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ORIGINAL ARTICLE – CANCER RESEARCH

Differential expression of p52 and RelB proteins in the metastatic and non-metastatic groups of uveal melanoma with patient outcome

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Abstract

Purpose Non-canonical NFκB (NC-NFκB) pathway plays an influential role in metastasis, which promotes cancer proliferation and progression. The aim of the study was to examine the expression of NC-NFκB proteins and their correlation with clinicopathological factors associated with metastatic cases of uveal melanoma (UM) and with the patient outcome.

Method Expression of NC-NFκB proteins (p52, RelB, and co-expression of p52/RelB) was evaluated in 75 formalin-fixed cases of uveal melanoma by immunohistochemistry. Validation of nuclear immunoreactivity was done by western blotting. Transcriptional status of NC-NFκB genes was assessed in 60 fresh tumor tissues by quantitative real-time PCR. Co-immunoprecipitation was performed to determine the presence of native p52/RelB heterodimer in UM. Prognostic relevance was determined using Cox proportional hazard and Kaplan–Meier methods.

Results Immunohistochemical expression of p52, RelB, and their co-expression was observed in 81%, 68.7%, 56.2% of metastatic cases, respectively, while their expression was seen only in 38%, 33% and 30% of non-metastatic cases. Loss of BAP-1 was correlated with expression of p52 and RelB proteins. Co-immunoprecipitation assay confirmed the putative interaction of p52 with RelB protein in metastatic cases of uveal melanoma. Co-expression of p52/RelB and expression of p52 protein was significantly correlated with decreased metastasis-free survival (MFS) ($p = 0.004$; $p = 0.002$) and overall survival (OS) ($p = 0.004$; $p = 0.032$), while the RelB expression only correlated with reduced MFS ($p = 0.003$).

Conclusion Our data showed that non-canonical NFκB proteins were significantly higher in metastatic cases and associated with poor outcome of the patients. Furthermore, the p52 protein could be used as a potential therapeutic biomarker for metastatic cases in uveal melanoma.

Keywords Uveal melanoma · Metastasis · p52 · RelB · Co-expression

Introduction

Uveal melanoma (UM), the most common adult primary intraocular tumor, is associated with the development of systemic metastases in approximately 50% of the cases (Kujala et al. 2003; Ehlers and Harbour 2006). Despite advances in the diagnosis and treatment of the primary tumor, the 5-year mortality rate of uveal melanoma patients has not significantly changed (Singh et al. 2011). The principal target organ for metastasis is the liver, which is involved in 71–87% of patients with metastatic disease (Kath et al. 1993; Lorigan et al. 1991). The root of traditional metastatic risk assessment has been associated with clinical and histopathological features of uveal melanoma tumors. Older age, increased tumor thickness (> 8 mm), largest basal tumor diameter (LBD) greater than 15 mm, ciliary body involvement,

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epithelioid tumor cell morphology, and extraocular tumor extension have all been identified as poor prognostic factors associated with increased risk of metastasis and disease-related mortality in UM (AJCC Ophthalmic Oncology Task Force 2015).

Mutation in tumor suppressor BAP-1 protein is strongly associated with metastatic risk (Koopmans et al. 2014). According to Bronkhorst et al., loss of one copy of chromosome 3 (monosomy) in UM cells may produce inflammatory mediators, which recruit and activate many lymphocytes as well as macrophages (Bronkhorst and Jager 2012). Maat et al. proposed a hypothesis that loss of chromosome 3 leads to inefficient suppression of the NFκB pathway by PPARγ (Maat et al. 2008). Dror et al. support the theory that the NFκB pathway is upregulated when it is no longer negatively modulated by PPARγ in uveal melanoma (Dror et al. 2010).

NFκB transcription factors are either homodimers or heterodimers of five proteins comprising p65 (RelA), c-Rel, RelB, p52(NFκB2) and p50 (NFκB1) which are regulated in various cellular activities. In healthy cells, inactive NFκB exists in the cytoplasm bound to inhibitory (IKK) proteins. The phosphorylation and degradation of this inhibitory protein resulted in the nuclear translocation of NFκB dimers (Rojo et al. 2016). Each NFκB dimer selectively regulates a set of target genes involved in cell proliferation and survival, inflammation and innate immunity (Saccani et al. 2003). In tumor tissues, the NFκB signaling pathway is constitutively active and has a role in tumorigenesis and resistance mechanisms towards treatment (Ben-Neriah and Karin 2011; Basseres and Baldwin 2006; Prasad et al. 2010).

Non-canonical pathway involves NFκB2 and RelB subunits, and the NFκB-inducing kinase (NIK) and IKK kinases (Miller et al. 2005). This pathway activates NF-kappa-B-inducing kinase (NIK), which subsequently phosphorylates inhibitor of nuclear factor kappa-B kinase subunit alpha (IKKα) which leads to ubiquitination of p100 and release of active p52/RelB dimers. This results in the translocation of p52/RelB dimer to nucleus where they drive the function of inflammatory genes, cell proliferation and negative regulators of NFκB. Dysregulation of these genes leads to tumorigenesis in a variety of cancers (Karin et al. 2002).

The activity of RelB and p52 has been demonstrated in numerous cancer types, and they regulate a diverse set of genes. When activated, these non-canonical NFκB transcription factors promote tumor initiation, growth, and survival. Non-canonical NFκB has been shown to regulate mammary gland development (Wang et al. 2012; Cao et al. 2001). The activity of the non-canonical NFκB (NC-NFκB) transcription factors RelB and p52 has been implicated in promoting breast cancer. Recently, the expression of RelB and p52 protein was shown to elevate estrogen receptor-negative (ER) tumors as compared to ER-positive breast carcinoma. Patients with higher expression of RelB and p52 had decreased disease-free survival as well as reduced overall survival (Rojo et al. 2016).

Therefore, the purpose of this study was to evaluate the expression of p52 and RelB protein in metastatic and non-metastatic groups of uveal melanoma patients by immunohistochemistry and quantitative reverse transcriptase PCR (qRT-PCR). The presence of p52/RelB heterodimer was investigated by co-immunoprecipitation and finally, all the results were correlated with clinicopathological characteristics and patient outcome.

Materials and methods

Clinical samples

A total of 75 histopathological confirmed uveal melanoma tumors, diagnosed between 2013 and 2016, were collected for this study. This was a prospective study conducted at the Dr. Rajendra Prasad Centre for Ophthalmic Sciences, New Delhi, India after approval of the institutional ethics committee, All India Institute of Medical Sciences (IESC/T-417/2015). Written informed consent for enucleation surgery, and participation in this study was obtained from all the patients. All procedures conformed to the tenets of the Declaration of Helsinki. Fresh tumor tissues and control tissues were collected for mRNA, western blotting and co-immunoprecipitation study. Fresh tumor tissues were fixed in RNA later (Sigma-Aldrich) to avoid degradation of RNA and were then stored at − 80 °C until further use.

