Genetics of Retinoblastoma

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Review Article

ABSTRACT

Background: Retinoblastoma (Rb) is the most common primary intraocular tumor in childhood. This review covers the causes, modes of inheritance, genetic testing, and recurrence risk of retinoblastoma, as well as disease management and the experience of the Centre of Excellence for Human Genetics at the National Research Centre in dealing with Rb. The incidence of Rb is approximately one in every 16,000-18,000 live births, affecting around 8,000 children worldwide each year. More than 80% of these cases are from low- and middle-income countries, with 50% of patients in Asia and 25% in Africa. The survival rate exceeds 95% in high-income countries, while the mortality rate can reach up to 70% in low-income countries.

Rb can affect one or both eyes. In hereditary cases, there is a 50% chance of transmitting the affected allele to the next generation. The primary gene responsible for Rb is the retinoblastoma gene (*RB1*), located on chromosome 13q14. Most mutations causing Rb are within the *RB1* gene, a tumor suppressor gene. Retinoblastoma requires mutations in both alleles (biallelic mutation) to develop into cancer. The *RB1* gene product, retinoblastoma protein, plays several roles in retina development, cell division checkpoints, DNA replication, and apoptosis. In some cases, other genes such as MYCN and BCOR, as well as epigenetic factors, may contribute to Rb.

Early diagnosis and appropriate treatment significantly improve survival rates. Recent advancements in molecular diagnosis have greatly impacted Rb management. This review aims to raise awareness about the disease, emphasizing the importance of early diagnosis and management to provide affected children and their families with better outcomes and the possibility of a complete cure.

Key Words: Hereditary, Mosaic cell line, *RB1* gene, Retinoblastoma, Retinoblastoma protein, Tumor suppressor gene.

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INTRODUCTION

The assumption of the inheritance of retinoblastoma (Rb) and the idea of tumor suppressor gene, was postulated by **Knudson, (1971)**. The two -hit hypothesis of Knudson postulated that one *RB1* allele is lost or mutated in germline and the second event is the somatic mutation in the other allele that occurs in the primitive cells in the retina which initiate the tumor. The 1st identified tumor suppressor gene was the *RB1* gene (**Lee *et al.,* 1987; Fung *et al.,* 1987**), then the relation of *RB1* to esterase D expression on chromosome 13 and deletion of chromosome 13q14 was discovered (**Sparkes *et al.,* 1983; Godbout *et al.,* 1983**). The loss of heterozygosity mapped to chromosome 13q14 and its relation to Rb and other tumors was approved (**Cavenee *et al.,* 1983; Hansen *et al.,* 1985**), then sequencing of the *RB1* gene was performed (**Lee *et al.,* 1987; Fung *et al.,* 1987**). The product of the *RB1* gene is the Rb protein, the nuclear phosphoprotein of Rb was the 1st cloned tumor suppressor gene (**Friend *et al.,* 1986**). The Rb protein involved in network interaction that regulate the cell cycle, check points before DNA synthesis, control cell division, apoptosis, and cell proliferations (**Medema *et al.,* 1995; Lee *et al.,* 2002**). The deficiency of the Rb protein is responsible for many malignant tumors e.g. Rb and osteosarcoma. There are two forms of Rb, the isolated and hereditary form. The hereditary form present when there is family history, tumor in both eyes, and multifocal affection (the sporadic case does not exclude the presence of the inherited mutation). The isolated form of Rb have 16% hereditary germ line mutation (**Shields *et al.,* 2021; Martínez-Sánchez, 2022**). Rb developed because of biallelic inactivation of *RB1* gene which is located on chromosome 13q14. Hypermethylation of promotors of *RB1* gene can lead to silencing of the *RB1* (tumor suppressive gene) and affect gene expression (**Holliday, 2006; Reis *et al.,* 2012**). The *RB1* gene itself

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has a role through epigenetic effect in the pathways of other oncogenes and tumor suppressive genes (**Choy *et al.*, 2002; Zhang *et al.*, 2012; Cruz-Gálvez *et al.*, 2022**).

Chemotherapy is the most important therapy in Rb to restore vision, chemo-reduction and intra-arterial chemotherapy can cure and reduce eye enucleations (**Shields *et al.*, 2015; Hahn *et al.*, 2016**), also the discovery of the role of some miRNA and lnc-RNA which can be used as tumor markers as well as for Rb therapy carry a great achievement for early diagnosis and cure (**Shen *et al.*, 2014; Ding *et al.*, 2014**).

In this review we are highlighting Rb, the gene, the protein role in cell cycle, cell division and the epigenetic effect. We also report the experience of the Clinical and cytogenetic departments in the Centre of Excellence for Human Genetics, National research Centre, in dealing with Rb.

***RB1* gene:**

RB1 gene has 27 exons spanning 180 kb exons on chromosome 13q14 (**Toguchida *et al.*, 1993; Friend *et al.*, 1986; Hong *et al.*, 1989; www.els.net**) (Figure 1) and has 4.7 kb messenger RNA transcript encoding 110 kDa phosphoprotein (**Lee *et al.*, 1987**).

