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ORIGINAL ARTICLE

Prognostic relevance of ATM protein in uveal melanoma and its association with clinicopathological factors

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Abstract

Purpose Uveal melanoma (UM) is an intraocular malignancy commonly arising from choroid which can cause visual loss or metastasis. Ataxia-telangiectasia mutated (ATM) protein is an activator of DNA damage response and its role in uveal melanoma (UM) is still unexplored. Therefore, the study aims to detect the expression and localization of ATM protein and its association with clinicopathological parameters

Methods Expression of nuclear ATM (nATM) was investigated on 69 formalin fixed paraffin embedded choroidal melanoma samples by immunohistochemistry and validated by western blotting. Results were then correlated with clinical and histopathological parameters. Prognostic significance was determined by the Kaplan–Meier analysis and the multivariate analysis by Cox's hazard proportional method.

Results Loss of nATM was observed in 65% of cases, which was statistically significant with the reduced disease-free survival ($p = 0.042$). This loss was more frequently found in cases with high-risk histopathological factors like epithelioid cell type, tumor infiltrating lymphocytes and high pigmentation which might help in the progression of melanoma. On multivariate analysis, extraocular spread and loss of nATM were found to be independent prognostic factors ($p < 0.05$).

Conclusion Our data suggest that loss of nATM protein might serve as a poor prognostic marker in the pathogenesis of uveal melanoma which may lead to increased risk of metastasis.

Keywords Uveal melanoma · ATM protein · Immunohistochemistry · Prognosis

Introduction

Uveal melanoma (UM) is an intraocular tumor in adults commonly arising from choroid followed by iris and ciliary body. Patients with uveal melanoma present with visual symptoms such as decreased vision, flashes, and floaters. Untreated uveal melanoma can cause visual loss and death from the intracranial spread or metastatic disease. Metastasis mostly occurs in the liver and is rarely detectable at the time of initial presentation [1].

Previous studies have suggested various clinical and histopathological parameters, which are associated with poor prognosis in patients with uveal melanoma. These parameters include large tumor size, epithelioid cell type, extrascleral extension, tumor infiltrating lymphocytes (TILs), and tumor staging which are at high risk of developing metastasis [1, 2]. There is no current effective therapy available to treat early metastatic spread in these patients. Therefore, it is important to find new biomarkers that help in early detection of metastasis of this disease [3]. In normal cells, DNA damage triggers DNA damage response (DDR), which leads to DNA repair. One of the key proteins inducing this response is Ataxia telangiectasia mutated (ATM).

Inactivation of ATM causes the development of certain common sporadic cancers [4]. It is a tumor suppressor gene and a member of phosphatidylinositol-3 kinase-like kinase family (PIKKs) predominantly found in the nucleus. It plays

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a central role in the repair of double-strand breaks (DSBs), which can be induced by various factors like chemotherapy drugs, radiation, or oxidative stress. Activated ATM involves triggering DNA repair machinery for regulating cell cycle checkpoints [5–7]. COSMIC database revealed that ATM is among the most aberrant genes in sporadic cancers and its alteration is associated with the development and progression of cancer [8]. Loss of nuclear ATM (nATM) protein has been observed in different cancers such as colon cancer, breast cancer, gastric cancer, and lung cancer and is associated with poor prognosis [9–11]. Its prognostic significance has been also observed in skin melanomas [12]. Therefore, loss of nATM is a potential biomarker useful for targeting therapies to promote DNA repair mechanisms.

Till date, there is no current study found in the literature on uveal melanoma and ATM protein expression. Therefore, our study aims to detect the localization of ATM protein and its association with clinicopathological parameters and prognostic outcome to determine the efficacy of ATM protein as a prognostic biomarker.

Materials and methods

Patient details

This prospective study is approved by the institutional ethics committee (IEC), All India Institute of Medical Sciences (AIIMS), New Delhi. Written informed consent for enucleation surgery and participation in this study was obtained from the guardians of all patients. A total of 69 patients with primary Uveal Melanoma who underwent enucleation in the Dr. Rajender Prasad Centre for Ophthalmic Sciences, All India Institute of Medical Sciences, New Delhi India, were included in this study and were followed up from 6 to 48 months period (2014–2018). Demographic, clinical and imaging data [computerized tomography (CT)] were obtained from the medical records. For follow up, postoperative surveillance was performed at the IRCH, AIIMS, India, including routine, clinical and laboratory examinations. Histopathological high-risk factors include analysis of the largest tumor diameter (LTD), tumor thickness, epithelioid cell type, the presence of scleral invasion, ciliary body involvement, mitotic count per 40HPF (high power field), the presence of closed vascular loops [13]. Clinical TNM staging system was used to classify the patients according to the American Joint Committee on Cancer (AJCC) system [14].

