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Clinicopathological relevance of NFκB1/p50 nuclear immunoreactivity and its relationship with the inflammatory environment of uveal melanoma

Mithalesh Kumar Singh[a], Lata Singh[b], Kunzang Chosdol[c], Neelam Pushker[a], Rachna Meel[a], Sameer Bakhshi[d], Seema Sen[a], Seema Kashyap[a,*]

[a] Dr. R. P. Centre for Ophthalmic Sciences, All India Institute of Medical Sciences, New Delhi, India
[b] Departrment of Biosciences, JMI, New Delhi, India
[c] Department of Biochemistry, AIIMS, India
[d] Department of Medical Oncology, IRCH, AIIMS, India

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ABSTRACT

Purpose: To analyze the activation of NFκB1/p50 in the inflammatory and non-inflammatory environment of uveal melanoma and its association with clinicopathological factors and patient outcome.
Methods: Activation of NFκB1/p50 was evaluated in 75 cases of uveal melanoma by immunohistochemistry. mRNA expression in 58 fresh UM specimen was measured by quantitative reverse-transcriptase PCR (qRT-PCR). Western blotting was performed to validate the immunohistochemistry results in representative cases.
Results: Forty-five cases showed both cytoplasmic and nuclear immunoreactivity of NFκB1/p50. Increased level of NFκB1/p50 activation was more frequent in the inflammatory environment group as compared to non-inflammatory environment group at both transcriptional and translational level. In multivariate analysis, infiltrating macrophages and nuclear immunoreactivity of NFκB1/p50 ($p < .05$) in tumor cells were found to be an independent prognostic factor for poor survival.
Conclusion: Our results suggest that nuclear immunoreactivity NFκB1/p50 may serve as a useful marker in assessing the prognosis of uveal melanoma patients.

1. Introduction

Uveal melanoma (UM) is a rare malignancy with a 5-year survival rate as low as 60%. High incidence of metastases results in increased mortality rate (Caminal et al., 2012; Singh and Topham, 2003). Clinical and metastatic behavior of UM differs from cutaneous melanoma because of its purely hematogenous dissemination and tendency to metastasize to the liver (Bakalian et al., 2008). Prognosis of uveal melanoma is poor when liver metastasis occurs, and life expectancy reduces to less than six months in the absence of treatment (Pons et al., 2011; Mariani et al., 2009).

Inflammation is considered the seventh hallmark of cancer (Colotta et al., 2009). Cancer cells may exploit immune evasion to survive and expand (Cavallo et al., 2011). The eye is considered as an immune-privileged organ. It has the unique ability to defend itself against uncontrolled inflammation that could damage vision (McKenna and Chen, 2010). This immune privilege influences the immune response against UM cells and provides escape mechanisms for UM. There is strong evidence that UM cells mimic the mechanisms that enable normal ocular cells to be immune-privileged, both in the eye and other metastatic locations (Niederkorn, 2009).

The term “inflammatory phenotype” was given by Jager et al. to define tumors carrying a high number of macrophages and lymphocytes, high level of HLC class I and class II expression, the presence of epithelioid cells and high MVD (microvascular density) in uveal melanoma (Jager et al., 2011). There are studies which described the biochemical pathway between inflammation and cancer and proves that inflammation increases the tumor progression and supports the metastatic spread (Mantovani et al., 2008; Aggarwal and Gehlot, 2009). In uveal melanoma, the tumor may take advantage of the inflammatory microenvironment by several mechanisms. Instead of a robust antitumor response, the immune cells in the microenvironment promotes the tumor progression. Activation of these infiltrating immune cells results in secretion of inflammatory mediators for generating tumor-promoting factors for inflammatory microenvironment. Some of the important intrinsic pathways include specific transcription factors such as nuclear factor-kappa B (NFκB) and signal transducer activator of transcription-3 and inflammatory cytokines (Bronkhorst and Jager,

* Corresponding author at: Department of Ocular Pathology, Dr. R. P. Centre for Ophthalmic Sciences, All India Institute of Medical Sciences, New Delhi, India.
E-mail address: dr_skashyap@hotmail.com (S. Kashyap).

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2012).

Many cellular functions are regulated by NFκB such as cell proliferation, survival and apoptosis, angiogenesis, immune response, cell adhesion and differentiation (Chen and Sawyers, 2002). The signaling pathway of NFκB plays an important role in linking inflammation to tumor development and progression (Wan and Lenardo, 2010). NFκB1/p50 has been shown to have an important function as an anti-inflammatory transcription factor through repressing expression of proinflammatory genes while enhancing the expression of anti-inflammatory genes (Beinke, 2004).

In vivo studies, mice models showed that absence of NFκB1/p50 reduces the number of plasmacytoid dendritic cells which are involved in viral immunity displaying defective activation (O'Keeffe et al., 2005). One more study revealed that knockdown of NFκB1/p50 results in damage to internal organs by increased inflammation (Oakley et al., 2005; Rolova et al., 2014). Overexpression of NFκB1/p50 has been observed in Hodgkin lymphoma, DLBCL, mucosa-associated lymphoid tissue lymphoma, primary effusion lymphoma and adult T-cell lymphoma/leukemia (Wang et al., 2017; Keller et al., 2000).

There are few studies in literature which assessed the protein expression of NFκB1/p50 in uveal melanoma (Dror et al., 2010; Singh et al., 2019). Hence we aim to detect the expression of NFκB1/p50 in inflammatory microenvironment factors and its correlation with patient outcome.

2. Material and method

2.1. Patient details

Seventy five patients with primary uveal melanomas were included in this prospective study after ethical approval from Institute's Ethical Committee, AIIMS (Ref. No. IESC/T-417/2015) and was carried out by the declaration of Helsinki principles. Uveal melanoma patients who didn't received chemotherapy or radiotherapy preoperatively were included in this study. Written consent was obtained from all the patients enrolled in the study, and their clinical and radiological details were noted. Enucleated uveal melanoma samples were collected from operation theatre of Dr. Rajendra Prasad Centre for Ophthalmic Sciences, All India Institute of Medical Sciences (AIIMS), New Delhi, India over a period of 3 years. Follow up ranged from 6 months to 60 months. Gross photograph of enucleated eyeball helps in identifying the growth pattern (dome, mushroom or diffuse shape), size and location of a tumor. Uveal melanoma samples from enucleated eyes were collected from the main tumor and stored at -70 °C for molecular experiments. Rest of the tissue was formalin fixed paraffin embedded (FFPE) for Hematoxylin and eosin staining (H&E) and immunohistochemical procedures.

2.1.1. High-risk factors

Histopathology slides were reviewed to determine the histological cell types, high-risk factors, and inflammatory parameters. High-risk factors (HRFs) include anterior location like ciliary body tumor, tumor height (> 8 mm), largest basal diameter > 15 mm (clinically), large tumor diameter > 15 mm (gross pathology), high mitotic figures (> 4/10HPF), scleral invasion and ciliary body invasion (Kaliki et al., 2015a) and status of BAP1 immunoreactivity (Johannes et al., 2016; Harbour et al., 2010). Inflammatory parameters include the presence of both tumor infiltrating lymphocytes (TILs), infiltrating macrophages (> 30% CD68 positivity), epithelioid cell type (> 50% epithelioid cell) and vascular mimicry (> 30% CD34 positivity) (Singh et al., 2019). American Joint Committee on Cancer (AJCC) staging system classified tumor staging according to the 8th edition uveal melanoma classification system (Kivelä et al., 2016).

