Expression of Ki67 as detected by MIB-1 and its association with histopathological high-risk factors among patients with retinoblastoma tumour: a cross-sectional study

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ABSTRACT

Objectives This study aims to investigate the expression of Ki67 in formalin-fixed paraffin-embedded tissue blocks from patients with a diagnosis of retinoblastoma tumour (RbT) as well as determining its association with histopathological high-risk factors (HHRFs).

Methods and analysis Retrospectively, a total of 194 eyeball specimens from 163 children with RbT were reviewed at Muhimbili National Hospital between 2009 and 2013. Immunohistochemical expression of Ki67 using MIB-1 antibody (Abcam, batch ab93680, Cambridge, UK) was determined and correlated with the conventional HHRFs. The predictors of Ki67 expression were determined using binary logistic regression model in multivariate analysis. A two-tailed p<0.05 was considered statistically significant.

Results Majority (67.5%) of the patients had leukocoria and exuacular disease was found in 20.9% of all the patients. High expression of Ki67 was present in 63.8% of the 80 eyeballs that were tested. Massive choroidal invasion (adjusted OR=9.32, 95%CI=2.82 to 10.89), positive retrolaminar optic nerve invasion (AOR=3.01, 95%CI=4.43 to 9.11), positive surgical margin (AOR=7.10, 95%CI=1.63 to 11.40) and pT4 (AOR=7.49, 95%CI=0.12 to 0.89) were the potential HHRFs that were associated with Ki67 overexpression.

Conclusion Overexpression of Ki67 may be of prognostic value for patients with RbT as it has been shown in the present study that high expression was common in tumours with massive choroidal invasion, positive retrolaminar optic nerve invasion, positive surgical margin and advanced tumour stage, which are the conventional HHRFs associated with prognosis of RbT.

INTRODUCTION

Retinoblastoma tumour (RbT) is the most common primary ocular malignant tumour affecting the paediatric population.1 It is caused by mutation of the retinoblastoma gene (Rb1), which is located on the chromosome 13q14.2 The mutations are 40% germline in nature and the other 60% of the mutations are sporadic.3 The annual incidence of RbT for children with age ranging from 0 to 4 years in high-income countries (HICs) is 10.0–11.8 per 100000 person-years.4 The incidence reported in the literature for Africa particularly in the sub-Saharan African region is 9000 new cases per year, which corresponds to the incidence of 1 in 15000 births.5 In low- and middle-income countries (LMICs), children who are diagnosed with RbT usually are older than those in HICs and also, they tend to have poor prognosis because they are diagnosed at late stage.6
Studies have outlined different histopathological features as high-risk factors (HHRFs) and are associated with tumour progression, metastasis and overall poor prognosis. A number of HHRFs has been found to be associated with higher risk of metastasis and poor overall survival including massive choroidal invasion, extrascleral extension and optic nerve involvement.\(^8\) Thaug and Karaa also reported tumour extension to the anterior chamber, iris, trabecular meshwork, Schlemm’s canal, ciliary body, choroid (above a certain threshold), sclera, extraocular structures, retrolaminar optic nerve (including the cut end) as the HHRFs in their series.\(^9\) In one study it was reported that, HHRFs, for example, massive choroidal invasion and positive retrolaminar invasion of the optic nerve were associated with recurrence, metastasis and poor overall survival.\(^10\)

Determination of the HHRFs in paediatric patients with RbTs is of utmost importance in assessing the prognosis of the patients as well as establishing the proper type of treatment for better clinical outcomes, although enucleation remains to be the main type of management for paediatric patients who are diagnosed with RbTs. Additionally, for those with advanced RbTs treatment of the patients usually must involve adjuvant chemotherapy.\(^11\) Complementing with biomarkers has shown to be of benefit in the determination of prognosis of the paediatric patients with RbTs. Studies have shown that increased expression of cell proliferation biomarkers such as Ki67 is associated with tumour progression, metastasis and overall survival.\(^7\) A number of HHRFs has been found to be associated with higher risk of metastasis and poor overall survival including massive choroidal invasion, extrascleral extension and optic nerve involvement.\(^8\)

**Materials and Methods**

**Study design and patients**

This was a cross-sectional analytical retrospective laboratory-based study. The study was conducted at the Central Pathology Laboratory of the Muhimbili National Hospital in Dar es Salaam, Tanzania. The study included review of 194 eyeball specimens from 163 children who were diagnosed with RbT between January 2009 and December 2013. Patients’ files and laboratory investigation request forms were used to extract the required clinical data and histological results. Only cases with histologically confirmed diagnosis of RbT were enrolled in the present study. All cases with missing formalin-fixed paraffin-embedded (FFPE) tissue blocks and those with insufficient tissue as a result of being spoiled by insects were excluded from the study.

