CHAPTER 2

LITERATURE REVIEW

2.1 Overview of Retinoblastoma

Retinoblastoma (OMIM 180200) is a paediatric cancer (Keith & Webb, 1985). It is a highly malignant tumor of the developing retina, the light-sensitive lining of the eye (Figure 2.1a & Figure 2.1b) (Gallie et al., 1999). It is the most common intraocular tumor that occurs during infancy and early childhood (Zucker et al., 1998). Retinoblastoma accounts for 3 - 4% of all paediatric neoplasms and contributes to approximately 1% of all cancer deaths under the age of 15 years (Albert et al., 2003; Bhagia et al., 2011; Leiderman et al., 2007). Sanchez (2008) estimated that about 14% of all malignancies in infants are represented by retinoblastoma.

Retinoblastoma is principally linked to mutations in the *RB1* gene on chromosome 13 (Vogel, 1979). In a recent report, Sachdeva and O'Brien (2012) stated that it remains unclear why the retina shows high susceptibility to mutation at the *RB1* locus. Available data demonstrates that retinoblastoma is a rare tumor that often arises in a cone cell of the retina, which provides colour vision (Yusof et al., 2010; Lewis 2012). In general, neoplasm or solid tumors, either benign or malignant, are classified into four main groups: epithelial tumor, mesenchymal tumor, neurogenic tumor and germ cell tumor. Of these, retinoblastoma falls in the neurogenic tumor category and it is also malignant (Fredga et al., 1990). The tumor cells are characteristically grouped into clusters known as rosettes (Figure 2.2) (Batterbury & Bowling, 2005).

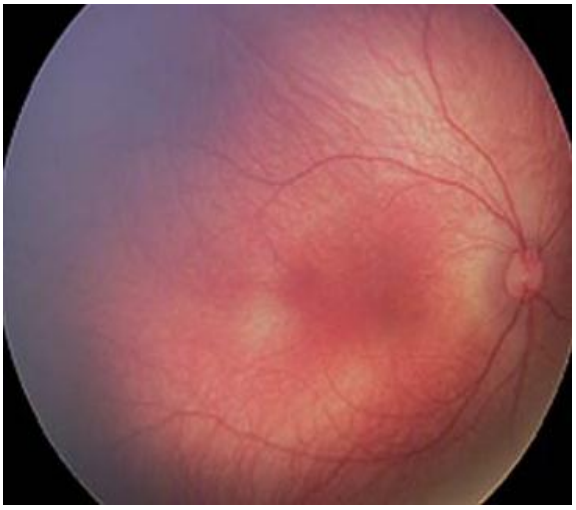

Figure 2.1a : Normal Retina. Source: “Recognizing the Signs of Retinoblastoma”, by C. Juliette, 2009, *Practice Nursing*, *20*(8), p. 396.

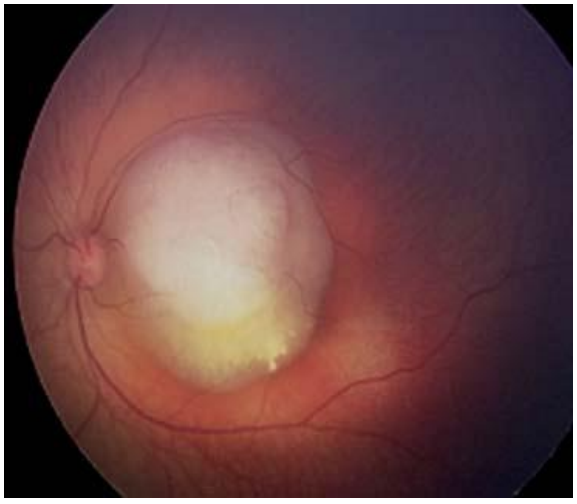

Figure 2.1b : Retinoblastoma Tumor Develops on Retina. Source: *Second Gene Causes Retinoblastoma*, by L. Ricki, 2013, Retrieved July 26, 2013, from http://blogs.plos.org/dnascience/2013/03/21/second-gene-causes retinoblastoma/

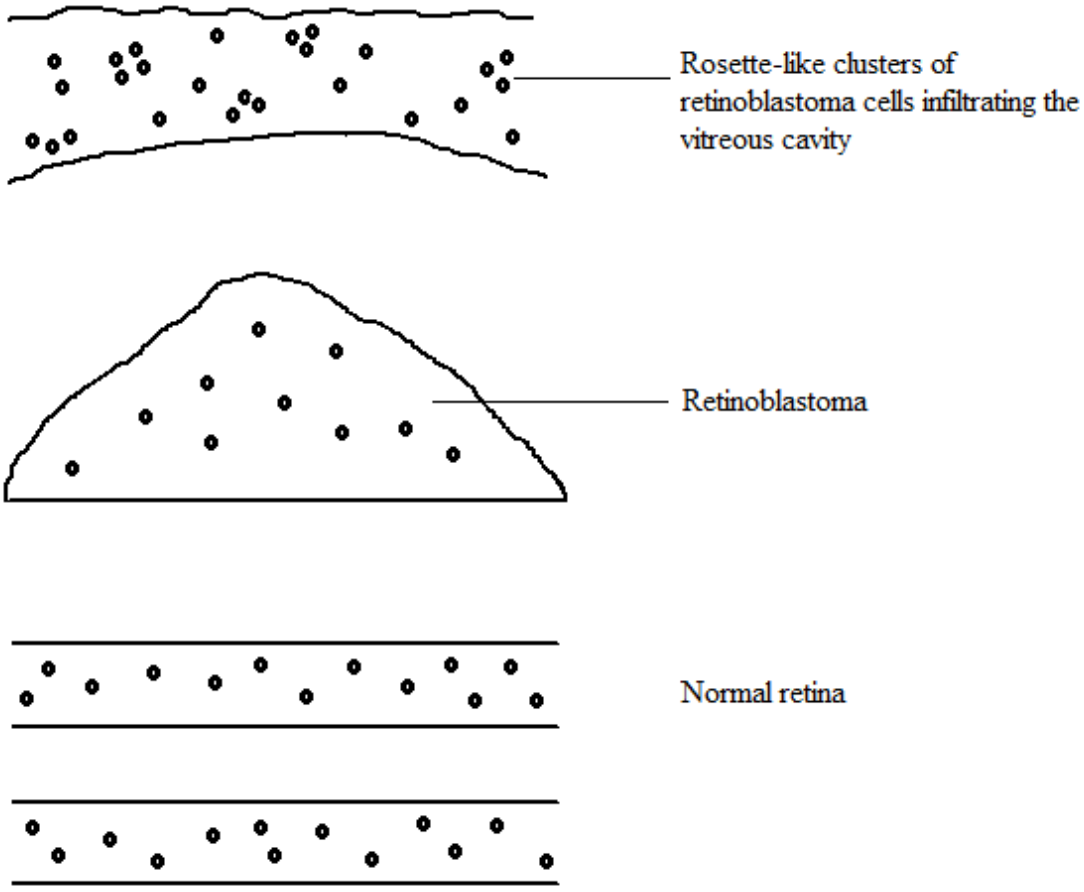

Figure 2.2 : Retinoblastoma. Adapted from *Ophthalmology: An Illustrated Colour Text* (p.19), by B. Mark & B. Brad, 2005, London: Elsevier Churchill Livingstone.

Retinoblastoma may affect one or both eyes of an individual (Knudson, 1971; Vogel, 1979). However, there is no predilection for any eye, i.e., the disease affects each eye equally (Albert et al., 2003). Retinoblastoma develops rapidly in comparison to other cancers that take years or even decades to form (Yusof et al., 2010; Newswire, 2012). When left untreated, retinoblastoma can lead to serious impact, including visual loss and death, with tumors disseminating throughout the retina, optic nerve, brain parenchyma and systemically (Kaufman & Saunders, 2006). Hence, Jamalia and colleagues (2010) described retinoblastoma as an aggressive tumor.

Some children are born with the disease, i.e., having retinoblastoma tumors at birth; whilst in the majority of children, the tumor develops anytime between birth until the age of approximately five years, by which time retinal development is complete (Carter, 2009). Similarly, Yusof et al. (2010) elucidated that the majority of cases are often discovered in children under four years old while Hung et al. (2011) argued that this disease is typically diagnosed much earlier, before the age of three years. Nonetheless, numerous studies reported a general finding that the disorder usually discovered in children under five years of age (Jamalia et al., 2010; Lohmann, 2010).

It is understood that retinoblastoma is a malignant transformation of primordial retinal cells before retinal differentiation. However, these primordial retinal cells disappear within the first few years of life. Hence, retinoblastoma is seldom seen after the age of three or four years. As such, the chances of carriers developing this malignancy later in life are relatively low (Keith, 1978; Quah, 2005). In addition, only a small number of retinoblastoma cases which were diagnosed after the age of five years had been published thus far excluding late presentations (Hung et al., 2011; Jamalia et al., 2010; Mastrangelo et al., 2009). Both males and females are equally affected by retinoblastoma. The disorder has both inherited and acquired forms (Vogel, 1979).

2.1.1 Epidemiology

Retinoblastoma is a relatively rare disease; it has a low incidence rate of 1 in every 15,000 to 20,000 live births (Lewis, 2012; Maricela et al., 2002; Murakami, 1991). Yusof et al. (2010) estimated the cumulative lifetime incidence rate of retinoblastoma as 1 in 18,000 to 30,000 live births worldwide. They reported an estimation of 250 to 300 new cases of retinoblastoma in the United States annually and 5000 new cases worldwide (Yusof et al., 2010; Wilson, 2009). Similarly, Newswire (2012) reported that retinoblastoma is diagnosed in 5,000 children each year worldwide. Nonetheless, these incidence values differ by global region although it has no racial or gender predilection (Leiderman et al., 2007; Zage et al., 2006).

Based on the Hospital Kuala Lumpur Retinoblastoma Registry Report (2004 and 2009), Jamalia and colleagues estimated an average of 14.5 new cases per year seen in Hospital Kuala Lumpur, being the main referral centre for retinoblastoma in Malaysia (Jamalia et al., 2010). In contrast, Ishak and colleagues (2010) believed that there were significant numbers of unreported cases of retinoblastoma in remote rural areas in Malaysia.

2.1.2 Clinical Presentation

Patients with retinoblastoma have four common presentations: leukocoria (56%), strabismus (24%), poor vision (8%) and family history (7%) (Leiderman et al., 2007). A child with retinoblastoma mainly presents a white pupillary reflex (Goldberg, 2000). The white pupillary reflex (leukocoria) is the white tumor in the retina that grows forward behind the lens and thus reflecting light out through the pupil (Rhee & Pyfer, 1999). The second most common sign is strabismus (squint) which may accompany or precede leukocoria (Carter, 2009; Lohmann, 2010).

Jamalia and colleagues (2010) observed that in a cohort of 84 Malaysian patients with retinoblastoma, leukocoria was the primary ocular presentation of the disease (80%) followed by strabismus (14%), proptosis (10%) and other signs (10%). In addition, leukocoria was found to be the most common presenting sign among 50 Malaysian patients with retinoblastoma by Ishak and colleagues (2010). Figure 2.3 shows a child with retinoblastoma, who presented with leukocoria or white reflex in the left eye.

Figure 2.3 : Leukocoria. The arrow shows the white reflex or leukocoria in the left eye of the patient, indicative of retinoblastoma. Source: Retinoblastoma, In *Crossword911.com*, 2013, Retrieved July 26, 2013, from http://crosswords911.com/retinoblastoma.html

2.1.3 Diagnosis

Diagnosis of retinoblastoma is made by examination of the fundus of the eye (Retcam) under general anaesthesia using indirect ophthalmoscopy (Voute et al., 1998). Other diagnostic tools such as computer tomography scan (CTS), magnetic resonance imaging (MRI) and ultrasonography are used for differential diagnosis and staging, whereas histopathology or analysis of tumor material confirms retinoblastoma (Albert et al., 2003).

The average age at diagnosis is 12 months for bilateral and 18 months for unilateral cases of retinoblastoma. Patients with either form of retinoblastoma have an overall 85% to 95% survival rate if diagnosed earlier but a worsening survival with increasing age at diagnosis. In infants with positive family history of retinoblastoma, the disease might be diagnosed early upon investigation performed shortly after birth or periodically by an ophthalmologist (Voute et al., 1998). A delay in diagnosis is often contributed by the retinoblastoma's usual localisation in the posterior pole of the eye (Alvarez et al., 2005).

Jamalia and colleagues (2010) had stated that timely diagnosis and improved treatment methods vastly improved prognosis for vision and survival. They also highlighted that late presentation with advanced stage is a common and a major problem in developing countries. The prognosis is said to be worse for children in developing countries because their cancer is usually advanced by the time it is discovered (Aerts et al., 2006; Jamalia et al., 2010). Intraocular disease beyond that of prognostic value is commonly seen in patients at time of diagnosis in developed countries (Abramson et al., 2003).

2.1.4 Treatment

Retinoblastoma is almost always fatal if the tumor spreads beyond the eye and left untreated. However, it is highly treatable because more than 95% of individuals survive when they are treated before the tumor spreads extraocularly (Shin and Grossniklaus, 2011; Sugano et al., 2004; Turnpenny & Ellard, 2012). Hence, the severity and impact of treatment are dependent on the size of tumor(s) and their location at the time of diagnosis (Carter, 2009).

The treatment of retinoblastoma is aimed at curing the disease whilst preserving the affected eye and maintaining vision, with the lowest possible morbidity. However, enucleation may be warranted if loss of vision is already present at diagnosis. Usually, eyes with large tumor mass and no promising vision are removed or enucleated (Figure 2.4). Although chemotherapy is the first option for a patient with tumors in both eyes, the surgeon and patient may decide to remove the more severely affected eye at presentation. For bilateral disease, the more severely affected eye is enucleated whilst the opposite eye is then treated with local therapy, radiotherapy and/or chemotherapy in order to prevent total blindness in the patient (Abramson et al., 2003; Carter, 2009; Goldberg, 2000; Keith, 1978).