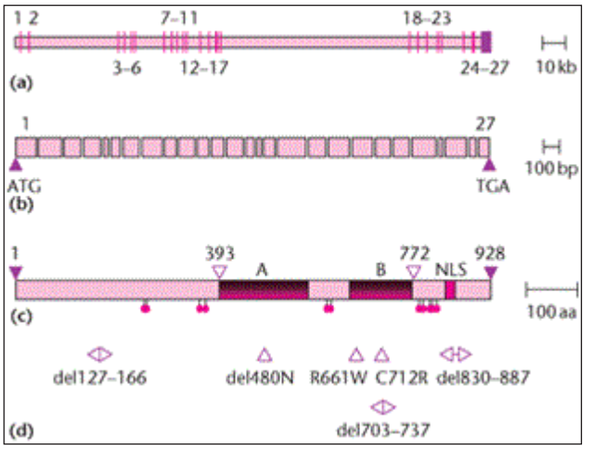

Figure 1: Organization of the retinoblastoma *RB1* gene and protein pRB (www.els.net).

RB Protein, pRB:

The protein product of the *RB1* gene is called pRB or p105. pRB has two paralogs, pRB2/p130 and p107. These three proteins form the RB protein family, sharing similar sequences and collectively regulating the cell cycle (**Claudio *et al.*, 1994**).

Function of pRB:

pRB/p105 plays a crucial role in regulating the G1/S transition of the cell cycle. It controls the cell cycle and prevents cells with genetic mutations from transitioning from the G1 phase to the S phase. This checkpoint ensures that normal cells can carry out DNA synthesis and cell division properly. Loss of pRB function leads to uncontrolled cell growth.

pRB/p105 binds to the E2F family of transcription factors, aiding cells in entering the S phase and initiating DNA synthesis (**Dyson, 1998**). When pRB/p105 is hypophosphorylated, it is in its active form and binds to E2F, keeping the cell in a quiescent state. Upon phosphorylation, pRB releases E2F, enabling the cell to progress to the S phase (**Weintrub *et al.*, 1995**).

Epigenetic effect:

Genomic imprinting is an epigenetic process that affects genes through parental-specific DNA methylation. It has been suggested that *RB1* is maternally imprinted. This imprinted expression of *RB1* is associated with a differentially methylated CpG island at CpG 85 in *RB1* intron 2. *RB1* is methylated on the maternal copy and acts as a weak promoter for other *RB1* transcripts on the paternal copy. Studies have shown that differential methylation of CpG islands skews *RB1* expression towards the maternal allele (**Kanber *et al.*, 2009; Buiting *et al.*, 2010; Kanber *et al.*, 2013; McEvoy and Dyer, 2015**).

Gene-specific hypomethylation plays a role in oncogene activation, while hypermethylation of tumor suppressor gene promoters leads to gene silencing (**Holliday *et al.*, 2006**). The *RB1* gene is involved in the regulation of various epigenetic mechanisms, including histone modification, DNA methylation, ATP-dependent chromatin remodeling, microRNA (miRNA), and long noncoding RNA (lncRNA) regulation (**Reis *et al.*, 2012**). Inactivation of the *RB1* gene can cause epigenetic dysregulation of pathways involving oncogenes and tumor suppressor genes. These epigenetic changes, which contribute to tumorigenesis, can also serve as therapeutic targets, particularly miRNA and lncRNA (**Zhang *et al.*, 2012**).

Hypermethylation of *RB1* promoters can lead to low expression of *RB1* and affect other tumor suppressor genes, such as RASSF1A, MGMT, and p16INK4A (**Livide *et al.*, 2012**). Hypermethylation of the MGMT promoter is associated with bilateral and poorly differentiated retinoblastoma (**Choy *et al.*, 2002**). *RB1* also plays a significant role in histone modification and methylation through histone methyltransferases (HMTs). Abnormal HMT expression has been observed in Rb (**Rivera *et al.*, 2014**).

Overexpression of certain miRNAs and lncRNAs is associated with tumorigenesis and tumor progression. Conversely, the discovery of other highly expressed miRNAs and lncRNAs that can regulate Rb progression and metastasis offers potential therapeutic avenues for Rb (**Shen *et al.*, 2014; Ding *et al.*, 2014; Sun *et al.*, 2020**).

Clinical Overview:

Retinoblastoma (Rb) typically develops before the age of 5 years and is a neuroblastic disease. In most children with Rb, the disease affects only one eye, though approximately one-third develop cancer in both eyes (**Castillo and Kaufman, 2003**). When diagnosed early, Rb is usually treatable and causes minimal pain. However, if not treated promptly or properly, this cancer can spread from the affected eye to other parts of the body and become life-threatening. The current goal of early treatment is not only to save lives but also to preserve vision (**Chantada *et al.,* 2011**).

There are two classification systems for Rb: the Intraocular Classification of Retinoblastoma (ICRB) and the International Intraocular Retinoblastoma Classification (IIRC), Philadelphia version. These systems consider the size, location, and extent of tumor spread to the vitreous body and classify Rb into five groups, labeled A to E (**Linn Murphree, 2005; Shields *et al.,* 2006; Fabian *et al.,* 2018**; **Ancona-Lezama *et al.,* 2020)** (Table 1).