Histopathology of uveal melanoma

Enucleation specimens were immediately fixed in formalin (10%) and embedded in paraffin wax. Five-micrometer-thick pupil-optic nerve sections were cut, deparaffinized with xylene and rehydrated through a graded series of ethanol and distilled water. The sections were stained with hematoxylin and eosin (H&E) and were reviewed by two pathologists (SK and SS). Histopathology slides were examined to determine the high-risk factors. High-risk factors (HRFs) included anterior location of the tumor–ciliary body tumor, tumor thickness (> 8 mm), large basal diameter greater than 15 mm (LBD > 15 mm), large tumor diameter greater than 15 mm (LTD > 15 mm), increased microvessel density (> 30% CD34 positivity), infiltrating lymphocytes (> 30% CD3 positivity), macrophages (> 30% CD68 positivity), high mitotic figures (> 4/40HPF), scleral invasion and ciliary body invasion (Demicco et al. 2005).

Immunohistochemical staining

The p52 and RELB immunohistochemistry was performed on 75 µm formalin-fixed paraffin-embedded tissue sections with the Fast Quanto red (Lab Vision™, Thermo Scientific; Kalamazoo, MI) using a red chromogen according to the manufacturer's protocol. Heat antigen retrieval was performed using citrate buffer pH 6.0 for 30 min. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide for 30 min, after being washed with phosphate-buffered saline (pH 7.4). The sections were incubated with primary monoclonal antibody against p52 (clone-E.212.1; Thermofisher Scientific; Rockford, USA; diluted 1:250) and polyclonal antibody against RelB (Thermofisher Scientific; Rockford, USA; diluted 1:300) overnight at 4 °C in a humidified moisture chamber. Secondary incubation was carried out with the horseradish peroxidase polymer for 25 min at room temperature. Immunoreactivity was visualized with Fast Quanto Red chromogen. Counterstaining was carried out with hematoxylin, and the sections were dehydrated by sequential treatment with water and xylene. Finally, the sections were mounted with DPX (BDH, Poole, UK) and examined by light microscopy. Appropriate positive and negative controls were performed. Human placenta was considered as a positive control for both p52 and RelB immunostaining (Kaliki et al. 2015). Negative controls were performed using a nonimmunized IgG replacing the primary antibody. Nuclear immunoreactivity was considered as positive immunoexpression of NC-NFκB proteins. Cytoplasmic immunoreactivity was considered a negative immunoexpression of NC-NFκB proteins.

Assessment of immunoreactivity scoring (IRS) of p52 and RelB

Microscopically, the sections were independently scored by two authors (MKS and LS) under the supervision of the experienced pathologist (SK) who established a semi-quantitative score for p52 and RelB proteins, and a consensus was reached for each IHC score. Nuclear immunoreactivity was recorded for p52 and RelB proteins.

Immunoreactivity score of nuclear immunoreactivity in UM

The immunoreactivity score (IRS) for p52 and RelB was evaluated by percentage positivity and staining intensity in the nuclei of tumor cells. The staining intensity was classified as negative, weak, moderate and strong (recorded as 0, 1+, 2+and 3+, respectively). In addition, the percentage of positive cells (400× magnification) was classified as 0–25%, 26–50%, 51–75% and 76–100% (recorded as 1, 2, 3 and 4). In the end, the scores of the two indexes were added. The tumors were regarded as immunopositive when an IRS was greater or equal to 3 and immunonegative when the IRS was less than 3 (Li et al. 2017).

Immunoreactivity of the p52/RelB co-expression

Nuclear immunoreactivity of p52 and RelB together was considered as a co-expression of p52/RelB. Nuclear immunoreactivity of both proteins was taken as an immunopositive co-expression of the p52/RelB, whereas the absence of nuclear immunoreactivity was considered as an immunonegative co-expression of the p52/RelB.

Quantitative reverse transcription real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from 60 fresh tumor tissues and 12 age-matched controls (normal uveal tissue) from enucleated specimens of staphylomatous eyes as non-tumor samples using RNA isolation kit (Purelink RNA Kit, Ambion, USA) according to manufacture protocol. 250 ng of RNA was used as a template for cDNA synthesis using Verso cDNA synthesis kit (Thermo Scientific, California, USA). mRNA level of p52 and RelB genes was determined by qRT-PCR using SYBR Green Master Mix (Thermo, Invitrogen, USA). β-Actin was used as a reference gene for the experiment. All reactions were carried out in a final volume of 10 µl. qRT-PCR primer sequences: NFκB2(sense): GGGGCATCAAAC CTGAAGATTTCT; NFκB2(antisense): TCCGGAACACAA TGCATACTGT; RelB(sense): AGGCAGTCACCTCCA CCTC; RelB(antisense): AGCATCCTTGGGGAGAGC. The PCR conditions were as follows: 95 °C for 10 min, followed by 35 cycles of 95 °C for 30 s, 56 °C (NFκB2)/58 °C (RelB) for 30 s and 72 °C for 30 s. Each PCR reaction was followed by a continuous melt curve analysis. All samples were run in triplicates and to assess contamination, a no template control was included in each PCR run. As negative controls, each sample was previously run with β-actin primers without reverse transcription to detect genomic DNA contamination All PCR reactions were performed on step one real-time PCR systems (Applied Biosystems). Data were normalized to the reference gene in all the cases, and the results were compared with the normalized expression in control (normal uveal tissue) to calculate a fold change value using the $2^{-\Delta\Delta CT}$ method. Relative mRNA expression of p52 and RelB was measured according to the fold change value. Fold change value ≥ 1.5 was considered as upregulation of the gene, whereas fold change value < 1.5 was considered as downregulation of the gene.

Western blotting

Nuclear proteins were extracted from 12 uveal melanoma tissues using NE-PER Nuclear and cytoplasmic extraction

kit (Pierce, Rockford, IL, USA). Protein concentrations were measured using BioRad protein assay (BioRad, Hercules, CA, USA). Twenty-five micrograms of nuclear lysates were resolved by SDS-PAGE (12% gel) and transferred to nitrocellulose membrane (MDI membranes Technologies, California, USA) at 75 V for 2.5 h. Nitrocellulose membrane was blocked in 5% of BSA in Tris-buffered saline containing 0.05% Tween-20 for 45 min at room temperature, followed by overnight incubation at 4 °C at the following dilutions: β-actin 1:10,000 (Sigma), anti-p52 1:3000 (Thermofisher Scientific; Rockford, USA) and anti-RELB 1:2500 (Thermofisher Scientific; Rockford, USA). After incubation with primary antibodies, blots were washed three times using TBS-T buffer (150 mM NaCl, 10 mM Tris–HCl (pH 7.6), 0.1% Tween-20) (Sigma-Aldrich) and then secondary antibody incubation was done at room temperature using anti-rabbit HRP (Cell Signaling Technology, Danvers, MA) at 1:4000 for 45 min. Blots were washed, and protein bands were visualized using the ECL plus detection kit (GE Healthcare).

Co-immunoprecipitation

Presence of heterodimer between p52 and RelB was examined using a co-immunoprecipitation technique (co-IP kit) (Thermo Scientific, Waltham, MA), following the manufacturer's protocol [canonical]. Briefly, Amino Link Plus coupling resin beads were used for 10 µg of p52 antibody (Thermofisher Scientific; Rockford, USA) or RelB (Thermofisher Scientific; Rockford, USA, clone-E.212.1) antibody conjugation and washed extensively. The resin was washed twice by adding IP lysis buffer to spin column containing the antibody-coupled resin. The flow through was discarded after centrifugation. Protein mixtures and controls were added to the appropriate resin and incubated with gentle mixing for 1–2 h. After extensive washing, bound proteins were eluted using elution buffer in 50 µl total volume for immediate analysis of total elution by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 12% gels and subsequently analyzed by western blotting.