Cryotherapy and systemic chemotherapy are targeted to tumors that require shrinkage, whereas local laser or cryotherapy is applied to treat small tumors (Kaufman & Saunders, 2006; Shields & Shields, 2004). When tumors are extraocular or become resistant to treatment, radiotherapy is considered. Even though radiotherapy is effective in treating retinoblastoma and has been used for decades, it is now not a preferred treatment option for it brings forth unfavourable consequences in later stage of a patient's life. Notably, there is an elevated risk of second cancer especially osteosarcoma to the irradiated site following treatment of retinoblastoma (Nale, 2009).

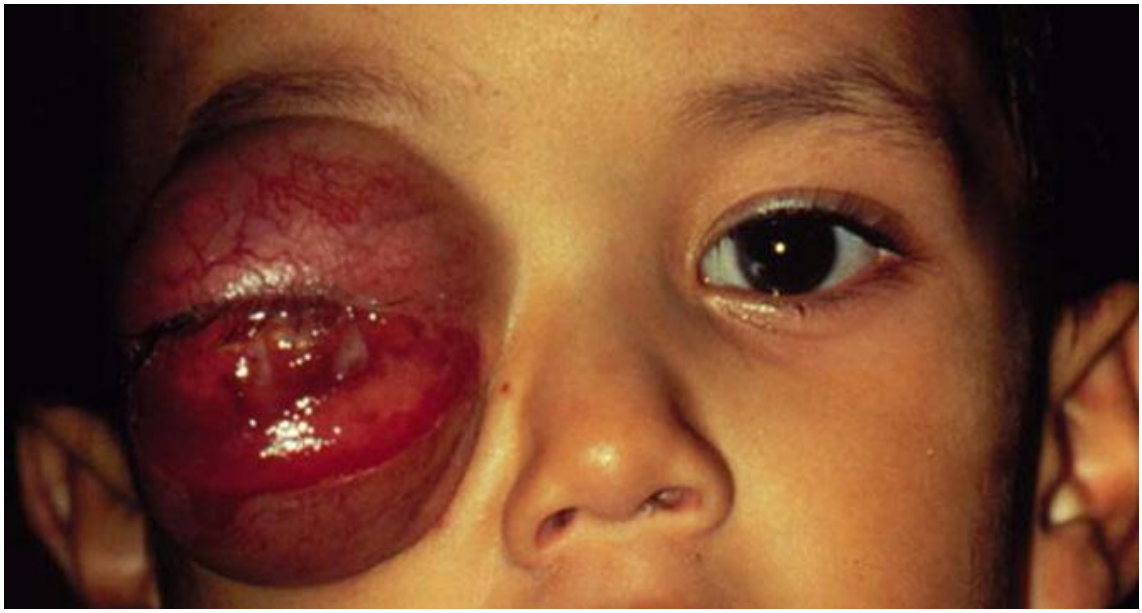

Figure 2.4 : Patient with Unilateral Retinoblastoma. The severely affected right eye with large tumor mass warrants enucleation. Source: Retinoblastoma, In *The Online Atlas of Ophthalmology*, n.d., Retrieved July 26, 2013, from http://www.eyeatlas.com/box/284.htm

2.1.5 Risk of Secondary Malignancies

Wilson (2009) estimated that majority of patients with retinoblastoma (approximately 30%) die from second, third and fourth malignancies rather than from retinoblastoma itself (only 5%). Since the 1970s, numerous reports have been documented on the high risk of second malignancies among survivors of retinoblastoma (Nale, 2009). In parallel with these findings, a recent review by Lewis (2012) highlighted that mutation in the *RB1* gene as causing other cancers.

Many children with retinoblastoma survive into adulthood and are prone to other non-ocular cancers (Serrano et al., 2011). *RB1* mutation carriers have a lifelong predisposition to non-ocular cancers, namely, osteosarcoma, melanoma and brain tumors (Abramson & Frank, 1998). Similarly, Genuardi and associates (2001) and Lohmann (1999) discovered second primary neoplasms, including bone and soft tissue sarcomas, malignant melanoma and neoplasms of brain and meninges in carriers of an *RB1* gene mutation and highlighted the increased risk of mortality from these second malignancies in patients with bilateral retinoblastoma. In an investigation of second malignancies among 14 patients with a history of bilateral retinoblastoma, Rubin and colleagues (1997) identified 17 locally aggressive tumors such as osteosarcoma, malignant fibrous histiocytoma, high-grade spindle cell sarcoma, malignant mesenchymoma, leiomyosarcoma and angiosarcoma in various parts of the body, particularly in the facial structures and in the lower extremities. This was in accordance with Carter's (2009) statement that second primary tumors may develop in any part of the body.

Approximately 60% of retinoblastoma cases are non-hereditary. In addition, almost all non-hereditary cases present as unifocal tumor in a single eye. These unilaterally affected patients are not at increased risk for developing second cancer later in life (Albert et al., 2003). On the contrary, patients with heritable retinoblastoma possess high risk of developing second new malignancies, namely, osteosarcoma, fibrosarcoma and chondrosarcoma (Turnpenny & Ellard, 2012). Nevertheless, these second neoplasms arise only in some patients and thus it was speculated that second tumors were induced by specific germ-line mutations (Genuardi et al., 2001). Furthermore, genetic predisposition and environmental factors also lead to an increased risk of second malignancies among survivors of retinoblastoma (Serrano et al., 2011). Valverde et al. (2005) recapitulated the increased risk for development of second primary tumors in an individual with a *RB1* germ-line mutation, with a cumulative incidence of 22% at the age of 25 years.

Dommering and colleagues (2012) pioneered the study of relation between specific *RB1* germ-line mutations and the risk of second primary malignancies in a cohort of patients with retinoblastoma. In numerous studies, parallels between predisposing *RB1* mutation and incidence of second neoplasm have been observed. Concluding from these findings, Valverde and colleagues (2005) illustrated that most of the second primary tumors were osteosarcomas (37.0%), other sarcomas (16.8%) and melanomas (7.4%), while brain tumors (4.5%), leukaemia (2.4%) and non-Hodgkin lymphomas (1.6%) were less frequent. They also highlighted that survivors of hereditary retinoblastoma have a lifetime risk of developing common epithelial cancers.

Pinkerton et al. (2004) and Sampieri et al. (2006) stated that patients with familial *RB* mutations are prone to retinoblastoma only in early life and in adolescence are at increased risk of osteosarcoma. In other words, the occurrence of second cancerous tumors accelerates with time (Gera et al., 1996). Based on past findings and research review, Pauser & Grimm, (2008) found that patients had a tumor free survival of about three decades between retinoblastoma and second malignancies. Gallie and Moore (as cited in Braggio, Bonvicino, Vargas, Ferman, Eisenberg & Seuanez, 2004) explained that patients with constitutional mutations exhibited a higher frequency of secondary tumors in adult life.

The survivors of hereditary retinoblastoma have an increased risk for metachronous malignancy due to prior treatment and genetic susceptibility of *RB1* (Abramson, 1999). Several studies indicated that second cancer arises in patients who receive radiation therapy as part of their treatment. Cowell (1994) emphasized that risk of second tumors is enhanced within the irradiated tissues following radiation treatment. Hence, the risk is greater for patients with germ-line mutations in *RB1* (Cowell, 1994). Bhagia et al. (2011) discovered three retinoblastoma survivors who received prior radiation therapy had developed sinonasal adenocarcinoma. Serrano and colleagues (2011) speculated that radiotherapy increases the risk of a second primary tumor by 3.1 fold, especially development of sarcomas. They also demonstrated that it is essential to consider the *RB1* mutations and genetic predisposition for bone and soft tissue sarcomas among carriers in the absence of radiotherapy treatment. When retinoblastoma is treated with external beam radiation (EBRT), the risk for second malignancies becomes greater than 50% by the age of 50 years (Chen et al., 2003; Shin & Grossniklaus, 2011). Similarly, Lohmann (2010) highlighted the enhancement of risk for second cancer among patients with hereditary retinoblastoma who receive external beam radiotherapy.

Nale (2009) reported that overall risk of second cancer among hereditary retinoblastoma survivors was 20-fold higher than that in the general population. For this, retinoblastoma gene offers a genetic basis for this prediction using molecular diagnostics as a platform. Irrespective of treatment, most children develop second malignant tumor owing to germ-line *RB* gene mutations (Gera et al., 1996). Osteosarcoma is the most common second malignancy observed in survivors of retinoblastoma (Gera et al., 1996). A case report by Pauser & Grimm (2008) highlighted the occurrence of a secondary malignancy identified as intramucosal leiomyosarcoma in a 37-year-old man following hereditary retinoblastoma during childhood. The patient was diagnosed with bilateral retinoblastoma at the age of 1 year old and had his right eye enucleated and his left eye treated with laser. The retinoblastoma was due to a germ-line mutation and there was no further case of retinoblastoma in his family history. Although, neither radiation nor chemotherapy was used in his case, the investigator explained that the secondary malignancy might be due to primary genetic or structural alteration in the *RB1* gene that would have conferred proto-oncogenetic effect on the development of secondary malignancy.

Nale (2009) also reported possible risk of epithelial cancers such as breast, lung and bladder cancer in middle-aged survivors of hereditary retinoblastoma. It is reported that retinoblastoma survivors with bilateral phenotype and with an inherited germ-line mutation stand a higher chance for the risk of second cancer especially melanoma which is a highly malignant tumor due to shared genetic aberrations when compared with those with a *de novo* germ-line mutation (Kleinerman et al., 2012). Prior studies by Kleinerman et al. (2005) reported that long-term hereditary Rb survivors were predisposed to many new cancers in the long run, with radiotherapy being an enhancer of the risk of developing tumors in the radiation field. Their observation showed hereditary patients were at a significantly higher risk for another cancer such as

sarcomas, melanoma and cancers of the brain and nasal cavities when compared to non-hereditary patients (Kleinerman et al., 2005). This work was supported by Chen and colleagues (2003) by correlating *RB1* germ-line mutations to the 500-fold increased risk for sarcoma. They postulated that about 6% of young patients with an *RB1* mutation will develop soft-tissue sarcoma by the age of 18 years.

In a retrospective study, Bhagia et al. (2011) acknowledged that the incidence of second cancers in survivors of both unilateral and bilateral retinoblastoma with *RB1* germ-line mutations have been reported previously. Gera and his team diagnosed a peripheral nerve sheath sarcoma and a spindle cell squamous carcinoma in patients with unilateral and bilateral retinoblastoma respectively (Gera et al., 1996). Lewis (2012) explained that mutant *RB* genes have been identified in patients with breast, lung or prostate cancers or acute myeloid leukaemia but not in patients who never had any eye tumors. She suggested that these occurrences could be due to the expression of the same genetic defect in different tissues.

2.2 Molecular Genetics of Retinoblastoma

2.2.1 Brief History

A Mayan stone from 2000 B.C. was discovered to be depicting a child with retinoblastoma, which was engraved to illustrate a child with an outgrowth from his eye (Lewis, 2005). In 1597, a Dutch anatomist described the eye cancer as a growth equivalent to the "size of two fists". By 1886, researchers had discerned some inherited cases of retinoblastoma (Figure 2.5) (Lewis, 2013; Lewis, 2007). However, the only treatment available for patients in that era was the removal of the affected eye (Lewis, 2012; Sihota & Tandon, 2007). The invention of flash photography aided the parents of the affected children as they could suspect the disease when they notice abnormal white spots in the pupil, from light reflecting off a tumor (Lewis, 2013).

Retinoblastoma was the first cancer to be studied to explain cancer causation. It was the origin of Knudson's idea of "two-hit" hypothesis (Knudson, 1971; Lewis, 2012). In 1971, Alfred Knudson presented the "two-hit" hypothesis based on empirical surveillance of the clinical genetics of retinoblastoma. This theory was pertinent to the role of tumor suppressor genes in human cancer (Alvarez, 2008; Kiaris, 2006). In parallel with this, *RB1* gene was the first tumor suppressor gene to be discovered and isolated in 1986 (Friend et al., 1986). Lewis (2012) stated that *RB* gene and its protein product were identified in year 1987 during when researchers aimed to find the cancer-causing gene in chromosome 13. They were led by the observation in children with retinoblastoma who had deletions in the same region of long arm of chromosome 13. The identification of the gene and the protein enabled the researchers to correlate the cancer with the control of cell cycle. The cloning of this gene substantiated Knudson's theory (Alvarez, 2008).

Figure 2.5 : Untreated Retinoblastoma, Circa 1806. Source: *Second Gene Causes Retinoblastoma*, by L. Ricki, 2013, Retrieved July 26, 2013, from http://blogs.plos.org/dnascience/2013/03/21/second-gene-causes-retinoblastoma/

In the past, the disorder was commonly known as ‘glioma retinae’. Later, it was revised and termed as ‘retinoblastoma’ since malignant proliferations of neuroglia were very rare in the retina (Sihota and Tandon, 2007). According to Sihota and Tandon (2007), retinoblastoma was the first cancer to be directly associated with a genetic abnormality, i.e., deletion or mutation of the q14 band of chromosome 13.

2.2.2 Knudson's Two-Hit Hypothesis

Knudson (1971) introduced the two-hit theory of carcinogenesis to describe the inheritance of retinoblastoma after a thorough epidemiological investigation carried out on large number of cases affected by unilateral and bilateral. With the introduction of this theory, he could explain the different clinical phenotypes of retinoblastoma and the incidence of retinoblastoma in patients with and without a positive family history.

