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RESEARCH

Correlation of High Mobility Group Box-1 Protein (HMGB1) with Clinicopathological Parameters in Primary Retinoblastoma

Mithalesh Kumar Singh[1] · Lata Singh[1] · Neelam Pushker[2] · Seema Sen[1] · Anjana Sharma[3] · Feeroj Ahamad Chauhan[1] · Seema Kashyap[1]

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Abstract HMGB1 is considered to be DNA chaperone as it binds without any specificity. It is the structural protein which alters nuclear homeostasis and genomic stability of chromatin. Its role in retinoblastoma (Rb) remains unclear. The aim of the present study was to evaluate the expression of HMGB1 protein in primary enucleated retinoblastomas. Expression of HMGB1 in 69 prospective cases of primary retinoblastoma were assessed by immunohistochemistry and reverse transcriptase PCR (RT-PCR) technique and correlated with clinicopathological parameters. Immunohistochemical staining revealed expression of HMGB1 in 55.07 % (38/69) cases. Semi-quantitative RT-PCR was performed on 31 fresh tumor tissues. mRNA expression was observed in 77.41 % (24/31) cases. Expression of HMGB1 was statistically significant with poor tumor differentiation (p=0.0440) & optic nerve invasion (p=0.0128). HMGB1 expression was frequently seen in poorly differentiated tumors and those with histopathological high risk factors. Therefore, HMGB1 may contribute to tumor invasiveness and could serve as a poor prognostic marker in Rb.

Keywords Retinoblastoma · Immunohistochemistry · HMGB1 · RT-PCR

✉ Seema Kashyap
dr_skashyap@hotmail.com

[1] Department of Ocular Pathology, Dr. R. P. Centre for Ophthalmic Sciences, All India Institute of Medical Sciences, New Delhi, India

[2] Department of Ophthalmology, Dr. R. P. Centre for Ophthalmic Sciences, All India Institute of Medical Sciences, New Delhi, India

[3] Department of Ocular Microbiology, Dr. R. P. Centre for Ophthalmic Sciences, All India Institute of Medical Sciences, New Delhi, India

Abbreviations

Rb	Retinoblastoma
PDRB	Poorly differentiated retinoblastoma
WDRB	Well differentiated retinoblastoma
AC	Anterior chamber
CB	Ciliary body
HRFs	Histopathological high risk factors
RL	Retrolaminar
IHC	Immunohistochemistry
RT-PCR	Reverse-transcriptase polymerase chain reaction

Introduction

Retinoblastoma (Rb) is a malignant tumor of developing retina in children. Incidence of retinoblastoma is 1 per 15,000 to 17,000 live births [1]. It is a genetic disease which is present in cells that have cancer-predisposing mutations in both copies of the RB1 gene [2–4]. Retinoblastoma ranks 4th in the list of mortality in Indian children. The estimated incidence of retinoblastoma in India is about 2000 a year [1].

The High Mobility Group proteins (HMG) are the non histone chromosomal proteins that are abundant in chromatin and bind non specifically to DNA. These high mobility group proteins ("High Mobility" refers to their electrophoretic mobility in polyacrylamide gels) play an important role in chromatin remodeling and transcriptional activation [5]. They are oncogenic chromatin factors. Acidic and basic domains of HMG proteins are present in the same molecule which is involved in gene regulation and maintenance of chromatin structure [6]. HMG protein family comprises of three isoforms such as HMGA, HMGB and HMGN [7].

HMGB1 is a chromatin-associated protein with high content of acidic and basic amino acid [8]. It is a nuclear DNA binding protein that binds and modifies DNA. It is responsible for the regulation of gene transcription [9]. HMGB1 proteins are expressed in the nucleus of both cancer and embryonic cells [10]. HMGB1 is localized on human chromosome 13 which is expressed in embryonic as well as differentiated stages [11].