Table 1: International classification of retinoblastoma (ICRB):

Group	Mnemonic	FeaturesA
A	Small tumor	Retinoblastoma ≤3mm in basal diameter or thickness
B	Bigger tumor Beside the macula or optic nerve	Retinoblastoma >3mm in basal diameter or thickness OR tumor location ≤3mm from foveola tumor location ≤1.5mm from optic disc tumor-associated subretinal fluid ≤3mm from tumor margin
C	Contiguous seeds	Retinoblastoma with subretinal seeds ≤3mm from tumor vitreous seeds ≤3mm
D	Diffuse seeds	Retinoblastoma with subretinal seeds >3mm from tumor vitreous seeds >3mm from tumor subretinal and vitreous seeds >3mm from tumor
E	Extensive tumor	Retinoblastoma occupying >50% of the globe OR neovascular glaucoma opaque media from hemorrhage in subretinal space, vitreous, or anterior chamber invasion of postlaminar optic nerve, choroid (>2mm), sclera, orbit, anterior chamber

Incidence and Prevalence:

Retinoblastoma (Rb) affects 1 in 16,000 to 18,000 births worldwide, with the incidence varying by country (**Moll *et al.,* 1997; Abramson and Schefler, 2004; Kivelä, 2009**). It accounts for approximately 4% of all cancers in children under 5 years of age and shows no gender preference (**Seregard *et al.,* 2004**). In industrialized countries, treatments for Rb are highly effective, resulting in the successful treatment of 90% of children (**Shields and Shields, 2021**). However, the survival rate in underdeveloped countries is lower and heavily influenced by socioeconomic factors (**Canturk *et al.,* 2010; Abdelazeem *et al.,* 2023**).

A global survey on the incidence of Rb reported the highest number of cases in Asia (4,027 per year), followed by Africa (1,792), with much lower numbers in Europe (414), North America (258), and Japan (59) (**Kivelä, 2009**). Mortality rates are highest in Africa (up to 70%) and Asia (39%), compared to much lower rates in Europe, North America, and Japan (3-5%). **The Global Retinoblastoma Group, (2020)**, led by **Fabian *et al.,*** found that 85% of Rb patients were from low- and middle-income countries. In these regions, the median age at presentation was 31 months, with 49% having extraocular tumors. This contrasts with high-income countries, where the median age at presentation was 14 months, with only 2% presenting with extraocular tumors (**Shields *et al.,* 2023**).

Approximately 6% of newly diagnosed Rb cases are familial, while 94% are sporadic. All individuals in families with Rb are at risk of passing the disease to their offspring, who will develop the tumor, although the penetrance varies (**Shields and Shields, 2002**).

Rb is caused by a genetic or acquired deletion or mutation in the *RB1* gene. The *RB1* gene is located on chromosome 13q14.1-q14.2, specifically at: arr[hg19] ch r13q14.1q14.2(48,877,883_49,056,026). Hundreds of *RB1* mutations have been identified in Rb patients (**Lohmann, 1999; Leiderman *et al.,* 2007; OMIM, 2011**). Most of these mutations prevent *RB1* from producing a functional retinoblastoma protein (pRB), which is essential for regulating cell division. Without functional pRB, some retinal cells can divide uncontrollably, forming cancer cells (**Chinnam and Goodrich, 2011**) (Figure 2).

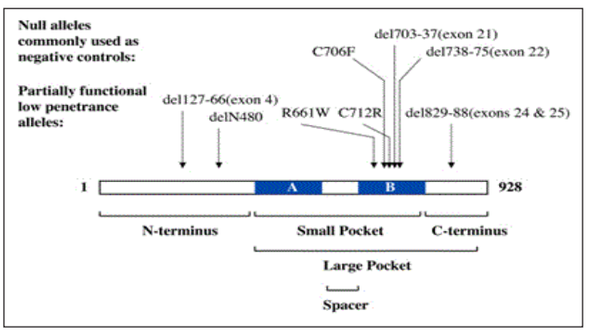

Figure 2: Frequent *RB1* mutations and pRB domain structure. The open reading frame of pRB is shown in conjunction with the positions of the A and B parts of the pocket. Arrows indicate the locations and changes found in frequently studied low penetrance and high penetrance mutant alleles. Below the open reading frame, identify the structural regions of pRB (**Dick, 2007**).

Inheritance:

There are two types of retinoblastoma (Rb): a genetic/hereditary form and a non-genetic/non-hereditary form. Parents of children with Rb should have their eyes checked for signs of undiagnosed, spontaneously regressed tumors. If such tumors are found, the chance of all their offspring being affected is up to 50%. If the parents are unaffected, there is no family history of Rb, and the child is the first in the family to be affected, the chance of another child being affected is less than 1 in 20 (**Tucker and Freidman, 2002**).

Researchers estimate that 60% of Rb cases are non-germinal (sporadic). This means the *RB1* mutation occurs only in the eye and cannot be passed to the next generation. Rb in these cases arises due to somatic non-hereditary mutations, typically affecting only one eye and with no family history of the disease. These individuals are born with two normal copies of *RB1*, but during childhood, both copies are mutated or deleted in retinal cells (**OMIM, 2011**). People with non-germinal Rb have no risk of passing these *RB1* mutations to their children (Table 1). Without genetic testing, it is challenging to determine whether a person with Rb in one eye has the germinal or non-germinal form. Unilateral Rb often involves a single lesion. Reports indicate that 7-33% of unilateral Rb cases have germline mutations (**Brichard *et al.*, 2006; Berry *et al.*, 2018; Shields *et al.*, 2023**). **Shields *et al.*, (2021)** found that 16% of patients with unilateral Rb might have germline mutations based on family history or the presence of other tumors.

The other 40% of Rb cases are caused by germline mutations, either due to inherited pre-existing germline mutations in the family (10%) or new germline mutations (30%). These mutations follow an autosomal dominant inheritance pattern and are referred to as germinal Rb. People with germinal Rb may have inherited a gene mutation from a parent or the mutation may result from a new mutation in the egg or sperm or a birth defect occurring after birth (**Shields and Shields, 2004; Athavale and Khetan, 2018**). If a parent has the *RB1* mutation, the risk for each sibling of the parent is 50% (Table 1). However, if the parent has germline mosaicism, the risk of Rb for the sibling is 3-5%. When parents do not have germline *RB1* mutations, the mutation in their child could be de novo (90-94%) or due to one parent having germline mosaicism (6-10%). For Rb to develop, a second mutation involving the other copy of *RB1* must occur in retinal cells during the individual's lifetime (**Lohmann, 2010**). Cancer occurs when cells become homozygous for the mutant allele, losing heterozygosity for the *RB1* gene (LOH). This second mutation typically occurs in childhood (Figure 3).

