GENETIC STUDY OF POCKET A AND B DOMAIN OF RB1 GENE AMONG MALAYSIAN CHILDREN WITH RETINOBLASTOMA

by

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KAJIAN GENETIK DOMAIN POKET A DAN B PADA GEN RB1 DI KALANGAN KANAK-KANAK RETINOBLASTOMA DI MALAYSIA

oleh

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Tesis yang diserahkan untuk
memenuhi keperluan bagi
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LIST OF ABBREVIATIONS

A_{260}/A_{280}	: ratio of 260 absorbance over 280 absorbance
ACCIS	: Automated Childhood Cancer Information System
ALS2	: amyotrophic lateral sclerosis
ANOVA	: analysis of variance
ASA	: allele specific amplification
ATF	: activating transcription factor
bp	: base pair
BRCA2	: breast cancer type 2 susceptibility protein
BRG1	: brahma-related gene 1
Buffer AE	: elution buffer
Buffer BL	: lyses buffer
Buffer BW	: wash buffer
buffer EB	: elution buffer
buffer NW	: wash buffer
buffer PB	: purification buffer
Buffer TW	: wash buffer
C/EBP	: CAAT/enhancer binding protein
Cdc2	: cell division cycle 2
cdk	: cyclin dependent kinase
cdk4	: cyclin dependent kinase 4
CDKN2A	: cyclin-dependent kinase inhibitor 2A
cDNA	: complementary deoxyribonucleic acid
c-myb	: cellular-myb
c-myc	: cellular-myc
CNS	: central nervous system
CT	: computerized tomography
dATP	: deoxyadenine triphosphate
dCTP	: deoxycytosine triphosphate
ddH_2O	: deionized distilled water

DGGE : denaturing gradient gel electrophoresis

dGTP : deoxyguanine triphosphate

dH_2O : distilled water

DHPLC : denaturing high performance liquid chromatography

DNA : deoxyribonucleic acid

dNTPs : dinucleotide triphosphates

dsDNA : double strand deoxyribonucleic acid

dTTP : deoxythymine triphosphate

E1A : early region 1A gene of adenovirus genome

E2 : adenovirus early region 2

E2F : E2 promoter binding factor

EUA : examination under anesthesia

FH : familial hypercholesterolemia

FISH : fluorescent in-situ hybridization

G_0 : gap 0

G_1 : gap 1

G_2 : gap 2

GSTP1 : gluthathione S-transferase pi 1

HCl : hydrogen chloride

HDAC1 : histone deacetylase 1

HGMD : Human Genome Mutation Database

Hi-Di : highly deionized

HPV : human papillomavirus

ICRB : International Classification of Retinoblastoma

IR-RP : ion pair chromatography reverse phase

KCl : calcium chloride

TBE : tris-borate-EDTA

kDa : kilo dalton

LDLR : low density lipoprotein receptor

LOH : loss of heterozygosity

LXCXE : Lys-X-Cys-XGlu

mg/ml	: miligram per mililiter
MgCl2	: magnesium chloride
ml/min	: mililiter per minute
mm	: milimeter
g	: gram
MRI	: magnetic resonance imaging
mRNA	: messenger ribonucleic acid
nm	: nanometer
NP-40	: nonyl phenoxylpolyethoxylethanol
OD	: optical density
PAGE	: polyacrylamide gel electrophoresis
PCR	: polymerase chain reaction
pRb	: retinoblastoma-associated protein
PSEN	: presenilin gene
QMPSF	: quantitative multiplex PCR for short fluorescent fragment
Rb	: retinoblastoma
Rb1	: retinoblastoma susceptibility gene
RBF-1	: retinoblastoma binding factor 1
RFLP	: restriction fragment length polymorphism
RNA	: ribonucleic acid
RP	: retinis pigmentosa
rpm	: round per minute
SD	: standard deviation
SNP	: single nucleotide polymorphism
SSCP	: single strand conformational polymorphism
ssDNA	: single strand deoxyribonucleic acid
Taq	: *Thermophilus aquaticus*
TEAA	: triethylammonium amine
TGGE	: temperature gradient gel electrophoresis
TNM	: tumor-nada-metastasize
TULP1	: tubby like protein 1