Statistical analysis

Level of significance for all tests was considered as $p < 0.05$ and all tests were two-sided to evaluate the statistical association of p52, RelB and their co-expression and mRNA expression with clinicopathological parameters and patient outcome. All statistical analyses were performed using Stata 9 software (Stata Corp LP, College Station, TX, USA). Overall survival (OS) of the patient was estimated with a positive or negative expression of protein markers using the log-rank test. Hazard ratios and their 95% confidence intervals (CI) were noted for each marker. Kaplan–Meier method was used to draw the survival curves. Overall survival (OS) and metastasis-free survival (MFS) of patients with positive or negative immunoreactivity were compared using the log-rank test for equality of survivor functions. Independent prognostic factors were identified through the Cox proportional hazards models (univariate and multivariate analyses).

Results

Baseline, demographics and clinicopathological characteristics

In this cohort, the male: female ratio was 1.7:1 and the mean age was 57.21 ± 13.25 years. The mean largest basal diameter was 14.17 ± 4.1 mm. Our cohort of the study was divided into metastatic and non-metastatic groups.

Metastatic group (*N* = 16)

Sixteen of the 75 patients (21.33%) had distant metastasis. The mean large basal diameter was 16.8 mm (median 21 mm, range 12.4–24 mm). Sixty-two percent of cases showed the presence of microvessel density, whereas infiltrating lymphocytes were found in 56% of cases. Similarly, the presence of infiltrating macrophages and scleral invasion was found in 62% of the cases, respectively. Extraocular spread was seen in 37.5% of cases, while 56% of cases belonged to advanced tumor staging. Ciliary body invasion was found in 43% of cases, whereas loss of BAP-1 was observed in 87% of cases. Thirty-one percent of cases died due to liver metastasis.

Non-metastatic group (*N* = 59)

Fifty-nine of 75 patients had no metastasis. In this group, the mean large basal diameter was 14.2 mm (median 12, range 9.5–18 mm). Presence of infiltrating macrophages was observed in 28% of cases, while 25% of patients had infiltrating lymphocytes. Increased microvessel density was found in 33% cases. Thirteen percent of the cases had scleral invasion, while 6% of cases had an extraocular spread. Thirty percent of cases belong to advanced tumor staging and ciliary body invasion was seen in 16% of cases. No death occurred in this group.

Immunohistochemistry expression pattern of p52 and RelB proteins

In tumor cells, immunolocalization of NC-NFκB proteins was observed mainly in the nucleus along with weak cytoplasmic expression seen in few cases. The intensity of nuclear expression of RelB was comparatively lower than

p52 protein. Weak expression of non-canonical NFκB (NC-NFκB) proteins was observed in normal choroid samples. Nuclear expression of p52, RelB, and p52/RelB co-expression was observed in 48% (36/75), 41% (31/75) and 36% (27/75) cases, respectively, while 13% (p52), 17% (RelB) and 5% (p52/RelB) of the cases showed only cytoplasmic expression (Figs. 1, 2). In the metastatic group, a positive expression of p52, RelB, and p52/RelB was found in 81% (13/16), 68% (11/16) and 56% (9/16) of cases, respectively. While in the non-metastatic group, expression of p52, RelB and p52/RelB was found in less than 40% of the cases.

Immunoblot of p52 and RelB was performed to validate nuclear immunoreactivity results. Six cases were taken each from the metastatic and non-metastatic groups. In the metastatic group, fold change value of p52 (3.6-fold), RelB (2.9-fold) was increased as compared to 2.5-fold change value of p52 and 1.8-fold change value of RelB protein in the non-metastatic group (Fig. 3a–d).

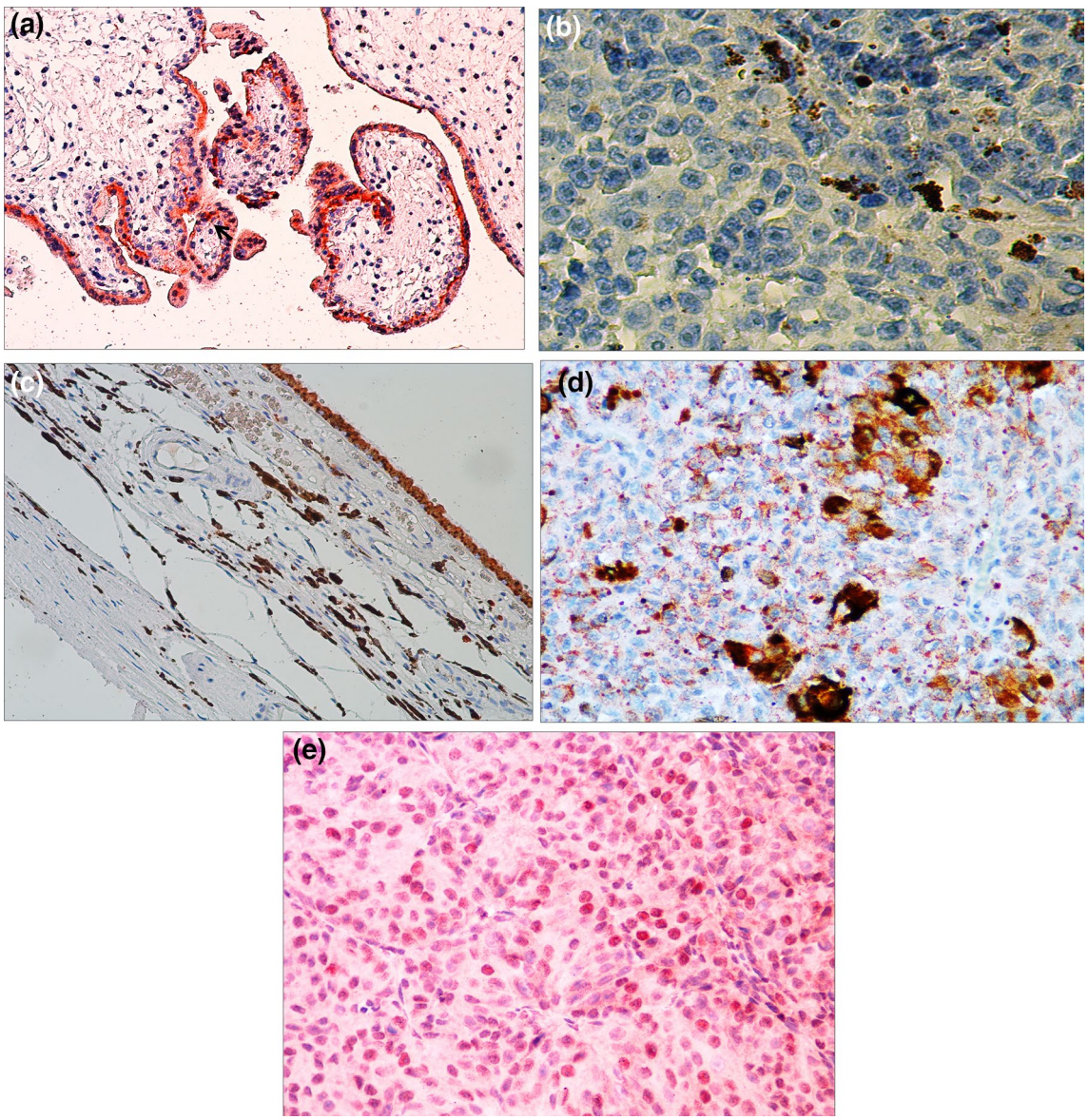

Fig. 1 Immunoreactivity of p52 in non-metastatic and metastatic cases of uveal melanoma (X200). **a** Immunoreactivity of p65 in human placenta taken as positive control; **b** negative control with omission of p52 in uveal melanoma; **c** weak immunoreactivity of p52 protein in the normal choroid; **d** weak cytoplasmic immunoreactivity of p52 (arrow) in the non-metastatic UM case; **e** strong nuclear immunoreactivity of p52 (arrow) in the metastatic UM case