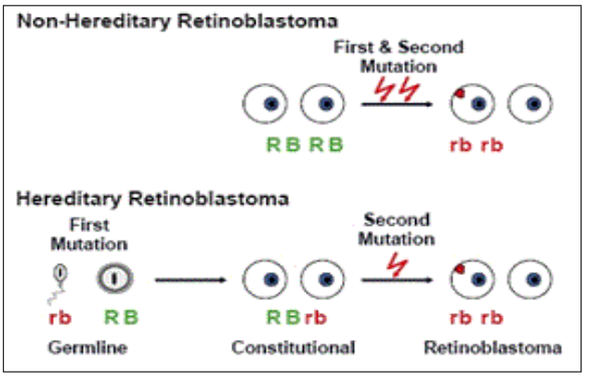

Figure 3: Diagram representation of the molecular genetic mechanisms that lead to non-hereditary and hereditary Rb. Rb development is initiated when the two *RB1* alleles are mutated (rb rb) (**Lohmann and Gallie, 2011**).

Hereditary Rb accounts for 10% of unilateral cases and all bilateral cases. Individuals with bilateral Rb usually have multifocal tumors in one or both eyes and are at high risk for other cancers outside the eye, such as pineal tumors, osteosarcomas, soft tissue tumors (including breast cancer), prostate cancer, and skin cancer (melanoma). Therefore, periodic medical follow-up is necessary throughout life to detect secondary lesions and avoid DNA-damaging agents (**Nichols *et al.*, 2009**). Trilateral Rb may develop in patients with bilateral Rb and germline mutations in the *RB1* gene, resulting in a poor prognosis. Follow-up of hereditary forms and early diagnosis can improve survival rates (**Shields *et al.*, 2001; De Jong *et al.*, 2020; Marković *et al.*, 2023**).

A small portion of Rb cases result from deletion or rearrangement of the region of chromosome 13 containing *RB1*, seen in approximately 5% of patients with unilateral Rb and about 7.5% of those with bilateral Rb. If a parent has a cytogenetically balanced chromosome 13 rearrangement, their siblings will be at greater risk of inheriting the unbalanced chromosome. Cytogenetic high-resolution analysis or fluorescence in situ hybridization (FISH) can detect these chromosomal aberrations (**Bamne, 2005**). These chromosomal changes often involve many genes beyond *RB1*, leading to intellectual disabilities, developmental delays, and distinctive facial features in affected children (Table 2).

Table 2: Summary of Rb types:

~ 60% of all Rb cases **Unilateral Rb with a mean age at diagnosis of 24 months**		**~ 40% of all RB cases** **Bilateral Rb with mean age at diagnosis of 15 months**
Nonhereditary form: 90% of unilateral cases	**Hereditary form:** 10% of unilateral cases Transmission risk: 50%	**Hereditary form:** 100% of bilateral cases With positive family history in ~ 10% Transmission risk: 50%

Counseling:

In individuals with bilateral retinoblastoma (Rb), genetic mutations can often be identified, enabling pre- or post-natal screening for their offspring and other family members. These services can also be offered to some unilaterally affected individuals, particularly when surgery provides a tumor sample for analysis (**Shields and Shields, 2004**).

For bilaterally affected individuals, molecular genetic analysis can identify the specific *RB1* mutation causing Rb. This indicates that the proband carries the genetic mutation and that one parent, although unaffected, has the *RB1* mutation. This adult carrier is at risk for secondary tumors, and all their children are also at risk for Rb. Brothers, sisters, and first cousins should be tested for the *RB1* mutation. Children with the mutation need frequent clinical examinations to detect tumors early, thus saving vision and life. Children without the mutation have the same risk as the general population and can be excluded from clinical surveillance. Rapid prenatal testing allows parents and doctors to plan treatment for infants with *RB1* mutations.

For unilaterally affected individuals, molecular testing can identify the two tumor-causing *RB1* mutations if DNA from fresh or frozen tissue and blood is available for analysis. Unilateral Rb in infants suggests germline mutations, while older children with unilateral Rb are more likely to have sporadic somatic mutations (**Shields and Shields, 2004**).

Family History:

Parents with a family history of Rb should receive genetic counseling. All children with a history of Rb should be examined immediately after birth, with examinations repeated every 4-6 weeks for up to 1 year, then every 2-3 months for up to 3 years. The risk of Rb varies depending on the relationship with family members and the type of mutation. *RB1* gene mutation testing should be considered for those at risk (**Lohmann, 2010**).

In a retrospective study of 1,831 patients with Rb, those with a family history of Rb who were monitored from birth were diagnosed at a younger age (mean age 8 months) and had better 5-year ocular survival compared to those with a family history who did not undergo surveillance (71% vs. 15% ocular survival for unilateral tumors and 67% vs. 43% for bilateral tumors) (**Abramson *et al.,* 2003**). **Brichard *et al.,* (2006)** suggested that screening for constitutional *RB1* mutations should be an essential part of Rb treatment, regardless of tumor laterality and family history.

