HIGH-RISK HISTOPATHOLOGIC FEATURES ANALYSIS OF PRIMARY ENUCLEATED RETINOBLASTOMA IN TANZANIA PATIENTS

Ndayisaba Marie Claire, MD

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By

Ndayisaba Marie Claire

A Dissertation Submitted in (Partial) fulfillment of the Requirement for the Degree of Master of Medicine (Anatomical Pathology) of

Muhimbili University of Health and Allied Sciences
October, 2018

CERTIFICATION

The undersigned certifies that he has read and hereby recommend for acceptance by Muhimbili University of Health and Allied Sciences a dissertation entitled: "***High-risk histopathologic features analysis of primary enucleated retinoblastoma in Tanzania patients"***, in (partial) fulfillment of the requirements for the degree of Masters of Medicine (Anatomical Pathology) of Muhimbili University of Health and Allied Sciences.

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Prof. James Kitinya

(Supervisor)

_____

Date

_____

Dr. Edda Vuhahula

(Supervisor)

_____

Date

DECLARATION AND COPYRIGHT

I, **Ndayisaba Marie Claire**, declare that this **dissertation** is my own original work and that it has not been presented and will not be presented to any other University for a similar or any other degree award.

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DEDICATION

To Almighty God

To my beloved parents

To my sisters and brothers

To my mentors and tutors

To all my friends and colleagues

I dedicate this work.

ABSTRACT

Introduction: In ocular retinoblastoma; massive choroid infiltration, post-laminar optic nerve invasion, invasion of the optic nerve to the surgical margin, sclera invasion, and extrascleral extension are now accepted as the high-risk factors for local recurrence, metastasis, and indication for adjuvant therapy. The presence of high-risk histopathologic features in enucleated eye specimen is an indication for adjuvant chemotherapy to limit tumor local recurrence and systemic metastasis.

Objective: To describe the histopathology of retinoblastoma and determine prevalence of high-risk histopathologic features in primary enucleated eye specimens of retinoblastoma in Tanzania.

Methods: This was a retrospective cross-sectional histopathologic review of 132 primary enucleated eyes with retinoblastoma during a 4-year period from January 2013 to December 2016 in the Histopathology Unit at Muhimbili National Hospital. We retrieved patients' records, slides and blocks for histology. Demographic, clinical and histopathologic data were collected using excel data collection sheet. Prevalence of high-risk histopathologic features, proportion of the patients that needed adjuvant therapy and proportion of completeness of histopathology reports were determined. Continuous variables were summarized using mean, median, mode, standard deviation and range. Frequency tables, bar charts and pie charts for categorical variables were done by excel. The association between demographic, clinical data, growth pattern and the need for adjuvant therapy was established by univariate and multivariate logistic analyses using Statistics and data (STATA) software, version 13. The association was considered significant for a p-value less than 0.05.

Results: Sixty-eight of 132 participants were male. The median age was 33 months, ranging from 3 to 108 months. Bilateral disease was noted in 23/132 participants (17.4%). Leukocoria was the predominant presenting sign. The mean duration of sign to surgery was 11.06 months. Eight two of 132 participants (62.1 %) were in clinical stage group E.

One or more high-risk histopathologic features were noted in 62.9% (83/132) participants. Massive choroid infiltration was recorded in (59.8%), post-laminar optic nerve invasion in

(7.5%), surgical margin optic nerve invasion in (30.3%), scleral invasion in (10.6%) and extrascleral invasion in (31.8%). The combination of surgical margin optic nerve invasion and extrascleral invasion was the overall high-risk feature encountered 30.1% (n=25/83).
Statistically significant association was noted between the need for adjuvant therapy and symptoms duration period more than six months, clinical stage group E and both mixed and exophytic tumor growth patterns. Half of the histopathology reports issued for our participants during the study period were found miss complete information about tumor extension and presence or absence of high risk features.

Conclusion: The prevalence of retinoblastoma high-risk histopathologic features in Tanzania is high. Though some demographic and clinical factors may predict the presence of high-risk histopathologic features, the histopathology remains the gold standard to determine who need adjuvant therapy in intraocular retinoblastoma after enucleation.

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LIST OF ABBREVIATIONS

Abl	Abelson proto-oncogene
AJCC	American Joint Committee on Cancer
C.I.	Confidential interval
CD	Cluster of differentiation
C-kit	protein-tyrosine kinase receptor
COG	Children's Oncology Group
CSF	Cerebrospinal fluid
CT	Computed Tomography
DAB	Diaminobenzidin
FFPE	Formalin-Fixed Paraffin-Embedded
GIST	Gastrointestinal stromal tumor
H&E	Hematoxylin and eosin
HP	Histopathology
HPF	High power field
HRP	Horseradish Peroxidase
ICRB	International Classification of Retinoblastoma
IHC	Immunohistochemistry
IRSWG	International Retinoblastoma Staging Working Group
kDa	kilodalton
M:F	Male: Female
MCI	Massive Choroid Invasion

MNH	Muhimbili National Hospital
MRI	Magnetic Resonance Imaging
MUHAS	Muhimbili University of Health and Allied Sciences
n	number of cases
N	Sample size
ONI	Optic Nerve Invasion
OR	Odds ratio
PDGFR	Platelet-derived growth factor receptor
pTNM	pathologic Tumor, Node, Metastasis stage
RB	Retinoblastoma
SD	Standard Deviation
SMONI	Surgical Margin Optic Nerve Invasion
SOP	Standard Operating Procedure
STATA	Statistics and data
TBS	Tris Buffer Saline
USA	United States of America

DEFINITION OF TERMS

Retinoblastoma: is a primary malignant intraocular neoplasm that arises from immature retinoblasts within the developing retina.

Leukocoria (leucocoria or leukokoria): also called white pupillary reflex or cat's eye is an abnormal condition of the eye in which the normal red reflex of the 11retina is replaced by a yellow or grayish white color.

Immunohistochemistry: is a process of detection and localization of antigens (e.g. proteins) in cells of a tissue section based on the principle of antibody-antigen binding reaction and visualized on microscopy.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Retinoblastoma (RB) is a rare malignant tumor but the most common retinal and intraocular neoplasm of childhood (1,2). Patient with RB may develop one tumor in one eye or multifocal tumors including bilateral involvement(3,4). RB accounts nearly 3% of cancers in children under 15 years(2,5–7) with an annual worldwide incidence of one case per 15 000 to 20 000 live births. There is no gender or racial predilection. RB is usually diagnosed between the ages of 16 to 60 months and is not common after the age of 10years(8–10). In Tanzania, every year around 75 new cases of RB are expected based on the worldwide incidence (11,12).

Genetically, RB results from inactivating mutation in both alleles of RB tumor suppressor gene *RB1* located at the long arm of chromosome 13 locus 14 (*RB1*, 13q14)(13,14). RB follows the Alfred Knudson's double hit hypothesis that stipulates "two separate losses of function mutational events must occur in both alleles of *RB* gene for the malignancy to develop"(15,16). However, other genetic and molecular alterations have been noted during RB development such as MYCN amplification, down-regulation of p16INK4A, overexpression of KI14 and so on(14,17).The *RB1*gene encodes 107 kDa RB protein that plays important role in cell cycle especially at the G1phase to S phase transition. The loss of function of both copies of the *RB1* gene is required for the tumor initiation(13,14). According to its etiology, RB can present in two forms: sporadic in 60% and hereditary in 40%. The hereditary form results from germ line mutations that may take place either *de novo* in most cases (30%) or before conception in parental germ cells giving rise to familial RB that represents 6-10%. The transmission in hereditary cases follows an autosomal dominant mode(3,4,14). The hereditary RB presents earlier and around 85% of individuals develop the tumor in both eyes. In the sporadic form both inactivating mutations of *RB1* gene are somatic mutations that occur after fertilization and affect one retinal cell. In these individuals, the relative risk to develop RB is lower compared to the hereditary form. The disease grows in later childhood and is usually unilateral(3,4,13).In Tanzania, previous authors recorded bilateral RB in one-third of the cases and these patients were below 3 years old (12).

Children with RB may present with leukocoria, strabismus, rubeosis iridis, pain or proptosis. Though leukocoria is the most common presenting sign in patients with RB, it can also be seen in other conditions like Coat's disease, persistent hyperplastic primary vitreous (PHPV), cataract and toxocariasis. In developing countries, a majority of patients presents with advanced lesion revealing enlarged eye or intense orbital extension. Indirect ophthalmoscopy examination of the fundus establishes the diagnosis in most cases. Other diagnostic methods are ocular ultrasound, computed tomography (CT)-scan, magnetic resonance imaging (MRI) and histopathology(11,18,19).The management of RB is guided by laterality and clinical stage at presentation and it involves a multidisciplinary approach. When the vision is preserved and the tumor is small local treatments are used namely brachytherapy, cryotherapy, photocoagulation, external radiation therapy. Systemic or intra-arterial chemotherapy may be added. When the eye cannot be preserved surgery is done with or without adjuvant chemotherapy and radiotherapy(1,18,20). Enucleation remains the treatment of choice for large RB tumor, in advanced disease with suspicion of optic nerve invasion or extrascleral extension. Loss of vision of the affected eye, glaucoma and long-standing retinal detachment also are indications for enucleation in RB. Once the tumor has spread beyond the eye, combination of chemotherapy and radiotherapy is given. (21–23). While in developed countries the 5-year survival of RB is over 90%; in developing countries, it is still less than 50% with a mortality rate of 50 to 90%(22,24). This poor prognosis is associated with late diagnosis, poor methods of diagnosis and management, tumor recurrence, and metastasis(11,25–28). Recurrence and metastasis can be predicted by presence of high-risk histopathologic features such as optic nerve involvement, choroid invasion, and anterior segment invasion, sclera invasion with iris neovascularization, necrosis, calcification and extrascleral invasion(29,30).

On gross examination of enucleated specimens, RB exhibits different growth patterns including endophytic, exophytic, mixed and diffuse infiltrative (2,29). The neoplasm can be unifocal or multifocal. Microscopically, the tumor is made up of uniform small round or polygonal cells with retinal differentiation. Necrosis sparing viable cells around blood vessels

and calcifications are also common. Fleurettes, Flexner-Wintersteiner, and Homer Wright rosettes are usually encountered (2,9).

Immunohistochemistry is one of the techniques in pathology that help to characterize tumor cell differentiation in cases of undifferentiated neoplasm, and is also used to determine the presence and quantity of specific targets for cancer treatment by personalized targeted therapies. Imatinib mesylate (Gleevec) is a targeted cytotoxic drug with mild side effects compared to other anticancer medications and it is effective in treatment of C-kit expressing tumors like Gastrointestinal stromal tumor (GIST) and Myelogenous Leukemia.

It acts by inhibiting tyrosine kinase enzymes especially the Abelson proto-oncogene (abl), C-kit and platelet-derived growth factor (PDGF) receptors. Different tumors including RB are under investigation to explore the use of tyrosine kinase inhibitors in their management. C-kit (CD117 or stem cell factor receptor) is a transmembrane protein receptor in the family of tyrosine kinase encoded by Kit gene on chromosome 4q11-21. The C-kit transmembrane tyrosine kinase protein plays role in cell proliferation, attachment, migration, differentiation and apoptosis Activating mutations of C-kit gene is associated with many tumors such as (GIST), myelogenous leukemia, synovial sarcoma and melanoma. The ligand for C-kit or stem cell factor receptor is also incriminated in tumor angiogenesis (1,2,26). Previous studies found that the expression of C-kit in RB was associated with advanced grade and poor prognosis.

In Tanzania, previous studies showed that the majority of patients with RB presents with an advanced stage of the disease. Some of studied patients had no timely histopathology reports and limited ancillary investigations. These contribute to incorrect staging of the disease and therefore inadequate management. RB histopathology reports, using synoptic template shows improved and complete report generation in different institutions (11,12,18,32,33). The present investigation was meant to determine the magnitude of high-risk histopathologic features in relation to other demographic, clinical and histopathologic characteristics in primary enucleated eye specimens with RB at MNH from January 2013 to December 2016. Knowledge and complete report on the evaluation of high-risk histopathologic features will greatly contribute to proper patients' selection for adjuvant chemotherapy and radiotherapyas well as for proper further follow-up(22,24,27).

1.2 Literature Review

1.2.1 Introduction

The human eyeball consists of three tunics specifically the outer protecting sclera and cornea, followed by a vascular layer urea and the inner neural layer called retina from which RB arises. There are three chambers in the eye: anterior chamber located between the cornea and anterior surface of the iris; posterior chamber situated from the posterior surface of the iris to the lens and lastly the vitreous cavity which is posterior to the lens. Both anterior and vitreous cavities are filled with aqueous humor. The retina consists of two distinct layers; the outer pigmented nonsensory layer made up of a single cuboidal cell layer and the sensory retina constituted by photoreceptor neurons, conducting neurons, association neurons and supporting neuroglia cells (34,35). RB is believed to arise from embryonic retinal cells specifically cone precursors (36). RB is the most common primary intraocular malignant neoplasm of childhood. It is an aggressive rapidly progressive tumor associated with less than 50% survival rate in developing countries. Late diagnosis, poor methods of management and poor healthcare infrastructure are the major contributing factors for poor outcome. Metastasis is one of the three major causes of death in RB and the most commonly encountered in developing countries (24%)(27). In developed countries, the incidence of metastasis in RB is less than 10%. Other major causes of death are second malignant neoplasms and pinealoblastoma. The tumor relapse in the first 12 months is high (97%) in patients with high-risk histopathologic features when there is no adjuvant therapy given(37,38). These high-risk features are determinant of the level of tumor extension. They are mostly encountered in older children, clinically advanced lesions and less differentiated tumor. Other associated factors include geographical location (developing and low-income regions), laterality and prior enucleation therapy(37,39–41).