Evidence reveals that disorders in HMGB1 are considered as a hallmark of cancer and enrichment of cancer development [12]. Overexpression of HMGB1 has been found in many cancers such as breast cancer [13], melanoma [14], gastric cancer [15], and non-Hodgkin lymphoma [16]. Among the three HMG proteins, HMGA1 and HMGA2 have been found to be overexpressed in retinoblastoma [17, 18]. Physiological role of HMGB1 in retinoblastoma is still unclear and further studies are required to determine the exact role.

The aim of the study is to investigate the expression of HMGB1 by Immunohistochemistry (IHC), semi-quantitative RT-PCR and to evaluate the prognostic significance and its correlation with clinical and histopathological features of retinoblastoma (Rb).

Materials and Methods

Clinical Details

The study was approved by the Institute Ethics committee AIIMS, New Delhi, India. Written informed consent for enucleation surgery and participation in this study was obtained from the guardians of all patients. Sixty nine prospective cases that underwent primary enucleation over two year period (Jan 2012-Dec 2013) were included, while patients who received chemotherapy and radiotherapy prior to enucleation were excluded from the study. Complete clinical and demographic data were obtained from medical records such as sex, age at presentation, laterality and clinical grouping. Hematoxylin and eosin stained slides were assessed for differentiation, necrosis, calcification and histopathological high risk factors. Tumor differentiation was categorized as well differentiated (>50 % Flexner-Wintersteiner rosettes) or poorly differentiated (<50 % Flexner-Wintersteiner rosettes). Tumor invasion was assessed on the basis of histopathological high risk factors (HRFs) such as invasion of choroid/anterior chamber/iris & ciliary body/optic nerve/scleral & extrascleral invasion. Follow up ranged from 6 to 24 months in our study. Staging was done according to the guidelines of American Joint Committee on cancer classification [19].

Table 1 Detail of clinicopathological parameters

Parameters	No. of patients (N=69) N (%)
Sex	
Male	44 (63.76 %)
Female	25 (36.23 %)
Age	
<24 months	30 (43.47 %)
≥24 month	39 (56.52 %)
Laterality	
Unilateral	57 (82.60 %)
Bilateral	12 (17.39 %)
Grouping	
Group E	52 (82.60 %)
Group D-A	17 (24.63 %)
pTNM staging	
T1NoMo	26 (37.68 %)
$T2aNoM_o$-$T4bNoM_o$	43 (62.31 %)
Tumor differentiation	
PDRB	53 (76.81 %)
WDRB	16 (23.18 %)
Necrosis	35 (50.72 %)
Calcification	20 (28.98 %)
Choroidal invasion	16 (23.18 %)
Anterior chamber invasion	2 (2.89 %)
Sclera invasion	6 (8.69 %)
Iris & ciliary body invasion	7 (10.14 %)
Optic nerve head invasion	34 (49.27 %)
Optic nerve retrolaminar and cut end invasion	16 (23.18 %)
Histopathological high risk factors (HRFs)	
HRFs≥1	31 (44.92 %)
None	38 (55.07 %)

RT-PCR

Semi-quantitative analysis for expression of HMGB1 mRNA was performed by RT-PCR technique, using GAPDH as an internal control. mRNA was isolated from 31 fresh frozen tissues and are subjected to reverse transcription, and analyzed by PCR. cDNA was synthesized from total RNA by reverse transcription using a cDNA kit according to the manufacturer's instructions (SuperScript III First Strand Synthesis System, Invitrogen, USA). PCR was performed in a total volume of 25 μl reaction mixture containing 1 μl of cDNA template, 1 Perkin Elmer PCR buffer, 1.5 mM $MgCl_2$, 0.8 mM deoxynucleotide triphosphates, 20 pmol of each primer for HMGB1, GAPDH, and 1 unit of TaqDNA Polymerase (AmpliTaq Gold; Roche Molecular Systems, Inc., Belleville, NJ). The sequences of these PCR primers were as follows:

