Update on pathology of retinoblastoma

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Abstract

Retinoblastoma is caused by mutational inactivation of both alleles of the RB1 gene, which maps to chromosome 13q14 and encodes retinoblastoma protein that acts as a tumor suppressor. Histopathological high-risk features of retinoblastoma are predictive of metastasis or local recurrence. The focus of this update is to emphasize the recent advances in pathology, various molecular key pathways and genome wide approaches for newer potential therapeutic future targets associated with retinoblastoma tumor biology. This review article highlights the new biomarkers expressed by the retinoblastoma tumor for the better survival of patients.

KEYWORDS: retinoblastoma; pathology; molecular biology

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RETINOBLASTOMA

Retinoblastoma is the most common intraocular malignancy of childhood and infancy accounting for 3% of all pediatrics cancers. It is caused by inactivation of RB1 genes commonly known as tumor suppressor gene[1]. Incidence of retinoblastoma ranges approximately worldwide at one case per 15 000-20 000 live births, which corresponds to about 9000 new cases every year[2]. There is no racial or gender predisposition. The sign of retinoblastoma is a white pupil, called leukocoria, strabismus, painful blind eye and loss of vision[3]. With the advancement in treatment and multidisciplinary approach, eye salvage is possible in group A through D intraocular tumours[4]. However, in group E tumours and group D (unilateral cases), enucleation always remains the choice of treatment. Higher incidences of histopathologic risk factors are reported from enucleated eyes from developing countries as opposed to developed countries[3].

GENETICS

Knudson’s putative tumor suppressor gene was cloned by Weinberg’s lab by studying genetic lesions and chromosomal aberrations in families with a history of retinoblastoma. This gene was the first human tumor suppressor gene to be cloned and it was named as RB1[5]. Initially, researchers considered that RB1 was important only for retinoblastoma susceptibility but Harbour et al[7] found that the RB1 gene was also mutated in lung cancer.

India has the largest number of retinoblastoma cases with an estimated 1500 new cases annually[8]. It was the first tumour in which cancer genetics was revealed[9]. Cases with heritable retinoblastoma (48%) carry a germline mutation in the RB1 gene and are likely to develop secondary cancers later in life like bone and soft tissue sarcomas, melanoma, brain tumours, etc. They have a 50% risk of transmitting their germline RB1 mutation to their offspring[10]. Cases with nonheritable retinoblastoma have normal RB1 gene. Some of the retinoblastoma cases are caused by RB1 gene mutation while others are caused by somatic amplification of the MYCN gene[11]. Recently, genetic laboratories have found that retinoblastoma may arise when the MYCN oncogene is amplified even in the presence of non-mutated RB1 genes. These cases are relatively rare, occurring in <3% of unilateral retinoblastoma cases[12]. Only 6% are familial while 94% are sporadic in newly diagnosed cases of retinoblastoma. All the cases of bilateral retinoblastoma involve germinal mutations[13].

Almost 15% of unilateral sporadic retinoblastoma is caused by germinal mutations affecting only one eye while the 85% are sporadic.

Knudson proposed the two hit hypothesis in 1971[9]. Knudson stated that two chromosomal mutations are needed for developing retinoblastoma. The initial hit is a germline mutation, which is inherited and is found in all cells in hereditary retinoblastoma. The second hit grows in the somatic retinal cells leading to the development of retinoblastoma. Therefore, hereditary cases are subjected to the development of non-ocular tumors such as osteosarcoma. In unilateral sporadic retinoblastoma, both the hits occur during the development of the retina and are somatic mutations[14]. Therefore, there is no risk of second nonocular tumors.

Genetic testing for RB1 mutations and counselling of the patient can improve disease outcome and management. There are definitive molecular tests which help in identifying children and their relatives who are at high risk for retinoblastoma,
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and need to be followed closely for the disease\[15\]. Recently, combinational approach of multiplex ligation-dependent probe amplification assay, deletion screening, direct sequencing, copy number gene dosage analysis and methylation assays provides mutational spectrum of \textit{RB1} gene mutation in retinoblastoma patients\[16\].

**PATHOLOGY**

**Gross** The gross appearance of retinoblastoma at cut section of the eye is somewhat variable, reflecting the stage of the disease at enucleation. The tumor has a white, encephaloid or brain-like appearance, with chalky areas of calcification and yellow necrotic areas\[17\]. The presence of calcium is often accentuated in eyes that have had prior radiotherapy or chemotherapy. Gross examination of the eyeball in the laboratory involves a total of 4 blocks. One block is the eyeball section with the optic nerve. Two blocks should contain the calottes. The fourth block consists of the resected margin of the optic nerve.