Knudson emphasized that two inactivating mutations are necessary for development of retinoblastoma. He explained that an affected individual with a positive family history had inherited a mutant or non-functional gene that was present in all cells of the individual, known as a germ-line mutation or the first 'hit' (Figure 2.6a). Tumor develops when the second gene at the same locus becomes inactivated somatically in a developing retinal cell. Hence, the second 'hit' is a single cell mutation which occurs during the mitotic cell cycle (Figure 2.6b) (Knudson, 1971).

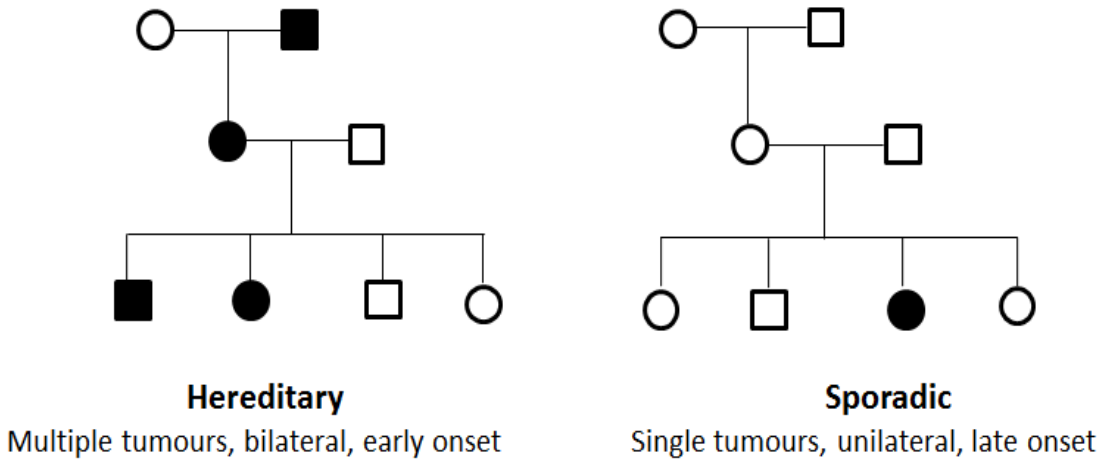

Figure 2.6a : Knudson's 'Two-Hit' Model for Retinoblastoma. Adapted from Retinoblastoma: Knudson, In *Retinoblastoma Genetics*, n.d., Retrieved May 23, 2013, from http://acad.depauw.edu/cfornari_web/DISGEN/retinoblastoma_website /public_html/Knudson.htm

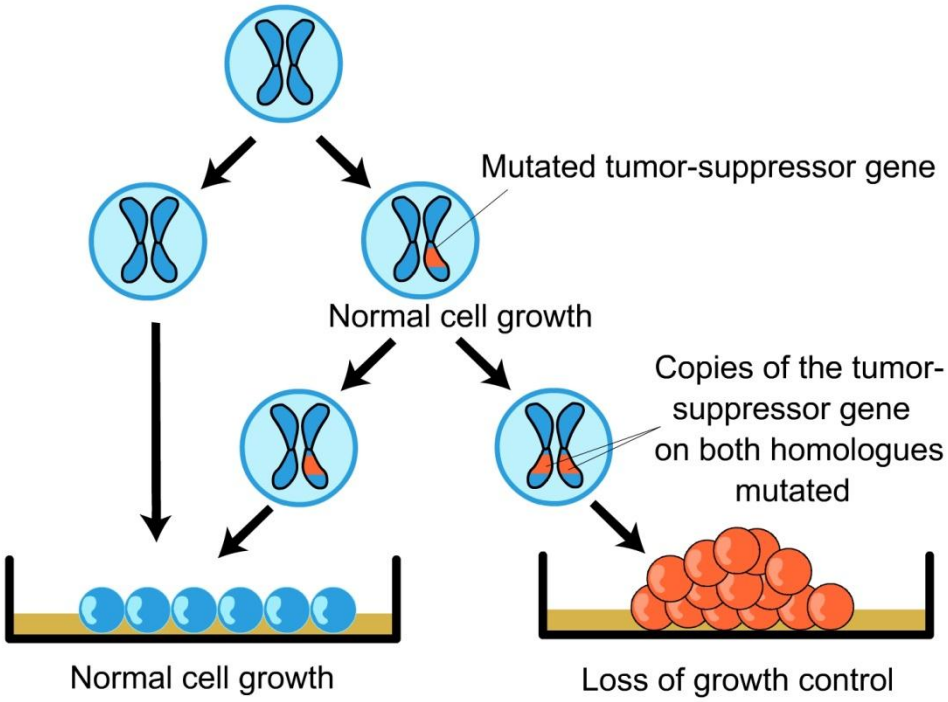

Figure 2.6b : Knudson's 'Two-Hit' Hypothesis. Source: *Retinoblastoma and The RB1 Gene*, by L. Justin, 2009, Retrieved July 26, 2013, from http://lengfeldgen677s09.weebly.com/

In the dominantly inherited form of retinoblastoma, the first mutation occurs in the germ cell and a second mutation is targeted in the retinal cell. On the contrary, in the nonhereditary form, both mutations occur in the retinal cells (Imbach, n.d., Mastrangelo et al., 2009). Naish et al. (2009) explained that approximately 100 retinoblasts are expected to experience a second hit owing to 1 in 1,000,000 chance of a second mutation. As a result, multiple tumors are usually formed in both eyes in the hereditary form of retinoblastoma. On the contrary, there is a small number of individuals who will not undergo the second hit and so will not inherit the disease. If two mutational events are needed for retinoblastoma, then the rate will be 10^{-12}. The incidence of sporadic mutation or single tumor in 1 in 10 000 cases is explained by the number of retinoblasts in an individual that is approximately 10^{8} (Naish et al., 2009).

Loss of heterozygosity is caused by a second mutation which occurs in any of the retinoblasts during embryonic life. As such, retinoblastoma develops in a homozygote retinoblast, carrying two abnormal chromosomes. The rate limiting step for the development of retinal tumors is either deletion or mutational inactivation of one *RB* allele (Vanderluit, Ferguson & Slack, 2006). Recent studies suggest that mechanisms such as chromosomal non-disjunction, gene conversion, mitotic recombination, methylation and second somatic mutations cause somatic loss of the second allele (Zage et al., 2006).

Table 2.1 illustrates the Knudson's 'two-hit' hypothesis explaining the genetic events that lead to non-hereditary and hereditary retinoblastoma (Knudson, 1971; 'Mode of Inheritance', n.d.).

Table 2.1 : Knudson's Two-Hit Hypothesis

In sporadic retinoblastoma	**In hereditary retinoblastoma**
Child starts with two wild type alleles (*RB*+/*RB*+)	Child starts with heterozygous allele (*RB*/*RB*+)
Both alleles must mutate to produce the disease	Only one additional mutation is required to produce the disease
The probability of occurrence of dual mutations is low, so the tumor will be in one eye	Mutations resulting in loss of heterozygosity (LOH) are more probable, so multiple tumors can be present in both eyes

2.2.2.1 Mode of inheritance

The inheritance pattern of retinoblastoma is autosomal dominant (Leiderman et al., 2007). This is because individuals that inherit the gene from an affected parent are likely to be affected too. In contrast, as far as the cell is concerned, it manifests in the recessive manner because the cell has to be homozygous for the abnormal gene in order for the tumor to develop (Naish et al., 2009; Turnpenny & Ellard, 2012). Figure 2.7 and Table 2.2 shows the principles and main characteristics of autosomal dominant inheritance respectively.

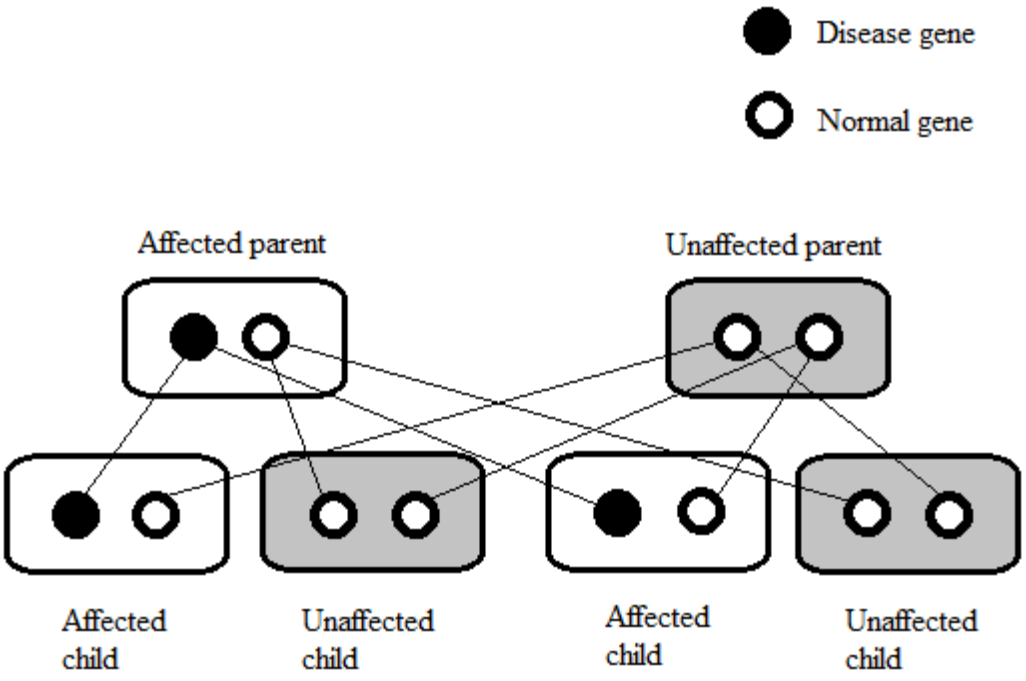

Figure 2.7 : Principles of Autosomal Dominant Inheritance

Table 2.2 : Characteristics of Autosomal Dominant Inheritance Pattern

• Sex-independent – It affects both sexes and can be transmitted by either sex
• One of the affected child's parents will also be affected with the same condition
• A child with an affected parent has a 50% chance of inheriting the disease genotype
• The mutant genotype, when present on only one of the autosomes, is sufficient to cause the disorder

In the case of retinoblastoma, however, it is not necessary that either parents of the affected child has to be affected with the disease because a mutation may occur *de novo* (spontaneously) in the *RB1* in germ-line of a child. Furthermore, inactivation of one *RB* gene is not sufficient to trigger the development of retinoblastoma since the other *RB* gene is still functioning. Thus, the disease only occurs when the second *RB* gene is also inactivated (Naish et al., 2009).

2.2.3 Retinoblastoma Susceptibility Gene (*RB1*)

Neoplasia is a mechanism whereby uncontrolled cell growth occurs, leading to formation of a mass of cells known as a neoplasm or tumor. A malignant neoplasm has the ability to invade adjacent tissues and always metastasise or spread to more distant parts of the body. A number of cancer genes have been implicated in carcinogenesis. They are known to have significant inherited component and/or somatically altered during tumor formation. Those cancer-relevant genes are tumor suppressors, oncogenes and DNA repair genes which are known to be affected by mutations in cancer cells (Weinberg, 1991).

Oncogenes (cancer ‘dominant’ genes) are activated when they harbour single gain-of-function mutation while tumor suppressor genes (cancer ‘recessive’ genes) are affected by two sequential loss-of-function mutations. This assumption forms the fundamental distinction between oncogenes and tumor suppressor genes (Fredga, Kihlman & Bennett, 1990). Similarly, DNA-repair genes are affected by loss-of-function mutations too, albeit the mutated genes have indirect role in cancer initiation and progression (Vogelstein & Kinzler, 2002). Tumor suppressors are also known as recessive oncogenes or anti-oncogenes (Fredga, Kihlman & Bennett, 1990; Morange, 1998; Traboulsi, 2006). As opposed to oncogenes, tumor suppressor genes are cellular genes whose normal function is to suppress inappropriate cell proliferation (Turnpenny & Ellard, 2012). Hence, it is also known as negative modulator (Leiderman et al., 2007).

Macdonald and Ford (1991) elucidated that the evidence for the existence of tumor suppressor genes came from studies of hereditary cancers, which exhibited clear pattern of inheritance, usually autosomal dominant, with a tendency for earlier age of onset than for sporadic tumors. Subsequently, the tumor suppressor gene was first described in 1971 by Alfred Knudson in the effort of validating his ‘two-hit’ hypothesis (Knudson, 1971). Vogelstein and Kinzler (2002) had highlighted that more than 20 tumor suppressor genes have been localized and identified thus far through various experimental approaches. Table 2.3 enlists some cancer syndromes caused by mutations in the tumor suppressor genes. Within neoplasms, the most common tumor suppressor genes with mutations are *TP53* and *RB1* (Hesketh, 1995; Kemp et al., 2008; Naish et al., 2009).

Table 2.3 : Cancer Syndromes Due to Tumor Suppressor Mutations

Disorder	**Gene**	**Locus**
Retinoblastoma	*RB1*	13q14
Familial adenomatous polyposis	*APC*	5q31
Li-Fraumeni syndrome	*TP53*	17p13
von Hippel-Lindau syndrome	*VHL*	3p25-26
Multiple endocrine neoplasia type II	*RET*	10q11.2
Breast-ovarian cancer	*BRCA1*	17q21
Breast cancer	*BRCA2*	13q12-13
Gastric cancer	*CDH1*	16q22.1
Wilms tumor	*WT1*	11p13
Neurofibromatosis I	*NF1*	17q12-22

RB1, the prototype of tumor suppressor gene was first isolated by positional cloning (Friend et al., 1986). Table 2.4 enlists some identified pathological genes in chronological order, among which *RB1* was first to be discovered in 1986 (Friend et al., 1986; Chen et al., 2010; Sumner & Chandley, 1993; Wikenheiser-Brokamp, 2006).