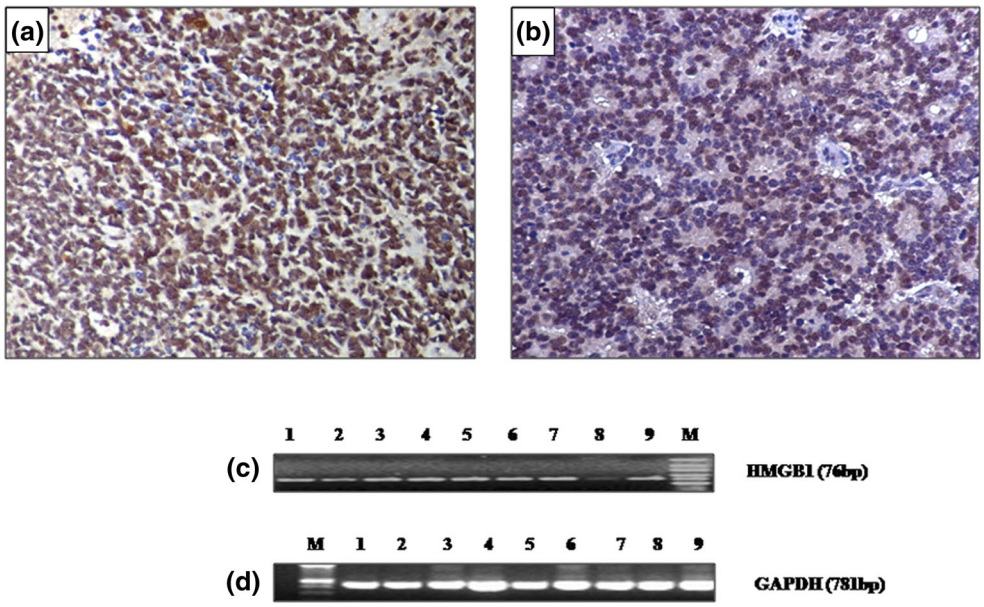

Fig. 1 Expression of HMGB1 in tumor samples; **a** Immunohistochemical detection of HMGB1 in poorly differentiated retinoblastoma (×200). **b** Immunohistochemical detection of HMGB1 in well differentiated retinoblastoma (×200). **c** Semi quantitative analysis of mRNA expression of HMGB1 in tumor samples by RT-PCR (76 bp). **d** Expression of GAPDH as an internal control (781 bp)

HMGB1(sense):5′-AATTTCACATAGCCCACTTACA TTTAC-3′and (antisense): 5′- TTGATTCTAATAATCC CATGCTTTGA-3′.
GAPDH(sense):5′-GTGGTGGACCTCATGGCCCACC AT-3′and (antisense): 5′-GAAATCCCAGCACCATCT TCCCAGG- 3′

The size of amplicons of GAPDH and HMGB1 were 781 and 76 bp, respectively. The PCR conditions were as follows: initial denaturing at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 58 °C for 45 s and 72 °C for 1 min, before a final extension at 72 °C for 10 min. 8 μl portion of each PCR product was electrophoresis on 2.5 % agarose gel.

Immunohistochemistry

For immunohistochemistry, paraffin embedded sections (5 μm thick) were dewaxed and rehydrated. Heat induced antigen retrieval (microwave oven method) was performed in citrate buffer (pH 6.0) for 20 min. Endogenous blocking was performed with 4 % Hydrogen peroxide (H_2O_2) in methanol (30 min) and the slides were incubated with the HMGB1 (Pierce Biotechnology, Rockford, USA) rabbit polyclonal antibody with a dilution of 1:250. Secondary incubations were performed with biotinylated linked secondary antibody and peroxidase-labeled streptavidin according to the manufacturer's protocol (LSAB+System-HRP kit; Dako Cytomation, Glostrup, Denmark). Immunostaining was visualized using 3′, 3′-diaminobenzidene (DAB) peroxidase substrate for 3 mins and counterstained with hematoxylin and analyzed under bright field microscopy. Breast carcinoma was taken as the positive control.

Evaluation of Immunostaining

All slides were assessed by two independent experienced pathologists. HMGB1 staining intensity was scored as 0 (negative), 1+ (weak), 2+ (moderate) and 3+ (strong) staining. HMGB1 staining intensity was grouped into two categories as: 0 & 1+ were considered as negative whereas 2+ & 3+ were considered as positive.