Immunohistochemistry

ATM immunoexpression was assessed by immunohistochemistry in primary uveal melanoma tissues from all 69 patients. The sections were deparaffinized in xylene and rehydrated with graded ethanol. For ATM detection, the sections were subjected to antigen retrieval for 25 min at room temperature in 0.01 M Tris–EDTA buffer, pH 9.0, and were subsequently incubated for overnight with a monoclonal mouse antibody to anti-ATM (G-12 Santa Cruz, Delaware, CA) at a 1:200 dilution. The reaction was visualized using the AEC (Vector Laboratories), and all sections were counterstained with hematoxylin. The placenta was taken as a positive control (Fig. 1a). For negative control, the primary antibody was omitted.

Evaluation of immunohistochemistry

Scoring of ATM immunoexpression was performed by JJ & MKS under the supervision of experienced pathologist SK. The analysis was done based on the expression of nuclear staining. The number of positive nuclear stained cells of $\leq 50\%$ were considered as negative expression and $> 50\%$ were considered as a positive expression [15].

Western blotting

Tumor samples were stored at − 80 °C for protein extraction. Protein was extracted in 60 fresh cases using NE-REP nuclear Extraction kit (Pierce, Rockford, IL USA). Protein concentrations were determined with the Bio-Rad protein assay (BioRad, Hercules, CA, USA). 20 µg of the total cellular protein extracts were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and were transferred onto a nitrocellulose membrane (MDI Membrane Technologies LLC, California, USA) at 90 V for 2 h. Blocking was performed with 5% BSA in Tris-buffered saline–Tween 20 (TBST) and was subsequently incubated with antibodies against non-phosphorylated ATM (G-12 Santa Cruz, Delaware, CA) 1:1000 overnight at 4 °C. Incubations were followed by TBST washes, incubation with secondary antibody conjugated with Horseradish Peroxidase (HRP labeled, 1:5000, 0.2 µg IgG1/ml dilution, Cell Signaling Technology) and further TBST washes. After three times rinses in TBST, membranes were finally treated by DAB-HCl reagent (Sigma, St. Louis USA). β-Actin was used as an internal control (1:5000; Sigma).

Statistical analysis

Statistical analyses were performed using Med Calc for windows V.15.0 (MedCalc software, Ostend, Belgium). χ^2 test was used for statistical analysis to compare categorical variables, i.e., immunohistochemical reactivity of ATM and clinicopathological characteristics. A p value of less than 0.05 was considered statistically significant. Disease-free