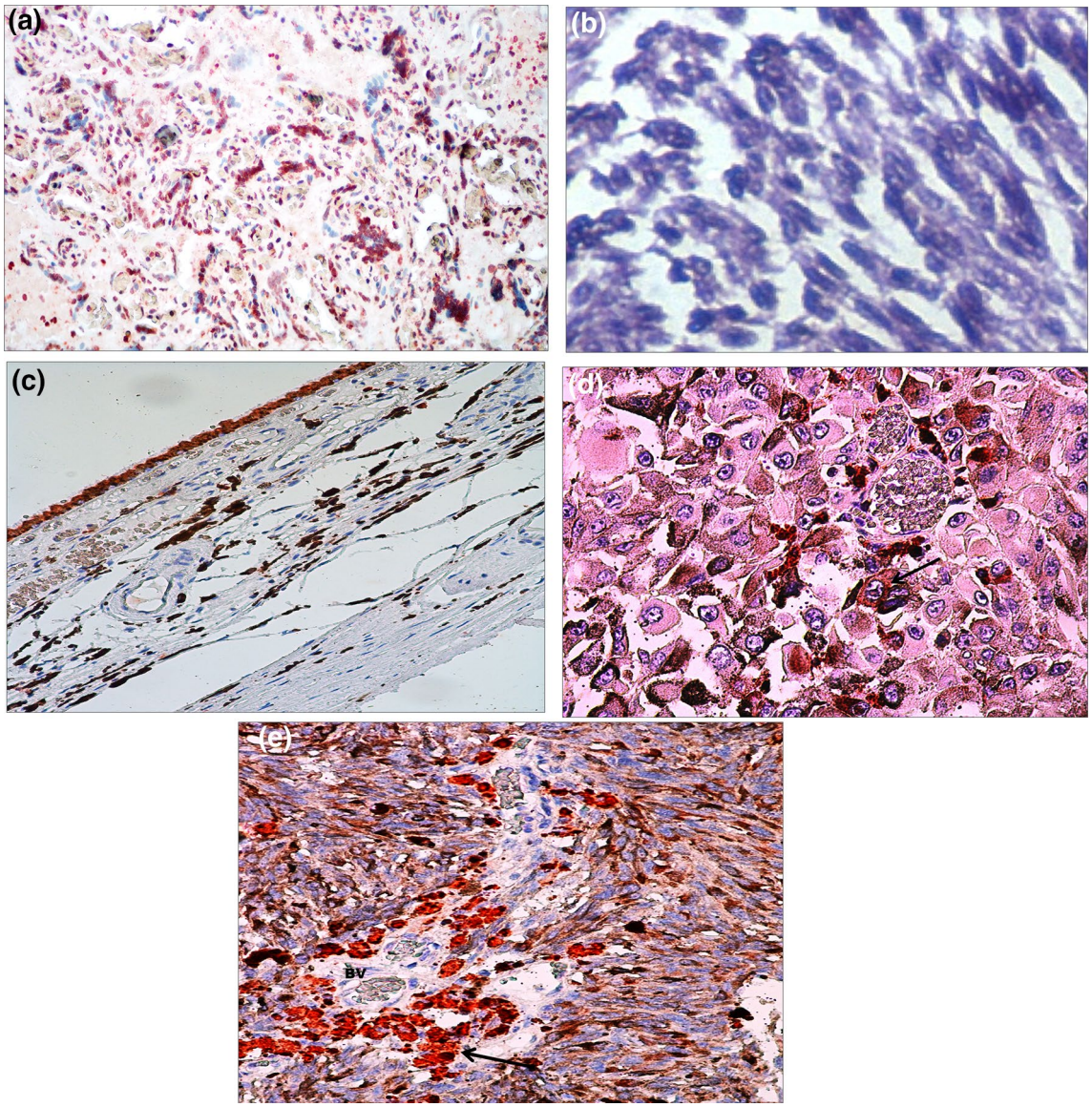

Fig. 2 Immunoreactivity of RELB in metastatic and non-metastatic cases of uveal melanoma (X200). **a** Immunoreactivity of RELB in human placenta taken as positive control; **b** negative control with omission of RELB in uveal melanoma; **c** weak RELB immunoreactivity in the normal choroid; **d** cytoplasmic immunoreactivity of RELB (arrow) in the non-metastatic UM case; **e** strong nuclear immunoreactivity of RELB (arrow) in the metastatic UM case

Statistical correlation of NC-NFκB proteins expression with clinicopathological parameters

In the metastatic group, co-expression of p52/RelB, p52, and RelB expression was statistically significant with regard to clinical factors such as LBD > 15 mm ($p = 0.029$; $p = 0.018$; $p = 0.004$), whereas co-expression of p52/RelB was significant as regards tumor thickness ($p < 0.05$) and clinical tumor staging ($p < 0.05$). None of the histopathological parameters were significant either with RelB or p52/RelB co-expression. While in non-metastatic group, expression of p52, RelB and co-expression of p52/RelB was statistically significant regarding LBD > 15 mm ($p < 0.05$). While histopathologically, NC-NFκB proteins correlated well with the infiltrating macrophages, and infiltrating lymphocytes ($p < 0.01$). Rest of the details is shown in Table 1.

Interaction of p52/RelB protein by co-immunoprecipitation

The presence of p52 and RelB as a heterodimer and how it regulates the NC-NFκB pathway in metastatic and non-metastatic cases of uveal melanoma was determined by co-immunoprecipitation (CO-IP). Nuclear-extracted proteins