1.2.2 RB epidemiology and clinical presentation

The annual incidence of RB is estimated at one case per 15 000 to 20 000 live births. In Europe and United States, the average age-adjusted incidence is 2 to 5 cases per million children while in Africa and Asia the annual incidence is estimated at 6 to 10 cases per million children. In Tanzania, every year around 75 new cases of RB are expected based on the worldwide incidence of 1/18,000-20,000 live births(1,8,11,12,15,18,20). Almost 90% of RB cases are diagnosed before the age of 5years worldwide which is similar to the picture in East Africa.

However, in the developing countries, Tanzania included, the lag period or time between the tumor development and management is longer, therefore, the patients present already with an advanced tumor. Clinical stage of the disease and the age of the patient can predict the presence of high-risk histopathologic features. There is no remarkable gender predilection according to different reviews however, some single studies showed gender variations. In advanced RB, patients present with enlarged eyes or intense orbital extension. Leukocoria and strabismus are the common presenting signs in the early stage of the disease(11,12,39,43–45).

1.2.2.1 Clinical staging of RB

The International Classification of Retinoblastoma (ICRB) was developed in 2005. This classification was developed to predict intraocular RB cases that are likely to be cured without the need for enucleation or radiation therapy. It is based on the size of the tumor and the presence and extent of tumor seeding. The classification consists of five stages ABCD and E and guide in the choice for treatment(46). Table 1 shows the ICRB details.

Table 1: The International Classification of Retinoblastoma

GROUP	ICRB FEATURES
GROUP A (Very low risk)	Small intraretinal tumours (≤ 3mm) away from foveola and disc
GROUP B (Low risk)	Tumors> 3mm, macular location ≤ 3mm to foveola or juxtapapillary location ≤ 1.5 mm to disc, or with subretinal fluid ≤ 3mm from margin.
GROUP C (Moderate risk)	Tumor with focal subretinal seeds ≤ 3mm from tumor or vitreous seeds ≤ 3mm from tumor or both subretinal and vitreous seeds ≤ 3 mm from tumor.
GROUP D (High risk)	Tumor with diffuse subretinal seeds > 3 mm from tumor or vitreous seeds > 3mm from tumor or both subretinal and vitreous seeds > 3mm from tumor.
GROUP E (Very high risk)	Extensive RB occupying >50% of the globe with or without neovascular glaucoma, opaque media from haemorrhage in anterior chamber, vitreous or subretinal space. Extension of tumor to post-laminar optic nerve, choroid > 2 mm, sclera and anterior chamber.

The management of RB is guided by clinical stage at the presentation and involves a multidisciplinary approach. In Tanzania, Salum et al. found that 76.7% of 133cases presented with an advanced extraocular disease which is similar in other African countries. Enucleation remains the treatment of choice for RB especially in case of large tumors (group D and E). High-risk histopathologic features are mostly found in an advanced stage and once present adjuvant chemotherapy and/or radiotherapy is mandatory (11,12,32,33,44).

1.2.3 RB histomorphology and C-kit immunoexpression

1.2.3.1 RB tumor growth pattern and high-risk histopathologic features

Four different growth patterns in RB were described including endophytic, exophytic, mixed and diffuse growth patterns. In endophytic growth, the tumor grows into the vitreous humor from the inner surface of the retina. It can progress to the aqueous humor venous channels and spread through lymphatics. Patient presents with leukocoria and vision symptoms. A white tumor in the vitreous humor is visualized by the ophthalmoscopy examination.

The tumor also may grow posteriorly into the subretina starting from the outer retinal sensory layer in front of retinal pigmented epithelium as the exophytic growth. In this case, the patient develops retinal detachment and proptosis, and the tumor may be missed on ophthalmoscopy in an early stage. The tumor then will spread through choroid invasion. In mixed growth pattern, the tumor exhibits both endophytic and exophytic growths. Lastly, few RB tumors grow in a diffuse infiltrative pattern. Usually, there is no obvious mass rather a diffuse growth within the retina. The patient may be misdiagnosed to have inflammatory conditions of the eye. In the literature, massive choroid invasion and optic nerve invasion are commonly associated with exophytic growth pattern while invasion to the anterior segment is mostly encountered as endophytic growth. The mixed growth pattern is associated with an advanced tumor stage; therefore, there is increased incidence of high-risk histopathologic features. The diffuse growth pattern is rarely encountered(47,48).

1.2.3.2 RB tumor differentiation and high-risk histopathologic features

On microscopy, RB is made up of uniform medium-sized round or polygonal cells with basophilic nuclei, scant cytoplasm, and high mitotic activity. The tumor forms fleurettes and rosettes. Flexner-Wintersteiner rosettes: cells line up around empty lumen delineated by a distinct eosinophilic circle composed of terminal bars analogous to outer limiting membrane of normal retina.

These are characteristic for the tumor differentiation and are more encountered than Homer Wright rosettes (nuclei are displaced away from lumen) and fleurettes. Tumor necrosis and calcifications are common, and usually spare viable cells around blood vessels (9,29,49). RB grading is based on the type and percentage of rosettes and mitotic index.

Well-differentiated RB shows fleurettes and Flexner-Wintersteiner rosettes in more than 2/3 of the tumor area and only few Homer Wright rosettes may be seen. Moderately differentiated RB shows fleurettes, Flexner-Wintersteiner and Homer Wright rosettes in one to two-thirds of the tumor area. Poorly differentiated RB shows Homer Wright rosettes and Flexner-Winterstainer rosettes in less than one-third of the tumor area. The undifferentiated RB shows diffuse tumor with a complete absence of rosettes and fleurettes. The presence of high-risk histopathologic features increases as the tumor loses the differentiation. In both developed and developing countries, poorly differentiated and undifferentiated RB are associated with high incidence of high-risk histopathologic features(33,48,50,51).

1.2.3.3 RB tumor pathologic staging and high-risk histopathologic features

Untreated RB a fills the eye, destroys the globe and spread beyond the eye limits. It may undergo complete regression in very rare cases. The tumor spreads through four major routes: local invasion, cerebrospinal fluid (CSF) dissemination, lymphatic spread and hematogenous spread.

In direct local infiltration, the tumor invades the optic nerve or the choroid, the sclera, orbital soft tissues and destroys the bone which results into brain invasion. The tumor cells also can infiltrate through the subarachnoid space of the optic nerve, reach the CSF and from there, they invade the brain and the spine. The hematogenous spread to the lung, bone, and brain follows the orbital and bone invasion as well as from the lymph nodes secondary to lymphatic dissemination. In lymphatic spread, the tumor involves the anterior chamber of the eye to the conjunctiva, eyelid and extraocular tissue. From there the conjunctiva and skin lymphatic vessels drain into the regional lymph nodes. High-risk histopathologic features are characteristics of tumor extension that are evaluated during the histopathologic examination of eye specimens removed due to RB. Once present, they predict a poor prognosis, local recurrence, and metastasis(32,50,52–54).According to the International Retinoblastoma Staging Working Group (IRSWG) and Children’s Oncology Group (COG), the high-risk histopathologic features in RB are defined as follows(41,55,56):Massive or significant choroid invasion means that either the invasive focus of tumor measures 3mm or more in thickness or width, or when the tumor reaches the inner fibers of the sclera tissue.

Optic nerve invasion is classified as pre-laminar, laminar, retro-(post)-laminar, or tumor at surgical margin. The optic nerve involvement posterior to the lamina cribrosa is considered as an independent high-risk feature while the pre-laminar involvement has to be coupled with posterior uveal involvement. Sclera invasion means the presence of tumor in the sclera (intrascleral involvement) or invasion beyond the sclera (extrascleral involvement). The American Joint Committee on Cancer (AJCC) pathologic staging provides summarized information about tumor extension. All RB patients with tumor stage 3 and 4 present with high-risk histopathologic features(57,58). The details of the 8th AJCC RB staging are given in table 2.

Table 2: Pathologic staging of RB

Primary tumor (pT)	
pTX	**Primary tumor cannot be assessed**
pT0	**No evidence of primary tumor**
pT1	**Tumor confined to eye with no optic nerve or choroid invasion**
pT2	**Tumor with intraocular invasion**:
	pT2a: Focal choroidal invasion and pre- or intra-laminar invasion of the optic nerve head
	pT2b: Tumor invasion of stroma of iris and/or trabecular meshwork and/or Schlemm's canal
pT3	**Tumor with significant local invasion**:
	pT3a: Choroidal invasion
	pT3b: Retrolaminar invasion of optic nerve without invasion of transected end of optic nerve
	pT3c: Partial thickness involvement of sclera within the inner two-thirds
	pT3d: Full thickness invasion into outer third of the sclera and/or invasion into or around emissary channels
pT4	**Extraocular extension:**
	Tumor invades optic nerve at transected end, in meningeal space around the optic nerve, full thickness invasion of the sclera with invasion of episclera, adipose tissue, extraocular muscle, bone, conjunctiva or eyelid
Regional Lymph Nodes (pN)	
pNX	Regional lymph nodes cannot be assessed
pN0	No regional lymph node involvement
pN1	Regional lymph node involvement
Distant Metastasis (pM)	
pM0	No distant metastasis
pM1	**Distant metastasis**
	pM1a Single or multiple metastasis to sites other than CNS
	pM1b Metastasis to CNS parenchyma of CSF fluid

1.2.3.4 C-kit immunoexpression and high-risk histopathologic features

C-kit (CD117 or stem cell factor receptor) is a type III transmembrane protein receptor in the family of tyrosine kinase encoded by Kit gene on chromosome 4q11-21. It is closely related to other receptors such as the platelet-derived growth factor receptor (PDGFR), the macrophage colony stimulating factor receptor (M-CSFR) and Flk receptors. In human, C-kit dependent cell type includes hematopoietic cells, germ cells, mast cells and Cajal cells in gastrointestinal tract. The C-kit transmembrane tyrosine kinase protein plays different roles in cell proliferation, attachment, migration, differentiation and apoptosis(1,2,31,46). Mutations in C-kit receptor gene may result into loss or gain of function; and may consist of deletions, point mutations, and duplication or insertion mutations. Loss of function mutation may result in disturbed gastrointestinal movement, depletion of mast cells while the gain of function mutations is associated with tumors development like GIST, mast cell tumor, myeloid leukemia, seminoma/dysgerminoma, nasal T-cell lymphoma, synovial sarcoma and melanoma. The ligand for C-kit or stem cell factor receptor is also incriminated in tumor angiogenesis. IHC is one the methods used to evaluate C-kit expression (2,31,47). RB is one of the tumors under investigation for potential use of Imatinib mesylate in its management though data are still few in the literature. Barry at al. in Canada found that RB cells show immunoexpression of C-kit while normal retinal cells do not. Similar findings are noted in Egypt by Yussef et al. where the expression was noted at 48.2% in a cohort of 56 RB(1,2). However, Bosch et al. found a lower C-kit expression in 19.2% of 73 cases and by using tissue microarray (59). On the other side, experimental study performed to evaluate effect of Imatinib mesylate on RB cell lines showed that it decreases cell proliferation of RB cell lines, their invasion capacity and increases their radiosensitivity. These RB cell lines however, did not express C-kit but showed immunoexpression to c-AbL and PDGFR (54). The C-kit is more expressed in advanced RB with high-risk histopathologic features therefore it may be considered as a marker of aggressive tumor(1,2). In the limits of our knowledge, in Tanzania as well as the East Africa region, there are no data about C-kit expression in RB. This therefore calls for more studies on the subject.

1.2.4 RB management and outcome

1.2.4.1 RB management and high-risk histopathologic features

The management of RB requires a multidisciplinary team and basically depends on the clinical stage. For an advanced disease with suspicion of optic nerve invasion or extrascleral extension as well as a large tumor, enucleation is the treatment of choice. Adjuvant chemotherapy regimen of a combination of Vincristine, etoposide, and carboplatin is administered in six cycles for patients presenting with one or combination of optic nerve post-laminar invasion, intrascleral invasion and massive choroidal invasion. In some institutions, anterior segment invasion is an indication for adjuvant chemotherapy. Extrascleral invasion and positive optic nerve margin are indications for intensive chemotherapy associated with orbital radiotherapy. However, protocols may differ in different institutions. Other histologic features that may be associated with risk of metastasis are tumor size, tumor vascularity, seeding of vitreous, degree of differentiation, and growth pattern however; they have not been approved for adjuvant therapy indication. These treatment modalities are available in Tanzania and RB patients benefit from them, but a standardized histopathology report is required for proper interaction(1,18,20,39,59).

1.2.4.2 Prevalence of high-risk histopathologic features in RB

In developed countries, the overall prevalence of high-risk histopathologic features is estimated at 20.4% according to different studies in North America and Europe. In developing countries, studies done in Latin America, Asia, and Africa showed a higher prevalence between 29 to 64.4% (24,39,40,45). In the majority of literature from both Western and developing countries, the incidence of specific high-risk histopathologic feature showed a remarkable variability but retro-laminar optic nerve invasion and massive choroid invasion were the most frequent (24,39,51). In Tanzania, previous studies showed that a majority of patients presents at advanced stage of disease, however, in the limits of our knowledge, the magnitude of high-risk histopathologic features was not known in this population. Some studied patients had no timely histopathologic reports and limited ancillary investigations (11,12,18,20,33).