2.2. Immunohistochemistry

Unstained FFPE sections (4 μm thickness) of all 75 cases were cut on poly-lysine coated slides for immunohistochemistry using the avidin-biotin indirect method. In brief, antigen retrieval was performed after deparaffinization and rehydration by microwave oven method in a citrate buffer solution at pH 6.0 for 30 min, followed by endogenous peroxidase blocking for 10 min. The sections were then incubated with primary monoclonal antibody against NFκB1/p50 (clone D4P4D; Cell Signaling). Subsequent incubations were performed with biotinylated secondary antibody and peroxidase-labeled streptavidin according to the manufacturer's protocol (Ultravision Fast Quanto red detection kit; Thermo Scientific, California, USA). Immunoreactivity was visualized using fast Quanto red substrate for 15 min, counterstained with hematoxylin and visualized by light microscopy. All experiments were carried out using positive and negative controls. Breast carcinoma taken as positive control tissues to verify the specificity of the antibody. Negative controls were performed using a nonimmunized IgG replacing the primary antibody.

Table 1
Clinical and histopathological characteristics of the uveal melanoma patients

Clinical parameters	*N* = 75 (N%)	Histopathological parameters	N = 75 (N%)
Sex		**Tumor pigmentation**	
Male	48 (64%)	High	66 (88%)
Female	27 (36%)	Low	9 (12%)
Age		**Cell type**	
≤40 years	19 (25.33%)	Spindle	40 (53.33%)
> 40 years	56 (74.66%)	Epithelioid	25 (33.33%)
		Mixed	10 (13.33%)
Tumor height		**Largest tumor diameter (LTD)**	
≤8 mm	42 (56%)	≤15 mm	20 (26.66%)
> 8 mm	33 (44%)	> 15 mm	55 (73.33%)
Largest basal diameter (LBD)			
> 15 mm	45 (60%)	Necrosis	26 (34.66%)
≤15 mm	30 (40%)		
Viterous Haemmorhage	8 (10.66%)	Scleral invasion	18 (24%)
Location of tumor			
Choroid	66 (88%)	Iris & ciliary body invasion	17 (22.66%)
Ciliary body	9 (12%)		
Clinical tumor staging			
T1-T2	48 (64%)	Optic nerve invasion	9 (12%)
T3-T4	27 (36%)		
Distant metastasis	15 (20%)	Extraocular spread	10 (13.33%)
		Mitotic count	
Death	5 (6.66%)	> 4 per 40HPF	25 (33.33%)
		≤4 per 40HPF	50 (66.66%)
High risk factor > 1 (HRFs > 1)	36 (48%)	Tumor infiltrating lymphocytes (TILs)	24 (32%)
Loss of BAP1 immunoreactivity	46 (61%)	Vascular mimicry	30 (40%)
-	-	Infiltrating macrophages	23 (30.66%)
-	-	**Tumor environment**	
		Inflammatory group	25 (33.33%)
		Non Inflammatory group	50 (66.67%)

2.3. Staining and scoring of immunohistochemistry

The samples were independently scored by two authors (MKS and LS) under the supervision of the experienced pathologist (SK) who established a semi-quantitative score for NFκB1/p50.

2.3.1. Grading of cytoplasmic and nuclear immunoreactivity in UM

The immunohistochemical staining for NFκB1/p50 was evaluated by percentage positivity and staining intensity in tumor cells. The staining intensity was classified as negative, weak, moderate and strong (recorded as 0, 1+, 2+ and 3+, respectively). In addition, the number of positive cells (magnification, ×400) classified as 0–25%, 26–50,

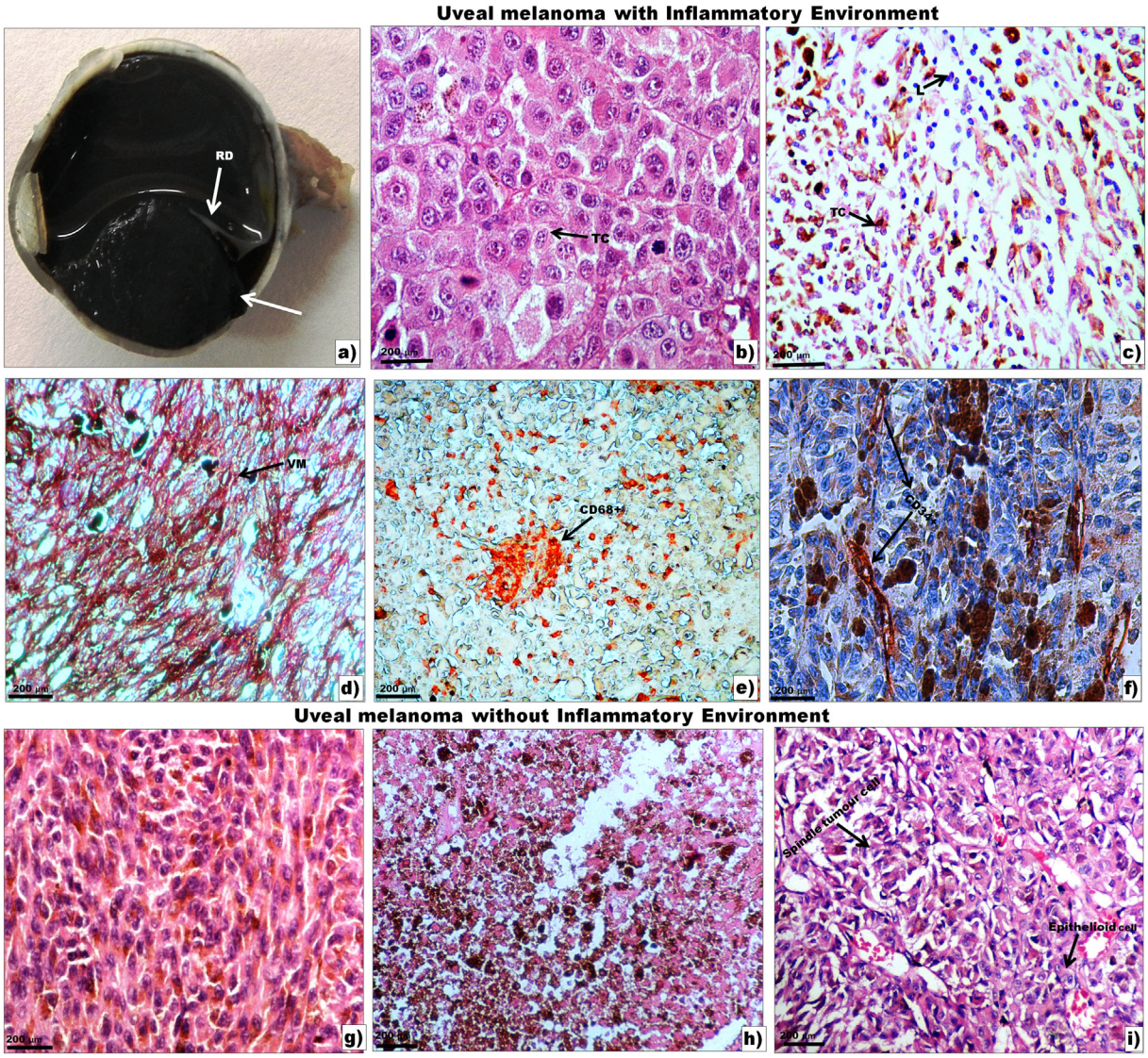

Fig. 1. Representative images of gross photograph, Hematoxylin &Eosin (H&E) staining of parameters associated with inflammatory (b-f) and non-inflammatory environment (g-i) (X200 magnification).
a) Cut section of enucleated eyeball showing dome-shaped melanoma; b) Epithelioid cell type uveal melanoma showing tumor cell (TC); c) Infiltration of lymphocytes (L) in the tumor (TC = tumor cell); d) presence of closed vascular mimicry (VL) in uveal melanoma; e) immunopositivity of CD68 staining showing the presence of macrophages; f) increased tumor pigmentation; g) spindle cell type melanoma; h) presence of necrotic areas (arrow); i) mixed cell type melanoma showing both spindle and epithelioid cells (arrow).