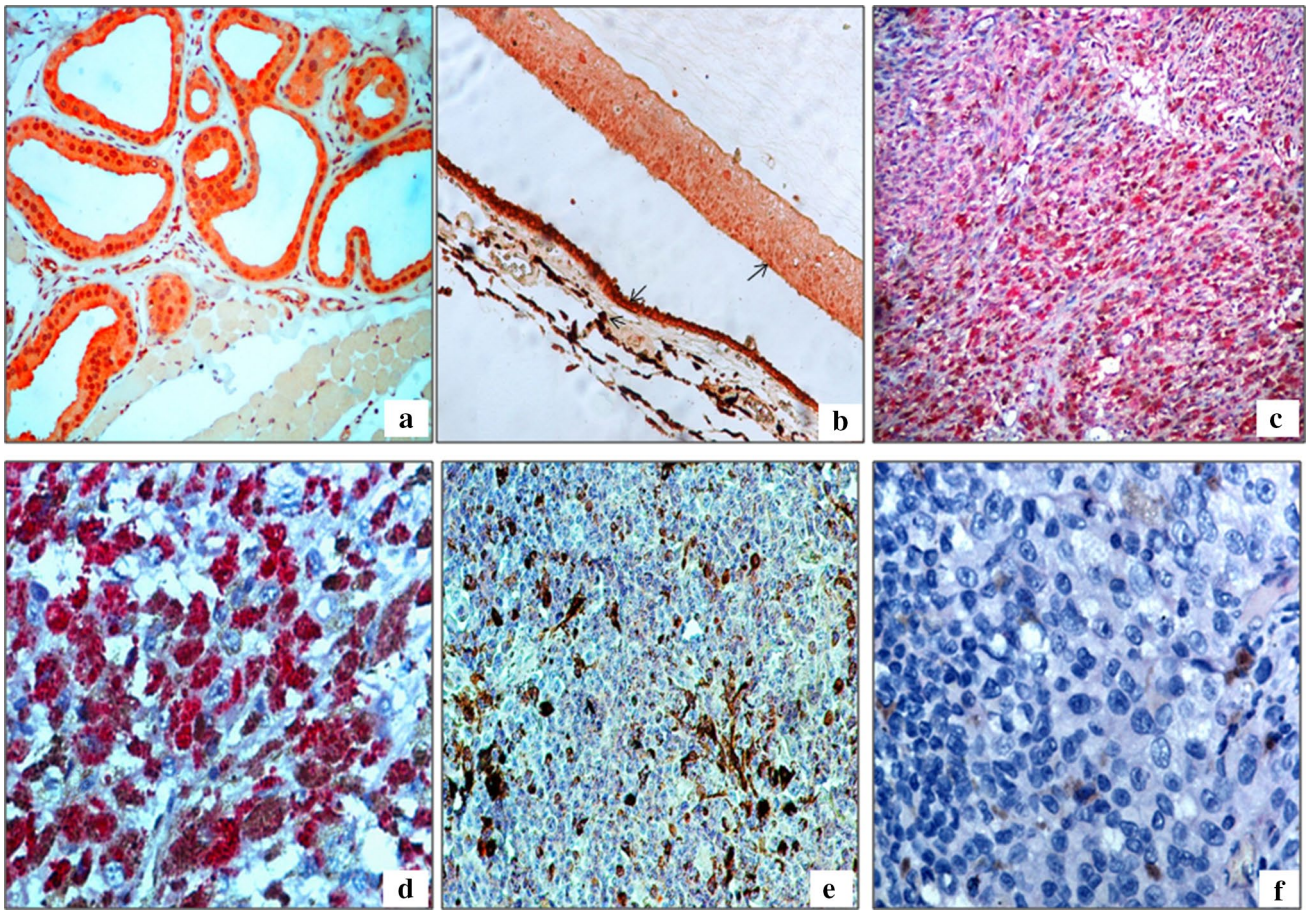

Fig. 1 Nuclear Expression of ATM protein in normal tissue and tumor cells of Uveal Melanoma. **a** Immunoexpression of ATM protein in Placenta as a Positive control (×200); **b** arrow showing immunoexpression of ATM protein in normal choroidal melanocytes, retinal pigment epithelial cells, and normal retina; **c** ATM expression in spindle cell type (×100); **d** high magnification of ATM expression in spindle cell type (×200); **e** low magnification of loss of nuclear ATM protein in epithelioid cell type (×100); **f** High magnification of ATM expression in spindle cell type (×100)

survival (DFS) of patients with positive or negative immunostaining was estimated by Kaplan–Meier survival analysis, starting from enucleation to the date of the last follow up and 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.

Results

Patient characteristics

Clinical data were available for all 69 patients as summarized in Table 1. There was a male preponderance (43/69) in our study. The ages were ranged 17–92 years, and the median age was 56 years. Choroidal Uveal melanoma was found in 88.40% cases whereas 14% of uveal melanoma originated from the ciliary body. Spindle cell melanomas were the most common, and only 14% of cases revealed epithelioid phenotype. Large tumor diameter ranged 9–28 mm (median 15 mm), and median tumor height was 10 mm. Vascular loops were present in 40.57% cases whereas tumor-infiltrating lymphocytes (TILs) were identified in 26% of the cases. The mean disease-free survival was 43 months. Of the 69 patients, 2 were lost to follow up. Eleven patients developed the metastatic disease out of which two died.