Risk to Family Members:

The risk to family members depends on whether the patient has a germline *RB1* mutation. First-degree relatives (parents, siblings, and children) share approximately 50% of their genes with the patient. Second-degree relatives (grandparents, grandchildren, aunts, uncles, nieces, nephews, and half-siblings) share approximately 25% of their genes with the patient (**Lohmann, 2010**).

Most patients with more than one affected family member or bilateral Rb have germline mutations. Therefore, molecular genetic analysis of peripheral blood DNA will detect *RB1* mutations in 90-95% of these cases. For patients with bilateral Rb and no family history, if no oncogenic mutation is found in peripheral blood, tumor DNA should be tested to identify the two mutations, suggesting the sporadic type of Rb (**Chantada *et al.,* 2011**).

In patients with unilateral Rb and no family history, molecular testing of tumor tissue can identify the two *RB1* mutations, indicating a sporadic type. In 14% of these patients, one tumor mutation is found in peripheral blood, indicating a germline mutation or mosaicism (**Lohmann and Gallie, 2011**).

Molecular testing of peripheral blood or tumor tissue confirms the diagnosis of Rb and is used for high-risk groups, prenatal testing, preimplantation testing, and predisposition testing. *RB1* mutation analysis can determine the genetic status of at-risk family members. Conventional molecular testing identifies *RB1* mutations in 90% of bilaterally affected individuals. The remaining 10% may have translocations, deep intronic splicing mutations, or low-level mosaic mutations, which might or might not be in the germline. One primary purpose of molecular testing is to eliminate high-risk individuals to justify deferring eye examinations.

Penetrance:

Hereditary retinoblastoma (Rb) is an autosomal dominant disease with high penetrance (90%) and carries an increased risk of bilateral Rb as well as other neoplasms. Most families with hereditary Rb (90%) segregate *RB1* null alleles, which are altered by frameshift or nonsense mutations. With few exceptions, *RB1* null alleles exhibit almost complete penetrance (nearly 99%) (**Lohmann *et al.,* 1996; Sippel *et al.,* 1998**).

Less than 10% of families show a low-penetrance phenotype with reduced expressivity, such as an increased prevalence of unilateral Rb and incomplete penetrance (25% or less). This low-penetrance phenotype is often associated with mutant *RB1* alleles showing in-frame or missense changes, distinct splice mutations, or mutations in the promoter region.

A third category of families exhibits reduced penetrance without reduced expressivity among family members with Rb (**Klutz *et al.,* 2002**). Some families demonstrate a low-penetrance phenotype with both reduced expressivity and incomplete penetrance of *RB1* (**Serrano *et al.,* 2011**). **Fernández *et al.,* (2007)** stated that the degree of penetrance of Rb inheritance depends not only on the

occurrence of the second mutation of the *RB1* gene but also on the extent of inactivation of the first mutation. Parental effects have been reported for *RB1*-associated phenotypes, including Rb penetrance and age of onset (**Kanber *et al.,* 2009**).

Prognosis of Rb:

The prognosis for retinoblastoma depends on several factors. Early diagnosis and timely treatment are crucial in reducing morbidity, extending life, and preserving vision. Additionally, patients with a history of Rb have an increased risk of developing other non-ocular tumors, which can be life-threatening. Another variable influencing prognosis is the experimental regulation of Rb proteins by viral infections, such as the human herpes virus method (**Hume and Kalejta, 2009**).

Mosaic cell line:

Mosaicism occurs when genetic changes, such as those affecting the *RB1* gene, happen during embryogenesis. In this scenario, some cells carry the mutation while others remain normal. The impact on various tissues depends on the timing of these changes during embryogenesis (**Paller *et al.,* 1994**). **Sippel *et al.,* (1998)** analyzed primary mutations in 156 Rb families and found that approximately 10% had mosaic gene mutations, resulting in reduced penetrance and expressivity.

A study by **Munier *et al.,* (1988)** reported that 20% of mosaic cell lines in Rb patients had a deletion of chromosome 13. **Mohamed *et al.,* (2009)** documented a case with developmental delay and a mosaic cell line with a large deletion of 13q. Additionally, **Mohamed *et al.,* (2009)** reported a family with three affected siblings from unaffected parents, suggesting a complex genetic scenario that may involve mosaicism.

In these cases, it is crucial to distinguish between germline mosaicism and low-penetrance hereditary Rb. Careful reexamination of the parents is essential to accurately determine the genetic status and potential risk to other family members.

Mosaic cell lines should be considered in genetic counseling for retinoblastoma (Rb). The ratio of white blood cells with mutations can predict the presence of mutant germ cells, though it would be more accurate to measure this ratio in sperm close to the time of conception. Germline DNA analysis can be employed to estimate the risk of recurrence in these cases.

Every detected germline mosaicism has an initial mutation that can be identified in leukocyte DNA, although the ratio can vary (**Munier *et al.,* 1988; Greger *et al.,* 1990; Sippel *et al.,* 1998; Barbosa *et al.,* 2008**). The recurrence risk in siblings of mosaic cases is low because these mutations occur during embryogenesis.

Cytogenomic of Rb:

The most common chromosomal abnormality observed in retinoblastoma (Rb) is the interstitial deletion of chromosome 13q14. Cytogenetic analysis of peripheral blood lymphocytes has revealed deletions or rearrangements involving 13q14.1-q14.2 in approximately 5% of patients with unilateral Rb and about 7.5% of patients with bilateral Rb. High-resolution karyotyping is the preferred method for detecting these abnormalities.

Offspring of patients with blood 13q14 deletions have a 50% chance of being susceptible to Rb (**Yunis and Ramsay, 1978; Lohmann and Gallie, 2011**). The identification of 13q14 deletions may aid in the early diagnosis of Rb (**Kenneerknecjt *et al.,* 1994**).