RB1 gene is permanently inactivated by loss-of-function mutation. This allows uncontrolled cell division to ensue. In other words, absence of the *RB1* gene product in the homozygous state leads to the development of tumor or retinoblastoma (Naish et al., 2009). It is well documented that retinoblastoma gene is also inactivated in many other cancers, i.e., familial retinoblastoma, osteosarcoma, breast cancer and small cell lung carcinoma (Hesketh, 1995; Kemp et al., 2008). The deregulation of this gene has been considered to be one of the hallmarks of human malignancies and thus became the focus of many cancer research (Wikenheiser-Brokamp, 2006). Besides, Naish et al. (2009) stated that this gene which was found on long arm (q) of chromosome 13 was the first gene to be studied in detail. Figure 2.8 shows an ideogram which represents the human chromosome 13.

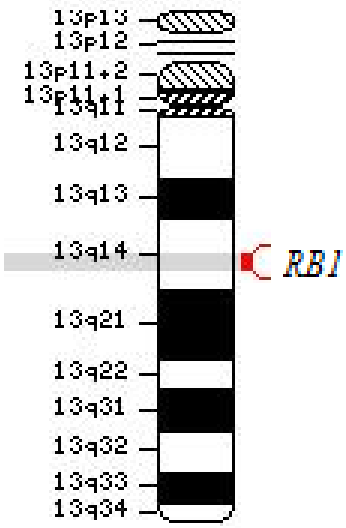

Figure 2.8 : Ideogram Illustrating Human Chromosome 13. Reprinted from 13q14.1q14.2, In *Retinoblastoma Genetics*, n.d., Retrieved May 23, 2013, from http://academic.depauw.edu/cfornari_web/DISGEN/retinoblastoma_website/public_html/Molecular%20Genetics.htm

Table 2.4 : Disease Genes Cloned by Gene Mapping

Year	Disease	Regional Assignment	Associated Chromosome Aberration	Local Candidate Gene Unknown
1986	Chronic granulomatous disease	Xp21.1	Deletion	No
	Duchenne muscular dystrophy	Xp21.2	Deletion, X-AUT	No
	Retinoblastoma	13q14.2	Deletion	No
1989	Cystic fibrosis	7q31-32	No	No
1990	Wilm's tumor/ Drash syndrome	11p13	Deletion	No
	Retinitis pigmentosa IV	3q21-24	No	Rhodopsin
	Neurofibromatosis I	17q11.2	Deletion, Translocation	No
	Malignant hyperthermia	19q13	No	Ryanodine receptor
	Testis determining factor	Yp11.3	X-Y Interchange	No
	Li-Fraumeni syndrome	17p13.1	No	p53 protein
	Choroideraemia	Xq21.1	Deletion	No
1991	Alzheimer disease	21q21.2	No	Amyloid P.P.
	Retinitis pigmentosa	6p12	No	Peripherin
	Fragile X syndrome	Xq27.3	Fragile site	No
	Familial adenomatous polyposis	5q21	Translocation	No
	X-linked spinal muscular atrophy	Xq11.2	No	Androgen receptor
	Marfan syndrome	15q21.1	No	Fibrillin
	Aniridia	11p13	Deletion	PAX-6
	Kallmann syndrome	Xp22.32	Deletion	No
1992	Waardenburg syndrome	2q35-37	Inversion	PAX-3
	Myotonic dystrophy	19q13.3	No	No
	Charcot-Marie-Tooth disease	17p11.2	No	Peripheral Myelin P.
	Norrie disease	Xp11.4	Deletion	No
	Lowe's oculocerebrorenal syndrome	Xq25-26X-AUT	No	

Note: From Chromosomes Today (p.13), by A.T. Sumner & A.C. Chandley, 1993, London: Chapman & Hall.

Upon the discovery of *RB* gene, Toguchida and his team (1993) sequenced this model tumor suppressor and published the complete genomic sequence of the human retinoblastoma gene to facilitate oncogenic studies as well as other studies. The sequence data of human retinoblastoma susceptibility gene is found at GenBank Accession No. L11910 (Toguchida et al., 1993).

2.2.3.1 Structure of RB1

Retinoblastoma 1 or *RB1* gene is found on the long arm of chromosome 13, located within chromosome band 13q14.2 (Friend et al., 1986). It has a promoter of about 1.5 kb. The gene has 27 exons across 183 kb (Toguchida et al., 1993). The 27 exons are clustered into three groups, each group separated by two relatively large introns (36 kb and 70 kb long respectively). Table 2.5 shows various genes that vary greatly in length with *RB* gene (Mange, E.J. & Mange, A.P., 1999). In *RB1*, CpG-island is normally found unmethylated at the 5' end. As for the promoter region, no TATA or CAAT elements are found except binding motifs for transcription factors such as Sp1 and ATF (Alvarez, 2008).

2.2.3.2 Function of RB1

The transcription of this gene yields a 4.8 kb messenger RNA (mRNA) that is finally translated into a protein, comprising 928 amino acids (Hung et al., 2011). This gene product is known as nuclear phosphoprotein (pRb or pRB) that weighs approximately 110 kDa. It plays a vital role in the control of cell proliferation by suppressing uncontrolled cell growth (Zhang et al., 2004). Burkhart and Sage (2008) added that *RB1* also has vital roles in regulation of apoptotic cell death, maintenance of permanent cell cycle arrest and preservation of chromosomal stability. Similarly, Serrano et al. (2011) specified that retinoblastoma susceptibility gene (*RB1*) has critical function on G1

checkpoint and regulation of cellular differentiation. Notably, RB has a critical role in regulation of gene expression. Despite being weak and non-specific, RB exhibits strong DNA binding activity by interacting with a couple of sequence specific DNA-binding transcription factors (Markey et al., 2007).

Alvarez (2008) denoted *RB1* as the dominant family member expressed in the developing human retina. Sihota and Tandon (2007) speculated *RB1* gene as the gene responsible for controlling retinal cell division. Being a potent inhibitor of cell cycle proliferation, the functional or bi-allelic inactivation of *RB1* gene causes the loss of *RB* which predictably promotes the hyperplastic proliferation associated with tumorigenesis (Markey et al., 2007). In children with retinoblastoma, retinal cell division occurs whereby it continues unchecked, causing the retinal tumor(s).

Table 2.5 : Classes and Length of Genes

Size class	Gene or gene product (disease)	Length of whole gene (kb)	Length of exons in kb (% of entire gene)	Number of introns
Small				
	Transfer RNA gene	0.2	0.2 (100)	0
	Histone H4	0.5	0.5 (100)	0
	β-globin (β-thalassemia; sickle-cell disease)	1.5	0.6 (38)	2
	Insulin (diabetes)	1.7	0.4 (33)	2
	Apolipoprotein E (Alzheimer disease)	3.6	1.2 (33)	3
Medium				
	Collagen type 1,α-1 chain (osteogenesis imperfecta)	18	5.0 (28)	50
	Albumin	25	2.1 (12)	14
	Adenosine deaminase (ADA deficiency)	32	1.5 (5)	11
	Clotting factor IX (Christmas disease)	34	2.8 (8)	7
	LDL receptor (hypercholesterolemia)	45	5.5 (17)	17
Large				
	Phenylalanine hydroxylase (PKU)	90	2.4 (3)	12
	BRCA1 (breast cancer)	100	5.6 (6)	24
	Factor VIII (haemophilia)	186	9.0 (3)	26
	RB1 (retinoblastoma)	200	2.8 (1)	27
	Cystic fibrosis transmembrane regulator (cystic fibrosis)	250	6.5 (2)	26
Giant				
	Dystrophin (Duchenne muscular dystrophy)	>2,000	16.0 (1)	>60

Note: From *Human Pedigrees*, In Basic Human Genetics, by J.M. Elaine & P.M. Arthur, 1999, Massachusetts: Sinauer Associates, Inc., p. 175.

2.2.4 Retinoblastoma Protein

The retinoblastoma protein (pRb) is the ultimate product of *RB1* gene (Figure 2.9). It has a molecular weight of approximately 110-kDa (Poznic, 2009). The role of retinoblastoma protein was initially comprehended based on bi-allelic inactivation in the childhood cancer, retinoblastoma (Markey et al., 2007; Poznic 2009). It was discovered that loss of retinoblastoma protein in progenitor retinal cells causes Rb (Sherr, 1996).

pRb is one of the key cell-cycle regulating proteins. It regulates critical G_1-to-S phase transition. It limits cell proliferation by arresting cells in the G1 to S phase of the cell cycle. This involves interaction of retinoblastoma protein with E2F family of transcription factors. These cell-cycle transcription factors repress transcription of genes required for cell-cycle check-point transition. In other words, Rb proteins are regulated by network sensing intracellular and extracellular signals which function to block or permit phosphorylation (inactivation) (Taya, 1997; Weinberg, 1995).

Being a cell cycle regulator, Rb protein is expressed in many tissues or most cell types and regulated in a cell-cycle-dependent manner. The protein can be detected in proliferating, quiescent and differentiated cells (Wikenheiser-Brokamp, 2006). pRb is described as a master regulator of the cell cycle for exhibiting control over several subordinate proteins. An example of this interaction is the reciprocal action between pRb and E2F family of transcription factors which permits entry into the S phase of the cell cycle (Weinberg, 1995). In addition to being a cell cycle regulator, it is also known to regulate other cellular processes such DNA replication, mitosis, DNA repair, DNA damage checkpoint control, cellular senescence, differentiation and apoptosis (Figure 2.10) (Fan & Steer, 1999; Sachdeva & O'Brien, 2012; Wikenheiser-Brokamp, 2006).

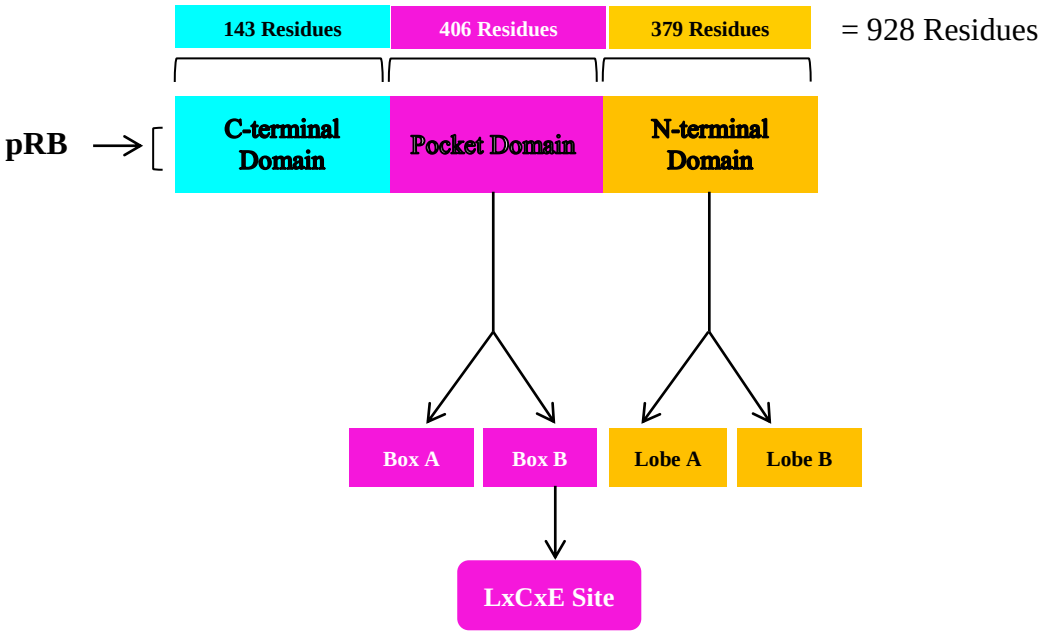

Figure 2.9 : pRB Structure. Adapted from *Retinoblastoma Protein*, by L. Ariel, 2008, Retrieved July 26, 2013, from http://maptest.rutgers.edu/drupal/?q=node/218

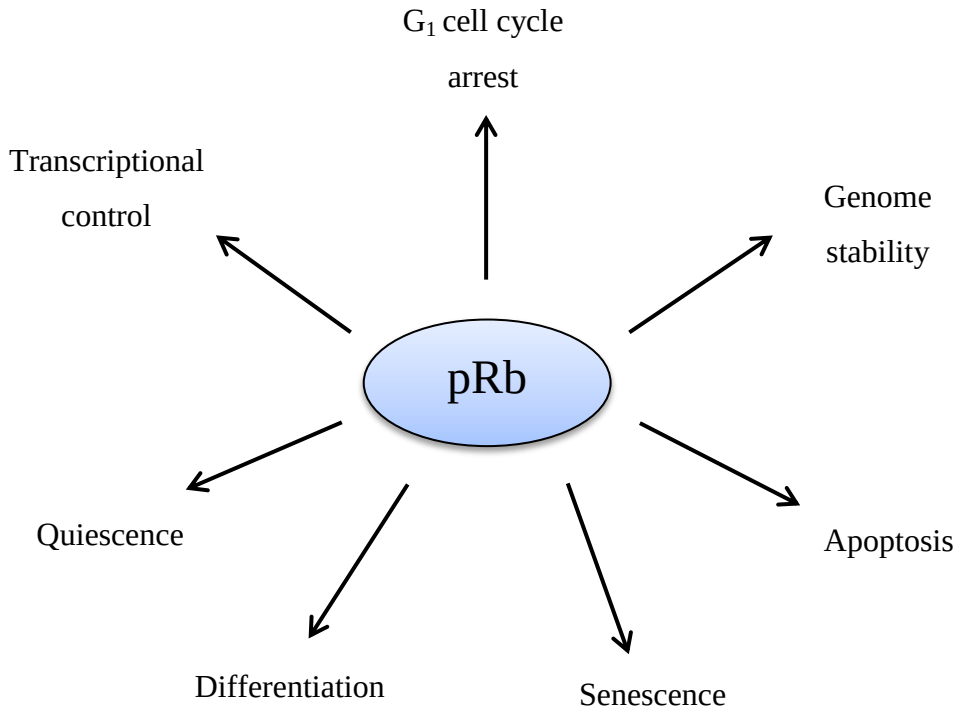

Figure 2.10 : Cellular Functions of pRb. Adapted from Understanding pRb: toward the necessary development of targeted treatments for retinoblastoma, by M.S. Uma & M.O. Joan, 2012, *Science in Medicine*, *122*(2), p.427

Specifically, Rb protein belongs to the 'pocket protein' family. All members of this protein family have a highly conserved sequence in the pocket domain. In general, proteins containing LXCXE motif are known to bind to A and B pocket domains (Fan & Steer, 1999). The pRb pocket domain is encoded by exons 21 and 22 while the C-terminal region is encoded by exon 23 of *RB1* gene. The highly conserved pocket domain is thus critical for biologic function (Henley et al., 2010; Wikenheiser-Brokamp, 2006). pRb polypeptide is homologous to other paralogues, i.e. p130 and p107 (Alvarez, 2008; Goodrich, 2006). Collectively, these polypeptides are grouped into RB family of proteins (Alvarez, 2008). Being part of a 'tumor surveillance'

mechanism, these proteins can actually supress tumorigenesis (Dannenberg & Riele, 2006). Sharing some functional domains, these proteins have coinciding functions in regulating growth control during development (Alvarez, 2008) eventhough p107/p130 and RB are distinguishable in features such as differences in expression pattern, interactions with the various E2F family members and associations with cyclin/cdk complexes. In comparison with p107 and p130, *RB1* is commonly mutated in human cancers (Burkhart & Sage, 2008).