Immunoexpression of ATM protein in uveal melanoma samples

In normal choroidal tissue, expression of ATM protein revealed nuclear staining by immunohistochemistry (Fig. 1b). Nuclear expression of ATM was seen in the viable tumor cells. Of the 69 cases, loss of nATM was detected in 65.21% of the cases. Nuclear expression of ATM protein

Table 1 Clinicopathological and demographic details of uveal melanoma patients

Clinical parameters	*N* = 69 (*N*%)	Histopathological parameters	*N* = 69 (*N*%)
Sex		Pigmentation	
Male	43 (64%)	High	61 (88.40%)
Female	26 (36%)	Low	8 (11.59%)
Age		Cell type	
≤ 40 years	18 (25.33%)	Spindle	33 (47.82%)
> 40 years	51 (74.66%)	Epithelioid	23 (33.33%)
		Mixed	13 (18.84%)
Tumor thickness		LTD	
≤ 8 mm	31 (44.92%)	≤ 10 mm	20 (28.98%)
> 8 mm	38 (55%)	> 10 mm	49 (71%)
Floaters	19 (27.53%)	Necrosis	27 (39.13%)
Retinal detachment	56 (81.15%)	Scleral invasion	16 (23.18%)
Iris neovascularization	25 (36.23%)	Iris and ciliary body invasion	15 (21.73%)
Decreased vision	53 (76.81%)	Optic nerve invasion	5 (7.24%)
Hyphaema	5 (7.24%)	Extraocular spread	7 (10.14%)
Viterous haemmorhage	9 (13%)	Mitotic count	
		≤ 4 per 40HPF	47 (68.11%)
		> 4 per 40HPF	22 (31.88%)
Location of tumour		TILs	18 (26%)
Choroid	61 (88.40%)		
Ciliary body	8 (11.59%)		
Clinical TNM staging		Vascular loops	28 (40.57%)
T1–T2	48 (69.56%)		
T3–T4	21 (30.43%)		
Distant metastasis	11 (15.94%)	HRFs > 1	31 (44.92%)
Death	2 (2.89%)		

LTD large tumour diameter, *TILs* tumour infiltrating lymphocytes, *HRFs* histopathological high risk factors

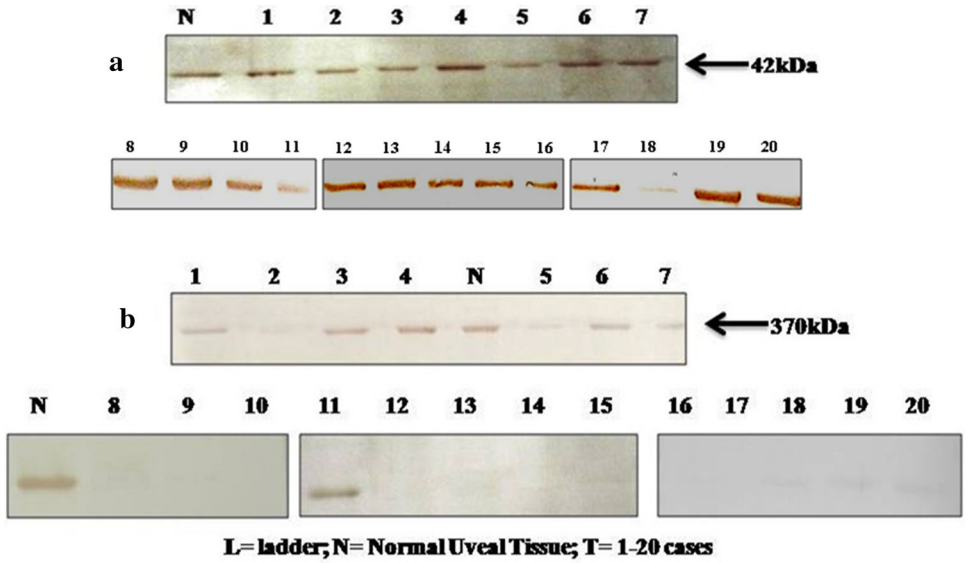

Fig. 2 Representative cases of western blot in nuclear extract of normal and tumor tissue protein. **a** Expression of β-actin protein as an internal control; **b** expression of ATM protein in normal uveal tissue (Lane: *N*) and tumor tissue (Lane: 1–20)

Table 2 Correlation of nuclear ATM (nATM) immunoexpression with the clinicopathological parameters of uveal melanoma cases