1.2.4.3 RB outcome and high-risk histopathologic features

The presence of any of the high-risk histopathologic features predicts tumor recurrence, metastasis, and poor prognosis. Therefore adjuvant chemotherapy and/or radiotherapy are mandatory to improve survival in patients with advanced RB (11,18,27). In general, worldwide literature, high-risk histopathologic features in RB have been studied by many in comparison with other prognostic factors and main variations were noted in geographic and socioeconomic distribution, clinical presentation, tumor differentiation, management, and outcome and survival rate. In Tanzania as well as in most of the developing countries these kinds of studies are still few.

1.2.4.4 Histopathology report and management of advanced intraocular RB

Patient older age, longer lag period before diagnosis and treatment, orbital cellulitis, staphyloma among others are clinical characteristics that can predict the presence of high-risk features for tumor recurrence or metastasis in RB however, histopathology assessment is more accurate. In RB management, pathologists have to confirm the diagnosis, stage the disease and determine who need adjuvant therapy. To assist pathologists to provide a complete report, standardized protocol has been developed by the IRSWG and the Royal College of Pathologists. The guideline highlights all the information about RB tissue specimen handling and dataset for histopathological reporting. Institutions that have adopted that protocol found that it helps to provide complete identification and description of high-risk histopathologic features in enucleated eyes due to RB. Narrative pathology reports showed more variability, inconsistence and incomplete histopathology reports in the literature (41,49,60–62). At MNH, synoptic reporting is not yet adopted in the routine practice. This study evaluated completeness of histopathology reports in the studied RB cases. Complete sampling during grossing, final diagnosis, comments on high-risk histopathologic features, grading and staging of the tumor were considered to evaluate the completeness of those reports.

1.3 Problem Statement

RB is the most common intraocular aggressive malignancy in childhood. When not well managed; advanced RB is associated with loss of vision, local recurrence, metastasis, and death. Chemotherapy and radiotherapy significantly improves the outcome of the tumor (15,55,66). Due to late diagnosis, insufficient diagnostic and management modalities, there is a high incidence of RB with poor outcome in developing countries(11). Pathologists in RB management play an important role to assist oncologists in selection of patients that need adjuvant therapy based on identification of high-risk histopathologic features. In Tanzania, every year around 75 new cases of RB are expected based on a worldwide incidence of 1/18,000-20,000 live births. The previous studies on RB, in Tanzania, found that some patients are treated based only on clinical diagnosis. This may lead to incorrect staging of the disease and therefore inappropriate management(11,12). To improve the management of patients with RB in Tanzania, high-risk histopathologic features have to be considered in patients' selection and personalized treatment should be applied whenever possible. Cancer management is becoming more personalized and targeted with new technologies and cytotoxic drugs with minimal side effects.

Tyrosine kinase inhibitors are one group of the targeted chemotherapy medications. They have found to improve survival rate with minimal side effects in the management of c-Kit-positive tumors (36). RB is one of the neoplasms under investigation for c-kit expression and potential use of Imatinib mesylate in its treatment. Some studies showed a significant correlation between high-risk histopathologic features and c-kit overexpression (1,2,29,31).In the limits of our knowledge, there were no data on RB c-kit expression and high-risk histopathologic features magnitude in Tanzania in the literature. A comprehensive study was therefore proposed to determine the magnitude of high-risk histopathologic features, determine other clinicopathologic characteristics associated with the need for adjuvant therapy and determine the proportion of complete histopathology reports for patients with RB treated by primary enucleation at Muhimbili National Hospital (MNH), Histopathology Unit.

1.4 Conceptual Framework

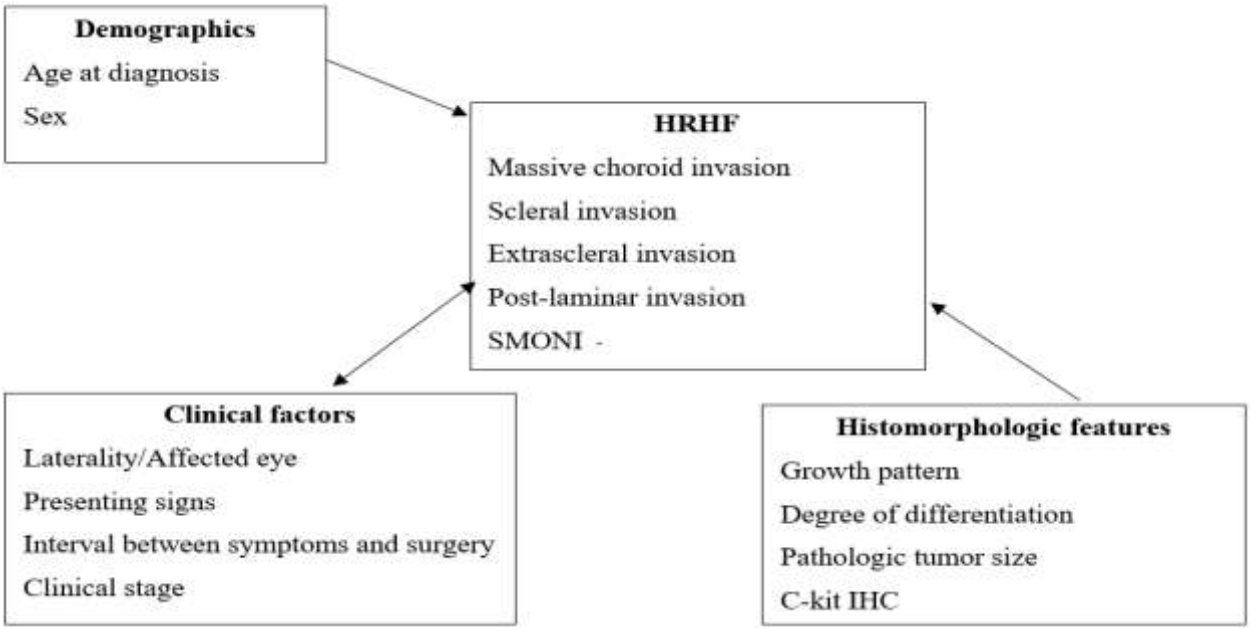


Figure 1: Diagram illustrating the conceptual framework of this study

1.5 Rationale

RB is one of the aggressive malignancies of childhood associated with a significant incidence of recurrence, metastasis, disability, secondary neoplasms and subsequent death in affected individuals. Evaluation and reporting of the presence of the high-risk histopathologic features in enucleated eye specimens due to RB allows selection of patients for adjuvant chemotherapy and radiotherapy. This study aimed to determine the frequency of high-risk histopathologic features, c-kit expression in RB using IHC, determine other clinicopathologic characteristics associated with the need for adjuvant therapy and determine the proportion of complete histopathology reports for patients with RB treated by primary enucleation at MNH, Histopathology Unit; for a 4-year period from January 2013 up to December 2016.This study provided baseline data for the RB management team at MNH. The results obtained are expected to raise awareness of the magnitude of high-risk histopathologic features in studied patients from MNH and to refocus on proper histopathology reporting of RB.

The results are also expected to call for future studies on different areas of RB to reduce its morbidity and mortality in Tanzania.

1.6 Research Questions

1. What are the demographic and clinical characteristics of RB patients who underwent primary enucleation and specimens sent to the Histopathology Unit at MNH from January 2013 to December 2016?
2. What are the histomorphologic characteristics and the profile of C-kit immunohistochemical expression of primary enucleated eye specimens for RB received from January 2013 to December 2016 at the Histopathology Unit, MNH.
3. What is the frequency of high-risk histopathologic features in primary enucleated eye specimens for RB received from January 2013 to December 2016 at Histopathology Unit, MNH?
4. What is the proportion of patients that needed adjuvant chemotherapy after primary eye enucleation for RB based on specimens received from January 2013 to December 2016 at Histopathology Unit, MNH?
5. Is there any association between the need for adjuvant therapy and age, sex, laterality, duration of symptoms, clinical stage and tumor growth pattern in RB patients who underwent primary eye enucleation and specimens sent to the Histopathology Unit, at MNH?
6. What was the proportion of complete histology reports for RB primary enucleated eye specimens received from January 2013 to December 2016 at Histopathology Unit, at MNH?

1.7 Broad Objective

To describe the histopathology of RB and determine prevalence of high-risk histopathologic features in primary enucleated eye specimens of RB in Tanzania.

1.7.2 Specific objectives

I. To determine demographic and clinical characteristics of the patients with primary enucleated eye specimens for RB, from January 2013 to December 2016 at the Histopathology Unit, MNH.

II. To determine the histomorphologic characteristics and C-kit immunohistochemical expression of primary enucleated eye specimens for RB received from January 2013 to December 2016 at the Histopathology Unit, MNH.

III. To determine the frequency of high-risk histopathologic features in primary enucleated eye specimens for RB received from January 2013 to December 2016 at the Histopathology Unit, MNH.

IV. To assess the association between the need for adjuvant therapy and age, sex, laterality, duration of symptoms, clinical stage and tumor growth pattern in RB patients who underwent primary eye enucleation and specimens sent to the Histopathology Unit, at MNH from January 2013 to December 2016.

V. To determine the frequency of complete histopathology reports issued for primary enucleated eye specimens for RB from January 2013 to December 2016 at Histopathology Unit, MNH.

CHAPTER TWO

2 MATERIALS AND METHODOLOGY

2.2 Study design

This was a cross-sectional study at the Histopathology Unit at MNH. All primary enucleated eye specimens received in the Histopathology Unit from the 1st January 2013 to 31st December 2016 and diagnosed as RB were retrieved and histologically reviewed to confirm the diagnosis. We retrospectively analyzed 132 primary enucleated eye specimens, confirmed to be RB during the study period.

2.3 Study settings

This study was conducted in the Unit of Histopathology at MNH, Dar es Salaam, Tanzania. MNH is the highest level institution of the Tanzania health system and receives referred patients from all over the country. MNH serves as a national consultant Hospital as well as a teaching hospital for the MUHAS. The Unit of histopathology is part of the Department of Diagnostic Laboratory Services at MNH. A quarter of all national practicing pathologists are working in this Unit and it has the capacity to do routine surgical pathology, cytology, histochemical special stains as well as immunohistochemistry (IHC). Paraffin-embedded tissue blocks, slides as well as patients' clinical information and morphology reports are kept in the archive of the Unit from where they can be retrieved for further studies. This Unit receives specimens from MNH and outside MNH, from private and public institutions.

2.4 Study Population

The study population included all enucleated eye specimens from 18-year-old and below patients (pediatric patients) received at MNH Histopathology Unit from the 1st January 2013 to 31st December 2016.

2.5 Inclusion criteria

Every primary enucleated eye specimen histologically diagnosed to be RB in 18-year-old or below patients.

2.6 Exclusion Criteria

Specimens exhibiting extensive tumor necrosis or severe artifact on preliminary H&E evaluation and enucleated eye specimens whose formalin-fixed paraffin–embedded (FFPE) blocks were not available, were excluded.

2.7 Sampling procedure

2.7.1 Convenience sampling

All enucleated eye specimens from 18-year-old and below patients received between1st January 2013 to 31st December 2016 were selected.

2.7.2 Sample size

The minimum sample size for this study was 132 cases. The population size of 200 was assumed based on a previous study performed in Tanzania that found 143 RB cases for a 3-year period(12). The known prevalence of high-risk histopathologic features in RB in developing countries literature varies between 29 to 64.4%(24,39,40,45). Therefore, the hypothesized frequency of the outcome factor in the population P was estimated at 50%. The sample size was achieved by the targeted period.

Sample size formula*:* $n = [DEFF*Np\ (1-p)]/\ [(d^2/Z^2_{1-\alpha/2}*(N-1) +p*(1-p)]$

N=Population size: 200

P=hypothesized% frequency of outcome factor in the population: 50%+/-5

D=confidence interval limits as % of 100 (absolute +/- %):5%

DEFF= Design effect: 1

The sample size for 95% confidence level was 132.

2.8 Laboratory methods

2.8.1 Patients records, tissue blocks and slides retrieval

Identification of cases was done by screening the specimens' register and histology reports kept in the archive of Histopathology Unit where they are arranged by the laboratory accession number (HP number). Slides of enucleated eye specimens for RB were retrieved for quality assessment and diagnosis. New sections for H&E staining were made if the quality of the original sections was not optimal. The patients' files were accessed through the MNH Medical Records Department to retrieve data that were missing on the request forms.

2.8.2 Microtomy and H&E staining

Paraffin-embedded blocks were retrieved, and microtomy of 4µ-thick sections was made. The sections were placed on the frosted glass slides then onto a hot plate at 60°C for 50 minutes, before being placed in xylene for de-waxing. Thereafter, H&E staining of sections was done, followed by clearing in xylene and mounting. The different steps of the procedure are detailed in appendix 2.