In general, the eight E2F family members are divided into two common subgroups: 'activating' and 'repressing' E2Fs. Rb protein preferentially interacts with the 'activating' E2Fs even though it can associate with both subgroups (Wikenheiser-Brokamp, 2006). Each of these proteins has distinct N-terminal, C-terminal and intervening A/B domains (Szijan *et al*., 1995). Of particular interest, A/B domain (small pocket) is conserved in all the gene family members. The small pocket and a part of C-terminus form the large pocket which facilitates interaction with endogenous proteins that mediate cellular growth and differentiation (Alvarez, 2008; Fan & Steer, 1999; Matsumoto et al., 2003).

Cyclin-cdk complexes phosphorylate pRb by recognizing localization signal and cyclin-cdk interaction motif at the C-terminal region of pRB. In this case, hypophosphorylated pRb can sequester a number of transcription factors by binding to them and inhibit cell-cycle progression. On the other hand, hyperphosphorylated pRb permits the liberation of bound E2F family members and therein access into the S phase of the cell cycle. To be precise, pRb binds specifically to the transactivation domains of E2F polypeptides. These domains act to mediate the binding between E2F and E2F-binding sequences of DNA found in promoters of genes associated with cell cycle regulation (Alvarez, 2008; Henley & Dick, 2012).

2.2.4.1 Effect of mutation on retinoblastoma protein

The mutations of *RB1* gene affect pRb or proteins that phosphorylate pRb, yielding hyperphosphorylation of pRb (Kemp et al., 2008). In the presence of mutant $p110^{RB}$, retinoblasts fail to differentiate normally (Turnpenny & Ellard, 2012). Poznic (2009) stated that sporadic cancers arise as a result of disruption of the sequence in the RB-coding gene that encodes the central 'pocket' domain of RB protein. Consequently, this yields an inactivating effect on the RB protein (Poznic, 2009). On this basis, oncogenic transformation takes place when alteration that inactivates RB protein occurs. When RB protein gets inactivated, a constant transcription of E2F-controlled genes involved in progression of the cell cycle causes uncontrolled cell proliferation (Dunn et al., 1989). An example of effects of point mutations in *RB1* gene is shown in Figure 2.11 (Loeb et al., 2003).

Matsumoto and partners (2003) discovered that in more than 90% of retinoblastoma cases in which *RB1* mutations were identified, the large pocket of pRb protein was affected. A truncated retinoblastoma protein is predicted to have lost the large pocket domain and thus results in the increased susceptibility to tumor cell proliferation. This is because a truncated protein cannot regulate cell cycle (Mamatha et al., 2006).

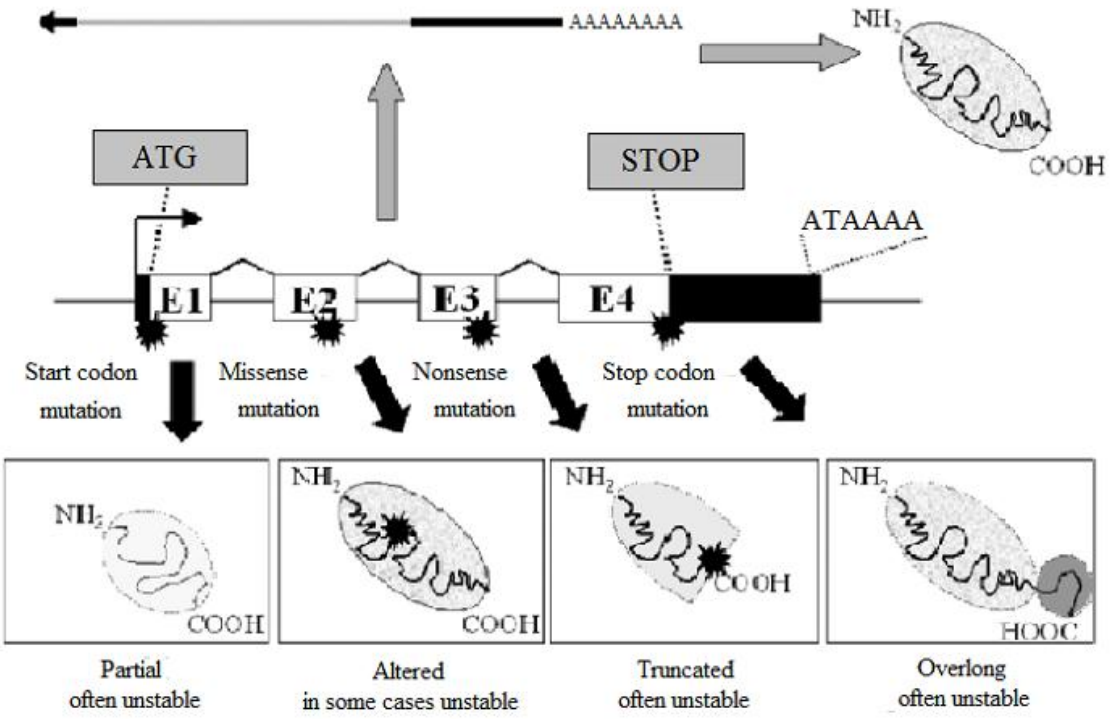

Figure 2.11 : Effects of Point Mutations in the Coding Sequence of a Gene. Source: *Chapter 2: Tumor Genetics* (p. 29), n.d., Retrieved August 31, 2012, from http://link.springer.com/content/pdf/10.1007%2F978-1-402031861_2.pdf

2.2.5 Development of Retinoblastoma (Tumorigenesis)

Mutations in *RB1* may result in either malignant retinoblastoma or benign retinoma (Hung et al., 2011). The tumor spreads throughout the eye, back along the optic nerve, to the brain, through the scleral channels to the orbit and by metastasis to the brain, skull, viscera, bones and lymph glands (Keith, 1978; McConkey, 1993). In the event of loss of heterozygosity in *RB1* gene, two copies of the weak allele are yielded. These alleles are regarded considerably active to prevent tumorigenesis. On the other hand, tumor arises when the second mutation is a null, causing a completely inactivated allele (Hung et al., 2011). So, when both alleles of *RB1* gene are inactivated in embryonal retinal cells, tumor develops (Heslop-Harrison & Flavell, 1993; Kumaramanickavel et al., 2003).

A tumorigenesis process involves subsequent genetic structural aberrations in pathways that control biological processes such as cell proliferation and cell survival. Pertaining to this, it is worth mentioning the key roles of Rb and p53 pathways: the former controls cell proliferation while the latter regulates responses to cellular insults such as DNA damage or oncogenic stress. Nevertheless, these pathways may be inactivated by alterations in their respective tumor suppressor genes, *RB1* and *p53* (also known as *TP53*) or in genes encoding modulators and/or effectors in these pathways (Laurie et al., 2006).

According to Voute and colleagues (1998), germ-line damage causes high susceptibility to cancer, and generally the targets of damage are tumor suppressor genes. Because the second copy of the tumor suppressor gene usually remains normal and active, tumorigenesis is suppressed and the development of the embryo is normal, due to the recessive character of the alteration. Tumorigenesis manifests when the genome of a somatic cell is affected (Voute et al., 1998).

2.2.6 Frequencies of Various Types of Retinoblastoma

Table 2.6 : Frequencies of Various Types of Retinoblastoma (Quah, 2005)

	Retinoblastoma		
	Unilateral	**Bilateral**	**Unilateral, multifocal**
Somatic	85%	0%	0%
Germ-line	15%	100%	100%
Frequency	60%	40%	

Quah (2005) observed that approximately 60% of retinoblastoma cases were non-hereditary in origin while the remaining 40% were hereditary (Table 2.6). The former requires two postzygotic mutations in the retinal cells for retinoblastoma to arise. Since it is rare to acquire two spontaneous somatic mutations affecting the same gene in a single cell, the tumors that occur are often unifocal and unilateral that appears later in life. In the case of unilateral and unifocal retinoblastoma where the eye is not removed, the frequency of the patient possessing a germ-line mutation is only about 15% (Rasheed et al., 2002). On the other hand, individuals with hereditary form of retinoblastoma are predisposed to early onset and multiple retinoblastoma tumors in both eyes. Thus, almost all bilateral tumors are hereditary. Only 10 – 15% of hereditary cases have a family history, the rest being new germ-line mutations which may be transmitted to future generation (Cowell, 1994; Quah, 2005).

2.2.7 Types of Retinoblastoma

Retinoblastoma can develop either sporadically or in a hereditary manner (Knudson, 1971; Vogel, 1979). Both non-inherited and hereditary forms of the disease are caused by mutations in the retinoblastoma gene. In accordance with this, Murakami (1991) predicted that retinoblastoma develops in 90% of carriers with a mutated *RB* allele. It is estimated that approximately 60% of cases are usually sporadic and unilateral, 15% are hereditary and unilateral, while 25% are hereditary and bilateral (Table 2.7) (Tibben, 2010; Vogelstein & Kinzler, 2002). Collectively, 40% of hereditary retinoblastoma is inherited in an autosomal dominant manner (Ashcraft et al., 2007). Table 2.8 shows the common features of each form of retinoblastoma (Naish et al., 2009).

Table 2.7 : Distribution of Retinoblastoma by Type and Laterality

	Bilateral	Unilateral	Total
Hereditary	25%-30%	10%-15%	35%-45%
Non-hereditary	0	55%-65%	55%-65%
Total	25%-30%	65%-70%	100%

Note: From "Retinoblastoma and the Genetic Theory of Cancer: An Old Paradigm Trying to Survive to the Evidence", by M. Domenico, H. Theodora, D.F. Sonia & L. Cosimo, 2009, Journal of Cancer Epidemiology, 2009, p. 2.

Table 2.8 : Common Characteristics of Sporadic and Inherited Retinoblastoma

Sporadic Retinoblastoma	Inherited Retinoblastoma
Parents are normal	Parent is normally affected
No risk to offspring	50% of offspring inherit the disease
Single tumor affecting only one eye	Several tumors, affecting both eyes

2.2.7.1 Non-hereditary retinoblastoma

A retinoblastoma case is classified as sporadic when no other case of retinoblastoma is known in the family medical history (Szijan et al., 1995). Most often, patients with unilateral phenotype have sporadic retinoblastoma (Carter, 2009; Lohmann, 2010). The sporadic form has a later onset of disease if compared with hereditary retinoblastoma (Turnpenny & Ellard, 2012).

Approximately 60% of patients are affected by sporadic or non-hereditary retinoblastoma (Sachdeva & O'Brien, 2012). In these patients, both *RB1* gene mutations are of somatic origins that occur during retinal development (Horsthemke, 1992). The dual mutations arise spontaneously in somatic cells and thus not transmitted to succeeding generation (Lohmann, 2010; Serrano et al., 2011). This is illustrated by a study on 16 Moroccan patients with sporadic unilateral retinoblastoma where mutational screening demonstrated the absence of *RB1* germ-line mutations (Abidi et al., 2011). The mutations are thus not found in other somatic tissues such as peripheral blood lymphocytes but can be detected in tumor material (Macdonald & Ford, 1991). Quah (2005) explained that if either of the two mutations which were identified earlier in the tumor material are found to be absent in the blood, then most likely the child has the non-heritable form of retinoblastoma. In this case, the risk to relatives is likely to be the same as in the general population.

2.2.7.2 Hereditary retinoblastoma

Retinoblastoma is one of the well-described cancer syndromes that have a hereditary component (Knudson, 1974). As such, a child inheriting the single gene predisposing to retinoblastoma will almost certainly develop the tumor (Horsthemke, 1992; Watson et al., 1986). Hereditary form accounts for 40% of overall cases of retinoblastoma. Of all heritable retinoblastoma cases, approximately 25% accounts for the familial form while the remaining 75% represents the sporadic form (Sachdeva & O'Brien, 2012). The familial form of retinoblastoma tends to appear at an earlier age than the sporadic form (Canty, 2009; Turnpenny & Ellard, 2012). Dundar and colleagues (2001) affirmed that about three quarters of hereditary cases are often marked by new or *de novo* mutations.