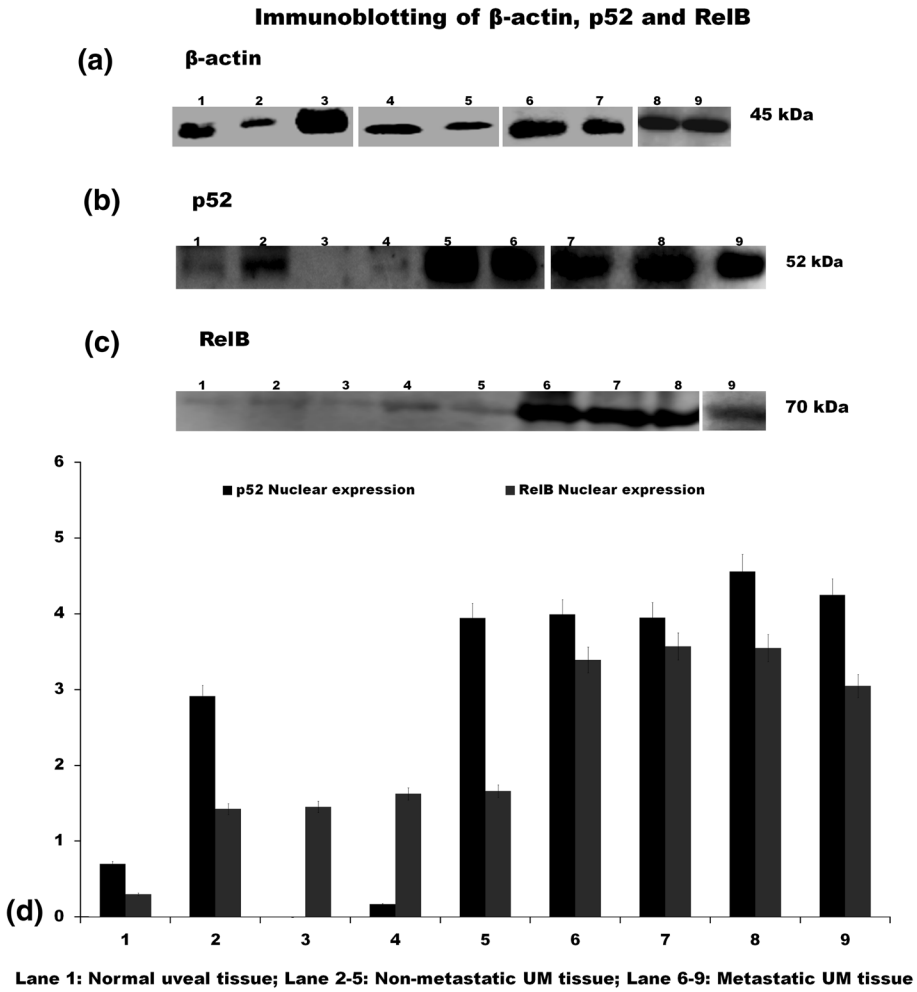

Fig. 3 Validation of p52, and RELB immunoreactivity results using western blotting: **a** β-actin protein endogenous control; **b** p52 expression in the nuclear extract of metastatic and non-metastatic tissues; **c** expression of RELB in the nuclear extract of metastatic and non-metastatic tissues; **d** relative levels of nuclear p52, RELB from immunoblot of eight UM patients (2–8) and one normal uveal tissue (1)

were taken from the metastatic and non-metastatic group. Our co-immunoprecipitation analysis showed that p52 predominantly binds to RelB in cases of metastatic UM as compared to non-metastatic cases (Fig. 4a, b).

mRNA levels of p52 and RelB genes and their statistical correlation with clinicopathological parameters

The average values of p52 (2.75-fold change) and RelB (2.05-fold change) mRNA expression were significantly higher than normal uveal tissue (Fig. 5). Upregulation of NFκB2 was observed in 58% cases, while 41% of cases showed upregulation of RelB gene. Upregulation of NFκB2 gene was statistical significant with loss of BAP-1 and infiltrating macrophages and lymphocytes. Clinically, RelB mRNA expression was significantly associated with none of the parameters, while histopathologically, it was significant regarding the loss of BAP-1 ($p=0.004$). Table 2 summarizes the details of mRNA expression. In Kaplan–Meier analysis, upregulation of NFκB2 ($p<0.01$) showed decreased metastasis-free survival (MFS) in 77% of cases, while upregulation of RelB gene showed decreased MFS in 87% of cases.

Univariate and multivariate analyses of clinicopathological factors for metastasis-free survival (MFS) and overall survival (OS)

The results of univariate analysis for metastasis-free survival (MFS) and overall survival (OS) are summarized in Table 3. Expression of p52 (hazard ratio (HR): 12, 95% confidence interval (CI): 1.65–103.62, $p=0.025$) and expression of RelB (HR: 5.06, 95% CI: 1.13–22.48; $p=0.034$) proved to be independent prognostic factors for MFS. In case of OS, expression of p52 (HR: 38.5; 95% CI: 5.38–175.05; $p=0.015$) was an independent prognostic factor.

Table 1 Correlation of immunoreactivity of p52, RelB and p52/RelB co-expression with the clinicopathological parameters of metastasis and non-metastasis groups of uveal melanoma by Fisher's exact test

Clinicopathological parameters ($N=16$) (N %)	Metastasis group								
	P52 immunoreactivity			RelB immunoreactivity			P52/RelB co-immunoreactivity		
	Positive (13)	Negative (3)	p value	Positive (11)	Negative (6)	p value	Positive (9)	Negative (7)	p value
Age									
> 40 years (9) (56%)	8	1	0.375	6	3	0.838	5	4	0.949
≤ 40 years (7) (43%)	5	2		5	3		4	3	
Large basal diameter (LBD)									
> 12 mm (13) (81%)	12	1	**0.018**	11	2	**0.004**	9	4	**0.029**
≤ 12 mm (3) (18%)	1	2		0	3		0	3	
Clinical tumor staging									
T3–T4 (9) (56%)	8	1	0.375	8	1	**0.049**	7	2	**0.049**
T1–T2 (7) (43%)	5	2		3	4		2	5	
Tumor thickness									
> 8 mm (11) (68%)	10	1	0.142	9	2	0.094	8	3	**0.049**
≤ 8 mm (5) (31%)	3	2		2	3		1	4	
BAP-1 expression									
Loss (14) (87%)	12	2	0.350	9	5	1.000	7	7	0.475
Present (2) (12%)	1	2		2	1		2	0	
Epithelioid cell type									
Present (10) (62%)	10	0	**0.013**	6	4	0.587	5	5	0.632
Absent (6) (37%)	3	3		5	1		4	2	
Large tumor diameter									
> 15 mm (11) (68%)	10	1	0.142	9	2	0.094	7	4	0.377
≤ 15 mm (5) (31%)	3	2		2	3		2	3	
CD68 + macrophage									
Present (10) (62%)	10	0	**0.013**	7	3	0.889	7	3	0.152
Absent (6) (37%)	3	3		4	2		2	4	
CD3 + T lymphocytes									
Present (9) (56%)	9	0	0.062	7	2	0.596	7	2	0.126
Absent CD3 (7) (43%)	4	3		4	3		2	5	
Ciliary body invasion									
Present (7) (43%)	6	1	0.687	6	1	0.197	4	3	0.949
Absent (9) (56%)	7	2		5	4		5	4	
CD34 expression (MVD)									
Present (8) (50%)	7	1	1.000	7	1	0.282	6	2	0.314
Absent (8) (50%	6	2		4	4		3	5	
Mitotic count									
> 4 per 40HPF (10) (62%)	8	2	0.869	8	2	0.210	6	4	0.696
≤ 4 per 40HPF (6) (37%)	5	1		3	3		3	3	
Extraocular spread									
Present (6) (37%)	4	2	0.247	4	2	0.889	3	3	0.696
Absent (10) (62%)	9	1		7	3		6	4	
Scleral invasion									
Present (10) (62%)	9	1	0.247	8	2	0.210	6	4	0.696
Absent (6) (37%)	4	2		3	3		3	3	

Table 1 (continued)