2.8.3 C-kit IHC

C-kit immunohistochemistry was done using Dako's Polyclonal Rabbit Anti-Human CD117 antibodies on formalin fixed paraffin sections. The antibody was 1/400 diluted. The same paraffin-embedded tissue blocks used for H&E stain were taken for microtomy of 4µ-thick sections. De-waxing also was done by placing the sections on a hot plate at 60°C for 50 minutes. They were thereafter placed in xylene before being hydrated in gradually decreasing concentration baths of ethanol (100%, 95% and 70%) to water. A ring was made around the section using Pap pen, to limit the spread of antibody solutions. Two drops of 0.5% Hydrogen peroxide solution were added to each section for 15 minutes to block endogenous peroxidase activity, and then the slides were washed into water. The slides were incubated in antigen retrieval solution of pH 9 in a pressure cooker at 120ºC and the slides were removed after 2 minutes of full pressure. The slides were put in water while waiting for addition of Tris Buffered Saline (TBS) to the sections for 3 minutes to reduce background staining.

TBS was drained from slides and the primary antibody CD117, C-kit, was added followed by incubating sections for 30 minutes at room temperature. The slides were washed with TBS for 5 minutes and then 2 drops of Horseradish Peroxidase (HRP) were added to each section for 30 minutes. The slides were again washed with TBS for 5 minutes. TBS was drained from the sections; the chromogen diaminobenzidin (DAB) was added to the sections for 5 minutes. The sections were washed into water, counterstained in hematoxylin for 10 seconds, differentiated by 2 dips into 1% acid alcohol. The sections were blued in warm water for 2 minutes, dehydrated through gradually increasing ethanol baths (70%, 95% and 100%), then cleared in xylene for 10 minutes. The sections were finally mounted for light microscopy evaluation. Appendix III shows the detail steps of the procedure. The cellular staining pattern for anti-CD117 is a diffuse cytoplasmic and membranous expression. The positive controls were used from a diagnosed CD117 positive GIST from the Histopathology Unit's archive. CD117 positivity grade was judged by determining the proportion of tumor cells with a cytoplasmic and/or membranous brown yellowish stain in 5 HPF. It was categorized into 4 grades: 3+ if strong positivity of the cell membrane or cytoplasm is seen in more than 50% of tumor cells; and2+ if moderate positivity of the cell membrane or cytoplasm is seen in 25 to 50% of tumor cells. 1+ if weak positivity of the cell membrane and cytoplasm is seen in less than 25 % of tumor cells. Negative if no cytoplasmic or membranous positivity is seen in all tumor cells.

2.8.4 Microscopy

Microscopy of both H&E and IHC stained slides was done by the primary investigator and slides were reviewed by the senior supervising pathologist. Leica DM750 microscope was used and representative images were taken with ICC50 HD camera and LAS EZ software.

2.9 Data collection

The MUHAS institutional review board approval and authorization from MNH were obtained for this study (Appendices 5 and 6 respectively). All patients' reports, files, slides and blocks of RB cases that underwent primary enucleation during the period of 4 years from January 2013 to December 2016 were retrieved form Histopathology Unit and MNH Medical record department. An excel dataset was designed for the study to record data. The questionnaire used is attached as appendix 4. The demographic data extracted from the request forms and files included age at surgery (months), gender, family history of RB or consanguinity (yes, no, or unknown) and laterality (unilateral, bilateral, trilateral or not provided). Clinical data recorded included first presenting signor symptom (leukocoria, strabismus, proptosis, red eye and others); affected eye (right, left, both, undetermined), duration of symptoms (first sign to surgery), ICRB group, enucleated eye (right, left, both or not specified). Histopathologic data recorded included the tumor growth pattern (endophytic, exophytic, mixed, diffuse or not applicable), the tumor pathologic grade (well differentiated, moderately differentiated, poorly differentiated and undifferentiated). Invasion to the anterior segment (negative, iris, ciliary body and anterior chamber, not sampled) was evaluated. The presence and extent of choroid invasion (negative, focal and massive), presence and extent of optic nerve invasion (negative, prelaminar, laminar, post-laminar, transection margin and not sampled) and the presence and extent of scleral invasion (negative, inner two-third, full thickness and extrascleral invasion) were assessed. The pathology tumor staging also was done according to AJCC 8th edition. In addition, the completeness of the report was recorded. All raw data were captured in the excel sheet followed by data cleaning and analysis.

2.10 Quality Assurance

The principal investigator has been trained in eye processing, histotechnology methods and pathology examination of ocular RB and anatomical pathology in general. The retrieved eye blocks and slides were clearly labeled.

The standard operating procedures (SOP) were followed during performance of procedures like microtomy, H&E staining, IHC staining, usage of archived documents and image taking. The senior pathologist confirmed the findings documented by the primary investigator. Data were carefully entered into the respective rows and columns in the excel data collection sheet with patient's specific identifier number to avoid mix-ups.

2.11 Study variables

The independent variables were age, sex, duration of sign or symptoms, laterality, growth pattern, completeness of the report. The dependent variables were ICRB stage group, tumor grade, tumor stage, the need for adjuvant therapy.

2.12 Data analysis

Data were entered into the Excel data sheet and analyzed using STATA software version 13. Data were summarized in the form of proportions and frequency tables, bar charts and pie charts for categorical variables. Continuous variables were summarized using mean, median, mode, standard deviation and range. Univariate and multivariate logistic analyses were done to assess characteristics of the study population. Pearson Chi-square test was used to assess association between the need for adjuvant therapy and age, sex, laterality, duration of symptoms, ICRB group and tumor growth pattern. Any association was considered significant at p-value less than 0.05.The results were compared with the findings from the literature; reasons for differences or similarities were discussed. Recommendations on useful good practices were highlighted.

2.13 Ethical consideration

Permission to conduct the study was granted from Ethics and Research Committee of MUHAS, as well as the MNH. Registration numbers instead of names were used for confidentiality except for academic purposes. The patients' information was strictly kept confidential. This study was expected to have no adverse effects on subjects' health. However, the safety measures were kept to follow the routine in the Histopathology Unit at MNH. The retrieved blocks, slides, patients' files and reports have been returned to their corresponding archives after processing.

CHAPTER THREE

3 RESULTS

3.2 Patients demographic and clinical characteristics

A total of 185 patients with RB were identified and 71% (n=132) were selected for further characterization. Most of the non-selected cases were secondary enucleation specimens (post neoadjuvant chemotherapy) and few cases had extensive necrosis or calcification with scant viable tumor cells. Five out of 185 (2.7%) patients underwent bilateral eye enucleation; only one eye with large tumor size was selected. Of the 132 participants, 51.5% (n=68) were male. The overall median age was 33 months, ranging from 3 to 108 months (mode was 36 months and mean age at surgery was 32.83 months ± 16.54 SD). The median age in bilateral disease was 24 months with age range of 3 to 60 months and represented 17.4% (n=23). The median age in the unilateral disease was 35 months ranging from 4 to 108 months. Leukocoria and strabismus were the most common noted features found in 69.7% and 14.4% respectively. The mean interval between the discovery of symptoms and surgery was 11.06 months ± 9.403 SD. The median duration of symptoms was 8months; the minimum lag period was one month and a maximum of 55 months. Using the ICRB, 62.1 % (n=82) patients were classified in clinical stage group E and the remaining 37.9% (n=50) were in group D. No patient was classified in groups A, B or C. Demographics and clinical data of all participants are described in table 3 below.

Table 3: Demographic and clinical characteristics of the participants

Characteristics	Frequency (N=132) n(%)
Sex	
Male	68 (51.5)
Female	64 (48.5)
Age group (in months)	
Under 24	40 (30.3)
25->60	87 (65.9)
61 and above	5 (3.8)
Laterality	
Unilateral	109 (82.6)
Right	*61 (46.2)*
Left	*48 (36.4)*
Bilateral	23 (17.4)
Signs and symptoms at presentation	
Leukocoria	92 (69.7)
Strabismus	19 (14.4)
Proptosis	12 (9.1)
Red eye	5 (3.8)
Others	4 (3.0)
Interval between onset of symptoms and surgery (in months)	
0-6	44 (33.3)
7-12	59 (44.7)
13 and above	29 (22.0)
Clinical stage group by ICRB	
D	50 (37.9)
E	82 (62.1)

3.3 Patients histomorphologic features and C-kit immunoexpression

Mixed growth pattern was observed in 63.6% (n=84) followed by endophytic growth presented in 20.5% (n=27).The anterior segment was involved in 45.5% (n= 60) while in 8 patients the anterior segment was not sampled. Twenty-six per cent (n=35) of the studied patients were free from choroid invasion. Absence of scleral invasion was noted in 57.6% (n=76).

The optic nerve invasion was identified in 52.27% (n=69).C-kit was expressed only in positive controls used for the IHC procedure and all the patients were negative. Histomorphologic features are described in table 4 and figures 2and 3 below:

Table 4: Histomorphologic characteristics of all participants

Characteristic	***Frequency (N=132) N/132 (%)***
Tumor growth pattern	
Mixed	84 (63.6)
Endophytic	27 (20.5)
Exophytic	21 (15.9)
Anterior segment involvement	
Anterior chamber, iris and ciliary body invasion	44 (33.3)
Iris and ciliary body invasion	5 (3.8)
Anterior chamber invasion	5 (3.8)
Ciliary body invasion	4 (3.0)
Anterior chamber and ciliary body invasion	1 (0.8)
Iris invasion	1 (0.8)
No anterior segment invasion	64 (48.8)
Incomplete sampling	8 (6.1)
Choroid invasion	
Focal	18 (13.6)
Massive	79 (59.8)
No invasion	35 (26.5)
Scleral invasion	
Inner two-third	14 (10.6)
Extrascleral	42 (31.8)
No invasion	76 (57.6)
Optic nerve invasion	
Prelaminar invasion	13 (9.8)
Laminar invasion	6 (4.5)
Postlaminar invasion	10 (7.6)
Surgical/ transection margin invasion	40 (30.3)
No invasion	41 (31.1)
Not sampled	22 (16.7)
C-kit IHC expression	0 (0)

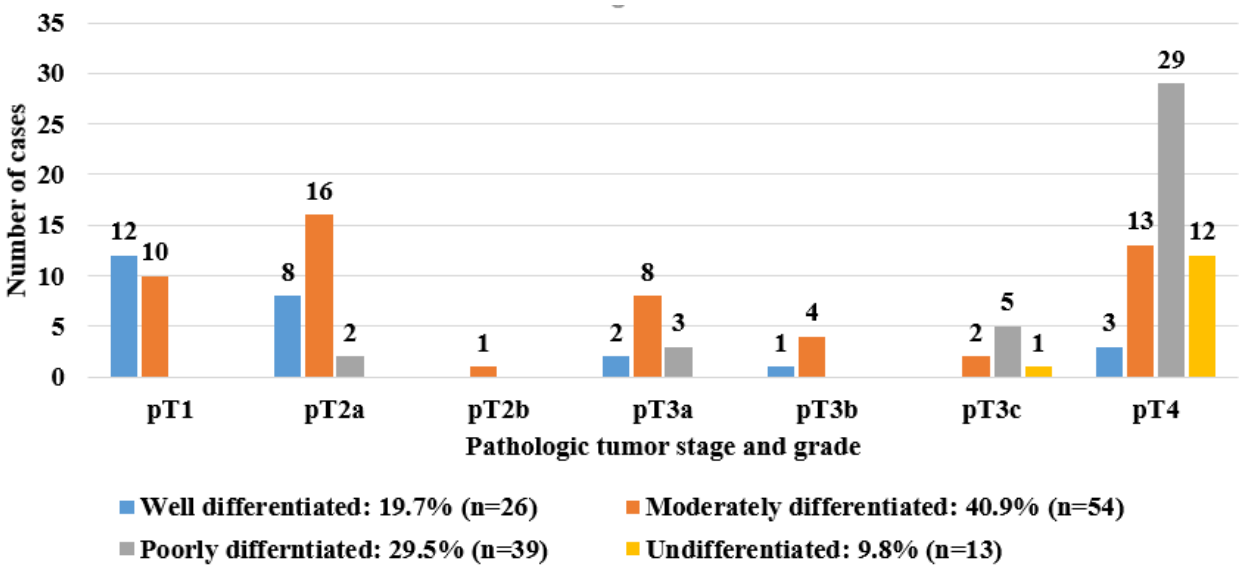
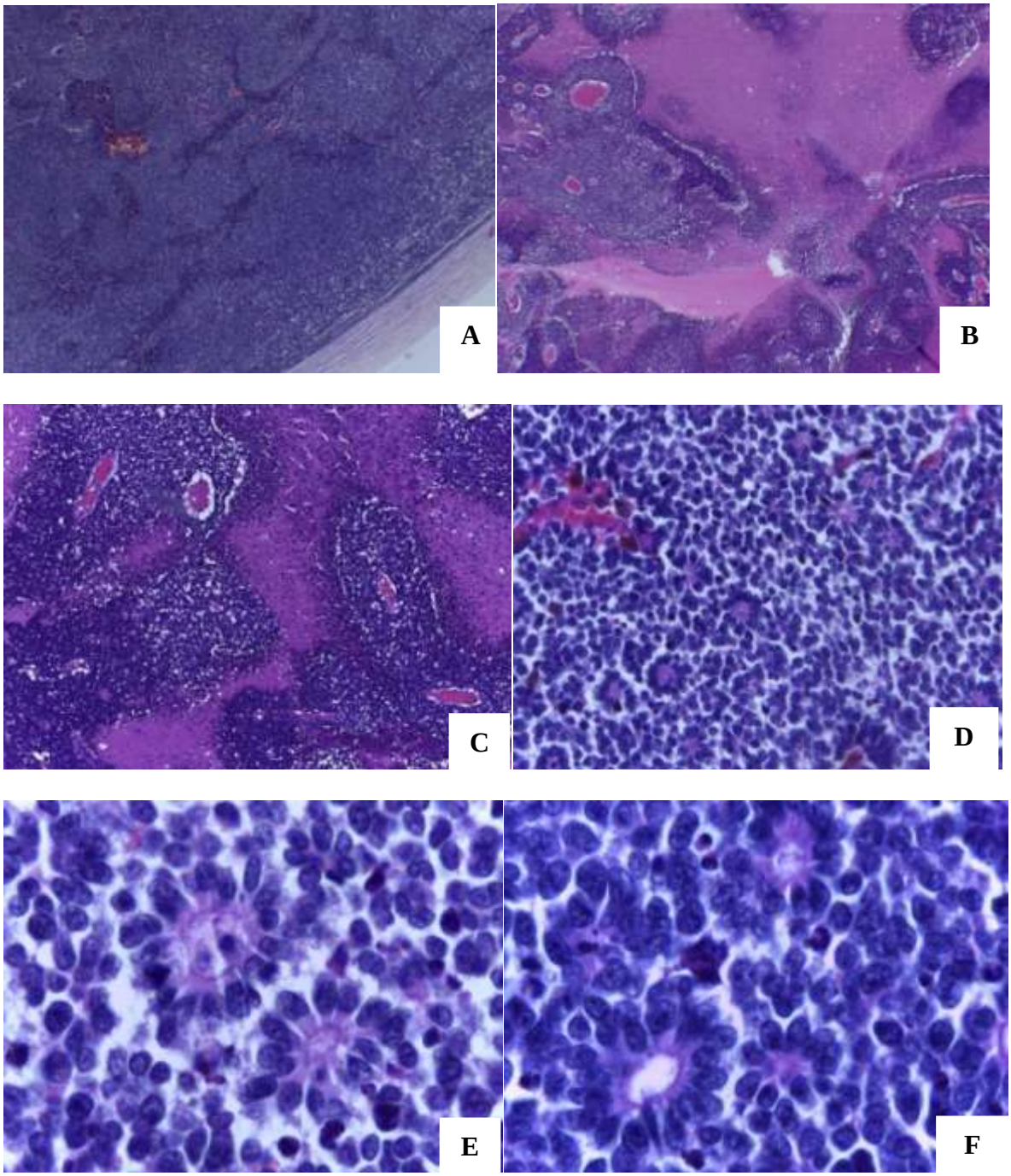


