)	Follow-up (months)	RKIP				pRKIP			
										IS	ES	IRS		IS	ES	IRS	
F	58	choroid	6.04	17.8	mixed	N	pT2a	63	64 (†)	0	0	0	L	1	3	3	L
M	71	choroid	13.14	17.1	epithelioid	N	pT3a	33	34(†)	2	4	8	H	0	0	0	L
F	74	choroid	5.7	12.1	spindle	N	pT2a	24	37(†)	0	0	0	L	0	0	0	L
F	60	choroid	8.25	16.5	spindle	N	pT2a	11	37(†)	0	0	0	L	1	3	3	L
F	72	choroid	6.7	15.2	epithelioid	N	pT2a	14	28(†)	0	0	0	L	0	0	0	L
M	69	choroid	7.21	15.8	mixed	N	pT2a	54	65	0	0	0	L	2	3	6	H
M	76	choroid	13.7	17.1	mixed	N	pT2a	14	54	1	2	2	L	2	4	8	H
F	57	choroid/cil.body	13.6	19	mixed	N	pT2b	6	39	1	1	1	L	3	4	12	H
M	72	choroid/cil.body	13.3	15.4	mixed	N	pT3b	0	35	2	2	4	L	3	3	9	H
F	50	choroid	7.36	15.6	epithelioid	N	pT2a	41	65	2	4	8	H	1	3	3	L
F	48	choroid	8.24	14.9	epithelioid	N	pT3a	1	13(†)	2	1	2	L	1	3	3	L
M	64	choroid	10.7	14.5	epithelioid	N	pT3a	32	45	1	4	4	L	2	3	6	H

DFS, disease free survival; RKIP, Raf kinase inhibitor protein; pRKIP, phosphorylated Raf kinase inhibitor protein; cil.body, ciliary body. (†) death

Table 3. Median (range) of demographics, tumour parameters, disease free time, follow-up, RKIP and pRKIP expression in primary uveal melanoma without and with systemic metastasis.

	Sex m-f	Age (yrs)	Location	Thickness	Largest diameter	Cell type	Extrascleral extension	Pathological T stage	DFS (months)	Follow-up (months)	RKIP	pRKIP
All (n=44)	22-22	64 (29-85)	Choroid 31 Chor/ cil.body 13	9.5 (5.7-14.2)	14.9 (9.2-20.1)	Epith: 6 Spindle: 12 Mixed: 26	No: 43 Yes: 1	pT1a: 1 pT2a: 22 pT2b: 10 pT2d: 1 pT3a: 7 pT3b: 3	41 (0-112)	50 (9-112)	8 (0-12)	5 (0-12)
Metastasis free (n=32)	17-15	63 (29-85)	Choroid 21 Chor/ cil.body 11	9.6 (6.4-14.2)	14.3 (9.2-20.1)	Epith: 1 Spindle: 10 Mixed: 21	No: 31 Yes: 1	pT1a: 1 pT2a: 15 pT2b: 9 pT2d: 1 pT3a: 4 pT3b: 2	55 (9-112)	55 (9-112)	9 (6-12)	6 (0-12)
Metastasis (n=12)	5-7	67 (48-76)	Choroid 10 Chor/ cil body 2	8.2 (5.7-13.7)	15.7 (12.1-19)	Epith: 5 Spindle: 2 Mixed: 5	No: 12	pT2a: 7 pT2b: 1 pT3a: 3 pT3b: 1	19 (0-63)	38 6 death (13-65)	1.5 (0-8)	3 (0-12)
p (metastasis free vs metastasis)		0.843*	0.438°	0.287*	0.150*	0.025*	0.601°	0.297*	0.006*	0.361*	<0.001	0.798

*: Kolmogorov-Smirnov test. °: chi-square test

Table 4. Number of uveal melanoma (with and without metastasis) with low and high RKIP and pRKIP.

	RKIP		pRKIP	
	Metastasis (n=12)	Metastasis free (n=32)	Metastasis (n=12)	Metastatis free (n=32)
Low	10 (83.3%) *	2 (6.3%)	7 (41.7%) °	15 (46.9%)
High	2 (16.7%)	30 (93.7%)	5 (58.3%)	17 (53.1)

RKIP, Raf kinase inhibitor protein; pRKIP, phosphorylated Raf kinase inhibitor protein. p (chi-square). *: $p<0.001$. °: $p=0.957$, ns

cell type, largest tumour dimension, extrascleral extension, and mitotic activity, followed by vascular patterns, presence of lymphocytes per 20 high-power fields, greater tumour pigmentation, and foci of necrosis. Clinical risk factors for survival are older age and sex of the patients (prognosis is worse in males).