Clinicopathological parameters ($N=59$) (N %)	Non-metastasis group								
	P52 immunoreactivity			RelB immunoreactivity			p52/RelB co-immunoreactivity		
	Positive (23)	Negative (36)	p value	Positive (20)	Negative (39)	p value	Positive (18)	Negative (41)	p value
Age									
> 40 years (47) (79%)	18	29	0.831	15	32	0.524	13	34	0.347
≤ 40 years (12) (20%)	5	7		5	7		5	7	
Large basal diameter (LBD)									
> 12 mm (37) (62%)	20	17	**0.002**	16	21	**0.049**	15	22	**0.030**
≤ 12 mm (22) (37%)	3	19		4	18		3	9	
Clinical tumor staging									
T3–T4 (18) (30%)	9	9	0.250	10	8	**0.020**	8	10	0.123
T1–T2 (41) (69%)	14	27		10	31		10	31	
Tumor thickness									
> 8 mm (22) (37%)	13	9	**0.015**	11	11	**0.044**	10	12	0.055
≤ 8 mm (37) (62%)	10	27		9	28		8	29	
BAP-1 expression									
Loss (32) (54%)	18	14	**0.003**	14	18	0.102	12	20	0.261
Present (27) (45%)	5	22		6	21		6	21	
Epithelioid cell type									
Present (17) (28%)	14	3	**0.001**	11	6	**0.001**	10	7	**0.003**
Absent (42) (71%)	9	33		9	33		8	34	
Large tumor diameter									
> 15 mm (34) (57%)	18	16	**0.010**	14	20	0.168	13	21	0.133
≤ 15 mm (25) (42%)	5	20		6	19		5	20	
CD68 + macrophage									
Present (17) (28%)	13	4	**0.001**	11	6	**0.001**	10	7	**0.003**
Absent (42) (71%)	10	32		9	33		8	34	
CD3 + T lymphocytes									
Present (15) (25%)	14	1	**0.001**	11	4	**0.001**	10	5	**0.003**
Absent (44) (74%)	9	35		9	35		8	36	
Ciliary body invasion									
Present (10) (16%)	5	5	0.433	4	6	0.655	4	6	0.474
Absent (49) (83%)	18	31		16	33		14	35	
CD34 expression (MVD)									
Present (20) (33%)	11	9	0.071	12	8	**0.002**	11	9	**0.003**
Absent (39) (66%)	12	27		8	31		7	32	
Mitotic count									
> 4 per 40HPF (16) (27%)	12	4	**0.001**	10	6	**0.005**	9	7	**0.009**
≤ 4 per 40HPF (43) (72%)	11	32		10	33		9	36	
Extraocular spread									
Present (4) (6%)	3	1	0.126	3	1	0.072	3	1	**0.045**
Absent (55) (93%)	20	35		17	38		15	40	
Scleral invasion									
Present (8) (13%)	4	4	0.492	4	4	0.301	4	4	0.198
Absent (51) (86%)	19	32		16	35		14	37	

Bold signifies statistically significant value

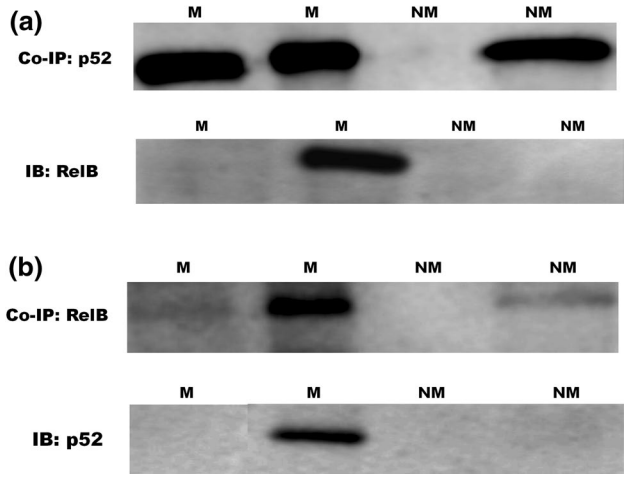

Fig. 4 Interaction of p52 and RELB as p52/RELB co-expression by co-immunoprecipitation (Co-IP): **a, b** co-IP from precleared lysates of the metastatic and non-metastatic uveal melanoma tissues using either p52-linked or RELB-linked agarose beads, followed by immunoblotting (IB) analysis for p52, RELB

Association of non-canonical NFκB (NC-NFκB) proteins with metastasis-free survival (MFS) and overall survival (OS) by Kaplan–Meier analysis (log-rank test)

In this study, increased p52 expression correlated with reduced MFS (p value = 0.003, HR = 4.67, 95% CI: 1.75–12.46) and a trend towards worse OS (p value = 0.002, HR = 7.31, 95% CI: 1.65–32.42) in UM patients was observed (Fig. 6a, b). As expected, nuclear RelB expression was also statistically correlated with MFS (p = 0.003, HR = 3.86, 95% CI: 1.39–10.70) with a trend towards worse OS (p = 0.166, HR = 2.58, 95% CI: 0.54–12.34) (Fig. 6c, d). Supporting these data, we analyzed the prognosis of the p52 and RelB subunits in UM patients (Fig. 6e, f). The presence of both subunits strongly correlated with MFS (p = 0.004, HR = 3.34, 95% CI: 1.14–10.31) and OS (p = 0.032, HR = 4.05, 95% CI: 0.71–22.56).

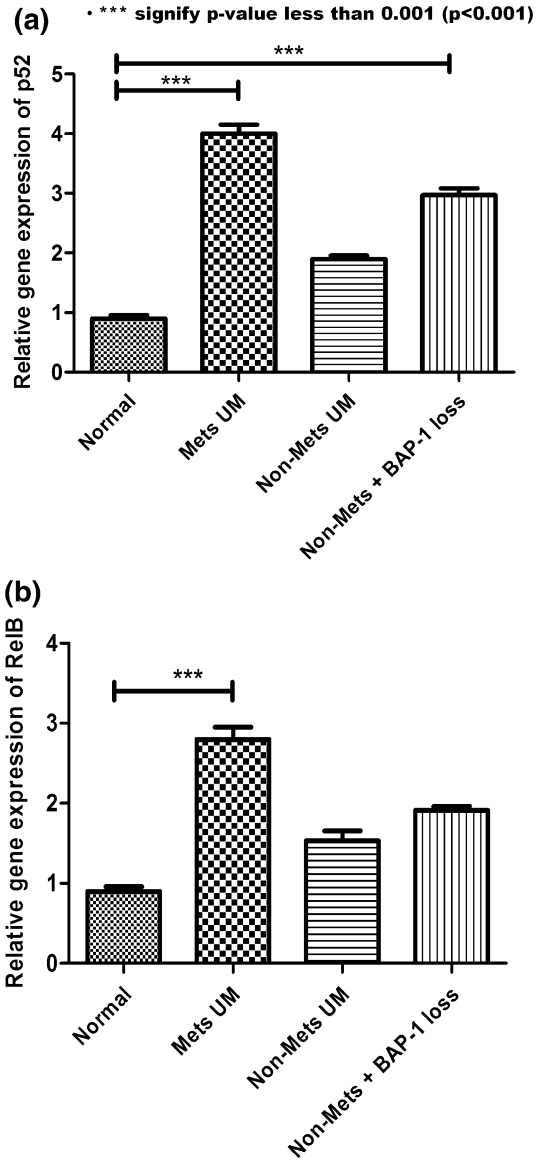

Fig. 5 mRNA expression of p52 and RELB genes by real-time PCR (qRT-PCR). Relative mRNA expression of **a** p52 and **b** RELB at mRNA level in normal uveal tissue, metastatic UM, non-metastatic UM and non-metastatic UM with loss of BAP-1 protein were evaluated using relative Ct method. β-Actin expression levels used as internal control

Discussion

Non-canonical NFκB (NC-NFκB) signaling pathway expression results in translocation of p52 and RelB subunits in the nucleus to activate gene transcription. Therefore, expression of NFκB is identified by positive nuclear immunostaining of p52, RelB, and p52/RelB co-expression. In this study, we performed immunohistochemistry which is a simple, reproducible method for the pathologists to investigate the expression of p52, and RelB proteins. The presence of the native heterodimer p52/RelB in human uveal melanoma was confirmed by co-immunoprecipitation experiments followed by western blot analysis. On extensive review of literature, only a couple of studies evaluated the expression of NC-NFκB proteins in cancer tissues mainly in breast carcinoma (Rojo et al. 2016), cutaneous melanoma (Ueda and Richmond 2006), and renal cell carcinoma (Lua et al. 2018) and has been implied in poor survival of these tumors.