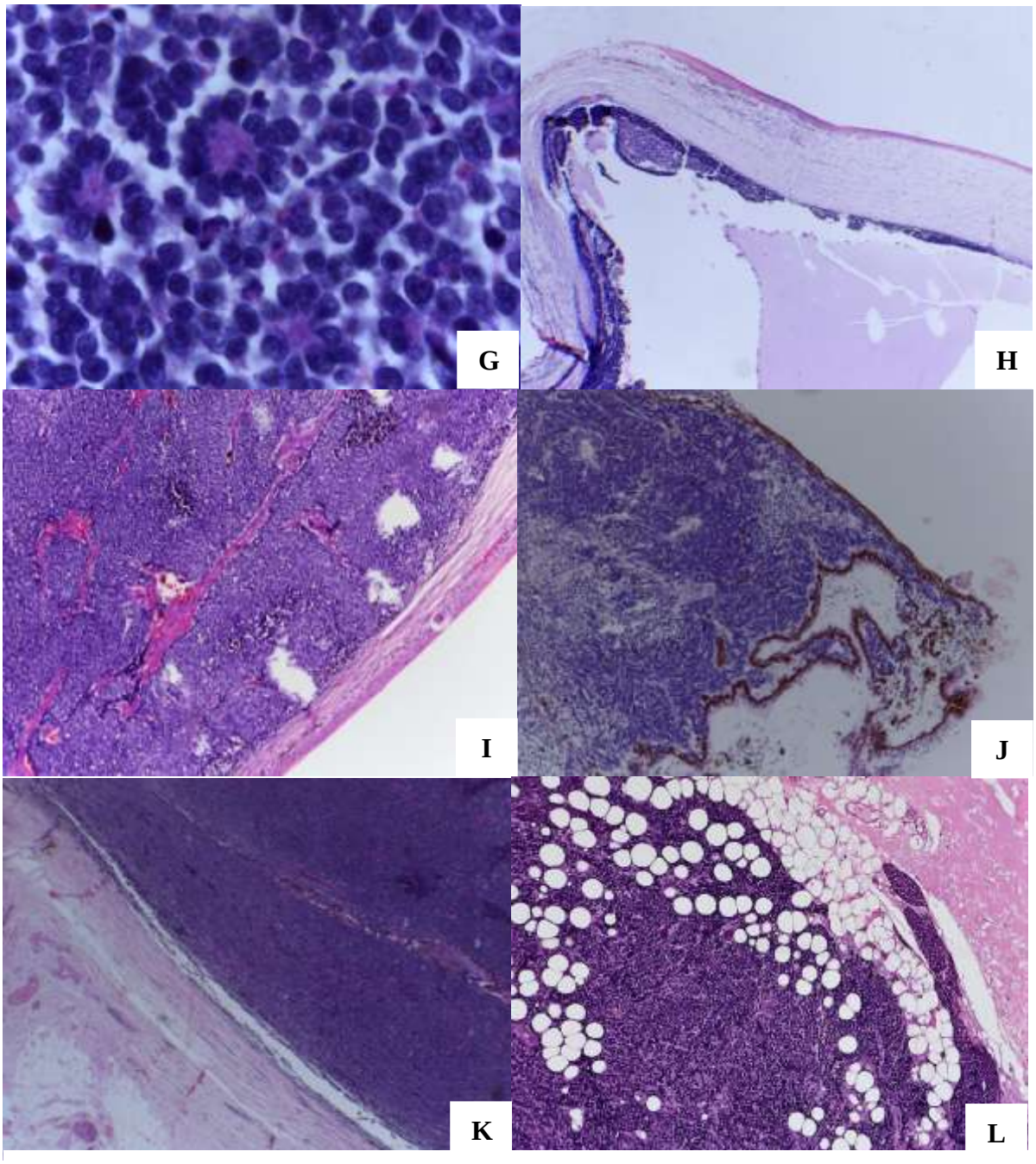
Figure 2: Distribution of pathology tumor stage and grade for all participants

In this study, 40.9% (n=54) of the studied participants showed moderately differentiated tumors and only 9.8% (n=13) had undifferentiated tumours of which 12 corresponded to pT4. No undifferentiated tumor was seen below stage pT3b. Well differentiated tumours were predominant in lower stages of the disease and this was statistically significant (p-value less than 0.001). The distribution of the participants by the pathologic stage and grade is described in figure 2 above.

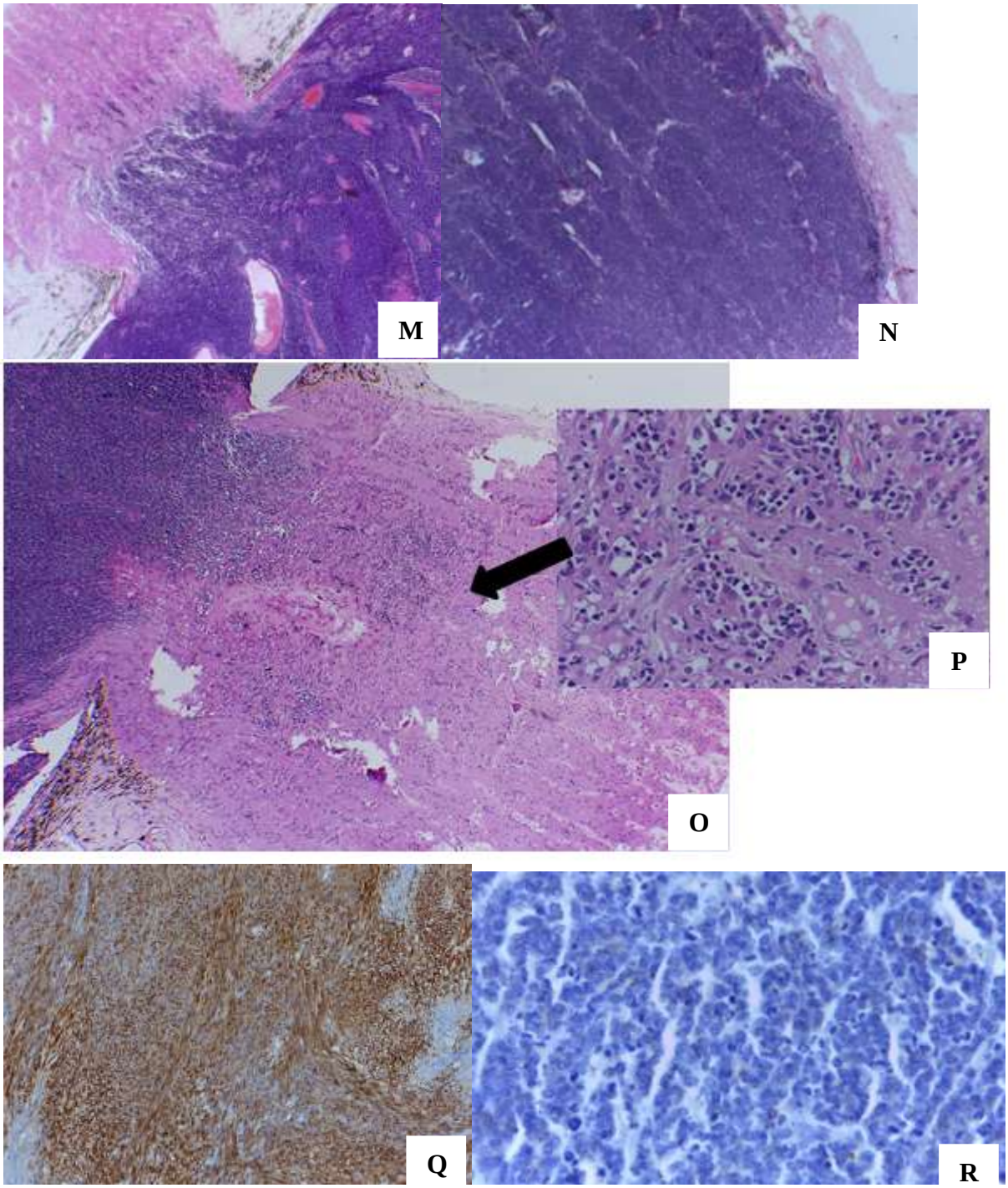
Figure 3: Photomicrographs of histomorphologic characteristics of RB



Photomicrographsof retinoblastoma stained with haematoxylin and eosin stain showing**;**
Fig. A: A small round blue cell tumor in sheets, trabeculae and nests. Original magnification: 40X scanning power field.
Fig. B and C: Extensive necrosis of tumor cells away from vessels and calcification. Viable cells remain around blood vessels. Original magnification: 40X scanning power field.
Fig. D: Small round blue cells with scant cytoplasm, hyperchromatic nuclei and scanty stroma and multiple rosettes. Original magnification: 100X low power field.
Fig. E and F: Well-formed Flexner-Wintersteiner rosettes, few Homer Wright rosettes and fleurettes. Original magnification: 400X high power field.



Photomicrographs of retinoblastoma stained with haematoxylin and eosin stain showing;
Fig. G: Well-formed Homer Wright rosettes. Original magnification: 400X high power field.
Fig. H and I: Anterior chamber invasion with malignant cells invading to the cornea. Original magnification: 40X scanning power field.
Fig. J: Invasion to the ciliary bodies.Original magnification: 40X scanning power field.
Fig. K: Massive choroid invasion. Original magnification: 40X scanning power field.
Fig. L: Extrascleral invasion with tumor cells invading the periorbital fat tissue. Original magnification: 40X scanning power field.



Photomicrographs of retinoblastoma stained with haematoxylin and eosin stain (M-O) and Immunohistochemistry (P and Q), showing

Fig. M: Lamina cribrosa invasion. Original magnification: 40X scanning power field.

Fig. N: Cross section surgical margin optic nerve invasion. Original magnification: 40X scanning power field.**Fig. O**: Post-laminar optic nerve invasion.Original magnification: 40X scanning power field and 100X low power field for the insert (P).

Fig. Q: CD 117 positive IHC in GIST control slide.Original magnification: 100X low power field.**Fig. R:** Negative to CD117 IHC, case of retinoblastomaOriginal magnification: 100X low power field.

3.4 Frequency and distribution of high-risk histopathologic features

3.4.1 Frequency of high-risk histopathologic features

We evaluated the frequency of massive choroid invasion, post-laminar optic nerve invasion, invasion of the optic nerve to the surgical margin, sclera invasion, and extrascleral extension as high-risk histopathologic features.