Lohmann (2010) speculated that the majority of patients with sporadic bilateral and almost all patients with familial retinoblastoma are heterozygous for *RB1* gene mutations, resulting in predisposition to hereditary retinoblastoma. A child with hereditary Rb is heterozygous for an *RB1* mutation that is either inherited from an affected parent or occurred *de novo* in one set of parental germ-line cells or occurred during embryonic development (Dryja et al., 1989). Although all the cells of an individual will be heterozygous for the mutation, not all the retinoblasts form tumors (Naish et al., 2009).

Lohmann (1999) predicted that in families with retinoblastoma, all members that have inherited the mutation are likely to develop bilateral retinoblastoma. In some rare cases or in exceptional families, unilateral retinoblastoma is frequent and some carriers remain unaffected owing to low-penetrance retinoblastoma (Lohmann, 1999). Schubert and colleagues (1997) described familial retinoblastoma as having high penetrance and high expressivity. Familial or germinal retinoblastoma is inherited in an autosomal dominant manner with high penetrance of over 90% (Aerts et al., 2006; Watson et al.,

1987). In line with this, there is a 50% chance that a mutation of the retinoblastoma gene in the germ-line is passed on to a child (Cooper, 1995; Knudson, 1971; Macdonald & Ford, 1991). Thus, patients inheriting *RB1* mutation are prone to develop more tumors because they carry a large number of retinal cells prone to a second *RB1* mutation. Since only one additional mutation is required in these cells and the chances of which are high, hereditary retinoblastoma is often characterised by the presence of multiple tumors in one or both eyes (Aerts et al., 2006; Carter, 2009; Cowell, 1994; Turnpenny & Ellard, 2012). However, Mastrangelo and colleagues (2009) refuted that bilateral Rb is always hereditary as they found 50% unilaterally affected children were born to affected parents in their study cohort.

A child who inherits susceptibility to the disorder will have one germ-line mutant allele for the *RB* gene in each of his or her cells. Cancer develops in a somatic cell where the second copy of the *RB* gene mutates (Knudson, 1971). As such, Lewis (2012) signified that hereditary retinoblastoma requires two point mutations or deletions, one germ-line and one somatic. As a result, the mutation is present in every cell of the body including all retinoblasts of the individual. Hence, the presence of constitutional *RB1* mutation in the blood signifies hereditary retinoblastoma (Quah, 2005).

Mutation and deletion of *RB1* gene causes malignant transformation of a retinal cell. As such, the genotype of an individual with inherited retinoblastoma is either *RB*/*rb* or *RB*/-. On having the genotype *RB*/*rb* or *RB*/-, the risk of developing retinoblastoma is observed to be 100,000 times higher than the general population. In addition, the risk for other types of cancer, especially osteosarcoma is also increased simultaneously (Lohmann, 2010; Therman & Susman, 1993).

2.2.8 Laterality of the Disease

2.2.8.1 Unilateral retinoblastoma

In 60% of patients, the tumor affects only one eye (unilateral Rb) (Buiting et al., 2010). More than 85% of patients with sporadic unilateral Rb have non-hereditary Rb. In these patients, both first and second mutations of the *RB1* gene are found only in the tumor (Cowell & Hogg, 1992). If neither of the two mutations found in the tumor is identified in the proband's blood, then the retinoblastoma is considered to be sporadic (Field et al., 2007). On the other hand, Cowell and Bia (1998) affirmed that patients with only unilateral, unifocal tumors and without a family history are generally considered not to have germ-line mutations.

Unilateral cases are often not transmissible (Knudson, 1971). However, Cowell's and Cragg's (1996) overview on "two-hit" hypothesis of retinoblastoma development suggested that patients with early presentation of unilateral retinoblastoma have predisposing mutations. The non-intermediary germ-line mutations often present as sporadic occurrence of unilateral retinoblastoma, lacking family history of the disease. Thus, it is estimated that approximately 15% of unilateral cases are heritable. Again, those with germ-line mutations have a 50% chance of passing the mutation to their future offspring; second malignancies are imminent in these patients (Knudson, 1971).

2.2.8.2 Bilateral retinoblastoma

Horsthemke (1992) & Murakami (1991) indicated that close to 40% of patients are hereditary cases of retinoblastoma. Dundar and his colleagues (2001) speculated that hereditary form of retinoblastoma is likely to attribute to bilateral phenotype of the disease. By correlating both notions, Buiting and her team (2010) postulated that 40% of patients have tumors in both eyes (bilateral Rb).

Generally, bilateral and multifocal tumors of retinoblastoma are considered severe phenotypes resulting from mutations in the *RB1* gene (Abidi et al., 2011). Owing to *de novo* mutations that arise in the germ-line or embryo, a large number of patients have sporadic bilateral retinoblastoma with no familial transmission (Parsam et al., 2009). Findings by Lohmann and associates (1996) refuted the hypothesis that some bilateral cases are non-hereditary.

Bader et al. (1982) indicated that few children with bilateral retinoblastoma also gradually develop trilateral retinoblastoma or pinealoblastoma. Pinealoblastoma is not a second cancer, but it is a brain tumor similar to retinoblastoma with respect to histological appearance and age at diagnosis. Children with bilateral Rb more often exhibit multiple tumor foci in both eyes. Cobrinik and colleagues (2006) estimated that patients with bilateral disease develop an average of five foci in their first 2 years of age. At the same time, these patients have more or less 1% per year likelihood of developing all other tumor types.

2.2.9 Genotype-Phenotype Correlation of Retinoblastoma

Since the gene involved is *RB* or *RB1* gene, the genotype of a normal person is *RB*/*RB.* Homozygosity or hemizygosity for the allele rb or nullosomy for the locus are prerequisites for tumor development. Thus, possible genotypes of retinoblastoma cells thus are *rb*/*rb*, *rb*/- or -/- (Therman & Susman, 1993).

Leiderman and colleagues (2007) stated that heterozygous carriers of an *RB1* mutation show variable phenotypic expression, i.e., laterality of the disease and degree of multi-focality in each eye. Although tumor predisposition and tumor development are the only phenotypic consequences of *RB1* mutations in human, some carriers do not develop tumors due to incomplete penetrance (Buiting et al., 2010; Leiderman et al., 2007).

These differences are due to the nature of the underlying *RB1* mutation and the chance occurrence of the second somatic mutation (Alvarez, 2008).

It is presumed that specific *RB1* mutations confer variation in phenotypic expression in accordance with the extent of loss-of-function of pRb *in vivo* (Table 2.9) (Alvarez, 2003; Leiderman et al., 2007). Valverde and colleagues (2005) reported that detailed analysis of relation between genotype and phenotypic expression suggest that hereditary retinoblastoma has features of a complex trait. Cowell and Bia (1998) stated that most of the mutations detected in patients with severe phenotype are the result of premature stop codons. Similarly, Kumaramanickavel et al. (2003) elucidated that alleles with premature termination in the coding sequence are responsible for complete penetrance and bilateral disease.

Table 2.9 : Genotype – Phenotype Correlates in Hereditary Rb

Mutation	**Resultant pRb activity *in vivo***	**Rb phenotype (assuming loss of heterozygosity)**
Nonsense	Nil	Hereditary (Multifocal; bilateral)
Frameshift	Nil	Hereditary
Aberrant splice mutation	Exonic - variable Intronic – variable; may yield reduced quantity of functional protein	Hereditary High to reduced penetrance and/or decreased expressivity
Missense and in-frame	Variable – reduced to normal quantities of stable transcript	Reduced penetrance and/or decreased expressivity
Mutations in *RB1* promoter	Variable – reduced to normal quantities of stable transcript	Reduced penetrance and/or decreased expressivity

In an attempt to screen for *RB1* germ-line mutations in 106 patients with hereditary retinoblastoma, Lohmann et al. (1994) identified 27 small length mutations. The majority of these mutations were found to result in premature truncation (Black & Hatchwell, 2002; Cowell, 1994). Although genotype-phenotype analysis of these patients with variable small length mutations did not reveal any significant connection, two patients who had in-frame mutations presented a high count of tumors consistent with normal-penetrance retinoblastoma.

Notably, majority of mutations in patients with bilateral retinoblastoma yield truncated protein. As a result, these patients on an average develop more than three tumors per eye (Kumaramanickavel et al., 2003). Lohmann (1999) speculated that heterozygous carriers of nonsense or frame-shift mutation customarily develop multiple foci in both eyes. In contrary, incomplete penetrance and reduced expressivity are caused by missense mutations, substitutions in the promoter region and some splice-site mutations.

Hereditary Rb marks different phenotypic expressions: the number of eyes affected (no Rb, unilateral Rb and bilateral Rb), age at diagnosis and the development of second tumors later in life (Alvarez, 2008). Phenotypic expression in hereditary Rb varies depending on the functional type of the first mutation or predisposing mutation in the *RB1* gene:

- Loss of function: most germ-line *RB1* gene mutations are point mutations that result in premature termination codons (nonsense, frameshift, splice mutations causing out-of-frame exon skipping) and trigger nonsense mediated decay. Families segregating a loss-of-function mutation almost invariably show complete penetrance and bilateral Rb (Alvarez, 2008; Serrano et al., 2011).
- Partial loss-of-function: patients heterozygous for mutations that do not result in premature termination (regulatory, missense, in-frame) develop fewer Rb foci and families segregating partial loss-of-function mutations often show

incomplete penetrance (low penetrance retinoblastoma) (Alvarez, 2008; Serrano et al., 2011).

In non-hereditary Rb, the spectrum of first somatic *RB1* gene mutations is almost similar to the spectrum of *RB1* germ-line mutations. The spectrum of second somatic mutations, however, is distinct in two respects. First, in about 70% of Rb tumors chromosomal mechanisms such as mitotic recombination have led to loss of heterozygosity and thus demasking of the mutant allele. Second, about 10% of Rb tumors show hypermethylation of the CpG island associated with the regular promoter of the *RB1* gene. In some instances, there are *RB1* carriers who exhibit a low-penetrance phenotype with reduced expressivity (i.e. unilateral and delayed onset tumors) or incomplete penetrance (very few carriers of *RB1* mutations that will develop the disease). The low-penetrance phenotype patients are rather difficult to be distinguished from sporadic retinoblastoma (Alvarez, 2008; Serrano et al., 2011).

2.2.10 Germ-line Mutation and Retinoblastoma

Lewis (2007) estimated the mutation rate of *RB* gene that cause inherited retinoblastoma as 5 to 12 mutations per million gametes. Knudson's "two-hit hypothesis" model proposed that an individual is consequently tumor-prone due to inheritance of cancer-associated gene mutation from any of the parent. The germ-line mutation is thus classified as the first of two hits required for tumorigenesis or tumor development (Knudson, 1971).

Patients with hereditary retinoblastoma have germ-line *RB1* mutations (Knudson, 1971; Vogel, 1979). Ribeiro et al. (1988) believed that patients with familial and bilateral retinoblastoma should possibly carry an *RB1* germ-line mutation. A germ-line mutation is the first inactivating mutation at the *RB1* locus. It may be inherited from an affected

parent or can arise sporadically as a *de novo* mutation. As the foetus develops, *RB1* mutation is propagated throughout the body. Consequently, *RB1* germ-line mutation is present in all cells of the individual. Retinoblastoma is initiated only after the loss of second allele (Knudson, 1975; Vogel, 1979). Tsai and colleagues (2005) speculated that 75% of heritable cases represent new germ-line mutations.

Hung and partners (2011) strongly suggested that high penetrance (90%) is achieved by these germ-line mutations as they segregate as autosomal dominant traits. It is estimated that 50% of patients with retinoblastoma are carriers of heritable germ-line mutations with high penetrance (Table 2.10) (Black & Hatchwell, 2002; Chen *et al*., 2010).

Table 2.10 : Germ-Line Mutation in Association with Family History and Tumor Type

Family history	**Tumor type**	**Probability of germ-line mutation**	**Risk to offspring**	**Risk to siblings**
Positive	Bilateral retinoblastoma	100%	50%	-
Negative	Bilateral retinoblastoma	95%	Assumed to be 50%	Around 3-5% (due to germ-line mosaicism)
Negative	Multifocal, unilateral retinoblastoma	Uncertain	Difficult to determine	Difficult to determine
Negative	Unifocal, unilateral retinoblastoma	5-10%	2-5%	1%

If a mutation is not identified in either parent, then it is termed spontaneous or *de novo* germ-line mutation. Blanquet et al. (1993) and Szijan et al. (1995) concluded that most of the germ-line mutations being implicit to hereditary retinoblastoma are often *de novo* and differ among patients. Similarly, sporadic germ-line retinoblastoma is termed such in the event of unaffected parents and a *de novo* germ-line mutation (Bunin et al., 2012). Bunin and his colleagues (2012) speculated that children born with a *de novo* germ-line mutation in the *RB1* gene have 95% chance of developing retinoblastoma.

Mills and associates (2011) discovered that 85% of new germ-line mutations were paternal in origin before they correlated advanced paternal age with the appearance of *de novo* retinoblastoma in a cohort of retinoblastoma survivors in United States. They hypothesised that a retinoblastoma survivor with a *de novo* germ-line mutation is most likely to have a father of older paternal age as compared with sporadic or familial retinoblastoma survivors and the general population. Their hypothesis was proven true when they found the mean parental age of retinoblastoma survivors with presumed *de novo* mutations was significantly higher than the mean parental age of the general population in United States. When maternal was compared with paternal effect, the latter suggested greater contribution to *de novo RB1* mutations, presumably during gametogenesis. In short, new germ-line mutation, be it small length mutation or major structural alteration, preferentially forms in male rather than female germ cell (Dryja, Morrow & Rapaport, 1997).