Loss of BAP-1 protein was associated with increased metastatic risk and considered as a poor prognostic marker in uveal melanoma (Harbour et al. 2010). In our study, loss of BAP-1 significantly correlated with expression of p52

Table 2 Correlation of transcriptional status of p52 and RELB genes with the clinicopathological parameters of uveal melanoma patients using Fisher's exact test

Parameters ($N=60$)	$N=60$	Transcriptional status of non-canonical NFκB members					
		p52 mRNA expression			RELB mRNA expression		
		Upregulation (35)	Downregulation (25)	*p* value	Upregulation (25)	Downregulation (35)	*p* value
Age							
> 40 years	45	29	16	0.096	20	25	0.450
≤ 40 years	15	6	9		5	10	
Large basal diameter (LBD)							
> 15 mm	39	24	15	0.493	16	23	0.891
≤ 15 mm	21	11	10		9	12	
Extraocular spread							
Present	6	4	2	0.663	3	3	0.663
Absent	54	21	33		22	32	
Clinical tumor staging							
T3–T4	19	11	8	0.963	7	12	0.606
T1–T2	41	24	17		18	23	
Tumor thickness							
> 8 mm	**28**	18	10	0.382	13	15	0.484
≤ 8 mm	**32**	17	15		12	20	
Metastasis							
Present	9	8	1	**0.044**	4	5	0.855
Absent	51	27	24		21	30	
Epithelioid cell type							
Present	21	17	4	**0.009**	9	12	0.891
Absent	39	18	21		16	23	
BAP-1 expression							
Loss	35	25	10	**0.018**	16	9	**0.004**
Present	25	10	15		9	26	
Large tumor diameter (LTD)							
> 15 mm	23	21	2	0.538	13	10	0.538
≤ 15 mm	37	14	23		12	25	
Ciliary body invasion							
Present	13	10	3	0.125	8	5	0.101
Absent	47	25	22		17	30	
Scleral invasion							
Present	13	10	3	0.125	4	9	0.368
Absent	47	25	22		21	26	
Infiltrating macrophage (CD68 +)							
Present	20	16	4	**0.016**	5	15	0.064
Absent	40	19	21		20	20	
Mitotic count							
> 4 per 40HPF	20	12	8	0.853	10	10	0.355
≤ 4 per 40HPF	40	23	17		15	25	
CD34 expression (MVD)							
Present	25	17	8	0.199	14	11	**0.047**
Absent	35	18	17		11	24	
CD3 + T lymphocytes							
Present	19	15	4	**0.027**	5	14	0.101
Absent	41	20	21		20	21	

Bold signifies statistically significant value

MVD microvessel density

Table 3 Clinical relevance of clinicopathological parameters of uveal melanoma by multivariate and univariate Cox proportional hazard analysis for metastasis-free survival (MFS) and overall survival (OS)

Clinicopathological parameters	Metastasis-free survival (MFS)				Overall survival (OS)			
	Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis	
	Hazard ratio (95% confidence interval)	*p* value	Hazard ratio (95% confidence interval)	*p* value	Hazard ratio (95% confidence interval)	*p* value	Hazard ratio (95% confidence interval)	*p* value
Loss of BAP-1	2.68 (0.83–8.36)	0.088	–	–	**10 (1.05–95.04)**	**0.045**	3.74 (0.85–25.75)	0.108
Advanced clinical tumor staging	2.92 (0.94–9.08)	0.063	–	–	2.87 (0.44–18.39)	0.265	–	–
Tumor thickness (> 8 mm)	**3.7 (1.13–12.05)**	**0.030**	2.70 (0.66–11.11)	0.166	5.65 (0.60–53.24)	0.130	–	–
Large basal diameter (LBD > 15 mm)	2.57 (0.66–10.05)	0.173	–	–	–	–	–	–
Scleral invasion	**10.62 (3.02–37.34)**	**0.001**	**5.13 (1.13–23.27)**	**0.034**	–	–	–	–
Ciliary body invasion	**3.81 (1.14–12.64)**	**0.029**	1.30 (0.26–6.47)	0.742	2.44 (0.37–15.98)	0.351	–	–
Large tumor diameter(LTD > 15 mm)	1.61 (0.49–5.24)	0.423	–	–	–	–	–	–
Extraocular spread	**8.25 (1.96–34.59)**	**0.004**	4.59 (0.79–26.62)	0.089	1.69 (0.16–16.90)	0.653	–	–
High mitotic count (> 4/40HPF)	**4.47 (1.39–14.33)**	**0.012**	2.39 (0.34–16.77)	0.378	8.72 (0.92–82.69)	0.059	–	–
Epithelioid cell type	**3.45 (1.10–10.82)**	**0.033**	0.07 (0.01–2.74)	0.157	**9.33 (0.98–88.56)**	**0.042**	–	0.361
CD3 + T lymphocytes	**4.47 (1.39–14.33)**	**0.012**	–	0.451	**8.82 (0.92–83.45)**	**0.045**	–	–
CD68-macrophage	**4.11 (1.29–13.11)**	**0.017**	**12.83 (0.15–70.94)**	**0.035**	**8.92 (0.92–84.69)**	**0.039**	**10.70 (0.65–71.36)**	**0.041**
CD34 expression (MVD)	**3.25 (1.03–10.23)**	**0.044**	0.80 (0.15–4.22)	0.796	–	–	–	–
p52 expression	**4.52 (1.65–12.42)**	**0.033**	**12 (1.65–103.62)**	**0.025**	**15.48 (4.48–53.38)**	**0.036**	**38.5 (5.38–175.05)**	**0.015**
RelB expression	**3.28 (1.44–10.63)**	**0.043**	**5.06 (1.13–22.48)**	**0.034**	4.66 (1.72–12.64)	0.322	–	–

Bold signifies statistically significant value

and RelB protein and both were seen more frequently in the metastatic group as compared to non-metastatic group. This suggests evidence that tumor suppressor proteins regulate genes that are involved in tumor-promoting inflammation and immune response by interacting with NF-κB pathway. In a recent hypothesis, loss of BAP-1 (tumor suppressor protein) reduces the suppression pathways leading to activation of the NFκB proteins which in turn produce cytokines and chemokines (Gezgin et al. 2017). Similarly, expression of NC-NFκB proteins was statistically significant regarding the presence of epithelioid cell type, infiltrating lymphocytes and macrophages. Increased numbers of microvascular densities along with the presence of infiltrating macrophages suggest that NC-NFκB proteins play an essential role in promoting the angiogenesis involved in the progression of the tumor (Mantovani and Sica 2005).