**Sampling method**

Convenience sampling method was applied to enrol all available FFPE tissue blocks due to limited RbT provided that they had met the inclusion criteria. Seventeen cases were excluded from the study (10 cases due to FFPE tissue blocks being spoiled by insects and 7 cases due to missing previous histological report).

**H&E staining**

After retrieving the FFPE tissue blocks, the tissue were sectioned and stained with H&E for re-evaluation of the previous histological diagnosis. Histological re-evaluation of the eyeball specimens was done by an independent and experienced pathologist (EAMV), who was blinded of the patients’ clinical details.

**Immunohistochemical staining of MIB-1 antibody**

The eyeball specimens for immunohistochemical (IHC) procedure were sectioned at the thickness of 4 μm by using a micrometre. The sections were de-waxed by placing them on a hot plate at a temperature of 60°C for 30min. This was followed by hydration by dipping the tissue sections in decreasing concentration of ethanol (100%, 95%, 80% and 70%). Two drops of 3% hydrogen peroxide solution were added to each section for 15min to block endogenous peroxidase for inhibiting background staining. Then the slides were put into running tap water for rinsing purpose. A 100X EDTA citrate buffer, pH 8.0 heat antigen retrieval solution was used. The antigen retrieval solution was heated in a pressure cooker until it started boiling followed by placing the slides in the retrieval solution and closing the lid of the pressure cooker from which the slides were removed 2min after full pressure. Then the slides were placed in tap water to prevent drying.

The slides were washed using phosphate-buffered saline solution (PBS) for 3min. PBS was then drained from the slides. A ring was made around the section placed on a glass slide using a hydrophobic Pap pen so as to limit spreading of the primary antibody solution. Then the primary antibody MIB-1 (Abcam, ab93680 model, Cambridge, UK) was applied and allowed to react with the antigen by incubating for 60min at room temperature. The slides were then washed with PBS for 5min and then two to three drops of horse radish peroxidase was added to each section for 30min. The slides were again washed with PBS for 5min. PBS was drained from the sections, and then one drop of chromogen diaminobenzidine was added to the sections for 5min as the detection system. The sections were washed with tap water for 1min, counterstained in haematoxylin for 5min, dehydrated and waxed by paraffin before embedding.

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The areas of highest proliferative activity (hot spots) were identified by scanning the tumour sections at low magnification of ×10. The method of determining the positive tumour cells in this study was adopted from the previous study which was done by Kouzegaran et al., in which approximately 1000 tumour cells from 20 systematically randomised fields were counted, and the proportion of positive tumour cells was determined. The percentage scoring of immunoreactive tumour cells was as follows: 0 for <1% positive tumour cells; 1 for 1%–10% positive tumour cells; 2 for 11%–50% positive tumour cells; 3 for 50%–75% positive tumour cells; 4 for >75% positive tumour cells. Then intensity was evaluated as follows: negative staining (0), weak staining (1+), moderate staining (2+) and strong staining (3+). The scores of intensity and percentage were added to obtain the final score which ranged from 0 to 7. The results of immunostaining were divided into two groups; 0–2 was considered negative and 3–7 was considered positive. Reporting of the IHC tissue slides was performed by an experienced pathologist who was blinded of both clinical and histopathological data.

**Quality assurance**

To ensure validity and reliability of the collected data as well as the findings of the study, stringent measures were adhered to all steps of data extraction including use of negative and positive controls and blinding during histological re-evaluation of the tissue sections.

**Statistical analysis**

Data were analysed by using SPSS V.23.0. The coded data were cross-checked for possible error and any missing data after running frequency tables and crosstabs. Categorical and continuous variables were summarised in proportions and mean±SD, respectively. Inferential statistics were used in determining the predictors of Ki67 immunohistochemical expression by using binary logistic regression. Variables that were statistically significant as well as those which had p≤0.2 in univariate analysis were then fitted in the multivariate analysis after adjusting for each confounding factor. A two-tailed p<0.05 was considered statistically significant.

**Patient and public involvement statement**

This work did not include direct interaction with patients or the public in designing and conceptualisation of this work.

**RESULTS**

**Selection process of the study subjects**

The process of selection of the study cases is presented in figure 1. For a period of 5 years (2009–2013), there were 173 cases of RbT that were diagnosed among all ocular tumours (OTs). Of the OTs that were retrievable, 17 cases were excluded due to either being spoilt by insects while in the archive room or missing previous histological diagnosis. Therefore, a total of 163 cases were retained for review in the present study.