Cytogenetic testing demonstrates that loss of 1 copy of chromosome 3 (monosomy 3) is predictive of poor likelihood of survival (Prescher et al., 1996). Onken (Onken et al., 2004) found uveal melanomas clustered into two groups, described as class 1 and class 2. Class 1 lesions were unlikely to undergo metastasis, have better prognoses and are associated with disomy 3 and a gain of chromosome 6p; class 2 lesions predicted a greater rate of metastasis and disease-related mortality, and are associated with a loss of heterozygosity of chromosome

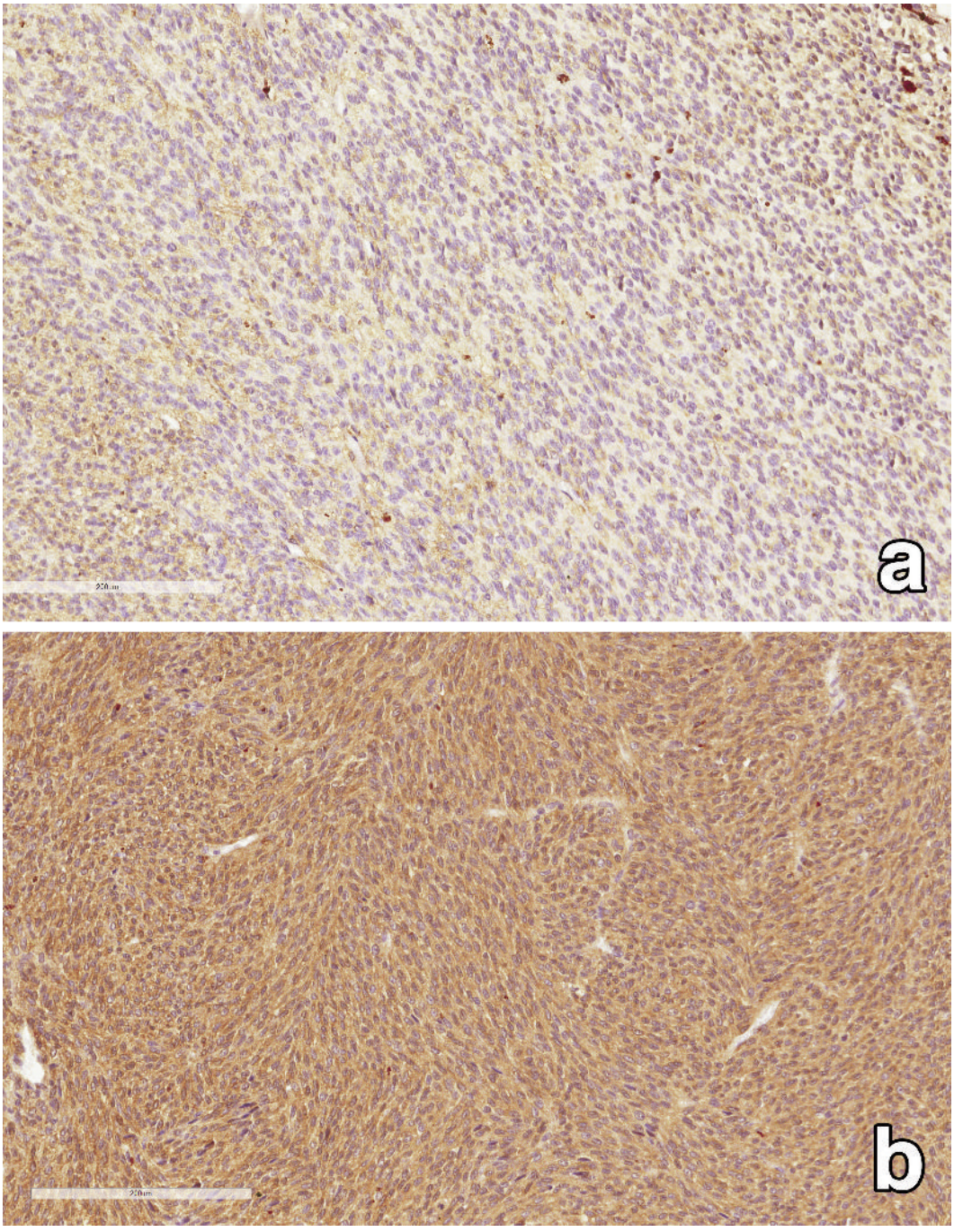

Fig. 1. RKIP in uveal melanoma. **a.** Mild and diffuse cytoplasmic positivity in spindle cell malignant melanoma. **b.** Strong and diffuse cytoplasmic positivity in spindle cell malignant melanoma. x 200