51–75% and 76–100% (recorded as 1,2, 3 and 4). In the end, the scores of the two indexes were added. Total score ≥ 3 was considered as positive expression and < 3 as a negative expression (Singh et al., 2019).

2.4. RNA isolation & reverse transcription

Total RNA from a tumor and control samples was isolated using the Purelink RNA Isolation kit (Ambion, Austin, TX, USA) according to the manufacturer's protocol. Full lengths of cDNA for mRNA analysis of NFκB1/p50 were synthesized in 20 µl reaction volume using Verso cDNA synthesis kit. 200 ng of RNA was used as a template for cDNA synthesis.

2.5. Real-time polymerase chain reaction (qRT-PCR)

Quantitative Real-time PCR was performed to measure the relative mRNA level of all the target genes in 58 fresh tumor tissues and 11 age-matched controls (normal uveal tissue) from enucleated specimens of staphylomatous eyes 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 sequence were NFκB1/p50 (sense) TGCCAACAGATGGCCCA TAC; NFκB1/p50(antisense):TGTTCTTTTCACTAGAGGCACCA. The PCR conditions were as follows: 95 °C for 10 min, followed by 35 cycles of 95 °C for 30 s, 56 °C (NFκB1/p50) for 30 s and 72 °C for 28 s. Each PCR reaction was followed by continuous melt curve analysis. Every sample was run in triplicates along with a no template control (NTC) and No-Reverse Transcriptase (NRT). All the reactions were performed on Step One Real-Time PCR Systems (Thermo, Applied Biosystems). Results were normalized to the reference gene in all the cases and compared to the normalized expression in control tissue to calculate a fold change value. The relative amount of gene expression was estimated using the $2^{-\Delta\Delta Ct}$ methods. Grading of NFκB1/p50 mRNA expression was performed according to the fold change value. Fold change value ≥1.5 was considered as an up-regulation of gene whereas fold change value < 1.5 was considered as down-regulation of the gene.

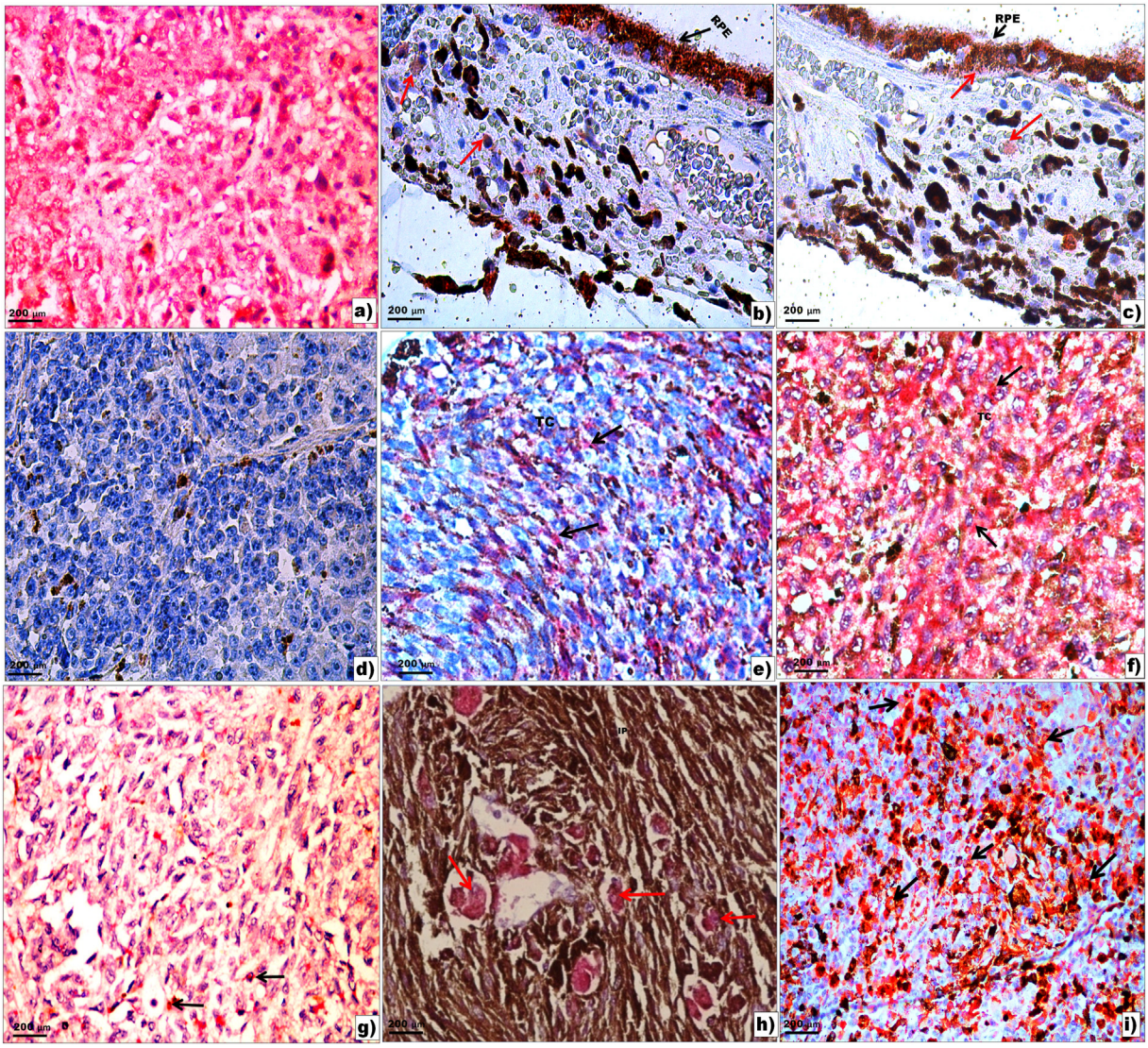

Fig. 2. Immunohistochemical staining of cytoplasmic and nuclear immunoreactivity of NFκB1/p50 in uveal melanoma samples (x200 magnification):
a) Immunoreactivity of NFκB1/p50 in positive control (Breast Carcinoma); b & c) weak cytoplasmic immunoreactivity of NFκB1/p50 (arrow) in normal choroid; d) negative control using IgG in epithelioid cell type; e) Weak cytoplasmic immunoreactivity (arrow) in spindle cell type; f) Strong cytoplasmic immunoreactivity (arrow) in epithelioid cell type; g) weak nuclear positivity (arrow) in spindle cell type; h) strong nuclear immunoreactivity (arrow) in increased pigmentation; i) strong nuclear immunoreactivity (arrow) in epithelioid cell type.

2.6. Western blotting

Twelve tumor samples were stored at −80 °C for protein extraction. Expression of NFκB1/p50 was evaluated by Western Blotting. Total Protein from UM tissue samples was extracted using nuclear and cytoplasmic extraction kit (NE-PER-Pierce, Rockford, Illinois, USA). The protein concentrations were determined using Bio-Rad Protein Assay Reagents (Bio-Rad, CA) and a microplate spectrophotometer. 25 µg of the cytoplasmic and nuclear protein extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane (Amersham Biosciences Inc., St. Albans, United Kingdom) at 90 V for two h. The blots were then stained with 0.1% Ponceau Stain and washed with water. Membranes were blocked with 5% BSA in TBST and incubated overnight at 4 °C with primary antibodies against NFκB1/p50 (1:2000 dilution) and β-actin (1:3500 dilution). They were then washed three times with TBST for 5mins and then incubated with horseradish peroxidase-labeled anti-rabbit secondary antibody (Cell Signaling Technology). Normalization was performed using beta-actin as an endogenous control. Protein bands were visualized using ECL detection kit (Takara Bio USA).