**Sociodemographic and clinical characteristics of the patients**

This study included a total of 163 patients, 80.9% (132/163) patients had unilateral disease and the remaining 19% (31/163) patients had bilateral diseases. The total number of eyeball specimens that were examined from the 163 patients was 194. The vast majority of the patients 65% (106/163) in the present study were aged 2 years and above and the mean age of the patients was 4.8±2.42 years. Males were slightly more than females and they consisted of 57.7% (94/163). The lag period of 3 months and above in terms of seeking medical attention among the patients was observed in 41.1% (67/163) of all the cases with an average of 2.1±1.94 months.

Table 1 presents the clinical characteristics of the patients. The varsity majority 67.5% (110/163) had leukocoria followed by proptosis, which consisted of 22.7% (37/163). Also, 20.9% (57/163) patients had extraocular extension (EOE). Majority (49/57) of those with EOE had optic nerve invasion. Additionally, 6.7% (11/163) of the patients had metastasis to distant organs; central nervous system—10 cases and bone marrow—1 case.

**Treatment given to the patients based on the local treatment guidelines**

Table 2 presents the treatment modalities as per eyeball which were given to the patients in the study based on the International Classification of Retinoblastoma (ICRB). All eyeballs with EOE for both bilateral 15.9% (31/194) and unilateral 21.6% (42/194) cases were exenterated (upfront enucleation), which involved removal of the eyeball, optic nerve (at least 2 cm) and clearing of the socket. There were as more eyeballs with a history of salvage attempt for bilateral disease 50% (31/62) than unilateral disease 31.8% (42/132). Chemoreduction involved a combination of carboplatin, vincristine and etoposide, which were given in six cycles at the interval of 3 weeks. Chemoreduction was given to shrink the tumour for patients with ICRB group B–D and some patients in whom the affected eyeballs were in ICRB class E. None...
of the cases with ICRB group A were included in the present study because they are not enucleated either upfront or after chemoreduction. Eyeballs found to have class A, usually were managed by focal therapy by either cryotherapy or thermotherapy alone for smaller tumours (<3 mm in diameter and height) located in visually non-crucial area. Cryotherapy was usually administered 3–6 hours prior to chemotherapy if systemic treatment was indicated.

Not all eyeballs had advanced disease for patients with bilateral RB, as summarised in table 2. In some eyeballs 4.1% (8/194), there was an attempt to salvage the affected eyeballs for vision preservation, and nevertheless, after assessment of improvement they were finally enucleated.

**Histopathological high-risk factors evaluated from the eyeball specimens**

Of all the eyeball specimens that we examined in the present study, massive choroidal invasion was present in 18.6% (36/194) and focal involvement of the choroid layer was found in 7.2% (14/194). Anterior chamber involvement by the tumour cells was observed in 4.6% (9/194) and 20.1% (39/194) of the eyeball specimens had scleral extension of the disease. Concerning spreading of the cancer cells to the optic nerve among the eyeball specimens evaluated, we found that 25.3% (49/194) of the eyeball specimens had retrolaminar optic nerve extension (online supplemental figure 1A) and involvement by the tumour for the surgical margin of the optic nerve (online supplemental figure 1B) was found in 4.6% (9/196). Also, 20.6% (40/194) showed advanced disease (29 for pT3 and 11 for pT4). Other HHRFs are shown in table 3.

**Clinical characteristics of patients with eyeballs stained with MIB-1**

Ki67 immunostaining was performed in 80 eyeball specimens and not for all 194 eyeball specimens due to lack of funds to purchase sufficient antibody (MIB-1). Table 4 shows the clinical characteristics of the 65 patients of whom the 80 eyeball specimens were obtained and stained with MIB-1. Of the 65 patients, 23.1% (15/65) and 76.9% (50/65) patients had bilateral and unilateral RB, respectively. The mean age of the patients whose
eyeball specimens were subjected to Ki67 immunostaining was 4.5±1.96 years.

**Expression of Ki67 as detected by MIB-1 and its association with histopathological high-risk factors**

The Ki67 labelling index (LI) was ranging from 3.2% to 70.8%. There was variation in expression of Ki67 in poorly differentiated cases (online supplemental figure 2A,B). The mean expression of Ki67 was 38±17.6%. Expression of Ki67 was present in 66.3% (53/80). Almost all the HHRFs had an increased chance for having high expression of Ki67 in the present study; however, massive choroidal invasion, retrolaminar optic nerve invasion positive surgical margin of the optic nerve and advanced tumour stage were the HHRFs that were significantly associated with high expression of Ki67.