Clinical parameters ($N=69$)	nATM immunoexpression		p value	Histopathological parameters ($N=69$)	nATM immunoexpression		p value
	Absent (45)	Present (24)			Absent (45)	Present (24)	
Sex				Pigmentation			
Male (43)	28	15	1.000	High (61)	43	18	**0.017**
Female (26)	17	9		Low (8)	2	6	
Age				Necrosis			
> 40 (51)	32	19	0.571	Yes (27)	15	12	0.203
≤ 40 (18)	13	5		No (42)	30	12	
Floaters				Scleral invasion			
Present (19)	15	4	0.167	Yes (16)	10	6	0.774
Absent (50)	30	20		No (53)	35	18	
Retinal detachment				LTD			
Present (56)	36	20	1.000	> 10 mm (49)	36	13	**0.029**
Absent (13)	9	4		≤ 10 mm (20)	9	11	
Iris neovascularization				Mitotic count			
Present (25)	17	8	0.796	> 4/40HPF (22)	14	8	1.000
Absent (44)	28	16		≤ 4/40HPF (47)	31	16	
Decreased vision				TILs			
Present (53)	36	17	0.550	Yes (18)	16	2	**0.020**
Absent (16)	9	7		No (51)	29	22	
Secondary glaucoma				Epitheloid cell			
Present (7)	4	3	0.687	Present (23)	19	4	**0.036**
Absent (62)	41	21		Absent (46)	26	20	
Hyphema				Iris and ciliary body invasion			
Present (5)	2	3	0.333	Yes (15)	11	4	0.550
Absent (64)	43	21		No (54)	34	20	
Viterous haemorrhage				Optic nerve invasion			
Yes (9)	5	4	0.709	Yes (5)	4	1	0.651
No (60)	40	20		No (64)	41	23	
Tumor thickness				Vascular loop			
> 8 mm (38)	29	9	**0.043**	Yes (28)	18	10	1.000
≤ 8 mm (31)	16	15		No (41)	27	14	
Location of tumour				Extraocular spread			
Ciliary body (8)	3	5	0.115	Yes (7)	6	1	0.407
Choroid (61)	42	19		No (62)	39	23	
Extra scleral invasion				HRFs > 1			
Yes (11)	9	2	0.306	Yes (30)	25	5	**0.010**
No (58)	36	22		No (39)	20	19	
Clinical TNM staging				BAP1			
T3–T4 (21)	18	3	**0.026**	Loss (46)	38	8	**0.001**
T1–T2 (48)	27	21		Expression (23)	7	16	
Metastasis							
Present (11)	11	0	**0.006**				
Absent (58)	34	24					

Bold signifies significant parameters ($p < 0.05$)

LTD large tumour diameter, *TILs* tumour infiltrating lymphocytes, *HRFs* histopathological high risk factors