UICC	: International Union Againts Cancer
US	: United States
UV	: ultra-violet
µl	: microliter

LIST OF SYMBOLS

<	: less than
>	: more than
^0C	: degree celcius
A>G	: A nucleotide substitute to G nucleotide
G>A	: G nucleotide substitute to A nucleotide
P_1	: proportion of retinoblastoma incidence based on previous study
P_2	: expected proportion of cases based on expert opinion
T>C	: T nucleotide substitute to C nucleotide
z_α	: value of the standard normal distribution cutting of probability α
Z_β	: value of the standard normal distribution cutting of probability β
α	: alpha, level of significance
β	: beta, power of study (1- β)

KAJIAN GENETIK DOMAIN POKET A DAN B PADA GEN RB1 DI KALANGAN KANAK-KANAK RETINOBLASTOMA DI MALAYSIA

ABSTRAK

Retinoblastoma (RB) adalah sejenis kanser mata yang sering terjadi di kalangan kanak-kanak yang berumur empat tahun dan ke bawah dengan kekerapan seorang pesakit di kalangan 15,000 hingga 20,000 kelahiran. Retinoblastoma berlaku dalam dua keadaan samada secara keturunan atau sporadik. Kanser mata yang boleh membawa maut ini memerlukan ketidakaktifan ke dua-dua alel pada gen penyekat tumor, RB1. Hasil penyelidikan terdahulu mendapati mutasi pada gen ini kebanyakkannya berlaku di kawasan domain poket A dan B. Tujuan penyelidikan ini adalah untuk mengenalpasti mutasi di kawasan domain poket A dan B pada gen RB1 dan kaitannya dengan fenotip klinikal pesakit retinoblastoma di Malaysia. DNA telah diekstrak daripada darah 50 orang pesakit retinoblastoma dan 50 sukarelawan normal (kumpulan etnik yang sama dengan kumpulan pesakit) dengan menggunakan kit ekstrak darah komersial sebelum diamplifikasikan melalui kaedah tindakbalas berantai polimerase (PCR) pada ekson 12 hingga 22 pada gen RB1 serta kawasan intron sekitarnya. Mutasi di dalam produk PCR disaring melalui teknik kromatografi cecair denaturasi berprestasi tinggi (DHPLC), yang kemudiannya disahkan dengan teknik penjujukan DNA. Didapati nisbah kanak-kanak lelaki kepada perempuan pesakit RB ialah 2:1 di mana 78% daripada pesakit adalah Melayu, 12% India dan 10% China. Leukocoria merupakan tanda klinikal awal pada sebahagian besar pesakit dan kebanyakkan melibatkan hanya sebelah mata sahaja. Secara purata, usia pesakit ketika diagnosa adalah 24.3 (16.9) [purata (SD)] bulan dan lebih awal iaitu dalam purata 19.47 (19.99) sekiranya kedua-dua belah mata dijangkiti

kanser. Setelah disaring, didapati 31 pesakit dan subjek kawalan mempunyai mutasi dan polymorphism nukleotida tunggal pada gen RB1. Semua mutasi adalah merupakan pertukaran nukleotida tunggal yang terletak di tujuh kawasan berbeza dalam jujukan intron dan juga di kawasan pemotongan intron-ekson. Dua mutasi baru juga telah ditemui iaitu IVS13+184C>T dan IVS17-133T>C di kalangan kumpulan pesakit dan kawalan. Walaubagaimanapun, tiada kaitan statistik yang signifikan ditemui antara semua variasi gen yang ditemui dengan fenotip klinikal pesakit. Namun, kaitan yang signifikan didapati antara IVS15-1G> A dan pesakit dari keturunan etnik China, serta IVS13+184 C> T, IVS17-133T> C dan IVS19-77A>G bagi kumpulan etnik yang berbeza dalam kumpulan kawalan. Berdasarkan penemuan ini, mutasi pada kawasan pemotongan intron-ekson iaitu IVS12+1G>A dan IVS15-1G>A mungkin memainkan peranan dalam mempengaruhi risiko seseorang untuk menghidap kanser ini. Sementara variasi lain mungkin mempunyai kepentingan sebagai penanda variasi genetik dalam populasi. Walaubagaimanapun, bilangan sampel yang lebih besar diperlukan bagi tujuan pengesahan. Sepanjang pengetahuan kami, ini merupakan laporan pertama mengenai mutasi pada kawasan intron domain poket A dan B dalam gen RB1 di kalangan pesakit retinoblastoma di Malaysia. Saringan pada keseluruhan gen RB1 adalah diperlukan bagi melengkapkan pengumpulan data genetik di Malaysia.