The most common chromosomal abnormality in retinoblastoma (Rb) is the interstitial deletion of 13q14. FISH analysis, using a probe derived from the *RB1* sequence, plays a crucial role in identifying individuals with mosaic deletions (detected frequency >8%). Mosaic and non-mosaic chromosomal deletions of the 13q14 region do not differ regarding the age at diagnosis, tumor laterality, or the presence of a family history (**Kivela *et al.,* 2003**). **Amare *et al.,* (2004)** suggested that mosaicism for 13q14 deletion should be considered in genetic counseling for unilateral Rb. Full-array comparative genomic hybridization (CGH) analysis, which identifies deletions/duplications across the genome, can also include the *RB1* gene/segment (**Lohmann and Gallie, 2011**).

Cytogenetically visible deletions involving 13q14 can also delete other genes in the same chromosomal region, potentially causing developmental delay and mild-to-moderate facial dysmorphism. In a small percentage of Rb patients, the disease develops due to inactivation of the *RB1* gene through chromothripsis of chromosome 13, involving multiple breaks in one or several chromosomes (**McEvoy *et al.,* 2014**).

Other genomic alterations commonly observed in Rb tumor include isochromosome 6p (i6p) and trisomy 1q. Gains on 1q22, 1q32, and 2p24 are also frequently observed (**Zielinski *et al.,* 2005**). Tetrasomy 6p rearrangement in the form of i6p is thought to be nearly specific to Rb, with extra 6p copies detected in 25-60% of cases (**Squire *et al.,* 1984; Turleau *et al.,* 1995; Oliveros and Yunis, 1995, Xu *et al.,* 2020, Stålhammar *et al.,* 2022**). Enhanced expression of genes mapped on 6p, such as tumor necrosis factor-alpha, can lead to tumor initiation or progression (**Imbert *et al.,* 2001**). Extra copy of chromosome 6p Correlates with Severe Anaplasia (**Stålhammar *et al.,* 2022**).

Other genetic alterations associated with Rb include monosomy 16 or del(16q), del(16q24), monosomy 17 or del(17p), i(17p), complete absence of pericentromeric heterochromatin on chromosome 9, and loss of X or Y sex chromosomes. Genetic alterations on chromosomes 19, 20,

21, 22, and X have also been detected in Rb tumor samples (**Huang *et al.,* 2003**).

These chromosomal aberrations which detected in enucleated eye can be well documented by FISH, CGH, and array CGH analysis. Abnormal amplifications of genes, such as N-myc and INT-1 cellular oncogenes, tumor necrosis factor-alpha, and RBKIN/KIF13A, have been discovered in Rb. Malignant development in homozygous *RB1* mutations is associated with increased levels of aneuploidy and chromosomal instability (CIN) in pRB-depleted cells (**Dimaras *et al.,* 2008; Amato *et al.,* 2009**). Therefore, aneuploidy seen in tumor cells is a byproduct of the inactivation of the pRB pathway. Recently these chromosomal abnormalities can be detected in aqueous humor Cell-Free DNA and can be used as a Prognostic Biomarker for Retinoblastoma (**XU *et al.,* 2020**)

Loss of pRB function results in aneuploidy either directly from disruption of normal chromosome segregation mechanisms, alteration in centromere number, defects in the spindle assembly checkpoint, or micronuclei formation, or indirectly through the development of cells that allow spontaneous aneuploidy formation. **Manning *et al.,* (2010)** described the underlying mitotic defects in pRB-deficient cells that cause chromosome mis-segregation. They reported that pRB depletion disrupted CAP-D3/condensin II centromere localization and chromosomal cohesion, leading to increased centromere spacing and disruption of centromere structure. This defective centromere function allows pRB-deficient cells to proliferate but disrupts mitotic fidelity, leading to aneuploidy.

Understanding the basis of chromosome segregation defects in cells lacking pRB will provide insights into the mechanisms and pathways that are dysregulated in human tumors (**Claudio *et al.,* 2011**). Additionally, characterizing these problems can recommend approaches to restore chromosomal stability and reduce metastatic potential. Conversely, treatments that potentiate these changes can cause severe damage and death in cells lacking pRB.

Tumor studies by array CGH revealed other somatic copy number variations besides the *RB1* deletion. These alterations affect tumor progression and prognosis. Gains in chromosomes 1p, 2p, and 6p22 lead to oncogene activation. Gains in 16p22, which are common in Rb, activate DEK and E2F3 oncogenes. Gains in 1q32 lead to the activation of MDM4 and KIF14, while gains in 2p24 activate MYCN. Alongside the loss of the *RB1* gene, the loss of the CDH11 tumor suppressor gene on 16q22-24 is also found in Rb (**MacPherson *et al.,* 2007; Marković *et al.,* 2023**).

Types of *RB1* mutations:

At the chromosomal level, deletions or rearrangements affect chromosome 13q, which contains the Rb locus at 13q14.

At the molecular level, large deletions, and insertions (including entire exons or a few exons), small deletions/insertions, a base change and hypermethylation of CpG islands can be detected. **Harbour, (1998)** review of *RB1* mutations reported 78% of nonsense and frame-shift mutations, while **Lohmann, (1999)** reported 62% of single-base substitution and **Lohmann *et al.,* (2002)** identified single-base substitutions and small-length mutations in 70%. -75% of all *RB1* mutations.

In small proportion of unilateral Rb other genes and micro-RNA have altered expression e.g., MYCN gene (**Gupta and Meena, 2020**).