3. Harbour (Harbour, 2010) recently found inactivating somatic and germ line mutations of BRCA1 associated protein-1 (BAP1) in 84% of metastasizing uveal melanoma lesions. Nevertheless, molecular genetic tests demonstrate some limitations, such as availability and costs. The ability to predict the risk of metastatic relapse is of paramount importance because it may allow the identification of patients who should be monitored more frequently and who could benefit from adjuvant chemotherapy, even if at present there is not a standardized adjuvant therapy for uveal melanoma.

RKIP is a promising metastasis repressor that regulates several physiologic functions. Current evidence indicates that RKIP also cross-talks with several important cellular signaling pathways, including NF-κB (Yeung et al., 2001) and G-proteins (Kroslak et al., 2001). RKIP also suppressed metastasis in prostate, breast and ovarian cancer models (Fu et al., 2003; Li et

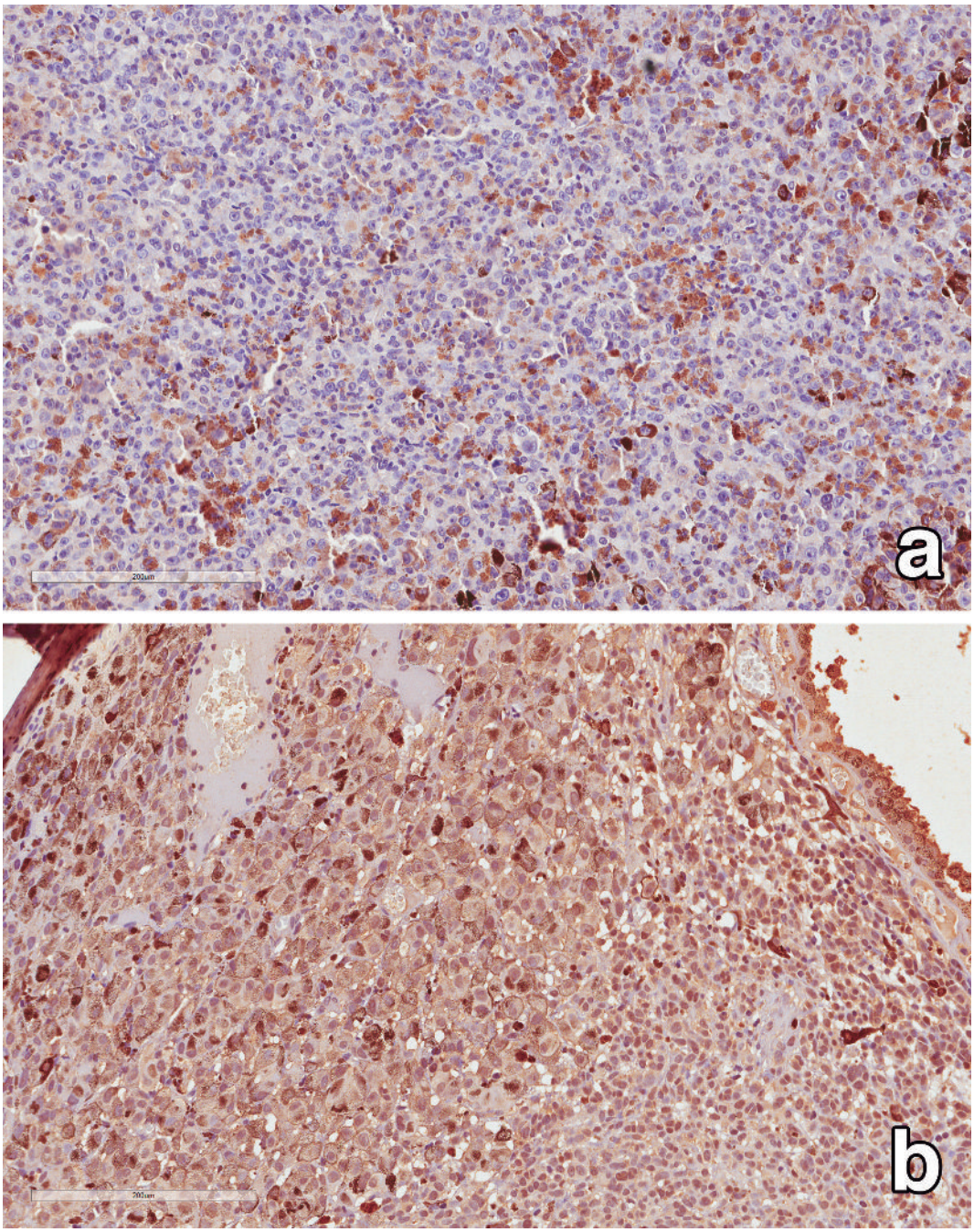

Fig. 2. pRKIP in uveal melanoma. **a.** Mild and focal cytoplasmic positivity in epithelioid cell malignant melanoma. **b.** Strong and diffuse cytoplasmic positivity in epithelioid cell malignant melanoma. x 200

al., 2008, 2009). Furthermore, loss of cytoplasmic RKIP has also been associated with colorectal carcinoma recurrence and with poor prognosis (Al-Mulla et al., 2006). In cutaneous nevi, almost all samples showed strong RKIP immunostaining, whereas RKIP expression was diminished or completely lost in primary malignant melanoma and in metastasis (Schuierer et al., 2004). In cutaneous melanoma, reduced levels of RKIP expression correlate with metastatic stage (Schuierer et al., 2004; Park et al., 2005), probably because RKIP acts as suppressor of metastasis by decreasing vascular invasion.

To the best of our knowledge, this is the first time that RKIP and pRKIP have been characterized in uveal melanoma. We found that tumour expression of RKIP was a prognostic factor for risk of metastasis. In our samples the median value of IRS for RKIP was 8, corresponding to a moderate staining in more than 75% of the neoplastic cells, or severe staining in more than 50% of the neoplastic cells. The patients with lower RKIP expression showed