Alvarez (2008) concluded that most of the germ-line mutations discovered in families with hereditary Rb were nonsense and frameshift mutations. He recapitulated that these mutations were scattered along exons 1-25 of *RB1* gene. He also pointed out that mutation in internal exons, namely exon 2-25, as causing bilateral retinoblastoma with very limited exceptions. Genuardi and colleagues (2001) acknowledged that most of the germ-line mutations identified were found to result in premature termination codons or

loss of considerable length of the coding sequence. On the other hand, Zage and colleagues (2006) affirmed the common types of germ-line mutations: partial or complete gene deletions, insertions and point mutations. These mutations attribute to premature transcription termination, dysfunctional protein, abnormal transcript splicing and promoter inhibition (Zage et al., 2006).

A germ-line mutation is usually identified by screening the genomic DNA extracted from blood. A screening test for germ-line mutation was carried out by Zajaczek and colleagues (1999) revealed *de novo* aberrations in *RB1* gene in 4 of 17 patients with sporadic unilateral retinoblastoma. Their report mentioned that one of the patients who was initially diagnosed with unilateral retinoblastoma and found to possess *RB1* germ-line mutation had subsequently developed tumor in the second eye after 35 months from time of early diagnosis (Zajaczek et al., 1999).

A person carrying a germ-line *RB1* mutation also possesses higher risk of developing second and non-ocular cancer. Past studies have revealed a 9% incidence of second malignancy by the age of 18 years and an increased risk of about 51% by the age of 50 years. In addition to this, individuals with germ-line mutations appear to be particularly sensitive to radiation oncogenesis, and therefore have an elevated risk of developing an osteosarcoma in any area treated with radiotherapy. Other metachronous malignancies such as soft tissue sarcomas, melanoma, pineoblastoma, lung and bladder cancer are also observed with increased frequency (Field et al., 2007).

2.2.11 Spectrum of Mutations in *RB1* Gene

In spite of large size of the gene and heterogeneity of mutations, *RB1* mutations are continuously being identified and can appear as either gross rearrangements (20%) or small mutations (80%) (Fernandez et al., 2007). Dehainault and associates (2007) professed that all kinds of mutations have been discovered in *RB1* which results in mutational screening to be an extremely challenging task. This is because the majority of *RB1* alterations are unique and distributed over the entire coding sequence.

By the year 2007, more than 500 distinct somatic and germ-line mutations have been identified in the effort of elucidating the genetic aberrations that inactivate *RB1* gene while new mutations were continuously detected (Fernandez et al., 2007). A recent review by Dommering and colleagues (2012) reported that over 600 pathogenic *RB1* mutations have been described thus far. Varverde and team (2005) investigated a spectrum of 932 reported *RB1* mutations and found that deletion and nonsense mutations frequently inactivate retinoblastoma protein although missense mutations are the main inactivating mechanism in most genetic diseases.

Wikenheiser-Brokamp (2006) highlighted that retinoblastoma gene mutations are usually observed in only a small number of human cancers, namely retinoblastoma and small-cell lung cancers. Retinoblastoma susceptibility gene is inactivated in the event of chromosomal deletion, single-nucleotide change, microdeletion, loss of heterozygosity or methylation of promoter region (Dunn et al., 1989, Ejima et al., 1988, Lele et al., 1963). Over the years, retinoblastoma has been associated with a wide spectrum of *RB1* aberrations ranging from single base substitutions in exonic regions (Abidi et al., 2011; Braggio et al., 2004; Hogg et al., 1992; Liu et al., 1995; Rashid et al., 2002; Szijan et al., 1995) and in intron-exon junctions (Braggio et al., 2004), small deletions (Liu et al., 1995; Szijan et al., 1995), small insertions (Liu et al., 1995; Mitter et al., 2009; Szijan et

al., 1995; Hogg et al., 1992), large deletions (Fernandez et al., 2007) and CpG island hypermethylation at the promoter region (Lohmann et al., 2011). In short, Lohmann (1999) concluded that the spectrum of both somatic and germ-line mutations in *RB1* gene are predominantly small mutations. Similarly, in Chinese population, mutations involving short base pairs in *RB1* gene were perceived to be common (Zhang et al., 1997).

Cowell (1994) speculated that missense mutations were rarely found in *RB1*. Maricela and colleagues (2002) supported that the commonly found mutations in this gene were point mutations. Findings by Lohmann et al. (1999) and Valverde et al. (as cited in Mitter, Rushlow, Nowak, Ansperger-Rescher, Gallie & Lohmann, 2009) described that the wide spectrum of oncogenic mutations in the *RB1* gene as heterogenous (Dundar et al., 2001), referring to the various types and locations of mutations (Blanquet et al., 1995; Mitter et al., 2009).

A Brazilian periodical (‘Lab Business Week’, 2006) reported that a wide spectrum of mutations varying from single base substitutions, insertions, deletions as well as small and large deletions were discovered in 36 Brazilian retinoblastoma patients. When 35 unrelated Italian patients with retinoblastoma were screened for novel mutations, the findings reflected a spectrum of mutations predominantly encompassing nonsense or frameshift and splicing mutations (Sampieri et al., 2006). Similarly, mutational screening of 16 Serbian patients affected by retinoblastoma revealed a few small length mutations, varying from nonsense mutations and frameshift deletions. These mutations were predicted to yield inactive truncated retinoblastoma protein lacking one or both pocket domains (Kontic et al., 2006). Abidi and his team (2011) successfully identified 10 germ-line mutations in 25 heritable cases of Moroccan patients with bilateral disease, among which 6 of these were nonsense mutations and thus directly associated to the severity of the disease.

Nonetheless, Mamatha and colleagues (2006) reported nonsense and frameshift mutations as being frequent in 78% of patients, intronic changes in 12% of patients, missense and small in-frame deletions in 8% of patients with retinoblastoma. The remaining 2% of patients were observed to have nucleotide changes in the promoter region. Dommering et al. (2012) stated that mutations in *RB1* promoter were rare. Similarly, Lohmann and team (1996) had not discovered any mutation in the *RB1* promoter in a cohort of 119 patients. Leiderman (2007) explained that mutations in *RB1* promoter sequences result in leaky transcription, yielding diminished quantities of functional proteins and thus lead to reduced phenotypic expression.

RB1 mutations have been discovered in 25 of the 27 exons. Oncogenic point mutations in the region beginning from codon 30 to codon 872 are known to result in premature termination codons (PTC) in exons 1 to 25 (Mitter et al., 2009). In turn, mutations have not been reported in two terminal exons (exon 26 and exon 27) of *RB1* gene thus far (‘Mode of inheritance’, n.d.). Of particular interest, Alvarez (2008) also pointed out the absence of mutations in the region of exons 26 and 27 regardless of having two CGA codons that could be possible hotspots for nonsense mutations. In addition, some studies reported that mutations were also not found in exon 25 of *RB1* (Babenko et al., 2002; Lohmann et al., 1996). Mutations are rarely found in these terminal exons suggesting that the C-terminal domain’s function is less essential for *RB1* as compared to central domains (Babenko et al., 2002). Furthermore, mutations in 3’- terminal region of *RB1* gene may not be oncogenic as suggested by Lohmann and colleagues (1996). In contrary, Mitter et al. (2009) reported the identification of a 2 bp frameshift insertion mutation in exon 27 (the final exon of *RB1* gene), even though no mutations were previously identified in both exons 26 and 27. They reported a point mutation in exon 27 which was found to result in termination at codon 917. Nevertheless, further investigation was not done to confirm whether it was an oncogenic mutant allele.

Concluding from past findings, Mitter et al. (2009) also stated that terminal exons of *RB1* gene rarely possess any oncogenic mutations. They correlated the presence of any mutation to incomplete penetrance and therefore bringing high rates of recurrence in family members.

Blanquet and colleagues (1995) enlisted exons 3, 8, 18 and 19 of *RB1* as preferential spots of aberration in the studied population. Whereas Ishak and team (2010) highlighted the findings of a study conducted in India (Bamne et al., 2005) which had reported somatic and germ-line *RB1* point mutations in exons 3, 17, 20 and 21. Ishak and colleagues (2010) suggested exon 20 as a potential 'hot spot' in the retinoblastoma gene when they discovered a mutation in the flanking region of intron 19.

Braggio et al. (2004) discovered CGA codons and E1A binding domains to be frequent mutational targets and therefore suggested the initial screening of Rb patients to mainly focus on these regions. This is because recurrence of single base substitutions was observed at CpG-dinucleotides that are found in CGA codons or splice donor sites (Lohmann, 1999). Kumaramanickavel et al. (2003) speculated that most of the reported nonsense mutations in the *RB1* gene were recurrent transitions, mainly in CGA codons of the open reading frame. According to Lohmann (1999), patients who are found heterozygous for mutations that result in premature termination codon develop bilateral retinoblastoma with few exceptions. He also highlighted the scarcity of missense mutations and in-frame deletions in *RB1* gene.

Braggio and colleagues (2004) found 10 mutations in 11 patients which characteristically were single base substitutions and occupied coding regions. In addition, eight of these mutations were C to T transitions and were primarily found in CGA codons. This aberration changed CGA (arginine) codons into TGA (stop) codons, resulting in truncated proteins (Lohmann, 1999). Although specific mutational hotspots

have not been declared in the literature (Braggio et al., 2004; Szijan et al., 1995), the afore-mentioned finding is very common and in agreement with the published data of Cooper and Krawczak, Tasheva and Roufa (as cited in Braggio, Bonvicino, Vargas, Ferman, Eisenberg & Seuanez, 2004) that *RB1* gene is not an exception in having CpG dinucleotides as frequent sites of mutation. Obviously, many papers have reported this type of mutation for different CGA codons in the *RB1* gene (Lohmann et al., 1996; Szijan et al., 1995). On the other hand, Shimizu and partners (1994) found a majority (57%) of mutations were residing in E1A binding domains of *RB1* gene. Thus, normal gene products were presumed to be truncated.

Rasheed et al. (2002) did not detect any mutations in about two-third of 21 retinoblastoma cases. These patients were assumed to have hemizygous deletions at the *RB1* locus or alterations outside the coding regions of *RB1*. On the other hand, all the mutations detected were speculated to result in premature termination based on previously reported predominant mutations. In addition, all mutations associated with bilateral disease were germ-line mutations while mutations discovered in patients with unilateral retinoblastoma were somatic (Rasheed et al., 2002).

Available data shows that there is no apparent 'hot spot' for mutations within *RB1* (Rasheed et al., 2002; Cowell, 1994; Ishak et al., 2010). Alvarez and colleagues (2005) discovered 26 novel mutations in 49 patients from Spain, Colombia and Cuba. This observation underlines the fact that no preferential mutation or 'hotspot' in the *RB1* gene (Alvarez et al., 2005). Nevertheless, in spite of its location all these mutations lead to alteration in function of the gene (Ishak et al., 2010).

An investigation by Lohmann and associates (1996) to search for germ-line *RB1* mutations in 119 patients with hereditary retinoblastoma reported a spectrum of *RB1* mutations comprising of 15% large deletions, 26% small length alterations and 42% base substitutions. Furthermore, they indicated that there were no correlations between the location of frameshift or nonsense mutations and phenotypic features such as age at diagnosis and number of tumor foci. In another study, Lohmann (1999) had specified that mutations detected in peripheral blood of patients with hereditary retinoblastoma were of single-base substitution (42%) and small length mutation (26%) types. He discovered that some of the mutations found in *RB1* gene are directly linked with a notable phenotype shown by incomplete penetrance (late onset of disease) and reduced expressivity (affected unilaterally) (Lohmann, 1999).

2.2.12 Clinical Specimen for Germ-Line *RB1* Mutation Screening

The main clinical difficulty is to distinguish the truly sporadic form from the inherited form of retinoblastoma. But this is achieved by conducting mutational analysis of DNA from peripheral blood (e.g., nucleated blood cells) and preferably tumor tissue from patient with retinoblastoma (Mastrangelo, 2003). Constitutional mutations are determined using genomic DNA from peripheral blood leukocytes while somatic mutations are confirmed using DNA from tumor tissue (Rasheed et al., 2002). Examination of retinoblastoma tumor tissue allows identification of genetic mechanisms by which both copies of the *RB1* gene are inactivated in the retinal progenitor cell. Concurrently, if any of this mutation is also discovered in peripheral blood, this confirms a germ-line mutation or heritable retinoblastoma regardless of laterality of the disease (Field et al., 2007).

DNA sample from peripheral blood of patients and relatives is preferred over DNA from tumor tissue. This is because DNA extracted from blood or leukocyte (white blood cell containing nucleus) DNA can be used to ascertain or exclude constitutional origin of mutations. For this, blood samples are normally obtained from both parents of patients. In cognizance, patients with hereditary Rb are usually screened for mutations through peripheral blood test (Abidi et al., 2011; Braggio et al., 2004). Alternatively, Murakami (1991) proposed the isolation of mRNA from peripheral blood cells for direct diagnosis of *RB* gene mutations in persons who are likely to inherit mutations in consideration with short period required for the entire procedure of obtaining blood sample and isolation of mRNA.

On the other hand, DNA is usually obtained from tumors of patients with isolated unilateral Rb. The tumor material is obtained at the time of enucleation and stored at -70°C until DNA extraction (Szijan et al., 1995). Many findings show that DNAs from tumor tissue tend to exhaust before patients are completely screened for mutations (Braggio et al., 2004 & Mitter et al., 2009). This finding was plausible as Braggio et al. (2004) discovered DNA isolated from formalin fixed, paraffin wax embedded tumor tissues after histological analysis were present in small quantity and in degraded form. Thus, tumor tissue would be an optimal source of DNA because the integrity of DNA could be severely affected in extracts of embedded tumor tissues.