The chance for eyeball specimens with massive choroidal invasion to have high expression of Ki67 was 9.32 times more than that without choroidal invasion (adjusted OR (AOR)=9.32, 95% CI=2.82 to 10.89). This showed a noticeable difference for eyeball specimens with focal choroidal invasion which even though had a 1.22-fold increased chance for high expression of Ki67 compared with the eyeball specimens without choroidal invasion, but the difference was not significant (AOR=1.22, 95% CI=0.49 to 2.82).

The odds of high expression of Ki67 in eyeball specimens with positive retrolaminar part of the optic nerve was 3 times more than that of the eyeball specimens that had no invasion of the retrolaminar part of the optic nerve and the difference was significant (AOR=3.01, 95% CI=4.43 to 9.11). Also, there was a 7.10-fold increased chance for the odds of having high expression of Ki67 for eyeball specimens with positive surgical margin of the optic nerve as compared with eyeball specimens (AOR=7.10, 95% CI=1.63 to 11.40). Interestingly, there was a linear increase of the percentage of high expression of Ki67 as per pT stage (pT1–pT2=18.5%, pT3=34.5% and pT4=75%). Eyeball specimens with pT4 had a 7.49-fold increased chance of having high expression of Ki67 compared with cases which were of pT1–pT2 (AOR=7.49, 95% CI=0.12 to 0.89). Despite the fact that other variables showed increased odds of high expression of Ki67, they showed no statistical difference between the compared groups of each variable (online supplemental table 1).

**DISCUSSION**

RbT being the most common intraocular malignancy in the paediatric population, it carries the highest mortality rate in LMICs unlike in HICs. This has been mainly associated with remarkable delay in diagnosis for the LMICs. In this series, the main HHRFs in a cohort of paediatric patients with RbT were described along with association with immunoreactivity of Ki67.

The key and promising finding which has further strengthened the evidence that Ki67 may be used as a prognostic biomarker is that, in this study Ki67 LI was associated with increased tumour aggressiveness as indicated by extent of invasion into various structures of the eyeballs. This observation is in agreement with findings in previously reported studies elsewhere.17 18 The Ki67 LI

| Laterality | ICRB | Treatment options | Frequency (n) | Percentage (%) |
|------------|------|-------------------|---------------|----------------|
| BL         | C    | Enucleation after salvage attempt | 3 | 1.5 |
| BR         |      |                    | 3 | 1.5 |
| BL         | D    | Enucleation after salvage attempt | 3 | 1.5 |
| BR         |      |                    | 3 | 4.1 |
| BL         | C    | Enucleation after chemoreduction | 5 | 2.6 |
| BR         | D    | Enucleation after chemoreduction | 2 | 1.0 |
| BL         | E    | Upfront enucleation | 3 | 1.5 |
| BR         |      |                    | 9 | 4.6 |
| BL         | EOE  | Upfront enucleation | 19 | 9.8 |
| BR         |      |                    | 12 | 6.2 |
| UL         | C    | Enucleation after salvage attempt | 20 | 5.2 |
| UR         | D    | Enucleation after salvage attempt | 25 | 11.3 |
| UL         | E    | Upfront enucleation | 19 | 19.6 |
| UR         | C    | Enucleation after chemoreduction | 15 | 7.7 |
| UL         | D    | Enucleation after chemoreduction | 11 | 5.7 |
| UR         | EOE  | Upfront enucleation | 29 | 14.9 |
| UL         | EOE  | Upfront enucleation | 13 | 17.0 |

BL, bilateral left; BR, bilateral right; EOE, extraocular extension; UL, unilateral left; UR, unilateral right.
of 66.3% in the present study was higher than 53.33% which was reported in the study by Kouzegaran et al., but lower than 73%, 90% and 91% which were reported in Mexico and other two studies in China, respectively. The discrepancies in the incidence of Ki67 LI observed across studies may be contributed by a number of factors including extensive necrosis in the FFPE tissue blocks, challenges with other pre-analytical phase procedures like delayed fixation and prolonged fixation which may be associated with antigen masking. Furthermore, it has also shown that neoadjuvant therapy affects the expression of immunohistochemical biomarkers. For example, in a study which was done by Wu et al., it was shown that the expression of oestrogen receptor (ER) and Ki67 before chemotherapy and after chemotherapy decreased from 65.3% to 42.6% for ER and from 51% to 43.4% for Ki67.