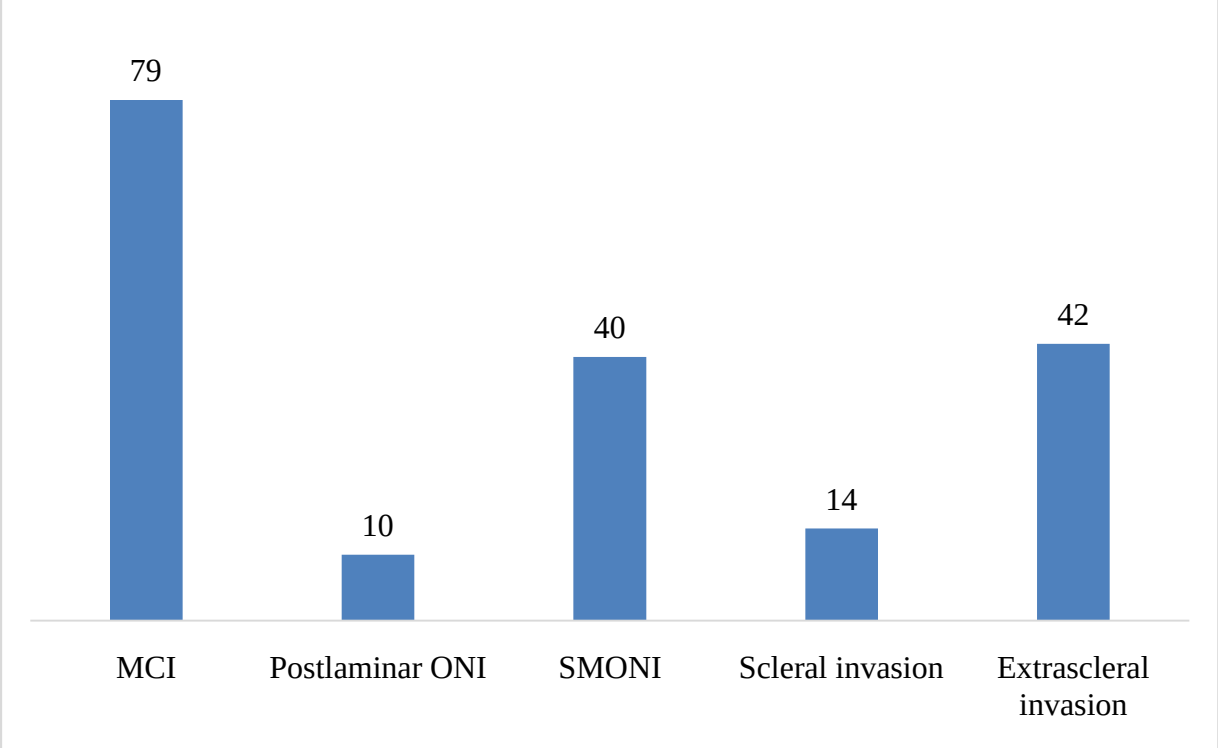


Figure 4: Frequency of high-risk histopathologic features

The cumulative frequency of high-risk histopathologic features was 185. Massive choroid invasion (MCI) was the commonest high-risk histopathologic features and was noted in 59.8% (n=79/185) of our patients. Extra-scleral invasion was the second high-risk histopathologic features followed by the surgical margin optic nerve invasion (SMONI) were noted in 31.8% (n=42/185) and 30.3% (n=40/185) of studied patients respectively. The post-laminar optic nerve invasion (ONI) was present in 10 patients (7.5%) and scleral invasion was seen in 10.6% (n=14/185). The high-risk histopathologic features frequencies are shown in figure 3.

3.4.2 Distribution of high-risk histopathologic features among 83 patients

Eight three (62.9%) patients showed high-risk histopathologic features and correspond to pathologic stage (pT) 3 to 4.

MCI was the most common single high-risk histopathologic feature (10.8%) while combination of SMONI with extrascleral invasion were the overall high-risk histopathologic feature encountered (30.1%). The details are given in table 5 below:

Table 5: Distribution of high-risk histopathologic features among patients and corresponding pT

High-risk histopathologic features	**Frequency n (%)**	**Corresponding pT**
MCI*	13(15.6)	pT3a
Post-laminal ONI and MCI	5 (6.0)	pT3b
Partial scleral invasion with MCI	1 (1.2)	pT3c
Partial scleral invasion with MCI and post-laminar	7 (8.4)	pT3c
SMONI**	4 (4.8)	pT4
SMONI with MCI	5 (6.0)	pT4
SMONI with partial scleral invasion	6 (7.2)	pT4
SMONI with extra-scleral invasion	25 (30.1)	pT4
Combined extra-scleral invasion and post-laminar	17 (20.4)	pT4
Total	***83(62.87)***	

Note: * Four patients also had prelaminar invasion

*****One patient also had focal choroid invasion***

3.5 Association between age, sex, clinical features, growth pattern and the need for adjuvant therapy

The need for adjuvant chemotherapy was seen in 83/132 (62.8%) of studied patients based on the presence of high-risk features. Among them 79.5% were more than 6 month-old. No significant gender difference was noted. The need for adjuvant therapy was higher in unilateral disease and the need increased as the patients were delayed for surgery. The need for adjuvant therapy also increased in patients with ICRB group E and in both mixed and exophyitic growth patterns. The details are given in table 6. Statistically significant (p-value less than 0.05) association was noted between the need for adjuvant therapy and symptoms duration period more than six months, clinical stage group E and both mixed and exophitic tumor growth patterns for both univariate and multivariate regression analyses as indicated in tables 6 and 7 below:

Table 6: Association between demographic and clinical features, growth pattern and need for adjuvant therapy

Characteristics	*No need for adjuvant therapy n(%)*	*Need for adjuvant therapy n(%)*
Age in months		
Under 24	23 (46.9)	17 (20.5)
25-60	24 (49.0)	63 (75.9)
61 and above	2 (4.1)	3 (3.6)
Sex		
Male	29 (59.2)	39 (47.0)
Female	20 (40.8)	44 (53.0)
Laterality		
Unilateral	37 (75.5)	72 (86.7)
Bilateral	12 (24.5)	11 (13.3)
Interval between onset of symptoms and surgery (in months)		
0-6	23 (46.9)	21 (25.3)
7-12	18 (36.7)	41 (49.4)
13 and above	8 (16.3)	21 (25.3)
Clinical stage group		
D	28 (57.1)	22 (26.5)
E	21 (42.9)	61 (73.5)
Growth pattern		
Endophytic	23 (46.9)	4 (4.8)
Exophytic	5 (10.2)	16 (19.3)
Mixed	21 (42.9)	63 (75.9)

Table 7: Association between demographic and clinical features, growth pattern and need for adjuvant therapy by univariate and multivariate analysis.

Explanatory factor	*Univariate*		*Multivariate*	
	OR (95% C.I)	p-value	OR (95% C.I)	p-value
Age (in months)				
Under 24	0.49 (0.74-3.28)	0.464	0.15 (0.01-1.96)	0.151
25-60	1.75(0.27-11.12)	0.553	0.66 (0.06-7.11)	0.738
61 and above	1.0 (ref)		1.0 (ref)	
Sex				
Male	1.0 (ref)		1.0 (ref)	
Female	1.63 (0.80-3.34)	0.177	1.96 (0.75-5.11)	0.165
Laterality				
Unilateral	2.12 (0.85-5.26)	0.105	2.01 (0.56-7.22)	0.281
Bilateral	1.0 (ref)		1.0 (ref)	
Interval between onset of symptoms and surgery (in months)				
0-6	1.0 (ref)		1 (ref)	
7-12	2.49 (1.10-5.61)	0.027	3.49 (1.10-1.05)	0.033
13 and above	2.87 (1.05-7.86)	0.040	2.56 (5.37-10.28)	0.164
Clinical stage group				
D	1.0 (ref)		1.0 (ref)	
E	3.69 (1.75-7.80)	0.001	3.76(1.41-10.00)	0.008
Growth pattern				
Endophytic	1.0 (ref)		1.0 (ref)	
Exophytic	18.4 (4.26-79.34)	0.000	24.31(4.34-136.18)	0.000
Mixed	17.25 (5.34-55.3)	0.000	24.34 (5.37-110.28)	0.000

3.6 Completeness of histopathology report

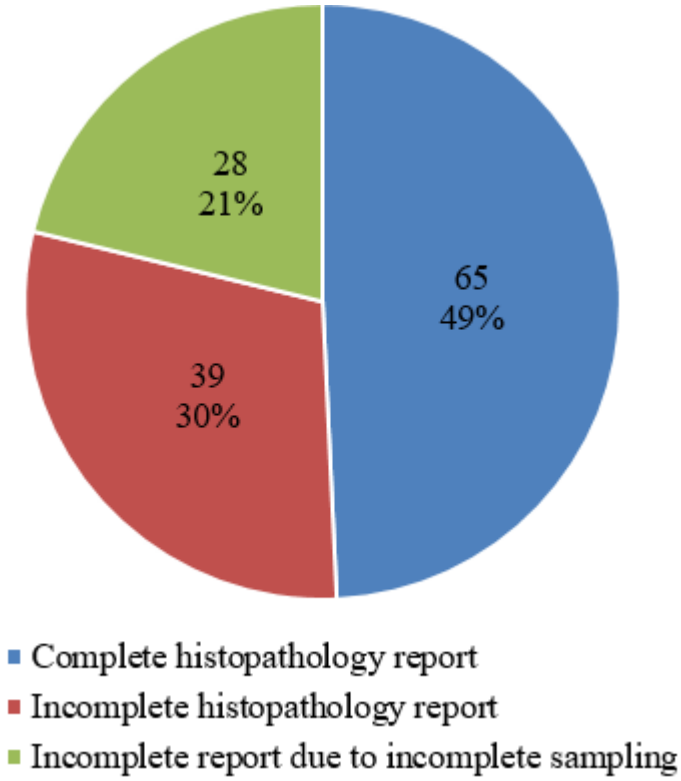


Figure 5: Completeness of histopathology report

Out of 132 total studied cases, 49.2% (n=65) had a complete histopathology report. Among 67 (50.8%) with incomplete reports; lacking comments about the presence or absence of high-risk features (tumor extension and staging). Among them, incomplete sampling was noted in 28 including the missing of the anterior segment alone (6 cases), optic nerve alone (20 cases) and 2 cases for both anterior segment and optic nerve. 39/132 cases (29.5%) had proper sampling but incomplete report on tumor extension. Among 67/132 patients with incomplete reports 73% (n=49/67) had high-risk histopathologic features and therefore, they needed adjuvant treatment.

CHAPTER FOUR

4 DISCUSSION

RB is the most common intraocular aggressive and life-threatening tumor with poor outcome in developing countries due to the delay in diagnosis. The worldwide annual incidence varies between 1 in 15,000 to 1 in 20,000 live births. Sub-Saharan Africa is one of the high incidence regions with expected 9,000 new cases per year(38,64–66). Massive choroid invasion, post-laminar optic nerve invasion, invasion of the optic nerve to the surgical margin, sclera invasion, and extrascleral extension are currently accepted as high-risk histopathologic features indication of local recurrence, metastasis, and adjuvant therapy. To the best of our knowledge, this is the first study to assess the magnitude of high-risk histopathologic features in patients with RB in Tanzania. The findings of this retrospective histopathology review show that majority of patients who underwent eye enucleation for RB had one or more high-risk histopathologic features and the need for adjuvant therapy was significantly increased for patients who were late for treatment, patients with advanced clinical disease, with less differentiated tumors and in patients with tumors exhibiting either exophytic or mixed growth patterns. Isolated massive choroid invasion was the most single high-risk histopathologic feature identified and in combined high-risk features the extrascleral invasion and positive surgical margin optic nerve invasion were the most prevalent. The present study also showed that 51% of the histopathology reports issued were missing complete information about high-risk histopathologic features and or tumor extension (stage).

The current study recruited 132 patients from January 2013 up to December 2016 at MNH, Histopathology Unit, there was no gender difference (M: F ratio was 1,06 :1). Majority of our patients (96. 2%) were below the age of 5 years at the time of surgery. These findings concur with the worldwide demographic characteristics of RB: generally, RB affects both boys and girls equally though some studies found a male predominance(11,27–32). In India, Singh et al. found 62% of 459 patients were male and a positive familial history was noted in 4.09%(69) as well as, Mali(70).Youssef et al. noted female predominance in their study performed in Egypt(1) and a study in Zambia found that girls were the most affected(54.4% of 57 cases)(71).

RB is less common after the age of five years however, median age at diagnosis varies considerably between developed and developing countries and disease laterality. In the present study, bilateral disease was found in 17.4% of studied patients, lower than in Kenya (32.5% of 160 cases)(72), Zambia (28.8% of 57 cases)(73) and in India (32.3% of 427 cases)(69).