2.2.13 Molecular Screening for Identification of *RB1* Gene Mutations

Genetic analysis in children affected with retinoblastoma may include the following molecular screening tests: direct search for germinal mutation of the *RB1* gene on the constitutional DNA, identification of intragenic or *RB1* flanking markers omnipresent in all affected family members, and evaluation of loss of heterozygosity via examination of tumor material to define *RB1* alleles (Aerts et al., 2006).

In recent years, multiplex PCR or mPCR are widely used to screen *RB1* gene for mutations because it offers more versatility and saves considerable reagents, time and effort. This is possible by simultaneously amplifying multiple sequences in a single reaction. It is ideal for mutational screening of *RB1* because the gene is large and has no mutation hotspots (Joseph et al., 2005; Orsouw et al., 1996). Joseph and colleagues (2005) argued that mPCR was useful in examining doubtful exonic deletions. Hence, they proposed mPCR as a penultimate to direct sequencing for better mutational screening of *RB1*.

Tibben (2010) stated that current molecular screening techniques as being able to identify about 90% of aberrations in *RB1* gene in bilateral and/or familial cases. In recent years, single-stranded conformational polymorphism (SSCP) analysis is being widely used in the *RB1* tumor suppressor gene mutational screening (Cowell & Cragg, 1996; Liu et al., 1995; Yu et al., 2001). Although direct DNA sequencing approach has been successful in identifying mutations by comparison with the normal sequence, it is further improved by using the single strand conformation polymorphism (SSCP) technique. With SSCP, one can pre-screen the PCR amplified exons before sequencing (Cowell, 1994). However, Liu and his team expressed their disappointment over the overall efficiency of the technique being only 48% in identification of germ-line mutations in patients with bilateral retinoblastoma when single-pass regimen was used

(Liu et al., 1995). With slight modification, Zhang and his colleagues (1997) combined PCR with non-isotopic heteroduplex-SSCP analysis to examine leukocyte DNA, exon-by-exon, for RB1 germ-line mutations excluding restriction endonuclease digestion. They concluded that a higher mutation detection rate was achieved rapidly when a combination of methods were used in comparison with the use of SSCP or heteroduplex analysis alone. A mutation in exonic region is suspected when PCR-SSCP for a particular exon shows bandshift. The mutation can thus be discerned upon sequencing of that particular exon (Yu et al., 2001). Conversely, Braggio and his team (2004) highlighted that SSCP results in 80 – 90% sensitivity, by which some mutations cannot be detected. Even so, SSCP results are usually verified by sequencing (Braggio et al., 2004).

Dundar and his team (2001) successfully discovered novel mutations in a cohort of five patients with sporadic bilateral retinoblastoma and therefore hoisted the approach used in their study, a combination of SSCP and AMD analysis for rapid detection of mutations in the *RB1* gene. Even so, the results were eventually verified by comparison with DNA sequence data. In another study by Hogg et al. (1992), SSCP technique was proven to be the fastest and simplest method for exon by exon screening for mutations in the *RB1* gene which mainly relied upon sequence-dependent migration of single-stranded DNA in a non-denaturing polyacrylamide gel. Nevertheless, DNA sequence analysis aided the revelation of causative mutations and therefore the overall approach was speculated as being powerful for rapid discovery of germ-line mutations in the *RB1* gene and thus suggesting the method to be applied on individuals with *de novo* mutations (Hogg et al., 1992). Furthermore, Murakami (1991) had developed a simple and rapid method to spot aberrations directly in retinoblastoma mRNA, namely, single nucleotide substitutions using an established combination of PCR and SSCP coupled with reverse transcriptase (RT) reaction (RT-PCR-SSCP). Maricela and her team (2002)

who were the first to conduct molecular study on *RB1* gene mutations in Mexican patients also preferred SSCP over other available molecular tools and revealed new mutations that caused frameshifts in ORF in all 19 cases they handled.

Analysis of *RB1* gene using RFLP is known to be useful in identifying the allele with a mutation. However, Shimizu and associates (1994) refuted the suitability of RFLP for identification of new germ-line mutations in hereditary cases as well as germ-line mutations in unilaterally affected patients. Parsam and colleagues (2009) demonstrated that molecular techniques such as fluorescent quantitative multiplex PCR, fluorescent genotyping of *RB1* alleles, PCR-RFLP and sequencing were feasible approaches and appropriate for identification of large deletions/duplications, small deletions/insertions and point mutations respectively. The combination of these different approaches yielded a detection rate of 83% in cases of bilateral retinoblastoma (Parsam et al., 2009).

RB1 constitutional mutations are highly detectable in hereditary form even though no preferential mutation or 'hot spot' has been identified thus far (Aerts et al., 2006). Blanquet and colleagues (1993) claimed that PCR coupled with denaturant gradient gel electrophoresis (DGGE) as a valuable technique for the detection of germ-line mutations in the *RB1* gene. They opted for this method over cytogenetics and Southern blotting which were generally known to have very poor detection rate of only 15% of constitutional mutations (Blanquet et al., 1993).

In lieu of this, Tsai and his team (2004) demonstrated the use of protein truncation test (PTT) in rapid discovery of *RB1* germ-line mutations. This test was based on the *in vitro* synthesis of protein from amplified RNA and carried out on probands from families with hereditary retinoblastoma. They explained that the test could be potentially used as an initial screen to increase the yield of other molecular screening methods such as sequencing and chromosomal analysis. This was because they had

discovered that focused DNA sequencing revealed presence of mutations in probands who tested negative for germ-line mutations by PTT. On top of this, they also found that PTT when accompanied by DNA sequencing had validated the positive results obtained through former technique.

Mutations located in the non-coding region of the gene are often not detectable using conventional mutation screening methods (Chen et al., 2003). Parsam and associates (2011) recommended cDNA analysis as a valuable option in the event of mutation is not detected by regular DNA analysis and to confirm deletions detected by quantitative PCR. Dehainault and colleagues (2007) reported the first intronic mutation in intron 23 of *RB1* gene. They revealed a 103 bp intronic insertion between exon 23 and 24 which led to subsequent frameshift and premature termination of translation. They also noted that none of the common mutational screening method revealed this mutation in the gene. The intronic exonisation was identified when *RB1* gene was investigated at cDNA level, particularly in hereditary retinoblastoma.

Cowell (1994) implied that a defined system such as molecular screening test for identification of germ-line mutations in patients with retinoblastoma would facilitate the clinical management of this disease. However, Joseph and associates (2005) explained that setting up a single genetic test is not applicable because of the comprehensive size and wide distribution of all types of mutations throughout the retinoblastoma susceptibility gene with no apparent mutation hotspots. Information gained from analysis of spectrum of *RB1* mutations with emphasis on molecular epidemiology and phenotype-genotype relationship is significant for the development of rapid procedures to identify mutations in patients and also to understand the molecular mechanism that leads to tumor development with variable degrees of penetrance or expressivity (Valverde et al., 2005). As such, Chen et al. (2010) employed 3 different molecular methods according to the needs: (1) RNA was extracted from the whole blood and

subjected to reverse transcriptase PCR to study *RB1* transcripts, (2) genomic DNA was used for PCR and direct sequencing to screen 27 individual exons of RB1 gene and (3) allele-specific PCR was performed to confirm the mutation.

2.2.13.1 Advantages of molecular screening in identification of RB1 mutations

The American Society of Clinical Oncologists had identified four maladies as Group 1 disorders: retinoblastoma, Von Hippel-Lindau (VHL) disease, familial adenomatous polyposis (FAP) and multiple endocrine neoplasia (MEN) types 2A/B. Essentially, for Group 1 disorders genetic testing was deemed as a standard part of the management of affected individuals and at-risk family members (Field et al., 2007). Data identified by Jamalia and colleagues (2010) also suggested the need for developing an awareness programme for early detection, to decrease the number of patients with advanced extraocular disease and offer less aggressive treatment with better outcomes.

Genetic testing is essentially a component of standard care for children in families with retinoblastoma. Pertaining to children, germ-line mutation testing for rare tumor susceptibility such as retinoblastoma influences the management of affected individuals and 'at-risk' family members (Field et al., 2007). In other words, a convincing identification of *RB1* mutations in patients with retinoblastoma facilitates and improves clinical management of affected children and family (Benedicte et al., 2006). In addition, the identification of a germ-line mutation in a proband allows predictive genetic testing of other at-risk family members (Matsumoto et al., 2003). Subsequently, individuals known to have inherited the family mutation can be offered an appropriate disease surveillance or prevention programme. In contrast, unaffected individuals can avoid unnecessary molecular screening for themselves and their offspring. Quah (2005) enlisted the main benefits of genetic tests as delineated in Table 2.11.

Table 2.11 : Use of Genetic Testing

1. Facilitates differentiation between hereditary and non-hereditary mutations
2. Identification of the carrier status of parents and risk to siblings are possible which avoids unnecessary and costly screening
3. Identification of mutation in the proband offers suggestion to future offspring to test for the mutation
4. The carrier status of any unborn fetus could be identified

Available data demonstrated that numerous children with heritable retinoblastoma do not exhibit clinical signs or family history suggestive of *RB1* germ-line mutation. As a result, none of the relatives is alerted about the risk of retinoblastoma or risk of other cancers in the affected individual in later life (Chen et al., 2003). Barbosa and associates (2009) affirmed that the nature of a mutation in the *RB1* gene could determine genetic penetrance, disease presentation and prognosis. In particular, the differences between heritable and non-heritable form of retinoblastoma are of great importance for prognosis and genetic counselling of family members. For instance, an affected family could be alerted of the increased risk for second neoplasms in patients with hereditary retinoblastoma (Albert et al., 2003). Furthermore, genotypic information is also important for establishing an accurate molecular diagnosis of retinoblastoma (Barbosa et al., 2009).

The identification of *RB1* gene mutations contributes to preliminary understanding of this neoplasia that primarily affects children. In addition, adequate information obtained from the medical investigation and molecular screening can be effectively used to deliver proper genetic counselling to patients and family members at risk (Maricela et al., 2002). Since the majority or three-quarters of hereditary retinoblastoma cases represent new mutations, the necessity for methods to accurately identify carriers remains vital to render genetic counselling to patients and close relatives (Dundar et al.,

2001). In parallel with this, Fernandez et al. (2007) also suggested that identification of sporadic germ-line mutations is important for risk-assessment in patients' relatives.

Macdonald and Ford (1991) stressed on the importance of early diagnosis and frequent examination among children in families with retinoblastoma to reduce both morbidity and mortality rate. An early identification of susceptibility to retinoblastoma in patients may guide the clinicians on health surveillance and/or timing of prophylactic surgery. In the event of successful detection of inherited cancer predisposition, the clinicians may consider whether the findings warrant further investigation, how to discuss the possibility of inheriting cancer with the affected family and how information gathered from genetic testing may be useful for the management of the extended family. Since the presence of a germ-line mutation in a genetic test clarifies the risk for all family members, an earlier identification of mutations in the *RB1* gene enables a genetic counselling session to include discussion of the chance of a child having affected siblings and affected offspring, and the risk of second cancers in hereditary retinoblastoma (Field et al., 2007). Table 2.12 enlists some important percentages of retinoblastoma genetic events that are often included in the process of genetic counselling (Quah, 2005; Wilson, 2009).

Following successful pre-symptomatic prediction in 2 newborn babies from families with hereditary retinoblastoma which was solely based on retinoblastoma gene diagnosis of exon by exon screening of leukocyte DNA using PCR combined with SSCP and heteroduplex analysis, Minoda (1995) suggested that molecular screening could also be carried out for diagnosis of unilateral cases to facilitate genetic counselling. In a study by Cowell and Cragg (1996), all the mutations discovered in unilaterally affected patients with early onset were reminiscent of those reported in patients with hereditary retinoblastoma. Through their findings, they affirmed that some of these unilaterally affected patients with early presentation convey constitutional

mutations. Thus, they highlighted the importance of genetic screening and counselling of these patients in overcoming the dilemma (Babenko et al., 2002).

Table 2.12 : Genetic Risks in Retinoblastoma

15% chance of an unilateral retinoblastoma patient having an *RB1* mutation
50% chance of a child of the retinoblastoma survivor inheriting the *RB1* mutation
90% penetrance for the gene in cases of hereditary retinoblastoma
10% chance of having the retinoblastoma gene, but being an unaffected carrier
95% accuracy of genetic screening
5% chance of an *RB1* mutation being missed

Note: From *Pediatric Ocular Tumors and Simulating Lesions*, In Pediatric Ophthalmology, by W.W. Matthew, 2009, Berlin: Springer-Verlag, p. 410.

When the cost of conventional screening was compared over cost of molecular testing, Lohmann (1999) recommended molecular mutational analysis to help mitigate the healthcare costs. Similarly, Chen and associates (2003) claimed that genetic testing for *RB1* mutations is much more cost-effective and thus preferred over conventional screening for retinoblastoma in all the family members of an affected child. Fernandez and colleagues (2007) agreed that molecular testing obviates the necessity for any clinical examination under anaesthesia during the first two years of life for children without *RB1* mutation. They explained that this not only helps reduce the cost of healthcare but also improves the quality of life for families with retinoblastoma. A comparison of the costs of molecular versus conventional screening approaches has indicated that mutational analysis will help to reduce healthcare costs (Noorani et al., 1996). However, in spite of numerous clinical advantages, molecular mutational screening of *RB1* gene persists to be difficult owing to unique alterations and their random distribution throughout the entire coding sequence (Benedicte et al., 2006).