GENETIC STUDY OF POCKET A AND B DOMAIN OF RB1 GENE AMONG MALAYSIAN CHILDREN WITH RETINOBLASTOMA

ABSTRACT

Retinoblastoma (RB) is the most common intraocular tumor mainly affecting children under four years of age, with a prevalence of 1 in 15 000 to 20 000 live births. Retinoblastoma is divided into hereditary and non-hereditary types. The development of this malignancy requires both alleles of the tumor suppressor gene RB1 to be knocked-out. Recent studies found mutation occurred more frequently in the pocket A and B domain of RB1 gene. The present study was undertaken to identify the mutation in the pocket A and B domain of RB1 gene among retinoblastoma patients in Malaysia, and the association of the mutation to the laterality and stage of the tumor. DNA was extracted from 50 RB patients and 50 healthy volunteers (ethnically matched to the patient group) blood using commercial blood mini kit prior to PCR amplification which was performed for exon 12 to 22 of RB1 gene and its intronic flanking region. The PCR products were screened for mutation through DHPLC analysis and sample with mutation was subjected to DNA sequencing analysis. The ratio of boy to girl patient were 2 : 1, where 78% were Malays, 12% Indian and 10% Chinese. Most of the patients presented with leukocoria and unilateral (only one eye affected) retinoblastoma. The mean age at diagnosis of patients is 24.3 (16.9) [mean (SD)] months with an earlier diagnosis among the bilateral cases [19.47(19.99)]. Thirty-one patient and control subjects presented with mutation and single nucleotide polymorphism (SNP), which then were identified as single nucleotide substitution which are located at seven distinct intronic regions, where two of the mutations affected splice site region. Two novel mutations were identified

(IVS13+184C>T and IVS17-133T>C) in both patient and control group. No significant association was found either between mutations and laterality or stage of the tumor. Significant association was found between IVS15-1G>A and patient of Chinese origin, while IVS13+184C>T, IVS17-133T>C and IVS19-77A>G were found to be associated with different ethnic groups in the control group. We postulated that the splice site mutations, IVS12+1G>A and IVS15-1G>A might play a role in the predisposition to retinoblastoma, as shown by previous studies. The other five mutations and SNPs are probably important as genetic variant markers for population studies. However, a larger sample size is needed for confirmation. To the best of our knowledge, this is the first report of the mutations found within intronic region of pocket A and B domain of RB1 gene among Malaysian children with retinoblastoma. In order to develop a more complete spectrum of RB1 mutations in Malaysian retinoblastoma patients, we suggest a larger scale of target screening region should be conducted in future research.

CHAPTER 1

INTRODUCTION

1.1 Retinoblastoma

Retinoblastoma (RB), a form of childhood cancer, is the most common intraocular tumor of early childhood, which is usually diagnosed before the age of five (Devesa, 1975). Generally, incidence of childhood cancer is less common than cancer in adult. It is present in less than 1% in industrialized country (Stiller and Draper, 2005). Nevertheless, childhood cancer has claimed the lives of many young children and is the second highest cause of death after road accident. The other most common childhood cancer is embryonic cancer (40%) such as acute leukemia, neuroblastoma, and Wilm tumor (Raja Khuzaiah, 1988).