**Histopathological** On microscopic examination, retinoblastoma reveals a tumor composed of small hyperchromatic cells with a high nuclear to cytoplasmic ratio with large areas of necrosis and multifocal area of calcifications. Tumour differentiation are categorized into well differentiated (>50% known as Homer-Wright (HW) rosettes) or poorly differentiated (<50% known as Flexner-Wintersteiner (FW) rosettes)\[18\]. In 2014, new rosettes were found which were comparatively larger than FW and HW and has an unusual anterior segment involvement\[19\]. Necrosis in the tumor is graded as none (<25%), mild (25%-50%), or extensive (>50%). Optic nerve invasion is graded as prelaminar, postlaminar and invasion of the resected margin. Postlaminar invasion is defined as tumor invasion beyond the lamina cribrosa of optic nerve\[20\]. Choroidal involvement is divided into focal invasion defined as a tumor focus of less than 3 mm in any diameter (thickness or width) or massive invasion defined as invasive focus of tumour measuring 3 mm or more in any diameter as per the Retinoblastoma Staging Working Group\[21\]. Artifactual seeding of tumour cells is seen at times in the sections which pose a problem to the pathologist. These are composed of small groups of tumour cells usually with many necrotic cells present within natural spaces of the eye. In contrast true tumour invasion comprises of solid nests of tumour cells with pushing or infiltrating borders without necrosis.

**Histopathological Prognostic High Risk Factors** The survival and management of high risk retinoblastoma has improved by identification of high-risk factors and appropriate adjuvant therapy. Histopathological high risk factors (HRFs) are evaluated and identified after enucleation for predicting metastasis. Prognostic factors like massive choroidal invasion, retrolaminar invasion and involvement of resected end of optic nerve, iris and ciliary body involvement, anterior chamber involvement, scleral and extrascleral involvement by tumour cells are associated with a greater risk of orbital recurrence and predictive of metastasis. There is a still debate regarding anterior chamber as a high-risk factor for retinoblastoma. Recently, Sreelakshmi et al\[22\] concluded in their study that anterior chamber seeds do not, by themselves, constitute an independent risk factor for metastasis in retinoblastoma. The reported incidences of HRFs are 7% to 56% for invasion of retrolaminar optic nerve and optic nerve to the transaction line; 12% to 42% for choroidal involvement; and 3% to 30% for scleral and extrascleral spread. Kashyap et al\[23\] described various clinical features like older age at presentation, longer lag period, presence of hyphema, pseudohypopyon, staphyloma, and orbital cellulitis. These factors were associated with occurrence of HRFs and may be a useful indicator for considering adjuvant chemotherapy especially in developing countries. Also poorly differentiated retinoblastomas present at a later age and are associated with presence of multiple HRFs and necrosis\[18\]. Cases with presence of HRFs need systemic adjuvant chemotherapy which improves the survival of children at risk for metastatic disease\[24\]. Therefore, histopathologic HRFs can provide important basis for clinicians to determine treatment plan.

**Pathological Tumor, Node, Metastasis Classification** Tumor, node, metastasis (TNM) classification is developed by the American Joint Commission on Cancer (AJCC) and the Union International Control Cancer (Table 1)\[25\]. Retinoblastoma is the first cancer in which role of germline predisposition is recognised by incorporating stage category “H” into the AJCC classification\[26\]. Table 2 describes the AJCC 2017 8\textsuperscript{th} edition tumor, node, metastasis, heritable trait (TNM(H)) clinical (c) and pathological (p) staging system which is known to be the first evidence-based system for predicting overall prognosis of both eye(s) and patients\[27\-28\].

**RECENT ADVANCES IN MOLECULAR PATHOLOGY OF RETINOBLASTOMA**

Ongoing studies \textit{via} higher resolution genomic technologies, gene expression profiling, direct gene sequencing, multiplex-polymerase chain reaction, mi-RNA microarray profiling, next-generation sequencing (NGS), microsatellite analysis for loss of heterogeneity, and \textit{in-situ} hybridization for chromosomal aberrations will continue to facilitate our exploration into the molecular intricacies of retinoblastoma and results in newer therapeutic approaches. The discovery of proto-oncogenes transformed our insight into mechanisms of cancer. More recent studies shows that retinoblastoma tumors may differ in the mutagenic pathway as some of retinoblastoma tumors are caused by \textit{RB1} mutation\[29\-32\], while some can also be initiated by amplification of \textit{MYCN} proto-oncogene.
Singh et al.[33] demonstrated prognostic significance of CDC25 phosphatases and polo-like kinases in retinoblastoma. They suggested that expression of CDC25B might be used as a potential prognostic marker in the pathogenesis of retinoblastoma and contribute to the development of the disease by causing genomic instability through deregulation of cell division. In their study, PLK1 was more frequently expressed and deregulated in poorly differentiated retinoblastoma tissue as compared to PLK3 protein that might serve as a poor prognostic marker in retinoblastoma.[20]