Next generation sequencing (NGS) nowadays are necessary for proper diagnosis and counseling, it can detect all types of point mutations, as well as structure variations like microdeletions, duplications and low level mosaicism (**Gudiseva *et al.,* 2019; Marković *et al.,* 2023**).

Genetic Testing:

Conventional testing cannot detect all mutations in retinoblastoma (Rb); it identifies approximately 90% of mutations using various methods. In cases of sporadic unilateral Rb, both blood and tumor samples should be tested to distinguish between germline and somatic mutations. Due to the variety of *RB1* mutations and the potential hypermethylation of CpG islands in the promoter region causing gene inactivation, a single genetic test is not feasible (**Lohmann, 1999; Richter *et al.,* 2003**). Comprehensive genetic diagnosis is essential for all children with Rb, as those with germline mutations require thorough follow-up and counseling (**Gupta and Meena, 2020**).

Genetic testing should include:

- Cases of bilateral Rb.
- Patients with unilateral multifocal tumors.
- Parents and siblings, especially when multiple tumors are present.
- This test should cover cases of unilateral sporadic Rb when the tumor tissue is available, and prenatal diagnosis in those with a positive family history.

Relatives of patients with familial Rb:

a. Molecular Cytogenetic Analysis to detect cases of large deletions/duplications

FISH is recommended to detect 13q14 microdeletions. The FISH technique is important in the detection of mosaic cell lines as it allows the detection of various metaphases and interphases. FISH is also important in prenatal diagnosis when there is a family history of 13q14 deletion, as FISH can be used to examine amniotic cells for *Rb1* deletion (**Mohamed *et al.,* 2009**). FISH is a sensitive technique used to identify the deletions in metaphase and interphase and to identify cell lines mosaicism (**Bamne *et al.,* 2005; Mohamed *et al.,* 2009**).

Multiplex ligation-dependent probe amplification (MLPA) can be used to identify whole exon deletions, insertions, and rearrangements that account for 16% of Rb mutations (**Richter *et al.,* 2003**). Additionally, MLPA is a great tool for evaluating the methylation status of genomic regions. Methylation-specific MLPA (MS-MLPA) is a type of MLPA that uses the methylation-sensitive endonuclease HhaI and lacks sodium bisulfite modification of unmethylated cytosine residues. It has been suggested that MLPA should be the first screening method for *RB1* CNVs as well as its methylation/inactivation status in Rb patients (**Eid *et al.,* 2022**). Moreover, large deletions can be detected using universal primer quantitative fluorescence multiplex PCR (UPQFM-PCR) (**Heath *et al.,* 2000**). Restriction fragment length polymorphism (PCR-RFLP) analysis can be used to identify different Rb types using exon-specific primers (**Parsam *et al.,* 2009; Soliman *et al.,* 2017**).

b. Protein Cleavage Testing

Tsai *et al.,* (2004) proposed protein truncation testing as an effective screen test for *RB1* mutations in Rb tumors.

c. Mutation Screening

Sequence analysis is done to detect small deletions, insertions, and base substitution in exons and splice sites. These mutations account for approximately 70% of Rb cases. This method uses bidirectional sequencing of all 27 exons and the promoter to detect point mutations (**Raiziz *et al.,* 2002; Parsam *et al.,* 2009**).

Bamne *et al.,* (2005) recommended the use of various diagnostic methods for *RB1* mutations, including karyotyping, FISH, PCR-SSCP, define-DNA sequencing. By using different methods, **Parsam *et al.,* (2009)** found mutations in 83% of patients with bilateral Rb and 21% of patients with unilateral Rb. Of all mutations identified, 22.4% were large deletions, 25% were small deletions/ insertions, 28.8% were nonsense mutations, 12.3% were splice mutations, and 10.2% were missense mutations.

d. Sequence analysis of RNA

It is used to identify mis-splicing that occurs due to mutations in the splice site or mis-splicing due to deep intron changes that cannot be detected by the conventional sequencing (**Rushlow *et al.,* 2013**).

e. Methylation Analysis

Assessing promoter hypermethylation is important when there is only one mutation or no mutation in the Rb. Sodium bisulfite conversion can detect methylation at CpG sites. Hypermethylation rates in the Rb promoter region are reported to be between 4-10% (**Strizaker *et al.,* 1997; Zeschnigk *et al.,* 1999; Joseph *et al.,* 2004; Eid *et al.,* 2022**).

Molecular alterations in retinoblastoma beyond *RB1*:

While typical Rb is due to biallelic alteration in the *RB1* gene, early-onset unilateral Rb may involve only one affected allele and MYCN gene amplification (**Rushlow *et al.,* 2013**). **Mendonça *et al.,* (2021)** identified deletions of genes like GATA2, AKT1, ARID1A, and others in tumors without *RB1* mutations, suggesting these genes might be important for Rb development regardless of *RB1* status. Understanding these molecular markers can provide insights into the malignancy and aid in developing targeted treatments.

Aqueous humor and retinoblastoma:

Direct tissue biopsy for retinoblastoma (Rb) is contraindicated due to the risk of tumor spread. A new noninvasive technique involves obtaining cell-free DNA (cfDNA) from the aqueous humor. CfDNA includes both genomic DNA and circulating tumor DNA, serving as an alternative to tissue biopsy (**Berry *et al.,* 2018; Singh *et al.,* 2016**).