2.7. Statistical analysis

Statistical analyses were performed using MedCalc for Windows V.15.0 (MedCalc software, Ostend, Belgium). The $\chi 2$ test was used for statistical analysis to compare categorical variables, i.e., immunoreactivity of molecular marker, mRNA status, and clinicopathological characteristics. A p-value $< .05$ were considered statistically significant. Metastasis-free survival was taken from the time of enucleation to the date of relapse or the last follow up. Metastasis-Free Survival (MFS) was estimated by Kaplan Meier survival analysis and then compared using the log-rank test for equality of survivor functions. Prognostic significance of clinicopathological features of UM was determined by univariate and multivariate analysis using Cox's Proportional Hazards Model.

Table 2
Correlation of NFκB1/p50 cytoplasmic and nuclear immunoreactivity with the clinicopathological parameters of uveal melanoma cases.

Clinical parameters (N = 75)	NFκB1/p50 cytoplasmic immunoreactivity		p-Value	NFκB1/p50 nuclear immunoreactivity		p-Value
	Positive (55)	Negative (20)		Positive (45)	Negative (20)	
Sex						
Male (48)	30	18	**0.005**	25	23	0.086
Female (27)	25	2		20	7	
Age						
> 40 (56)	44	12	0.13	38	18	**0.028**
≤40 (19)	11	8		7	12	
Largest basal diameter						
> 15mm (50)	44	6	> **0.001**	40	10	> **0.001**
≤15mm (25)	11	14		5	20	
US (tumour height)						
> 8mm (33)	28	5	0.065	25	8	**0.017**
≤8mm (42)	27	15		20	22	
Location of tumour						
Ciliary body (9)	8	1	0.429	6	3	0.733
Choroid (66)	47	19		39	27	
Clinical tumor staging						
T3-T4 (27)	24	3	**0.029**	23	4	**0.001**
T1-T2 (48)	31	17		22	26	
Metastasis						
Present (15)	14	1	0.057	14	1	**0.025**
Absent (60)	41	19		31	19	
Event						
Death (5)	5	0	0.316	5	0	0.079
Alive (70)	50	20		40	30	
Tumor pigmentation						
High (66)	52	14	**0.009**	43	23	**0.025**
Low(9)	3	6		2	7	
Necrosis						
Yes (26)	20	6	0.784	34	15	**0.028**
No (49)	35	14		11	15	
Scleral invasion						
Yes (18)	12	6	0.544	14	4	0.100
No (57)	43	14		31	26	
Large tumor diameter						
> 15mm (55)	46	15	**0.036**	39	16	**0.002**
≤15mm (20)	15	5		6	14	
Mitotic count						
> 4/10HPF (25)	22	3	0.054	21	4	**0.002**
≤4/10HPF (50)	33	17		24	26	
TILs						
Yes (24)	23	1	**0.002**	23	1	< **0.001**
No (51)	32	19		22	29	
Epitheloid cell present						
Yes (25)	23	2	**0.012**	23	2	< **0.001**
No (50)	32	18		22	28	
Iris & ciliary body invasion						
Yes(17)	14	3	0.533	12	5	0.403
No (58)	41	17		33	25	
Optic nerve invasion						
Yes (9)	7	2	1.000	6	3	0.733
No (66)	48	18		39	27	
Vascular loops						
Yes (30)	25	5	0.181	25	5	< **0.001**
No (45)	30	15		20	25	
Extraocular invasion						
Yes (10)	8	2	1.000	7	3	0.730
No (65)	47	18		38	27	
Infiltrating macrophages						
Present (23)	21	2	**0.023**	20	3	**0.002**
Absent (52)	34	18		25	27	
HRFs > 1						
Yes (36)	33	3	< **0.001**	31	5	< **0.001**
No (39)	22	17		14	25	
NFκB1/p50 nuclear expression						
Nuclear positive (45)	45	0	< **0.001**	-	-	**-**
Nuclear negative (30)	10	20				
Tumour environment						
Inflammatory Group (25)	23	2	**0.012**	23	2	< **0.001**
Non Inflammatory Group (50)	32	18		22	28	
BAP1 immunoreactivity						

(*continued on next page*)

Table 2 (*continued*)

Clinical parameters (N = 75)	NFκB1/p50 cytoplasmic immunoreactivity		p-Value	NFκB1/p50 nuclear immunoreactivity		p-Value
	Positive (55)	Negative (20)		Positive (45)	Negative (20)	
Absence (46)	30	16	0.061	33	13	**0.014**
Presence (29)	25	4		12	17	

Bold signify statistical significant value.