Evasion of apoptosis is a hallmark of human cancers that leads to cancer development, progression and treatment resistance. The Bcl-2 family members are important regulators of the mitochondrial pathway of apoptosis. Bax and Bcl-2 are proteins that regulate programmed cell death and apoptosis. Recently, Singh et al.[34] revealed higher expression of Bcl-2 in 66% of cases whereas Bax expression was found only in fewer cases (30%) of retinoblastoma tissue by immunohistochemistry, mRNA and Western blotting techniques. According to the author, differential expression of apoptotic regulatory proteins might represent poor response to patient outcome and have potential for tumor invasiveness.

Grotta et al.[35] used a combined approach of next-generation sequencing (NGS) and RB1 custom array-comparative genomic hybridization (aCGH) on a cohort of retinoblastoma patients. NGS and RB1 custom aCGH have demonstrated to be an effective combined approach in order to optimize the overall diagnostic procedures of retinoblastoma. Devarajan et al.[36] demonstrated for the first time that targeted next generation sequencing is an efficient approach for the identification of wide spectrum of pathogenic variants in retinoblastoma patients. Using this approach, an array of pathogenic variants including single nucleotide variants, InDels (small insertions/deletions) and copy number variations were detected in retinoblastoma patients. This comprehensive approach reduces the time and number of assays required for the detection of pathogenic variants by conventional methods which is sensitive (0.97) and efficient for RB1 screening.

The application of genomics to the study of cancer is rapidly shifting toward the analysis of tissue samples to discover new biomarkers for early detection of cancers. Mitochondria have been implicated in tumor progression, cell differentiation, and apoptotic pathways. The identification of mitochondrial DNA mutations and its associated proteins as a biomarker has been used to help understand not only gene function but also the underlying molecular mechanisms of mitochondrial biology in retinoblastoma. This strategy relies on the hypothesis that if mutations in mtDNA cause physiological aberrations
specifically in a particular tissue, the gene is more likely to be selectively expressed in that tissue. Currently, the role of mitochondria in retinoblastoma biology is still poorly understood.

Recently, Singh et al. have described and analyzed the morphological changes of mitochondria in retinoblastoma tumor by transmission electron microscopy. Poorly differentiated retinoblastoma cases showed fewer mitochondria, scant cytoplasm, disorganized organelles (mitochondria), and necrosis, whereas well-differentiated retinoblastomas had larger number of mitochondria and more organized organelles.

Understanding the structural and functional characteristics of mitochondria in retinoblastoma might be essential for the design of future therapeutic strategies. They have also studied the expression of mitochondrial oxidative phosphorylation complexes in retinoblastoma tumor tissues. Among all the complexes, loss of mitochondrial complex I immunoreactivity proved to be a useful independent prognostic biomarker to identify high-risk retinoblastoma patients. Role of mitochondrial DNA and its protein biomarkers necessitate careful experimentation to adequately assess its contribution in retinoblastoma which might prove of diagnostic and prognostic value, and serve as a basis for the development of better long term therapeutic strategies. This provides an insight into molecular mechanisms of mitochondrial dysfunction, and also helps to find novel cancer biomarkers in retinoblastoma.
molecular pathways in fatty acid synthase (FASN) silenced retinoblastoma cells and revealed that FASN silencing reduced the invading property of retinoblastoma cancer cells by scratch assay. Venkatesan et al[40] studied computational and in vitro investigation of miRNAs-gene regulation in retinoblastoma pathogenesis by an in silico approach. They concluded downregulation of miR-486-3p and miR-532-5p in primary retinoblastoma tissues, which might implicates their role in tumorigenesis.

Cytoplasmic expression of FOXO3a (transcription factor) has been found to be associated in pathogenesis of retinoblastoma. Relocation of FOXO3a from cytoplasmic to nucleus activates non-mutated retinoblastoma and might be a therapeutic target for retinoblastoma[41]. Reactive oxygen species and free radicals are associated with cancer development and its progression, which might suggest potential avenues of therapeutic intervention. Expression of NOX4 protein might be a source of reactive-oxygen species production in tumor cells, leading to oxidative stress and associated with less overall survival rates in patients with extraocular disease. Current treatment strategies contribute significantly to vision salvage in patients harbouring intraocular diseases, and overall survival rates in patients with extraocular disease. However therapies such as chemotherapy, brachytherapy and plaque therapy do result in significant morbidity. A better understanding of pathobiology of retinoblastoma may lead to therapeutic agents which will act synergistically in the control of retinoblastoma tumors.

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