There was no known family history of RB or consanguinity in all of studied patients as per records. These patients were one year much younger than patients with unilateral disease. The median age at surgery in bilateral disease was 24months with the age range of 3 to 60 months. The overall median age at surgery was 33months, (range of 3 to 108 months, mode 36 months).These values are similar to those from various countries such as South Africa (median age 35), Ghana(36.2 mean age), India, (mean age of 34.7 ± 24.6 months; median = 30) Malaysia, (younger median age at presentation of 22 months). In developed countries, the median age at diagnosis is less than 12 months for bilateral RB and 24 months for unilateral disease(1,32,33,43,66,69,74–76). We found that leukocoria was the most common feature (69.7%) followed by strabismus. Proptosis was noted in 9.1% (n=12). These findings are similar to several studies done all over the world (2,20,39,56,66,69,77).However, some studies in developing countries found that proptosis was the most common feature due to delays in referring patients to the specialized health facility(70,78); like in Zambia (47.4%of 57 cases)(71), and in Nigeria, fungating orbital mass was the most common feature in identification seen 46% of 42 cases (79). In our series, the median lag or interval period between the discovery of the first symptoms and surgery was 8 months; (mean 11.06 months, mode 7), similar to what was found by Yao et al. in Congo and Ivory Cost(66), and close to Zambia (mean 9.27 months)(71), Kenya (mean 10.5 months) (72), India (7.2 and 8.3) (69,80) and Brazil (8.29 months for unilateral and 5.6 months for bilateral disease)(81). Contrary to developing countries, studies found short symptoms duration in developed countries. Butros et al. recorded 1.5 (range: 0–46) and 2.25 months (range: 0–24 months) from presenting signs to diagnosis for patients with unilateral and bilateral disease respectively(20). However, Kaliki et al. in their study comparing Indians and Americans from two major referral centers, found no significant difference in both groups in terms of duration of symptoms (4 versus 3 months); but Indians were much older and had larger tumor size with more increased risk of having

high-risk histopathologic features. Subramaniam et al. also noted that 82.1% of 119 cases presented within 6 months from the onset of symptoms presenting in Malaysia (82).Using the ICRB, 62.1 % (n=82) patients were classified in clinical stage group E and the remaining 37.9% (n=50) were in group D. No patient was in group A, B or C. Enucleation is the treatment of choice in large advanced tumor with suspicion of optic nerve invasion or extrascleral invasion whereas small tumor are conservatively managed(56,83,84). In Tanzania, patients with extraocular RB receive neoadjuvant therapy and exenteration or palliative therapy according to the RB management protocol in Tanzania.

However, unpublished data from the ophthalmology department at MHN, done by Ngalula J. et al., indicate that RB is diagnosed late due to lack of awareness of the disease by the general population and poor knowledge of the disease by health care providers at primary health care facilities. Of ninety (90) RB patients followed for one-year period 84/90 (93.3%) had an advanced disease from group D to the unclassified. Late presentation also was reported in other developing countries(32,44,66,71,82).

The majority of tumors in our series (63.6%) exhibited both endophytic and exophytic (mixed) growth. The endophytic growth toward the vitreous cavity was noted in 20.5%. The exophytic growth toward the choroid was the least encountered. There was no reported case with diffuse growth pattern. Usually the mixed growth pattern is the commonest encountered pattern though the reported proportions of different patterns varies from study to study. The predominance of mixed growth pattern was also noted in USA by Mendoza et al. (44.7% of 266 cases), in Canada by Barry et al. (85.71% of 63 cases) in Egypt by Youssef et al (55.4% of 56 cases) as well as in other studies (1,2,48,59), however, Soliman et al. and Manjandavida et al. found a higher proportion of endophytic growth pattern over the mixed one(70,85). Exophytic growth pattern predominance was noted in more than a half (55.3% of 403 cases) in India(86). Paternler et al. noted association of mixed pattern with more advanced disease and presence of high risk histopathologic features (47). In our study, majority of the patients presented with advanced disease and extraocular extension was reported in 43.2%. Few patients 16.7% had small tumors confined to the eyeball. Choroid invasion was the most common (73.5%) intraorbital invasion, followed by optic nerve invasion (69%).

There was a statistically significant association between tumor differentiation and tumor size. Less differentiated tumors were larger and invasive. This association also was noted by Kashyap et al. in India(65) as well as by other studies performed in Taiwan(68).

In the present study, we performed IHC staining for CD117/ C-kit, and all of the cases showed no expression. This finding contrasted to those from Canada where Barry et al. found that RB cells expressed C-kit while normal retinal cells do not(1). Similar findings were noted in Egypt by Yussef et al. where the expression of CD117 was at 48.2% in a cohort of 56 cases of RB (2). Bosch et al. have found lower expression in 19.2% in 73 cases by using tissue microarray (26). In all those records, the CD117 expression was associated with the presence of high-risk histopathologic features and advanced disease hence it was suggested to be a marker for aggressive tumor and further targeted personalized chemotherapy(1,2).

In the present study, the lack of expression of CD 117 by IHC may be considered either as true negative, meaning that our RB patients did not present the amplification of CD 117 or it may be false negative that may results from tissue alterations in the pre-analytical phase which could not be controlled due to the retrospective nature of the study.

There is variation in defining high-risk histopathologic features in different literature. In the present study we defined high-risk histopathologic features based on the dataset for histopathological reporting of ocular RB, by the Royal College of Pathologists January 2018 and the IRSWG 2009(41,57,87). We considered and evaluated the frequency of massive choroid invasion, post-laminar optic nerve invasion, invasion of the optic nerve to the surgical margin, sclera invasion, and extrascleral extension as high-risk histopathologic features. Anterior segment invasion was excluded based on different studies that found that the anterior segment invasion alone is not a sufficient indication for adjuvant chemotherapy though in some institutions adjuvant treatment may be given(88,89).We found one or more high-risk histopathologic features in 62.9% of studied patients corresponding to pathology tumor size from stage 3 to stage 4. The isolated massive choroid invasion was the most common single high–risk feature while combination of surgical margin optic nerve invasion and extrascleral extension was prevalent in combined risk features.

The findings in the present study are supported by the previous data in the literature, where a higher incidence of high-risk histopathologic features was noted in developing countries from 29 to 64.4% (Latin America, Asia, and Africa) while the overall incidence of high-risk histopathologic features approximates 20.4% in developed countries like USA and France(11,37,39,90). However, the incidence is slightly higher than what was noted by Bowman et al. who found choroid invasion in 62% of 31 cases in Tanzania however, in their study it was not specified that it was either focal or massive choroid invasion (11). In Ghana, Essuman et al. found 75% of 12 cases(33), Gupta et al. in India (54.2%) of 142 eyes(91); Yue et al in China, 53% of 104 cases. Kaliki et al. found a 2-fold greater risk to have high-risk intraocular RB for Indians compared with Americans (35% of 331 cases against 23% of 193 cases (76).

In these recorded studies, massive choroid and post-laminar optic nerve invasions were the most prevalent single high-risk feature at different proportions(92). Variable proportions of different single high-risk feature also were noted in these studies. Clinical predictive factors for high-risk histopathologic features also have been identified and reported in different studies.

They include older age at diagnosis, longer lag period before diagnosis and treatment, hyphema, pseudohypopyon, staphyloma, and orbital cellulitis(38).

In the present study, the need for adjuvant therapy was noted in 62.9% of 132 patients and it was associated with longer symptoms duration of more than six months, clinical stage group E and both mixed and exophitic tumor growth patterns. These associations were statistically significant ($p \leq 0.05$) in both univariate and multivariate regression analysis. Matthew et al. also found significant correlation of high-risk histopathologic features and clinical stage group E(74) however, Yousef et al. found that clinical staging criteria alone cannot predict the need for adjuvant therapy because up to 50% of Group E patients in their cohort didn't have high-risk histopathologic features(93). Kaliki et al. found association of the presence of high-risk features with prolonged duration of symptoms more than 6months(76) but Yue et al. found no statistical significant correlation of gender, laterality, family history, onset age, symptoms duration, as well as stage group D or E with the high-risk histopathologic features (94).

Timely complete pathology report is a key element in controlling advanced RB after enucleation. RB standardized reporting protocols and standard operating procedures on handling enucleation specimens were developed to assist pathologists for consistency and easy recording of all necessary information for further management (41,57,95). In the present study, half of the 132 histopathology reports issued were incomplete. This may largely be attributed to the traditional narrative reporting system and non-regular histopathology audits. Insufficient clinical information and incomplete sampling also were noted. According to previous studies and experiences from different institutions, the synoptic reporting provides an easy and rapid way to issue complete and consistent pathology reports(49,60,96). Unpublished findings from a study done in Kenya by Midingo et al., showed increased identification and reporting on high-risk histopathologic features in RB once there porting checklist or audit tool is used. Similar findings also were noted by Sengupta et al., in India in a study done comparing RB pathology reports before and after introduction of the IRSW protocol (87,97).

4.1 Study limitations and mitigations

To our knowledge this is the first RB histopathology review in Tanzania, it provided general baseline information about high-risk histopathologic features in the primary enucleated eye specimens for RB. However, our study has inherent limitations that can be acknowledged. This is a retrospective study that used archived patients' materials.

At MNH there is no computerized cancer registry, the retrieval of RB cases was laborious. However, the use of histopathology specimens' registration books facilitated to retrieve all pediatric enucleated eye specimens prior consultation of other patients' records. The present study used archived tissue blocks therefore the pre-analytical factors that affect the quality of routine and IHC stains could not be controlled. In addition, the recommended enucleated eye tissue handling guidelines for RB have not been followed: for example, in regard to the required number of blocks (4 blocks) but one antero-posterior section of the globe including the optic nerve stamp and the tumor was available in the majority of our cases. Due to time constraint, this study only determined the frequency of high–risk histopathologic features in RB at MNH, long period follow up of our cohort would have highlighted the treatment provided and outcome after the enucleation.

CHAPTER FIVE

5 CONCLUSION AND RECOMMENDATION

5.2 Conclusion

Our study evaluated the magnitude of high-risk histopathologic features in enucleated eye specimens for RB in Tanzanian patients. The prevalence of RB with high-risk histopatologic features in Tanzania was found to be high. Our study shows that long duration of symptoms more than six months, clinically advanced disease (group E), exophytic and mixed growth pattern are more likely associated with the presence of high-risk histopathologic features. Though some clinico-demographic factors may predict the presence of high-risk histopathologic features, the histopathology remains the gold standard for diagnosis and determine who need adjuvant therapy after enucleation of intraocular RB. The pathologist is responsible to provide complete reports for proper selection of RB patients eligible for adjuvant chemotherapy and radiotherapy. This study calls for further studies to prospectively evaluate the outcomes of patients with RB in Tanzania. Synoptic reporting system and regular audits are suggested to improve pathology reporting however, prior adoption and validation studies are required.

5.3 Recommendations

To the Ministry of Health:

- To establish a functional cancer registry that can help in determining the magnitude and the prognosis of ocular RB, secondary RB cancers and other malignancies
- To prioritize educative and screening programs to raise awareness on RB and other pediatric cancers to the health care personnel and the public at large.

To the clinicians at MNH and other Hospitals:

- To be aware of the magnitude of high-risk histopathologic features in RB in Tanzania.
- To provide necessary and sufficient clinical information accompanying the tissue biopsy specimen to facilitate complete histopathology reporting.
- To collaborate with the pathologists for better management plan of RB patients.

To Histopathology Unit and pathologists at MNH:

- To be aware of the magnitude of high-risk histopathologic features in RB in Tanzania.
- To produce complete RB complete reports for better management of patients with RB in Tanzania.

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APPENDICES

Appendix I: Reporting check list for ocular retinoblastoma

A. PATIENT IDENTIFICATION

1. Patient identifier
    a. Names: .....
    b. Date of birth: .....
    c. Sex: M / F
    d. Hospital: .....
    e. Hospital no: .....
    f. Histopathology no: …….
2. Date specimen taken/ Date of procedure: .....
3. Responsible surgeon: .....
4. Date of receipt:

B. CLINICAL INFORMATION

1. Relevant History
    a. Clinical findings
    b. Status of the other eye
    c. Previous treatment
    d. Family history
2. Relevant investigations findings
3. Clinical diagnosis (including clinical stage)
4. Anatomic site (s)
5. Procedure: Enucleation, Partial exenteration, Complete exenteration, Other
6. Operative findings

C. MACROSCOPIC EXAMINATION

1. Specimen Type

___ Enucleation

___ Limited exenteration

___ Complete exenteration

___ Other (specify): _____

___ Not specified

2. Laterality

___ Right

___ Left

___ Not specified

3. Specimen fixation

___ Fixed (specify the fixative)

___ Unfixed though in fixative

___ Received flesh

4. Specimen Size

<u>For Enucleation</u>

___ Orientated globe (based on identification of extraocular or medial muscle insertions

and other landmarks)

___ Non oriented

Anteroposterior diameter: ___ mm

Horizontal diameter: ___ mm

Vertical diameter: ___ mm

Length of optic nerve: ___ mm

Diameter of optic nerve: ___ mm

___ Cannot be determined (see Comment)

<u>For exenteration</u>

___ Orientated globe (based on identification of extraocular or medial muscle insertions and other landmarks)

___ Non oriented

Greatest dimension: ___ cm

*Additional dimensions: ___ x ___ cm

___ Cannot be determined (see Comment)

5. **Tumor Site and Extent (macroscopic examination/transillumination) (check all that apply)**

___ Cannot be determined

___ Superotemporal quadrant of globe

___ Superonasal quadrant of globe

___ Inferotemporal quadrant of globe

___ Inferonasal quadrant of globe

___ Anterior chamber

___ Extrascleral extension

___ Optic nerve

6. **Tumor Basal Dimensions on Transillumination**

___ Cannot be determined

Size: ___ x ___ mm

7. **Number of tumor foci**

___ Cannot be determined

___ Univocal

___ Multifocal

8. **Tumor color, consistency and shape (free text)**

9. **Tumor Dimensions After Sectioning**

___ cannot be determined

Base at cut edge: ___ mm

Height at cut edge: ___ mm

Maximal tumor height: ___ mm

10. Tumor Location After Sectioning:

___ Cannot be determined

Distance from anterior edge of tumor to limbs at cut edge: ___ mm

Distance of posterior margin of tumor base from edge of optic disc: ___ mm

11. Tumor Involvement or Gross Pathology of Other Ocular Structures (check all that apply)

___ Cannot be determined

___ Optic disc

___ Choroid minimal (Bruch's membrane destroyed by 3 or less microscopic cell clusters

Without deeper penetration)