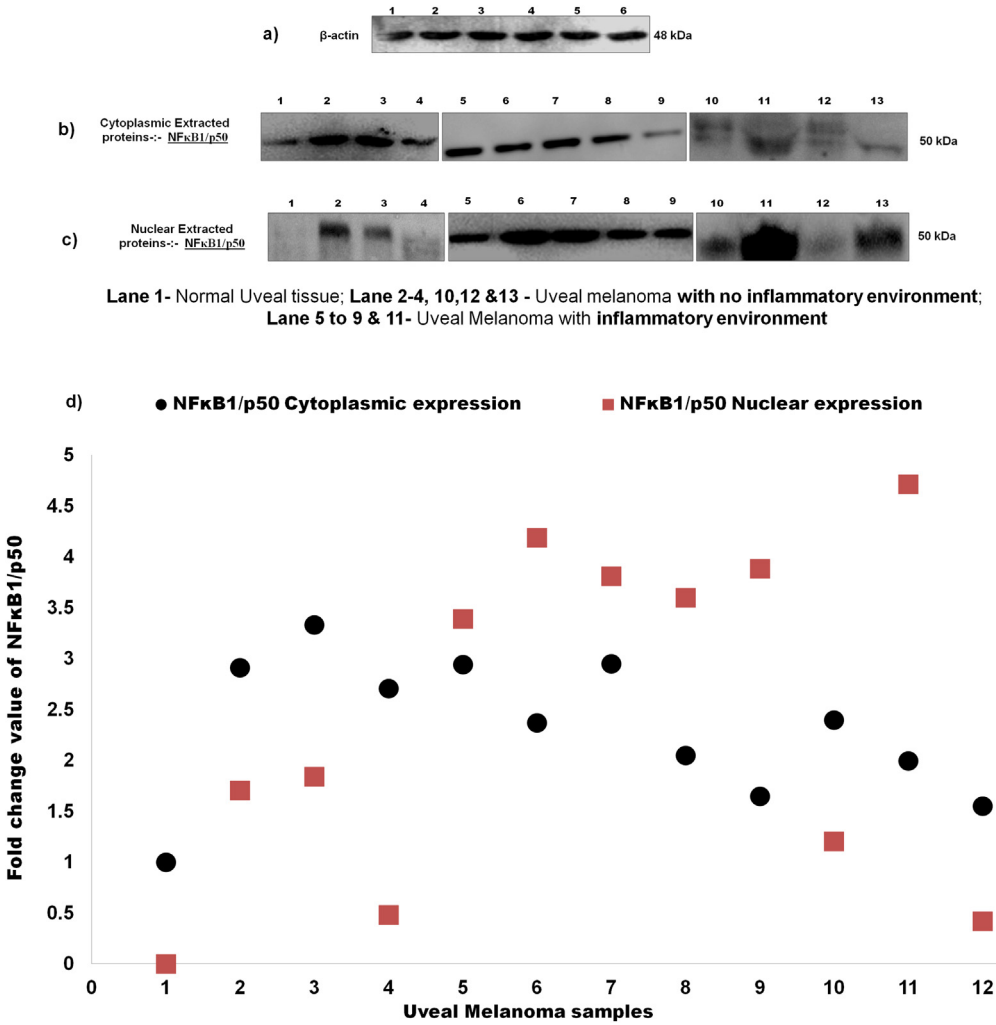

Fig. 3. Validation of immunoreactivity of NFκB1/p50 protein by western blotting in uveal melanoma tissue:
a) Expression of β-actin protein as an internal control; b) immunoblot of NFκB1/p50 (50 kDa) in cytoplasmic extract; c) immunoblot of NFκB1/p50 (50 kDa) in nuclear extract of tumor samples; d) relative levels of nuclear & cytoplasmic NFkB1/p50 from western blot of all 12 patients on dot plot.

3. Result

3.1. Demographic details

A total of 75 patients with histopathologically proven uveal melanoma were included in our study. There were 48 men and 27 women, with age range of 20–92 years. Seventy patients were alive at the last follow-up, 15 patients developed metastatic disease out of which 5 died. Detailed clinicopathological data are provided in Table 1. The majority of uveal melanoma were of choroidal origin (88%). Spindle cell was the most frequent (53%) cell type. The tumors ranged from 7 to 25 mm in diameter (mean of 14.2 mm), and thickness ranged from 2 to 17 mm (mean of 8.2 mm). Forty-eight percent of patients had more than one HRFs. Follow up data was obtained in seventy three patients and two patients were lost to follow up.

3.2. Gross pathology and histopathology

Histopathologically, tumor-infiltrating lymphocytes (TILs), infiltrating macrophages, epithelioid cell type, and vascular mimicry (Fig. 1a-f) are present in the inflammatory environment. By these parameters, our cohort of the study were divided into two groups: UM with an inflammatory environment and non-inflammatory environment. UM with a non-inflammatory environment had neither TILs nor infiltrating macrophages. Most of them showed spindle cell type with areas of necrosis while others showed mixed cell type (Fig. 1g-i).

3.3. Uveal melanoma with inflammatory and non-inflammatory environment group

The inflammatory environment was found in 33% (25/75) UM

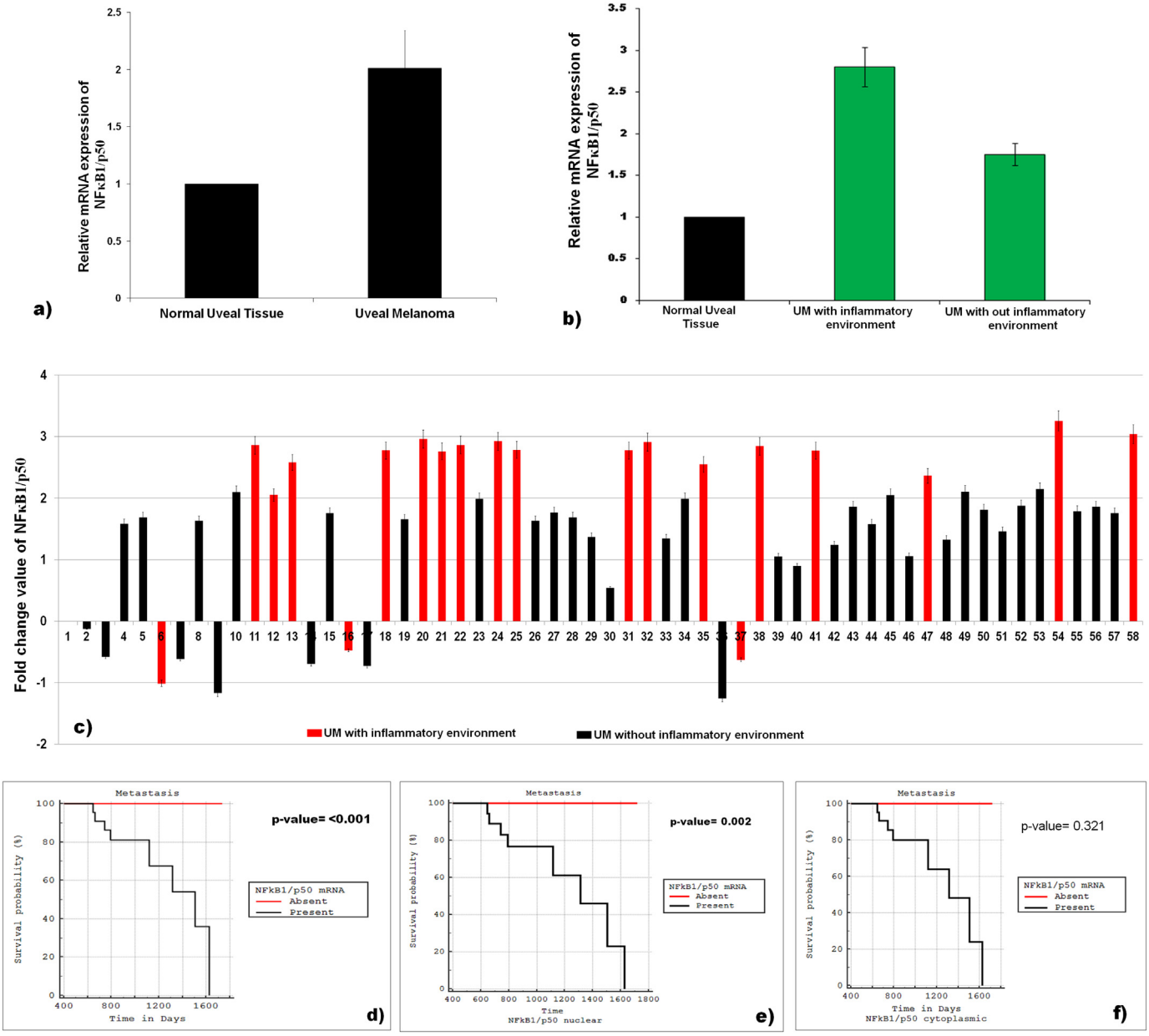

Fig. 4. Relative mRNA expression of NFκB1/p50 in uveal melanoma along with survival curves.
a) mRNA expression of NFκB1/p50 with respect to normal uveal tissue using of relative Ct method. β-actin was used as an internal control for normalization; b) mRNA expression of NFκB1/p50 in inflammatory environment and non-inflammatory environment with respect to normal uveal tissue using relative Ct method; c) fold change values of all 58 cases demonstrating relative expression of NFκB1/p50 with respect to normal uveal tissue; d) mRNA expression of NFκB1/p50 with metastasis-free survival; e) metastasis-free survival curve showing association of nuclear immunoreactivity and mRNA expression NFκB1/p50; f) metastasis-free survival curve showing association of cytoplasmic immunoreactivity and mRNA expression NFκB1/p50.

cases. All of the cases showed the presence of epithelioid cell type and vascular mimicry. Infiltrating macrophages and tumor-infiltrating lymphocytes (TILs) were present in 23 and 24 cases respectively. Increased tumor pigmentation found in 21 cases. Metastasis occurred in 48% (12/25) cases in the inflammatory environment group.