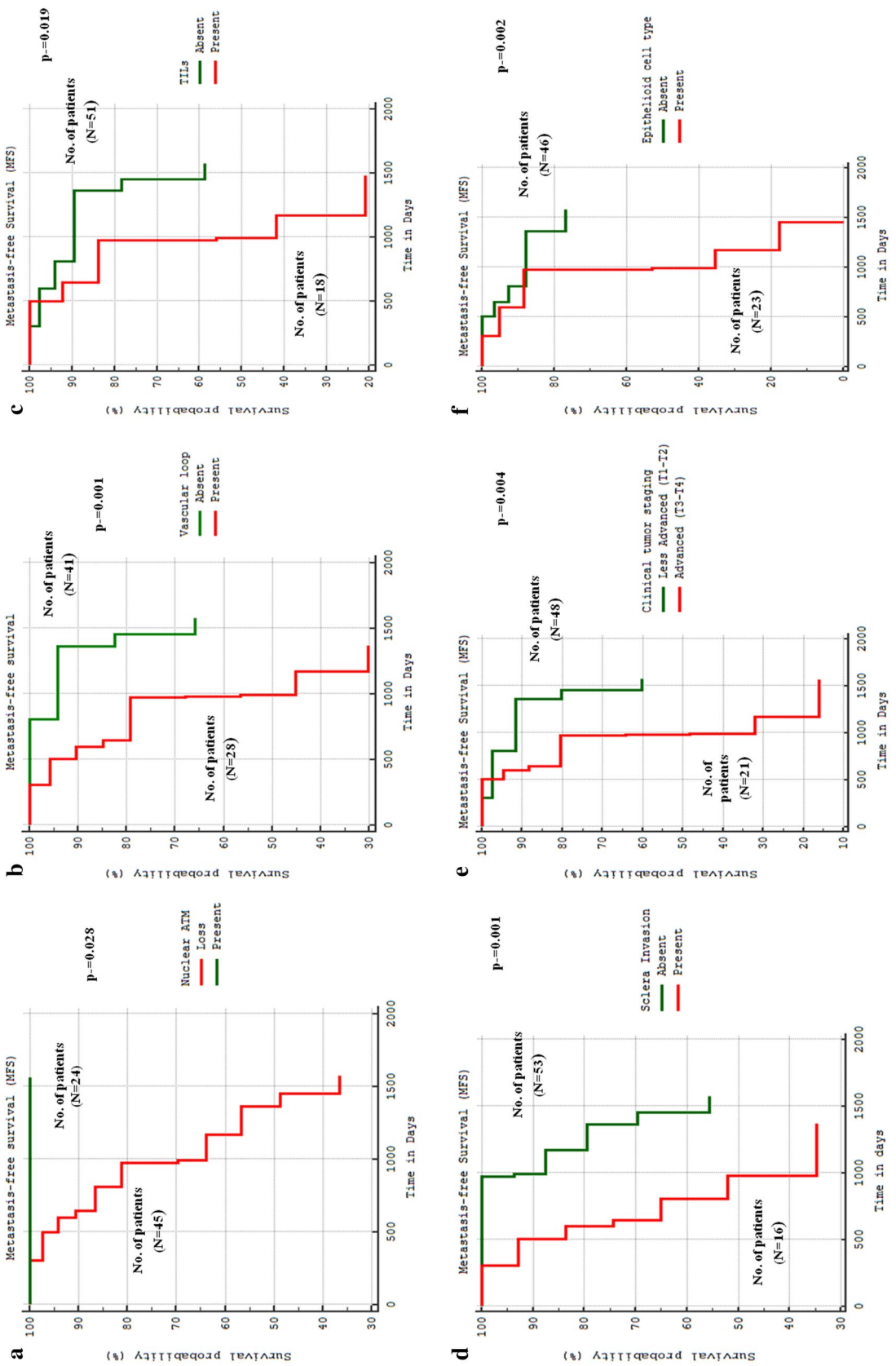

Fig. 3 Overall survival of uveal melanoma patients by Kaplan–Meier survival analysis. **a** Loss of nATM protein, **b** ultrasound-tumour height (> 8 mm), **c** clinical tumour staging, **d** large tumour diameter (> 10 mm), **e** Epithelioid cell type and **f** tumour infiltrating lymphocytes (TILs)

Table 3 Prognostic significance of clinicopathological features of uveal melanoma by univariate and multivariate analysis (Cox's proportional hazards model)

Clinicopathological parameter	Univariate analysis		Multivariate analysis	
	Hazard ratio (95% CI)	*p* value	Hazard ratio (95% CI)	*p* value
Sex (male)	0.93 (0.24–3.56)	0.922	–	–
Age (> 40 years)	0.55 (0.14–2.18)	0.401	–	–
Tumor thickness (> 8 mm)	4.5 (0.89–22.65)	0.068	–	–
Neovascularization	2.46 (0.66–9.10)	0.177	–	–
Location of tumour	0.72 (0.08–6.58)	0.715	–	–
Clinical tumour staging	5.5 (1.40–21.59)	**0.015**	4.33 (0.92–22.29)	**0.043**
Pigmentation	1.37 (0.15–12.41)	0.778	–	–
Epithelioid cell type	4.59 (1.18–17.83)	**0.028**	2.26 (0.74–12.17)	0.342
LTD > 10 mm	2.02 (0.39–10.33)	0.396	–	–
Necrosis	2.66 (0.64–11.06)	0.177	–	–
Scleral invasion	5.76 (1.46–22.63)	**0.012**	3.21 (0.79–19.34)	0.090
Optic nerve invasion	4.07 (0.59–27.87)	0.152	–	–
Extraocular spread	5.06 (0.95–26.91)	0.057	–	–
Mitotic count (> 4/40HPF)	2.00 (0.53–7.48)	0.298	–	–
TILs	4.6 (1.19–17.67)	**0.026**	1.60 (0.31–8.19)	0.570
Vascular loop	5.06 (1.20–21.23)	**0.026**	1.52 (0.24–9.64)	0.653
Loss of BAP1	1397 (1.37–93.46)	**0.004**	1.60 (0.08–10.36)	0.999
Loss of nATM	5.95 (1.67–27.28)	**0.004**	4.75 (1.08–26.85)	**0.041**

Bold signifies significant parameters ($p < 0.05$)

LTD large tumour diameter, *TILs* tumour infiltrating lymphocytes

was found to be more frequent in spindle cell melanoma as compared to epithelioid cell melanoma (Fig. 1c–f).