Ki67 expression in the present study was found to increase with invasion of the disease. This has also been reported in other studies. For example, Kim et al. showed that Ki67 expression was associated with advanced tumour stage. This is also similar to the finding that was reported by Kouzegaran et al. Ki67 being a nuclear cell proliferation biomarker, is usually associated with tumour invasiveness and metastasis. It indicates how fast the tumour cells tend to quickly divide when the tumour is at advanced stage. Furthermore, Orjuela et al. in Mexico also reported that Ki67 expression was associated with clinical stage of RbT.

Massive choroidal invasion has been reported to correlate with poor prognosis in patients with RbT. It is one of the most important HHRFs with short survival and other associated adverse effects for patients with RbT. In the present study, children in whom the eyeball specimens had massive choroidal invasion they had ninefold increased odds of having Ki67 expression compared with other cases in which there was no massive choroidal invasion. This finding was also reported before chemotherapy and after chemotherapy decreased from 65.3% to 42.6% for ER and from 51% to 43.4% for Ki67.

Table 3  Histopathological high-risk features in the eyeball specimens included in the study (n=194)

| Histopathological features | Frequency (n) | Percentage (%) |
|---------------------------|--------------|----------------|
| Schlemm's canal           |              |                |
| Present                   | 42           | 21.7           |
| Absent                    | 152          | 78.3           |
| Trabecular meshwork       |              |                |
| Present                   | 55           | 28.4           |
| Absent                    | 139          | 71.6           |
| Choroidal invasion        |              |                |
| No choroidal invasion     | 144          | 74.2           |
| Focal                     | 14           | 7.2            |
| Massive                   | 36           | 18.6           |
| Ciliary body involvement  |              |                |
| No                        | 181          | 93.3           |
| Yes                       | 13           | 6.7            |
| Anterior chamber involvement|             |                |
| No                        | 185          | 95.4           |
| Yes                       | 9            | 4.6            |
| Involvement of the iris   |              |                |
| No                        | 183          | 94.3           |
| Yes                       | 11           | 5.7            |
| Scleral involvement       |              |                |
| No                        | 155          | 79.9           |
| Yes                       | 39           | 20.1           |
| Retrolaminar optic nerve involvement | | |
| No                        | 145          | 74.7           |
| Yes                       | 49           | 25.3           |
| Optic nerve surgical margin status  | | |
| Negative                  | 162          | 83.5           |
| Positive                  | 32           | 16.5           |
| pTNM                      |              |                |
| T1–T2                     | 154          | 79.4           |
| T3                        | 29           | 14.9           |
| T4                        | 11           | 5.7            |

Table 4  Clinical features for patients with eyeball specimens stained with MIB-1 (n=65)

| Clinical features | Frequency (n) | Percentage (%) |
|-------------------|--------------|----------------|
| Age (years)       |              |                |
| <2                | 26           | 40.0           |
| ≥2                | 39           | 60.0           |
| Sex               |              |                |
| Male              | 40           | 61.5           |
| Female            | 25           | 38.5           |
| Lag period (months) |             |                |
| <3                | 17           | 26.2           |
| ≥3                | 48           | 73.8           |
| Laterality        |              |                |
| Unilateral        | 50           | 76.9           |
| Bilateral         | 15           | 23.1           |
| Leukocoria        |              |                |
| Present           | 47           | 72.3           |
| Absent            | 18           | 27.7           |
| Proptosis         |              |                |
| Present           | 25           | 38.5           |
| Absent            | 40           | 61.5           |
| Treatment         |              |                |
| Upfront enucleation | 24          | 36.9           |
| Exenteration after chemoreduction | 41 | 63.1 |
| Metastasis        |              |                |
| Yes               | 11           |                |
| No                | 54           |                |

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in the study by Kouzegaran et al, in which under univariate analysis, the expression of Ki67 was associated with massive choroidal invasion.17

This study had some limitations including the following: the relatively small number of cases analysed for Ki67 limits the strength of the study. Also, considering the methodology used in selecting the cases for the study which was convenient, this might have contributed to selection bias and therefore making the inferences to be biased.

In conclusion, the present study further reports the importance of Ki67 to be a biomarker, which has shown prognostic role by virtue of its expression to be associated with HHRFs including massive choroidal invasion, positive retrolaminar and surgical margin of the optic nerve as well as advanced tumour stage. Use of Ki67 immunoreactivity may be supplemented as a prognostic biomarker in the planning of the management of the patients. This may help to improve the prognosis of the patients with RbT.

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