higher incidence of metastasis after enucleation. At the same time, patients that had a high level of RKIP expression were significantly less likely to develop metastasis. This is consistent with the data reported by Li and colleagues (Li et al., 2008) which documented that the overexpression of RKIP suppressed the ability of human ovarian cancer cells to metastasize when transplanted into nude mice. However, the molecular mechanism of the antimetastatic function of RKIP is poorly defined and requires clarification. In melanoma, Schuierer and colleagues (Schuierer et al., 2004) showed that RKIP expression is lost in transformed cells and significant downregulation or complete extinction of RKIP occurs in melanoma metastases. They also documented that loss of RKIP expression in metastasis is not due to genomic loss, because no alterations of the genomic region of RKIP on human chromosome 12q23 and its adjacent areas have been reported for any cancer, supporting the hypothesis that it is not chromosomal changes, but other alterations that may lead to dysregulation of RKIP. RKIP has been reported to inhibit NF-κB activation by several distinct mechanisms. First, RKIP inhibits NF-κB activation as a result of its inhibitory effects on Raf-1 by interfering with MEK phosphorylation and subsequent activation by Raf-1 (Yeung et al., 2000). Second, RKIP interferes directly with NF-κB activation by interacting and blocking NIK and TAK1, which are necessary for NF-κB activation (Wu and Bonavida, 2009). RKIP can also inhibit IKKa and IKKb kinase activities in vitro (Wu and Bonavida, 2009). A potential mechanism underlying this activity in metastatic melanoma cells could be the cross-talk between RKIP and the prometastatic protein melanoma differentiation associated gene-9 (MDA-9), also known as syntenin, and therefore blocking c-Src activation and NF-κB activation (Das et al., 2012). As reported in the literature (Gangemi et al., 2012), the gene expression profiles of primary human uveal melanomas showed high expression of the gene encoding for syntenin in patients with recurrence, and immunohistochemistry showed that high expression of MDA-9/syntenin protein in primary tumors was significantly related to metastatic recurrence.