Fifty cases (67%) had no inflammatory environment. Presence of vascular mimicry and increased tumor pigmentation was found in 10% and 90% of the cases respectively. Metastasis was found in 6% (3/50) cases. Spindle cell type was present in 80% cases. TILs and infiltrating macrophages were not found in this group.

3.4. Immunoreactivity of NFκB1/p50 in tumor tissue

The immunoreactivity of NFκB1/p50 was observed in the cytoplasm and nucleus of the tumor cells. However, the immunoreactivity of NFκB1/p50 in normal uveal tissue was found only in the cytoplasm. Fifty-five out of seventy-five (73%) showed positive immunoreactivity for NFκB1/p50, of which forty-five (60%) cases displayed both cytoplasmic as well as nuclear immunoreactivity. Remaining 10 cases (13%) showed cytoplasmic immunoreactivity only (Fig. 2).

Strong nuclear immunoreactivity of NFκB1/p50 was found more frequently in the inflammatory environment group (23/25) (92%) in comparison to the non-inflammatory environment group (22/50) (44%).

Table 3
Correlation of NFκB1/p50 mRNA expression with the clinicopathological parameters of Uveal Melanoma cases.

Clinicopathological parameters (N = 58)	NFκB1/p50 mRNA expression		p-Value	Clinicopathological parameters (N = 58)	NFκB1/p50 mRNA expression		p-Value
	Up regulation (35)	Down regulation (23)			Up regulation (35)	Down regulation (23)	
Sex				**Iris & ciliary body invasion**			
Male (33)	24	9	**0.033**	Yes(15)	10	5	0.760
Female (25)	11	14		No (43)	25	18	
Age							
> 40 (43)	22	21	**0.029**	**Vascular loops**			
≤40 (15)	13	2		Yes (28)	21	7	**0.035**
				No (30)	14	16	
Tumour height				**Extraocular spread**			
> 8mm (27)	22	5	**0.003**	Yes (7)	4	3	
≤8mm (31)	13	18		No (51)	31	20	1
Location of tumour				**Infiltrating macrophages**			
Ciliary body (7)	5	2	0.691	Present (18)	15	3	**0.021**
Choroid (51)	30	21		Absent(40)	20	20	
Extra scleral invasion				**HRFs > 1**			
Yes (8)	5	3	1.000	Yes (32)	27	5	< **0.001**
No (50)	30	20		No (26)	8	18	
Clinical tumor staging				**NFκB1/p50 cyto immunoexpression**			
T3-T4 (15)	12	3	0.123	Yes (45)	29	16	0.335
T1-T2 (43)	23	20		No (13)	6	7	
Large basal diameter				**NFκB1/p50 nuclear immunoexpression**			
> 15mm (39)	28	11	**0.020**	Yes (38)	27	11	**0.027**
≤15mm (19)	7	12		No (20)	8	12	
Metastasis				**Tumour Environment**			
Present (7)	5	2	0.691	Inflammatory Group (25)	20	3	< **0.001**
Absent (51)	30	21		Non Inflammatory Group (50))	15	20	
Tumor pigmentation				-	-	-	-
High (45)	30	15	0.106				
Low (13)	5	8					
Necrosis				-	-	-	-
Yes (25)	15	7	0.413				
No (33)	20	16					
Scleral invasion				-	-	-	-
Yes (16)	13	3	0.070				
No (53)	22	20					
Large tumor diameter				-	-	-	-
> 15mm (38)	32	6	< **0.001**				
≤15mm (20)	3	17					
Mitotic count				-	-	-	-
> 4/10HPF (22)	16	6	0.171				
≤4/10HPF (36)	19	17					
TILs				-	-	-	-
Yes (19)	18	1	< **0.001**				
No (39)	17	22					
Epitheloid cell type				-	-	-	-
Present (23)	18	5	**0.030**				
Absent (35)	17	18					

Bold signify statistical significant value.

3.5. Correlation of NFκB1/p50 immunoreactivity with clinicopathological parameters

Nuclear and cytoplasmic immunoreactivity of NFκB1/p50 was statistically significant in patients with inflammatory parameters (TILs, infiltrating macrophages, epithelioid cell type, and increased pigmentation), presence of more than one HRFs ($p \leq .001$) and clinicopathological parameters such as neovascularization, male gender, largest basal diameter (> 15 mm), tumor height (> 8 mm)advanced clinical staging and largest tumor diameter (> 15 mm).

On the contrary, nuclear immunoreactivity of NFκB1/p50 was significant with older age, vascular mimicry, and various high-risk factors as shown in Table 2.

In our study, we evaluated immunoreactivity of BAP1 protein in all 75 cases. Absence of BAP1 was observed in 61% (46/75) of cases. On statistical correlation of absence of BAP1 with cytoplasmic and nuclear immunoreactivity of NFκB1/p50 protein, we found that strong statistical correlation of absence of BAP1 with nuclear immunoreactivity of NFκB1/p50 ($p = .014$) but not with cytoplasmic.

3.6. Validation of immunoreactivity of NFκB1/p50 using western blotting

Western blot analysis for NFκB1/p50 was performed in 12 representative cases of uveal melanoma samples along with the internal control (β-actin) to validate the immunohistochemistry results (Fig. 3 a-c). Six cases were taken from the inflammatory environment group, and other six taken from a noninflammatory group of UM patients. Expression of NFκB1/p50 was found only in cytoplasmic extracted in a normal uveal tissue sample. Among 12 UM samples, cytoplasmic and nuclear expression of NFκB1/p50 found in 9 cases. In the inflammatory environment, expression of NFκB1/p50 in nuclear and cytoplasmic extract showed 3.9-fold and 2.7-fold respectively, whereas in the non-

Table 4
Prognostic significance of clinicopathological features of uveal melanoma by univariate analysis and multivariate analysis (Cox's proportional hazards model).

Clinicopathological parameters	Univariate analysis			Multivariate analysis		
	Hazard ratio (HR)	95% CI	p-value	Hazard ratio (HR)	95% CI	p-value
Advanced tumor staging (T3-T4)	3.5	1.08–11.29	**0.036**	7.47	0.52–78.57	0.138
Tumor height (> 8 mm)	4.75	1.34–16.73	**0.015**	0.37	0.02–4.76	0.451
Extraocular spread	16.62	3.55–77.68	< **0.001**	–	–	0.632
High mitotic count (> 4/40HPF)	1.23	0.75–1.99	0.399	–	–	–
Scleral invasion	8.50	2.42–29.74	**0.001**	4.54	0.43–47.59	0.206
Ciliary body invasion	2.96	0.87–10.08	0.081	–	–	–
Necrosis	0.55	0.49–4.27	0.501	–	–	–
Optic nerve invasion	0.77	0.36–7.53	0.509	–	–	–
Large tumor diameter (LTD > 15 mm)	2.78	0.56–13.63	0.206	–	–	–
High risk factors (HRFs) > 1	24.18	2.97–116.60	**0.003**	0.48	0.01–18.97	0.701
Cytoplasmic NFκB1/p50 immunoreactivity	2.78	0.60–13.63	0.177	–	–	–
Nuclear NFκB1/p50 immunoreactivity	13.09	1.61–105.99	**0.016**	20.06	0.69–121.97	**0.034**
NFκB1/p50 upregulation mRNA expression	0.58	0.85–8.25	0.083	–	–	–
Epithelioid cell type	2.04	0.64–6.47	0.226	–	–	–
Increased pigmentation	1.04	0.20–11.86	0.673	–	–	–
Tumor infiltrating lymphocytes (TILs)	16.00	3.88–65.82	< **0.001**	16.63	0.81–40.67	0.068
Infiltrating macrophages	32.5	6.32–166.90	< **0.001**	25.88	3.18–151.65	**0.011**
'Vascular mimicry'	2.78	0.87–8.89	0.084	–	–	–

Bold signify statistical significant value.