Statistics

The Fisher's exact test was used for statistical analysis. All the values were two-sided, and p-values less than 0.05 were regarded as statistically significant. All statistical analyses were performed using SPSS 11.0 for windows (SPSS Inc., Chicago, IL, U.S.A.). Fischer's exact test was performed for analyses of the expression of these proteins with clinical and histopathological parameters.

Results

Clinical and Histopathological Details

Age of patients ranged between 4 and 96 months with the median age of 30 months. There was male preponderance (63.76 %) & bilateral involvement was seen in 17.39 % cases. Clinical and histopathological findings are summarized in Table 1. Most of the patients had higher pTNM staging

Table 2 Correlation of HMGB1 protein with clinicopathological factors

Pathological parameters N: 69	HMGB1 protein expression		
	Positive N: 38	Negative N: 31	*P*-Value*
Sex			
Male (44)	23	21	0.6186
Female (25)	15	10	
Age			
<24 months (30)	12	18	*0.0316*
≥24 months (39)	26	13	
Laterality			
Unilateral (57)	28	29	0.0527
Bilateral (12)	10	2	
Grouping			
Group E (52)	24	28	*0.0116*
Group D-A (17)	14	3	
Staging			
$T_{2a}N_0M_0$-$T_{4b}N_0M_0$ (43)	19	24	*0.0281*
$T_1N_0M_0$ (26)	19	7	
Tumor differentiation			
PDRB (53)	33	20	*0.0440*
WDRB (16)	5	11	
Necrosis			
Yes (35)	20	15	0.8106
No (34)	18	16	
Calcification			
Yes (20)	9	11	0.3010
No (49)	29	20	
Choroidal invasion			
Massive (16)	6	10	0.1525
Focal (53)	32	21	
AC invasion			
Yes (2)	2	0	0.4979
No (67)	36	31	
Scleral invasion			
Yes (6)	3	3	1.0000
No (63)	35	28	
Iris & CB invasion			
Yes (7)	3	4	0.6925
No (62)	35	27	
Optic nerve (Head)			
Yes (34)	24	10	*0.0155*
No (35)	14	21	
Optic nerve (RL & Cut end)			
Yes (16)	13	3	*0.0128*
No (53)	25	28	
HRFs			
Yes (31)	13	18	*0.0166*
No (38)	25	13	

Italics means statistically significant value of $p<0.05$

* Signify Fischer exact test

Table 3 Correlation of HMGB1 mRNA expression with clinicopathological parameters

Pathological parameters N: 31	HMGB1 mRNA expression		
	Positive N: 24	Negative N: 7	*P*-Value*
Sex			
Male (20)	16	4	0.6757
Female (11)	8	3	
Age			
<24 months (15)	11	4	0.6851
≥24 months (16)	13	3	
Laterality			
Unilateral (26)	21	5	0.0527
Bilateral (5)	3	2	
Clinical grouping			
Group A-D (10)	5	5	*0.0218*
Group E (21)	19	2	
Staging			
$T_1N_0M_0$ (13)	7	6	*0.0124*
$T_{2a}N_0M_0$-$T_{4b}N_0M_0$ (18)	17	1	
Tumour differentiation			
PDRB (24)	21	3	*0.0292*
WDRB (7)	3	4	
Choroidal invasion			
Massive (9)	9	0	0.0766
Focal (22)	15	7	
AC invasion			
Yes (2)	2	0	1.0000
No (29)	22	7	
Scleral invasion			
Yes (4)	4	0	0.5497
No (27)	20	7	
Iris & CB invasion			
Yes (3)	2	1	0.5497
No (28)	22	6	
Optic nerve (RL & Cut end)			
Yes (11)	11	0	*0.0331*
No (20)	13	7	
HRFs			
Yes (16)	15	1	*0.0373*
No (15)	9	6	

Italics means statistically significant value of $p<0.05$

* Signify Fischer exact test

(75.36 %). Poor differentiation & necrosis were found in 76.81 & 50.72 % cases respectively. Choroidal invasion & optic nerve invasion were found in 23.18 % each respectively. One patient died due to progressive disease. There was no recurrence/metastasis in any patient during this time period.