___ Choroid, massive (anything beyond minimal)

___ Vitreous

___ Retinal detachment

___ Ciliary body

___ Iris

___ Lens

___ Anterior chamber

___ Angle

___ Sclera

___ Cornea

12. Sections submitted for microscopic evaluation (specify by identifying letter on the block)

___ Pupil-optic (p-o) nerve section

___ Surgical margin cross section of the optic nerve (inked)

___ Margins (exenteration, specify)

___ Other sections (specify)

D. MICROSCOPIC EVALUATION

Retinoblastoma present: ___ Yes ___ No

Retinocytoma present: ___ Yes ___ No

1. **Histologic Features (check all that apply)**
   - ___ Cannot be determined
   - ___ Undifferentiated
   - ___ Differentiated
     - *___ Homer Wright rosettes
     - *___ Flexner-Wintersteiner rosettes
     - *___ Fleurettes
   - ___ Necrotic
2. **Growth Pattern**
   - ___ Cannot be determined
   - ___ Endophytic
   - ___ Exophytic
   - ___ Combined exophytic/endophytic
   - ___ Diffuse
3. **Extent of Optic Nerve Invasion**
   - ___ Cannot be determined
   - ___ Not identified
   - ___ Anterior to lamina cribrosa
   - ___ At the lamina cribrosa
   - ___ Posterior to lamina but not to end of nerve
   - ___ To cut end of optic nerve
4. **Choroid invasion**
   - ___ Cannot be determined
   - ___ Not identified
   - ___ Focal
   - ___ Massive

5. Scleral invasion

___ Not identified

___ Inner two-third

___ Full thickness into the outer third or invasion around the emissary channels

___ Extrascleral invasion

6. Involvement of Other Structures (check all that apply)

___ Cannot be determined

___ Vitreous

___ Ciliary bodies

___ anterior chamber

___ Iris

___ Other(s) (specify): _____

7. Pathologic Staging (pTNM)

Primary Tumor (pT)

___ pTX: Primary tumor cannot be assessed

___ pT0: No evidence of primary tumor

___ pT1: Tumor confined to the retina, the vitreous, or subretinal space. No optic nerve or choroidal invasion

pT2: Minimal invasion of the optic nerve and/or optic coats

___ pT2a: Tumor invades optic nerve up to, but not through, the level of the lamina cribrosa

___ pT2b: Tumor invades choroid focally

___ pT2c: Tumor invades optic nerve up to, but not through, the level of the lamina cribrosa and invades the choroid focally

pT3: Significant invasion of the optic nerve and/or optic coats

___ pT3a: Tumor invades optic nerve through the level of the level cribrosa but not to the line of resection.

___ pT3b: Tumor massively invades the choroid

___ pT3c: Tumor invades the optic nerve through the level of the lamina cribrosa but not to the line of resection and massively invades the choroid

___ pT4: Extraocular tumor extension that includes any of the following: invasion of optic nerve to the line of resection; invasion of orbit through the sclera; extension both anteriorly or posteriorly into the orbit; extension into the brain; extension to, but not through, the chiasm; extension into the brain beyond the chiasm

Regional Lymph Nodes (pN)

___ pNX: Regional lymph nodes cannot be assessed

___ pN0: No regional lymph node metastasis

___ pN1: Regional lymph node metastasis

Distant Metastasis (pM)

___ pMX: Cannot be assessed

pM1: Distant metastasis

___ pM1a: Bone marrow

___ pM1b: Other sites

*Specify site(s), if known: _____

8. Margins

___ Cannot be assessed

___ No tumor at margins

___ Tumor present at surgical margin of optic nerve

___ Extrascleral extension (for enucleation specimens)

___ Other margin involved

Specify: _____

9. Resection margins (for exenterations):

___ Cannot be assessed

___ Not involved

___ Involved (specify)

___ Other structures involved (specify)

10. *Additional Pathologic Findings (check all that apply)

*___ None identified

*___ Calcifications

*___ Mitotic rate: Number of mitoses per 40x objective

with a field area of 0.152 mm^2:___

*___ Necrosis

*___ Apoptosis

*___ Basophilic vascular deposits

*___ Inflammatory cells

*___ Hemorrhage

*___ Neovascularization (specify site): _____

*___ Response to prior therapy (specify): _____

*___ Other (specify): _____

11. Histology conclusion and comments

- Conclusion: Site, procedure, Diagnosis, grade, Pathological staging pT pN pM (TNM 8th edition)
- Recommendation/comments
- Reported by: Names of the pathologist
- Signature…… Date…….

Appendix II: SOP for H&E histochemistry

1. Retrieve blocks and make tissue sections using microtome [3-5 microns]
2. Pick the section on a slide from water floatation and place a hot plate 60^0C for 50 minutes
3. Deparaffinise sections, hydrate through graded alcohol to water
4. Remove fixation pigments if necessary
5. Stain in alum haematoxylin for a suitable time.
6. Wash well in running tap water until section ‘blue’ for 5 minutes
7. Differentiate in 1% acid alcohol (1% HCL in 70% alcohol) for 5-10 seconds.
8. Wash well in tap water until sections are again ‘blue’ (10-15 minutes)
9. Stain in 1% eosin Y for 10 minutes.
10. Wash in running water for 1-5 minutes
11. Dehydrate through alcohols (70, 95, 100%), clear and mount

Appendix III: SOP for IHC

Make tissue block sections 4 μm thick, put on immunohistochemistry (IHC) slides

1. De-wax by placing the slides on hot plate at 60°C for 50 minutes
2. Immerse the slides in xylene in 2 batches for 5 minutes each.
3. Rehydrate through alcohols (100, 95, and 70%).
4. Rinse the slides with deionized water.
5. Surround the tissue with a hydrophobic barrier using a barrier pen.
6. To quench endogenous peroxidase activity, incubate the sample with 2 drops of peroxidase blocking reagent (3% H_2O_2 in water or methanol) for 15 minutes.
7. Bring the slides into water for 5 minutes.
8. Prepare antigen retrieval solution to unmask the antigens
9. Heat antigen retrieval solution in pressure cooker (not closed) until it starts boiling
10. Place slides in antigen retrieval solution in pressure cooker and close the lid
11. Time for 2 minutes at full pressure
12. Remove the slides from pressure cooker and place in water (never allow slides to dry)
13. Add Tris buffer solution (TBS) wash buffer for 3 minutes
14. Drain slides of TBS, and wipe away any excess wash buffer, add the primary antibody
15. Incubate the section with the antibody for 30 minutes
16. Wash 3 times with wash buffer for 5 minutes each, and drain the slides.
17. Incubate the sample with 1-3 drops of HRP (Horseradish peroxidase) for 30 minutes.
18. Rinse with wash buffer 3 times for 15 minutes each and drain the slides.
19. Incubate the sample with 1-3 drops of biotinylated secondary antibodies (HRP) for 30-60 minutes (time depends on thickness of the section).
20. Rinse and wash 3 times in wash buffer (TBS) for 2 minutes each.
21. Calculate the required working volume of DAB Chromogen Solution given that 100-200 μL is required to cover the entire tissue section on a single slide. Make up DAB: 1 drop of DAB for each 1 mL of substrate buffer.

22. Add 1-5 drops of DAB Chromogen Solution to cover the entire tissue section and incubate for 3-20 minutes. Colored precipitate will localize to the sites of antigen expression as the chromogenic substrate is converted by HRP enzyme into insoluble end product.

Note: DAB and AEC are hazardous materials. Gloves and safety glasses should be worn and all steps performed inside a fume hood.

23. Rinse the sample with wash buffer (TBS) 3 times for 10 minutes each.
24. Rinse in deionized H_2O and drain the slides.
25. Counter stain in hematoxylin for 10 seconds.
26. Quick dip in 1% acid alcohol, 2 dips
27. Blue in warm water for 2 minutes
28. Dehydration through ascending concentrations of alcohol (70, 95, 100%)
29. Clear in xylene in 2 batches for 3 minutes each
30. Cover slip
31. Visualize staining of tissue under a microscope using a bright-field illumination.

Appendix IV: Data collection checklist

Patient's Particulars and demographics

1. Data Collection Sheet Number:

2. Laboratory number:

3. Hospital Registration Number:

4. Age at surgery (Months):

5. Sex: 1. Male, 2. Female, 3. Not provided

6. Family history of retinoblastoma or consanguinity: 1. Yes, 2. No, 3. Unknown

7. Laterality: 1. Unilateral, 2. Bilateral, 3. Trilateral, 4. Not provided

Clinical findings

8. S&S at presentation: 1. Leukocoria 2. Strabismus 3. Proptosis 4. Red eye 5. Others

9. Affected eye: 1. Right 2. Left 3. Both 4. Undetermined

10. Interval between first sign and surgery (in months)

11. Clinical stage by ICRB: 1. Group A, 2. Group B, 3. Group C, 4. Group D, 5. Group E, 6. Not provided

12. Eye enucleated: 1. Right eye, 2. Left eye, 3. Both, 4. Not specified

Histopathology findings

13. Growth pattern: 1. Endophytic, 2. Exophytic, 3. Mixed, 4. Diffuse 5. Cannot be specified

14. High risk histopathological features (HRHF): 1. Yes, 2. No

15. Completeness of the report: 1. Yes 2. No

16. Anterior chamber invasion: 1. Yes, 2. No, 3. Not sampled

17. Iris invasion: 1. Yes, 2. No, 3. not sampled

18. Ciliary body invasion: 1. Yes, 2. No, 3. not sampled

19. Anterior segment invasion: 1 yes, 2. No

20. Choroid invasion: 1. None, 2. focal, 3. Massive

21. Scleral invasion: 1. No invasion 2. Inner two-third 3. Full thickness into the outer invasion around emissary channels 4. Extrascleral invasion

22. Optic nerve invasion: 1. None, 2. pre-laminar, 3. Laminar, 4. Post-laminar, 5. Surgical margin invasion, 6. No optic nerve submitted

23. Extrascleral/Extra ocular disease: 1. Yes, 2. No

24. Grade: 1. Retinocytoma, 2. Well diff., 3. Moderately, 4. Poorly, 5. Undifferentiated

25. pTumor: 1. T1, 2. T2a, 3. T2b, 4. T3a, 5. T3b, 6. T4a, 7. T4b

26. Need for adjuvant therapy: 1. pTless than 3, 2. pT3 or more

27. C-kit/ CD117 immunoexpression: 1. Negative, 2. Weak and moderate positive, 3. Strong

Appendix V: A copy of the approval ethical clearance

MUHIMBILI UNIVERSITY OF HEALTH AND ALLIED SCIENCES
OFFICE OF THE DIRECTOR OF POSTGRADUATE STUDIES

P.O. Box 65001
DAR ES SALAAM
TANZANIA
Web: www.muhas.ac.tz

Tel G/Line: +255-22-2150302/6 Ext. 1015
Direct Line: +255-22-2151378
Telefax: +255-22-2150465
E-mail: dpgs@muhas.ac.tz

Ref. No. MU/ PGS/SAEC/Vol. IX/

23rd June, 2017

Dr. Marie Claire Ndayisaba
MMed. Anatomical Pathology
MUHAS.

RE: APPROVAL OF ETHICAL CLEARANCE FOR A STUDY TITLED: "HISTOMORPHOLOGIC HIGH-RISK FEATURES AND C-KIT IMMUNOEXPRESSION IN RETINOBLASTOMA TANZANIAN PATIENTS."

Reference is made to the above heading.

I am pleased to inform you that, the Chairman has, on behalf of the Senate, approved ethical clearance for the above-mentioned study. Hence you may proceed with the planned study.

The ethical clearance is valid for one year only, from 22nd June, 2017 to 21st June, 2018. In case you do not complete data analysis and dissertation report writing by 21st June May 2018, you will have to apply for renewal of ethical clearance prior to the expiry date.

Prof. Andrea B. Pembe
DIRECTOR OF POSTGRADUATE STUDIES

cc: Director of Research and Publications
cc: Dean, School of Medicine

Appendix VI: A copy of permission to collect data at MNH

MUHIMBILI NATIONAL HOSPITAL

Cables: "MUHIMBILI"
Telephones: +255-22-2151367-9
FAX: +255-22-2150534
Web: www.mnh.or.tz

Postal Address:
P.O. Box 65000
DAR ES SALAAM
Tanzania

In reply please quote:

MNH/TRC/Permission/2017/110

19th July, 2017

Dr. Marie Claire Ndayisaba,
MUHAS

RE: PERMISSION TO COLLECT DATA AT MNH: 2017

Name of Student	Dr. Marie Claire Ndayisaba
Title	"HISTOMORPHOLOGIC HIGH-RISK FEATURES AND C-KIT IMMUNOEXPRESSION IN RETINOBLASTOMA TANZANIAN PATIENTS".
Institution	Muhimbili Univeristy of Health and allied Sciences
Supervisor	Dr. Edda Vuhahula, Prof. James Kitinya
Period	17/07/2017 to 16/01/2018

You have been permitted to collect data in respect to the undertaking of the above mentioned study.

Please ensure that you abide to the ethical principle and other conditions of yours approval.

Sincerely,

HEAD, TEACHING RESEARCH & CONSULTANCY UNIT
MUHIMBILI NATIONAL HOSPITAL
P. O. BOX 65000
DAR ES SALAAM

Dr. Faraja S. Chiwanga
Head of Teaching, Research and Consultancy Coordination Unit