Table 5
Metastasis-free survival for clinicopathological factors with cytoplasmic and nuclear immunoreactivity of NFκB1/p50 estimated by Kaplan-Meier survival analysis and comparison using log-rank test.

Clinicopathological parameters	Metastasis	Median survival (no. of days)	Standard error	p-Value
Cytoplasmic NFκB1/p50 immunoreactivity				
Positive (55)	13	1916	93.48	0.163
Negative (20)	2	-	79.1	
Nuclear NFκB1/p50 immunoreactivity				
Positive (45)	13	1126	106.62	**0.019**
Negative (30)	2	1916	53.42	
Cytoplasmic NFκB1/p50 with infiltrating macrophages				
Positive (22)	10	1085	112.69	0.135
Negative (1)	0	1626	0	
Nuclear NFκB1/p50 with infiltrating macrophages				
Positive (20)	9	989	113.61	**0.024**
Negative (3)	1	1734	108	
Cytoplasmic NFκB1/p50 with TILs+				
Positive (21)	11	1105	123.49	0.594
Negative (3)	1	999	43.02	
Nuclear NFκB1/p50 with TILs+				
Positive (19)	9	1077	128.09	0.334
Negative (5)	3	1312	-	

Bold signify statistical significant value.

inflammatory environment, expression of NFκB1/p50 was reduced to 1.1-fold in nuclear and 2.6-fold in the cytoplasmic extract (Fig. 3d).

3.7. Correlation of transcriptional status of NFκB1/p50 with the clinicopathological parameters of UM

The average amount of NFκB1/p50 mRNA was significantly higher in uveal melanoma (2.01- fold; range = −1.6 to 3.3-fold) in comparison to normal uveal tissue. Upregulation of NFκB1/p50 observed in 60% of UM cases. UM with the inflammatory environment (2.9-fold) had higher NFκB1/p50 mRNA expression as compared to non-inflammatory UM cases (1.85-fold) (Fig. 4a-c). Upregulation of NFκB1/p50 at transcriptional level was statistically significant in patients having more than one high-risk factors, male gender, older age, tumor height > 8 mm and inflammatory parameters (Table 3). Upregulation of NFκB1/p50 at transcriptional level correlated well with nuclear immunoreactivity of NFκB1/p50 but not with the cytoplasmic immunoreactivity of NFκB1/p50. Patients having upregulation of NFκB1/p50 gene showed decreased metastasis-free survival ($p \leq .001$). Reduced survival was found with mRNA upregulation of NFκB1/p50 having nuclear immunoreactivity ($p = .002$) but not with cytoplasmic immunoreactivity ($p = .321$) (Fig. 4d-f).

3.8. Identification of independent prognostic marker by using univariate and multivariate analysis (Cox's proportional hazards model)

On univariate analysis, nuclear NFκB1/p50 immunoreactivity, neovascularization, advanced tumor staging, tumor height > 8 mm, extraocular spread, ciliary body invasion, HRFs > 1, epithelioid cell type, TILs, infiltrating macrophages and vascular mimicry emerged as significant risk factors. On multivariate analysis, two parameters as an independent prognostic indicators of metastasis: nuclear NFκB1/p50 immunoreactivity (HR = 20.06; 95%CI = 0.69–121.97; $p = .034$), and infiltrating macrophages (HR = 25.88; 95%CI = 3.18–151.65; $p = .011$) (Table 4).

3.9. Prognostic outcome of NFκB1/p50

Kaplan–Meier analysis by the log-rank test was carried out to evaluate the prognostic significance of NFκB1/p50. Table 5 summarizes the metastasis-free survival for clinicopathological parameters and NFκB1/p50 immunoreactivity. Fig. 5 shows the metastasis-free survival curves of uveal melanoma patients with NFκB1/p50 expression. The median survival time for patients with nuclear NFκB1/p50 immunoreactivity was 1126 days compared with 1916 days for cytoplasmic NFκB1/p50 immunoreactivity. There was a statistically significant difference in the metastasis-free survival of patients with nuclear NFκB1/p50 immunoreactivity ($p = .019$) in comparison to cytoplasmic NFκB1/p50 immunoreactivity. These survival differences were even more pronounced in the presence of TAM-CD-68+ and TILs patients showing a reduced survival for patients with nuclear NFκB1/p50 immunoreactivity compared to cytoplasmic NFκB1/p50 immunoreactivity.

4. Discussion

The transcriptional factor Nuclear factor-κB (NFκB) is considered as an essential regulator of the inflammatory response, which is associated with the pathogenesis and proliferation of cancer. NF-κB1/p50 plays a