Immunohistochemistry of HMGB1

Nuclear expression of HMGB1 was seen in viable tumor cells (Fig. 1a and b). Expression of HMGB1 was identified in 55.07 % cases. Of the sixty nine cases, 31 (44.92 %) cases had presence of HRFs whereas rest did not have HRFs.

HMGB1 expression statistically correlated with clinical and histopathological parameters as summarized in Table 2.

mRNA Expression of HMGB1 by RT-PCR

To verify the immunohistochemical results, we studied HMGB1 expression by semi-quantitative RT-PCR in 31 fresh cases of which 77.41 % cases showed HMGB1 mRNA expression. The same reverse transcription products with primers targeted to GAPDH acted as controls, and the product was identified in the samples (Fig. 1c and d). HMGB1 mRNA expression was correlated with clinicopathological parameters summarized in Table 3. HMGB1 mRNA expression correlated with tumor staging (P=0.0124), clinical grouping (P=0.0218), poor tumor differentiation (P=0.0292) and optic nerve invasion (P=0.0331).

Discussion

High Mobility Group (HMG) proteins play a major role in tumor biology. HMGB1 interacts with various cell cycle regulators and is associated with cancer development [12]. Interaction of HMGB1 with Rb protein induces the activity of HMGB1-mediated transcriptional repression, cell growth inhibition, G1 cell cycle arrest, apoptosis induction, and tumor growth suppression [20]. Downregulation of microRNA MIR34A-dependent high mobility group box 1 (HMGB1) enhances chemotherapy-induced apoptosis in the retinoblastoma cell [21]. Thus, such interaction with HMGB1 makes it as an important gene in the cause of retinoblastoma.

In the current study, HMGB1 protein expression was significantly higher in the tumor tissues with presence of HRFs as compared to the tumors with no HRFs by immunohistochemistry and RT-PCR. Our results showed HMGB1 expression in 55 % cases which are similar to studies in human bladder cancer [22], gastric adenocarcinoma [23]. Protein expression of HMGB1 statistically correlated with tumor differentiation, optic nerve invasion, age less than 2 years, pTNM staging and clinical grouping. mRNA expression of HMGB1 was also statistically significant with clinical grouping, tumor staging, poor tumor differentiation and optic nerve invasion. In our study, discrepancy between mRNA and the protein expression of HMGB1 was found. Possible explanation could be type of tissue sample taken as RNA was isolated from fresh tissue whereas immunohistochemistry was performed on formalin fixed paraffin embedded sections.

Hao et al. also found higher levels of mRNA and IHC expressions of HMGB1 in cervical squamous epithelial carcinomas (CSEC) samples than normal tissue and these correlated with tumor stage, invasion and metastasis but not with tumor size and differentiation [24]. Similarly high expressions of HMGB1 were found in colorectal cancer [25], nasopharyngeal carcinoma [26] and hepatocellular carcinoma and all these studies showed correlation of HMGB1 with poor prognostic features such as higher tumor staging, invasion and poor survival rate [27, 28].

Our results are supporting the hypothesis of Weinberg and Hananhan which states that any deregulation in the expression of HMGB1 may induce loss of differentiation, invasiveness and cancer progression [12]. Similarly, our findings explain that this protein might be responsible for the progression of retinoblastoma as they were significantly associated with histopathological high risk factors, poor tumor differentiation and staging. Thus, it is required to understand the molecular mechanism of HMGB1 in cancer development and invasiveness.

To conclude, the present study is the first study in literature, possibly to assess the clinicopathological correlation of HMGB1 protein expression in retinoblastoma in large cohort of patients. Our results demonstrate that expression of HMGB1 could possibly have a role in tumorigenesis of retinoblastoma. However, these are preliminary observation in understanding the role of HMGB1 protein in pathobiology of Rb. Further molecular and functional studies are required to prove the contribution of HMGB1 in tumorigenesis of Rb and identify ways to exploit therapeutically.

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Conflict of Interest None.

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