Western blotting

Western blot analysis for ATM protein was performed in 20 fresh cases to validate immunohistochemistry results of uveal melanoma along with the control (β-actin). Among 20 cases, nATM protein was expressed only in 35% cases, whereas loss was seen in 65% of the cases (Fig. 2).

Association of nATM expression with clinicopathological parameters

On correlation with histopathological parameters, loss of nATM was statistically significant with epithelioid cell type, high pigmentation, large tumor diameter greater than 10 mm and patients having more than one histopathological high-risk factors (HRFs > 1) ($p < 0.05$). On the other hand, loss of nATM was statistically correlated with clinical parameters such as tumor height ($p = 0.043$), TILs ($p = 0.020$) and tumor staging ($p = 0.026$) (Table 2).

Prognostic outcome of ATM expression in uveal melanoma patients

Kaplan–Meier analysis by the log-rank test was carried out to determine the prognostic significance of ATM protein. There was reduced disease-free survival (84%) in patients with loss of nATM. This was found to be statistically significant ($p = 0.046$) (Fig. 3). In univariate analysis, clinical tumour staging ($p = 0.001$), scleral invasion ($p = 0.021$), extraocular spread ($p = 0.009$), TILs ($p = 0.012$), neovascularization ($p = 0.008$), epithelioid cell type ($p < 0.001$) and loss of nATM ($p = 0.023$) emerged as significant risk factors (Table 3). On performing multivariate analysis, extraocular spread and loss of nATM were found to be independent prognostic factors (hazard's ratio 39.154; 95% CI 1.389–1103.455) (Table 4).

Discussion

Early detection of metastatic spread in uveal melanoma will help to salvage the vision and increase survival of patients. The effectiveness of treatment for metastatic uveal melanoma seems to be very limited. Once it metastasises, it becomes highly resistant to traditional systemic

Table 4 Metastasis-free survival (MFS) of clinicopathological parameters for nATM protein estimated by Kaplan–Meier survival analysis

Clinicopathological parameters	($N=69$)	MFS (%)	Hazard ratio (95% confidence interval)	p value
Age				
> 40 years	51	77.28	1.88 (0.46–7.65)	0.300
≤ 40 years	18	86.27	0.52 (0.13–2.14)	
Tumor thickness				
> 8 mm	38	76.32	3.85 (1.17–12.61)	0.060
≤ 8 mm	31	93.55	0.25 (0.07–0.84)	
Neovascularization				
Present	25	76	4.02 (0.98–16.41)	**0.011**
Absent	44	88.64	0.24 (0.06–1.01)	
Clinical tumour staging				
Advanced (T3–T4)	21	66.67	4.85 (1.27–18.54)	**0.004**
Less advanced (T1–T2)	48	91.67	0.20 (0.05–0.78)	
Pigmentation				
High	61	83.61	1.94 (0.12–30.10)	0.504
Low	8	87.50	0.51 (0.03–7.96)	
Epithelioid cell type				
Present	23	69.57	5.23 (1.33–20.49)	**0.002**
Absent	46	91.30	0.19 (0.04–0.74)	
LTD				
> 10 mm	49	81.63	2.54 (0.74–8.71)	0.213
≤ 10 mm	20	90	0.39 (0.11–1.34)	
Necrosis				
Present	27	81.48	1.21 (0.37–3.95)	0.743
Absent	42	85.71	0.82 (0.25–2.68)	
Scleral invasion				
Present	16	62.50	6.07 (1.23–29.83)	**0.001**
Absent	53	90.57	0.16 (0.03–0.80)	
Optic nerve invasion				
Present	5	60	8.64 (0.19–57.02)	**0.001**
Absent	64	85.94	0.11 (0.02–5.07)	
Extraocular spread				
Present	7	57.14	3.93 (0.48–32.04)	**0.027**
Absent	62	87.10	0.25 (0.03–2.06)	
Mitotic count				
Present (> 4/40HPF)	22	77.27	2.24 (0.59–8.49)	0.163
Absent (≤ 4/40HPF)	47	87.23	0.44 (0.11–1.68)	
TILs				
Present	18	66.67	3.68 (0.93–14.54)	**0.019**
Absent	51	90.20	0.27 (0.06–1.07)	
Vascular loop				
Present	28	71.43	6.15 (1.70–22.30)	**0.001**
Absent	41	92.68	0.16 (0.04–0.58)	
nATM				
Loss	45	75.56	7.84 (1.45–21.36)	**0.028**
Present	24	100	0.28 (0.15–0.85)	

Bold signifies significant parameters ($p < 0.05$)

LTD large tumour diameter, *TILs* tumour infiltrating lymphocytes

chemotherapy [16]. Patients having more than one histopathological high-risk factor should be kept on longer follow up to detect metastasis.