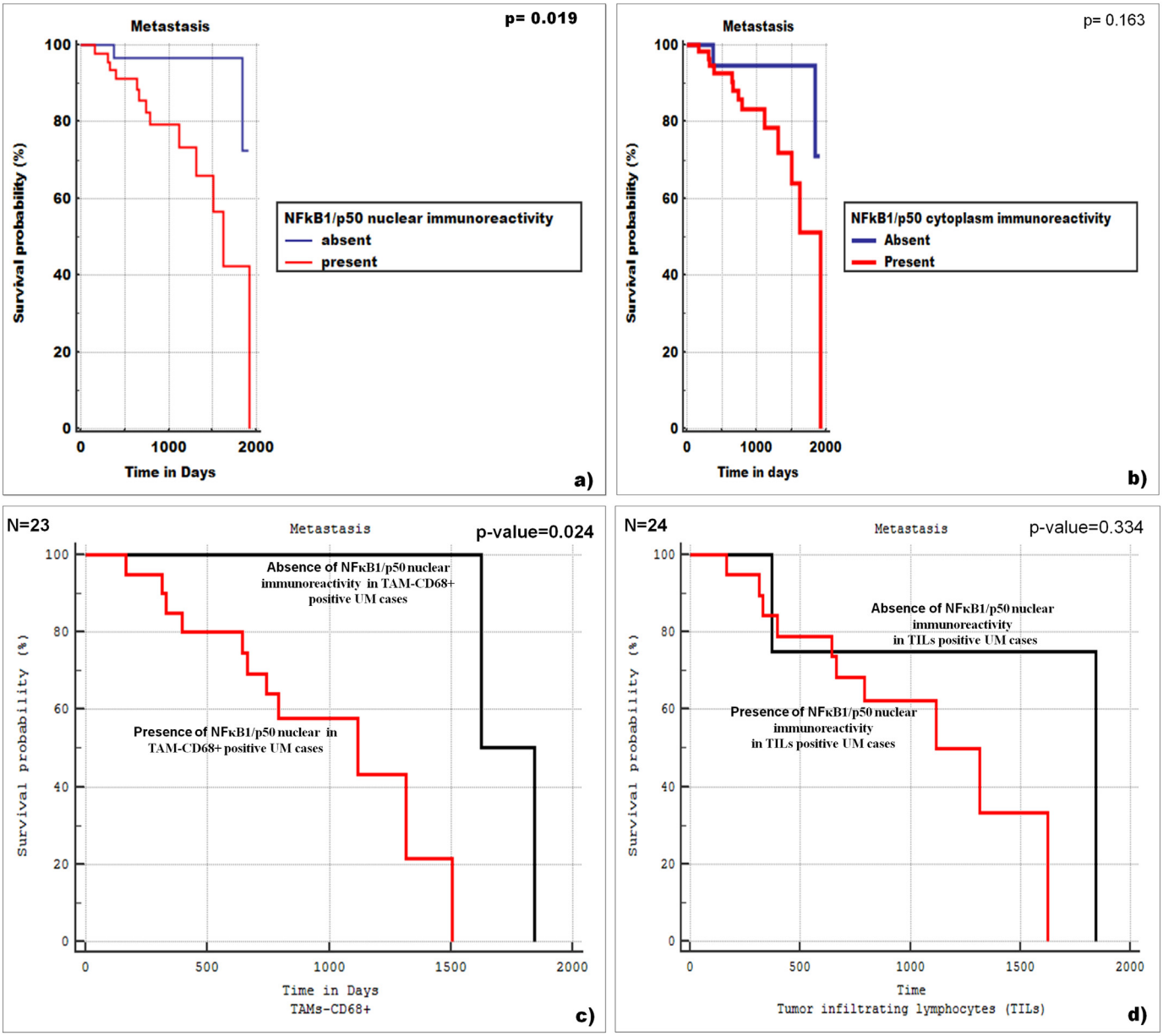

Fig. 5. Metastasis-free survival curves of uveal melanoma patients with a) NFκB1/p50 cytoplasmic immunoreactivity; b) NFκB1/p50 nuclear immunoreactivity; c) TAM-CD68 positive UM cases showing NFκB1/p50 nuclear immunoreactivity; d) TILs (Tumor infiltrating lymphocytes) positive UM cases showing NFκB1/p50 nuclear immunoreactivity by Kaplan Meier survival analysis.

vital role in the proliferation of melanoma cells and helps in metastatic spread (Carlsen et al., 2004). In the current study, we evaluate the activation of NFκB1/p50 by using immunohistochemistry and western blotting. Transcriptional status of NFκB1/p50 also assessed by quantitative reverse transcriptase real-time PCR (qRT-PCR).

Immunoexpression of NFκB1/p50 found in the cytoplasm as well as nuclei of the tumor cells which is similar to the immunoreactivity pattern found in gastric cancer (Che et al., 2015; Levidou et al., 2007) and ovarian cancer (Shuang et al., 2016). High cytoplasmic immunoreactivity of NFκB1/p50 was observed in 55/75 (73.3%) cases. The nuclear localization was present in 60% of UM cases which might be seen as the first sign of NFκB activation in UM. Our results are in line with other cancers which showed increased activation of NFκB involved in the development of various malignancies such as colon cancer (Charalambous et al., 2009), cutaneous melanoma (Gao et al., 2006), neuroblastoma (Zhi et al., 2015).

According to Fridman et al., the presence of infiltrating immune cells in and around a tumor led to the hypothesis that inflammatory microenvironment is an important prognostic parameter in cancers (Bronkhorst and Jager, 2012). Most of the cancers such as cutaneous melanoma, non-Hodgkin's lymphoma, non-small-cell lung cancer, and breast cancer associated with a good prognosis in the presence of inflammatory cells (Mlecnik et al., 2011). In contrast to other cancers like squamous cell carcinoma (Xu et al., 2017) renal cell carcinoma (Li et al., 2009), and uveal melanoma (Bronkhorst and Jager, 2012) where infiltrating immune cells are related to poor prognosis of the disease.

In our study, strong nuclear and cytoplasmic immunoreactivity of NFκB1/p50 was more frequently observed in the inflammatory group of uveal melanoma (UM). Out of 75 cases, the inflammatory environment was found in 33% (25/75) of UM cases, 12 of these 25 cases (48%) had distant metastasis. At the transcriptional level, upregulation of NFκB1/p50 was more pronounced in the inflammatory environment group compared to the non-inflammatory environment group.

Cytoplasmic and nuclear immunoreactivity of NFκB1/p50 were

significantly associated with inflammatory parameters including increased tumor pigmentation (0.009, 0.025), presence of epithelioid cell (0.030, < 0.001), TILs (0.002, < 0.001) and TAMs (0.023, < 0.001) whereas vascular mimicry (< 0.001) was significant only with nuclear NFκB1/p50 immunoreactivity. These results are supporting the hypothesis of De ward et al. which proves that the presence of inflammatory factors in UM results in poor prognosis (De Waard-Siebinga et al., 1996). Similar to these results, increased mRNA expression of NFκB1/p50 was significantly associated with inflammatory parameters except tumor pigmentation.

According to Kaliki et al., there are various clinical, histopathological and inflammatory parameters of UM which are considered as high-risk factors (HRFs) which are helpful in identifying those patients who should be followed up regularly for developing of metastasis. Patients are having more than one high-risk factors are highly prone to metastasis and become resistant to chemotherapy (Kaliki et al., 2015b). Our results support the above statement as an increased expression of NFκB1/p50 at protein and RNA level was seen in our patients having greater than one HRFs, and this was statistically significant.

A study by Kujala et al. showed that 5-year survival rates remain unchanged even after the effective advancement in the local therapies and almost 50% of UM patients develop metastasis (Kujala et al., 2003). At transcriptional and translational level increased expression of NFκB1/p50 was observed with many clinicopathological parameters which are associated with metastatic mortality (Carvajal et al., 2017).

This preliminary data indicates that increased transcriptional activity of NFκB1/p50 expression is paralleled by enhanced protein expression. This confirms that deregulation of NFκB1/p50 subunit results in activation of the NFκB signaling pathway in UM. Similarly, upregulation of NFκB1/p50 at transcript level was also seen in ovarian cancer (Guo et al., 2009).

In our study we found absence of BAP1 strongly correlated with the nuclear reactivity of NFκB1/p50 protein ($p = .010$). On review of literature, we found two studies which showed the relationship of NFκB pathway with absence of BAP1. Similar results observed in the study of Brouwer et al., who too reported the association of loss of BAP1 with increased expression of NFκB pathway proteins (Brouwer et al., 2019). According to Gezgin et al., who hypothesized that loss of BAP1 alleviates the suppression pathways leading to activation of NFκB pathway. This results in production of cytokines that attracts tumor specific T-cells (Gezgin et al., 2017).

Multivariate logistic regression analysis showed that nuclear immunoreactivity of NF-κB1/p50 and infiltrating macrophages were independent risk factors associated with the inflammatory environment of UM. Cox's regression analysis indicates that nuclear immunoreactivity of NFκB1/p50 in comparison to cytoplasmic immunoreactivity of NFκB1/p50 is an independent risk factor that strongly correlates with poor prognosis of UM. The Kaplan–Meier analysis indicated that survival rate was significantly decreased in the UM cases showing strong nuclear immunoreactivity.

The presence of activated NFκB in the tumor is not necessarily causal since NF-κB signaling pathway is complex and involved in many physiological functions and in modulating inflammation. Thus, inhibition of the pathway may have unanticipated adverse effects. An ideal NF-κB inhibitor should prevent NF-κB activation in the absence of any adverse effects on other signaling pathways and should not cause long-term immune suppression (Brouwer et al., 2019). In the present study, we did find the evidence of significantly increased expression of NFκB1/p50 protein and decreased metastasis-free survival in the inflammatory environment as compared to non-inflammatory environment. Therefore, it is likely that the inflammatory environment might play a role in the pathogenesis of uveal melanoma in our cohort of cases.

To conclude, the above results reveal that nuclear immunoreactivity of NFκB1/p50 are closely related to the poor prognosis of uveal melanoma and could serve as an prognostic marker in identifying inflammatory environment of UM. Further translational/in-vivo studies are necessary for the potential role of this protein to become a clinically useful inflammatory marker in uveal melanoma patients.

Compliance with ethical standards

- Disclosure of potential conflicts of interest: NONE
- Research Involving Human Participants
- Informed Consent: Yes

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