We present evidence that the expression levels of pRKIP were not significantly different between primary uveal melanoma with metastasis and primary uveal melanoma without metastasis. Therefore, the level of pRKIP had no predictive value for uveal melanoma metastasis. The underlying mechanism of the differential expression of pRKIP and RKIP is not known. We had expected that relatively lower levels of pRKIP might correlate with higher RKIP levels, which would inhibit

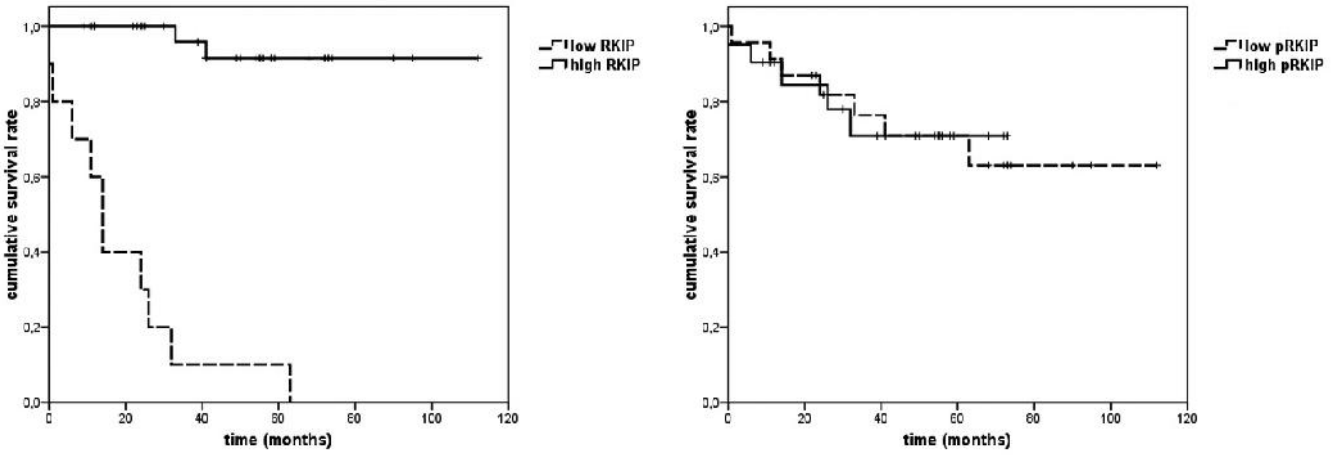

Fig. 3. Kaplan-Meier survival analyses for low and high RKIP expression (left), and low and high pRKIP expression (right). The logrank test showed significant differences between the two groups in RKIP ($p<0.001$) but not in pRKIP ($p=862$, ns).

the Raf-1 and NF-κB pathways and presumably result in better prognosis. Likewise, we expected relatively higher levels of pRKIP to correlate with the residual lower level of active RKIP, resulting in minimal inhibition of survival pathways, thus resulting in poorer prognosis. However, interestingly, our present data are not concordant with these expectations. Nevertheless, we emphasize the importance of examining the expression of both the non-phosphorylated active RKIP and phosphorylated inactive RKIP as already reported in the literature.

In conclusion, RKIP expression is a suitable and easily determinable marker in the primary tumour that could predict the risk of uveal melanoma to metastasize, and hence guide strategies for monitoring and therapy. The relationship between RKIP expression in primary tumours and metastatic relapse and overall survival gains in significance given the lack of effective markers of metastatic risk. A simple test such as RKIP expression in the primary tumour could provide an economically viable and immediately available decision aid. A limitation of the current study is the paucity of specimens available for evaluation. This is due to changes in the clinical management of uveal melanomas, many of which are treated with brachytherapy or proton beam radiotherapy rather than undergoing enucleation. Thus, while these preliminary results point to an association between low expression of RKIP and the development of metastasis, further studies with larger series are needed to validate RKIP expression as a prognostic marker in uveal melanoma. Given that fine-needle aspiration biopsy (FNAB) of uveal melanoma is becoming more commonplace, it will also be interesting in future to assess the expression levels of RKIP in the biopsies of uveal melanoma.

This study did not address the relationship between RKIP expression and patients' response to chemotherapy. An evaluation of RKIP expression in melanoma metastasis could be of interest given the recent data showing that chemotherapeutic drug treatments induce RKIP expression, which in turn sensitizes tumoural cells to apoptosis (Chatterjee et al., 2004).

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Accepted April 24, 2014