This is the first study showing the impact of ATM function in uveal melanoma patients. In cancer, loss of ATM resulted in increased genomic instability and compromised checkpoint regulation. Therefore, we assessed the immunohistochemical expression of ATM protein with the clinical and histopathological parameters. Our study revealed a loss of nATM protein in 65.21% of the cases, which is consistent with other published studies [17, 18]. The frequency of loss of nATM was more common in patients with high-risk histopathological factors like epithelioid cell type, large tumor diameter, high pigmentation, and TILs.

Histopathologically, spindle cell type is associated with a better prognosis than epithelioid cell type [13]. Nuclear expression of ATM protein was identified in spindle cell type. Loss of nATM was found more frequently in epithelioid cell type and this was statistically significant ($p < 0.001$). Also, high pigmentation is considered to be an essential risk factor for metastatic uveal melanoma. Extensive studies have shown that high pigmentation is triggered by DNA damage via DDR subunits such as ATM, PARP-1, ATR, etc. [19]. Our findings showed that loss of nATM was frequently found in highly-pigmented cases. Thus, pigmentation might have direct or indirect relation with DDR subunits in uveal melanoma.

Advanced tumor staging might be associated with the altered expressions of DDR-related proteins and poor prognosis [20]. We found a higher incidence of loss of nATM in advanced tumor stage, and it correlated well with poor survival. These findings were consistent with previous studies on breast cancer, gastric cancer in which loss of ATM was seen in high-grade tumors and advanced cancer [21].

While analyzing our results, loss of nATM statistically correlated with large tumor diameter (> 10 mm) as well as tumor height (> 8 mm). Tumour size is often used as a relevant clinical prognostic parameter for the selection of the treatment in UM. Carol Shields et al. reported that with an increase in each millimeter of tumor thickness, there is an increased risk of metastasis by 5% [22]. The mortality rate in 5 years for small (< 2–3 mm height), medium (3–8 mm height) and large (> 8 mm height) melanomas was 16%, 32%, and 53% respectively, and has not changed in recent years [23].

Interaction of DNA damage response (DDR) with an inflammatory environment is a new dimension in the field of tumor biology by altering the immune balance in the tumor microenvironment [24]. Our study found that Loss of nATM was more commonly found in cases having tumor-infiltrating lymphocytes (TILs). Generally, lymphocytic infiltration in most cancers has a favorable response with patient survival, but the presence of TILs in uveal melanoma is associated with a poor prognosis [16]. This indicates that loss of nATM correlated with aggressiveness and poor prognosis of the tumor.

BAP1 (BRCA-associated protein 1) is a tumor suppressor protein and a known poor prognostic marker of uveal melanoma. Our study revealed that loss of nATM was statistically significant with loss of BAP1 ($p = 0.001$).

Loss of nATM was associated with reduced disease-free survival in patients with UM. These findings suggest that patients who have loss of ATM may be less sensitive to treatment and thus more likely to have poor survival. Our results are in agreement with those cancers where loss of nATM has been associated with shorter disease-free survival [4].

On statistical correlation, loss of nATM was significant in patients having more than one histopathological high-risk factor ($p = 0.010$). Our findings are in line with other studies, which showed loss of nATM in metastatic cancers such as neuroendocrine cancer, prostate cancer, breast carcinomas resulting in increased cell division and proliferation [6]. It emphasizes the fact that patients with larger melanomas are at a higher risk of metastasis.

Conclusion

Our findings demonstrate an essential role of ATM protein and its loss may serve as a poor prognostic marker and a potential drug target in the treatment of uveal melanoma. However, further studies are required in a larger cohort of patients with longer follow up, and translational validation needs to be performed.

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Author contributions JJ executed the study, data analysis and in association with SK; MKS contributed to the design and draft of the manuscript. LS helped in the experiments and coordination of the manuscript. NP and MSB provide the enucleated specimens. SS and SK were responsible for histopathological examination. All authors read and approved the final manuscript.

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Compliance with ethical standards

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

Informed consent Informed consent was obtained from all individual participants included in the study.

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