Aqueous humor (AH) can be easily and safely used to assess disease-specific biomarkers in ophthalmic diseases such as Rb and serves as a surrogate for tumor tissue. Additionally, aqueous humor is considered superior to blood-based liquid biopsy for Rb (**Berry *et al.,* 2020; Raval *et al.,* 2022**). One study evaluated different protein biomarkers in the AH of patients with Rb at various disease stages and provided a proteome database related to Rb, which could be used as a source of biomarkers after further validation (**Galardi *et al.,* 2022**).

Treatment: Chemotherapy is the most important therapy for Rb, aiming to restore vision. Chemoreduction and intra-arterial chemotherapy can reduce the need for eye enucleations (**Shields *et al.,* 2015; Hahn *et al.,* 2016; Ancona-Lezama *et al.,* 2020; Shields *et al.,* 2020; Bas *et al.,* 2021**).

The treatment of Rb depends on the International Classification of Retinoblastoma (ICRB), which considers Rb laterality, size, and localization

Stages A and B (unilateral Rb): Treated with cryotherapy or transpupillary thermotherapy (TTT).

Stages B with involved macula, C, D, and E (unilateral Rb): Treated with intra-arterial chemotherapy (IAC).

Bilateral Rb: Treatment depends on intravascular chemotherapy (IVC) (**Ancona-Lezama *et al.,* 2020**).

The Experience of the Human Cytogenetics Department with Retinoblastoma (Rb):

The Cytogenetic Department at the National Research Centre has extensive experience in dealing with

retinoblastoma (Rb) patients and their families, providing essential genetic counseling. **Mohamed *et al.*, (2009)** utilized fluorescence in situ hybridization (FISH) to detect deletions in the *RB1* gene, studying patients and their families to differentiate between hereditary and sporadic Rb cases, which is crucial for accurate genetic counseling. Their research also addressed mosaic cell lines.

The Centre of Excellence for Human Genetics at the National Research Centre has made significant advancements in Rb diagnosis. Dr. **Eid *et al.*, (2022)** employed Multiplex Ligation-dependent Probe Amplification (MLPA) to diagnose Rb, comparing its efficacy with FISH. Using the SALSA MLPA probemix P047-E1 *RB1*, their study included 72 patients referred from the Children's Cancer Hospital to the National Research Centre. The FISH technique detected a 5.5% deletion rate in this cohort, whereas the SALSA MLPA probemix P047-E1 *RB1* identified a 16.6% mutation and methylation defect rate. This probemix contains probes for 26 of the 27 *RB1* exons, excluding exon 15.

The MLPA technique is capable of detecting both complete and partial deletions of *RB1*, as well as the gene's methylation status. The research team identified complete or partial deletions in *RB1* in 15.7% of the patients studied. Additionally, MLPA detected methylation in six patients. One patient exhibited no deletion but had hypermethylation at intron 2 of the *RB1* gene.

This body of research underscores the importance of advanced genetic techniques in improving the diagnosis and understanding of retinoblastoma, facilitating better patient management and genetic counseling.

The Egyptian experience in retinoblastoma has primarily focused on the clinical diagnosis and management of the disorder Rb (**Elzomor *et al.*, 2017; El Zomor *et al.*, 2021**). However, there is limited experience in Egypt regarding the molecular diagnosis of both hereditary and sporadic Rb. Public awareness of the early symptoms of Rb like leukocoria, strabismus, different-colored irises, poor vision and painful eyes is crucial for early diagnosis and can help save eyes and lives.

CONCLUSION

Retinoblastoma: Genetic Insights and Diagnostic Approaches:

Retinoblastoma (Rb) is a neuroplastic tumor and the most common cancer in childhood, typically occurring before the age of 5. Rb arises from the inheritance or acquisition of a deletion or mutation in the *RB1* gene, located on chromosome 13q14.1q14.2. Approximately 60% of Rb cases are non-germinal, meaning they cannot be transmitted to the next generation. The remaining 40% are due to germline mutations, which can be either inherited from a parent (10%) or occur de novo (30%). This genetic form of Rb is transmitted as an autosomal dominant trait with high penetrance (90%). Mosaicism occurs when mutations arise during embryogenesis.

Various molecular methods are available for the genetic testing of Rb, but due to the extensive distribution of mutations within *RB1* and the presence of hypermethylation, no single genetic test can detect all possible mutations. Current tests detect approximately 90% of *RB1* mutations using different methods. For accurate genetic counseling and diagnosis, multiple techniques must be employed. Cytogenetics and fluorescence in situ hybridization (FISH) can detect large deletions and mosaicism. Analysis of DNA from blood and tumor samples is crucial for patients with unilateral Rb and no family history of the disease.

Multiplex ligation-dependent probe amplification (MLPA) can determine the copy number of *RB1* exons and their methylation status. Sequence analysis of the 27 *RB1* exons and promoter regions can identify 16% of *Rb1* mutations. RNA sequencing (RNA-seq) is used when splice site mutations are suspected. These comprehensive diagnostic approaches are essential for effective genetic counseling and management of retinoblastoma.

We recommend a national decision to establish a comprehensive road map for the diagnosis and management of retinoblastoma (Rb) in Egypt. The program should begin with familial history and pedigree analysis to differentiate hereditary from sporadic types of retinoblastoma. Identifying the types of mutations is crucial in Rb, and achieving an accurate diagnosis requires a national strategy.

The diagnostic process can start with MLPA. In the hereditary Rb, mutations can be diagnosed using Rb panel or whole exome sequencing of DNA from blood samples.

Recently, accurate molecular diagnosis can be performed through aqueous humor cell-free DNA (cfDNA), which allows for the identification of mutations in *Rb1* gene and can detect mutations in other chromosomes.

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