Studies have demonstrated that LBD (> 15 mm), tumor thickness (> 8 mm) and advanced clinical tumor staging promote tumor growth and metastatic risk in uveal melanoma (Nguyen et al. 2018). In the present study, we found that expression of p52 and RelB was significantly higher in metastatic cases having LBD > 15 mm, tumor thickness greater than 8 mm and clinically advanced tumor staging, suggesting that these proteins may contribute to tumor invasion. These data are consistent with Oya et al., who demonstrated that increased activity of NFκB proteins plays a role in the progression of advanced stage tumors and increased inflammation in renal cell carcinomas (Oya et al. 2003).

Some of the studies suggested that non-canonical NF-κB showed invasive phenotype by induction of RelB gene which may promote survival of tumor cells after chemotherapy (Mineva et al. 2009). Therefore, RelB and p52 have been associated with reduced overall survival, poorer tumor differentiation, tumor invasion, lymph node metastasis, distant metastasis, and TNM stage in non-small cell lung cancer and bladder cancer (Qin et al. 2015). Our study showed that expression of p52, RelB and their co-expression was seen more frequently in cases with metastasis at transcriptional and protein levels and found to be associated with poor prognostic factors. Our results are consistent with the gene expression pattern study by Meir et al. who identified the NFkB2 as a potential regulator of metastasis-associated genes in cases with liver metastasis from uveal melanoma (Meir et al. 2007). These findings suggest that the non-canonical pathway might facilitate tumor progression in uveal melanoma.

Only few studies explained the importance of non-canonical pathway with patient survival. The Kaplan–Meier analysis of our data indicates that overall survival was significantly decreased in cases with an increased expression of p52 and p52/RelB co-expression, while metastasis-free survival was significantly reduced in cases with the expression of p52, RelB, and p52/RelB co-expression. This association

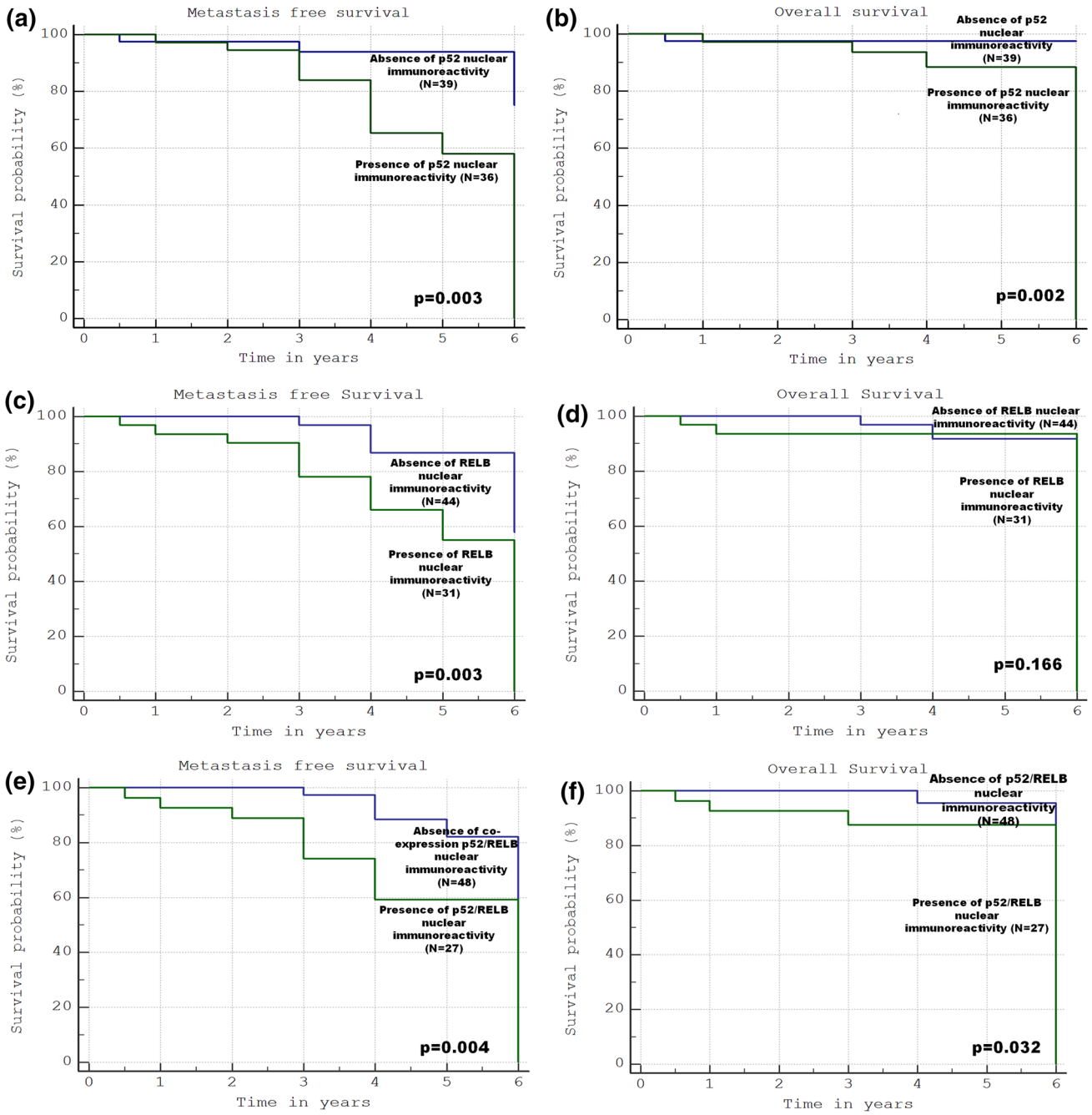

Fig. 6 Kaplan–Meier survival curve in dependence of clinicopathological parameters and immunoreactivity pattern of p52, RELB and p52/RELB. Metastasis-free survival (MFS) and overall survival (OS) dependent on nuclear immunoreactivity of the whole study population (**a**, **c**, **e**) as well as a metastatic subgroup (**b**, **d**, **f**)

is strongly related to the patient outcome in response to their expression suggesting the clinical relevance of these proteins alone as well as heterodimer form in uveal melanoma. These results are in line with breast carcinoma study in which metastasis-free survival was found to be reduced with the co-expression of p52/RelB and the expression of p52, RelB proteins is independently associated with poor survival in ER + breast cancer patients (Maat et al. 2008).

To conclude, our data are the first to report the transcriptional and translational status of p52, and RelB in metastatic and non-metastatic patients with uveal melanoma. Further, we showed that the expression of p52, RelB, and their co-expression were more frequently found in the metastatic group as compared to the non-metastatic group. Our results revealed that the non-canonical NFκB (NC-NFκB) pathway plays an influential role in tumor

progression and metastasis of uveal melanoma. Further in vitro and in vivo studies are required to explore whether NC-NFκB proteins might serve as a therapeutic target for uveal melanoma cases with metastasis.

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Author contributions All authors made substantial contributions to the study and they have approved the current version and agreed to publication.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interests.

Informed consent Yes.

Ethical approval The study was conducted following the protocol approved by the institutional ethics committee, All India Institute of Medical Sciences (IESC/T-417/2015).

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