RB tumor arises from retinal cells, which line the back of the eye (**Figure 1.1**). Retina is composed of 10 layers, with the ganglion cells that lie innermost closest to the lens and front of eye, and photoreceptors that lay outer most of the retina (**Figure 1.2**). The photoreceptor is composed of light-sensitive cells known as rods and cones. The photoreceptor is responsible for converting the light array into electro-chemical

signal that is later sent to the brain through the optic nerve, where they are turned into images.

The formation of retina begins in the early stage of human development. *Retinoblast*, the infantile cells of retina were divided during mitosis and later developed to become mature retinal cells. After that, the development of new retinal cells just occurs to replace the old damaged retinal cells. However, sometimes the divisions of cells can be interrupted, thus affect the production of new cells. In RB disease, the production of new cells cannot be stopped although the body has sent the stop signals. Consequently, the cells keep on growing and multiplying, thus developing the retinal tumor.

RB is caused by genetic mutation in a gene, known as retinoblastoma susceptibility gene (RB1 gene) in the retinal cells. RB1 gene is responsible to encode retinoblastoma phosphoprotein, (known as pRb protein), a regulatory protein that functions in cell cycle activity. pRb protein normally suppresses the cell cycle activity when the cell sends the signal to stop the production of new cells. Presence of mutation in RB1 gene is believed to be the main factor leading to loss of function of pRb protein in cell cycle activity by altering pRb structure, thus initiating the tumor development (Knudson, 1971; Lohmann, 1999). However, until now, the factors influencing the genetic changes in RB1 gene that caused RB still remains unclear.

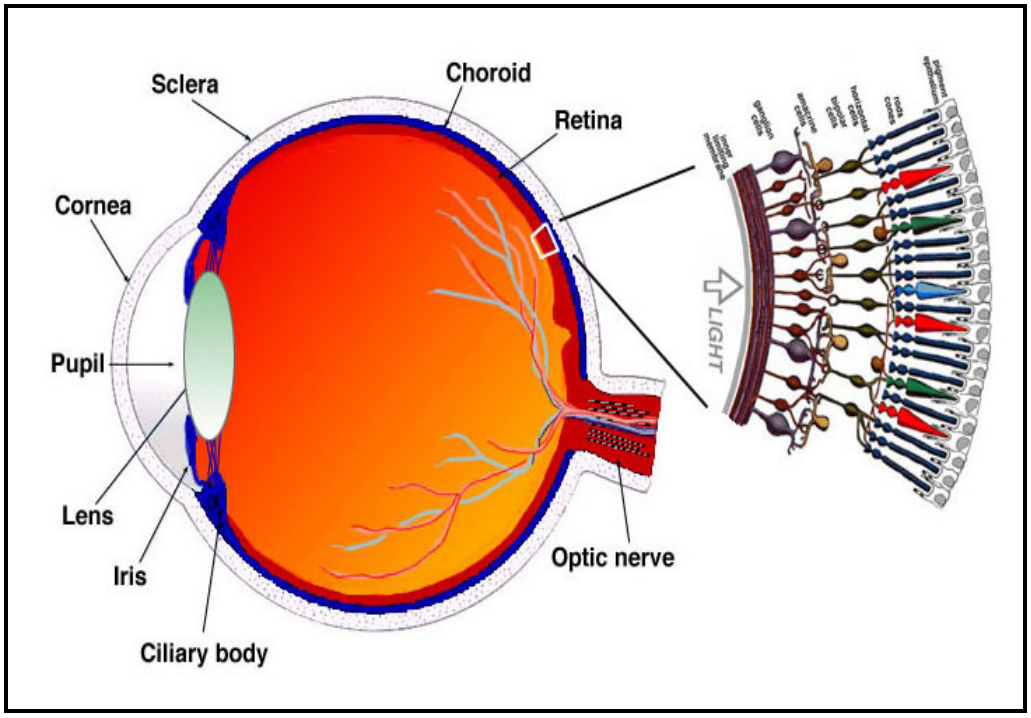

Figure 1.1 : Schematic diagram of eye. Retinal cells located at back layer of the eye and connected with the optic nerve to transfer the light rays to electrical impulse and sent to the brain. (webvision.med.utah.edu/sretina.html)

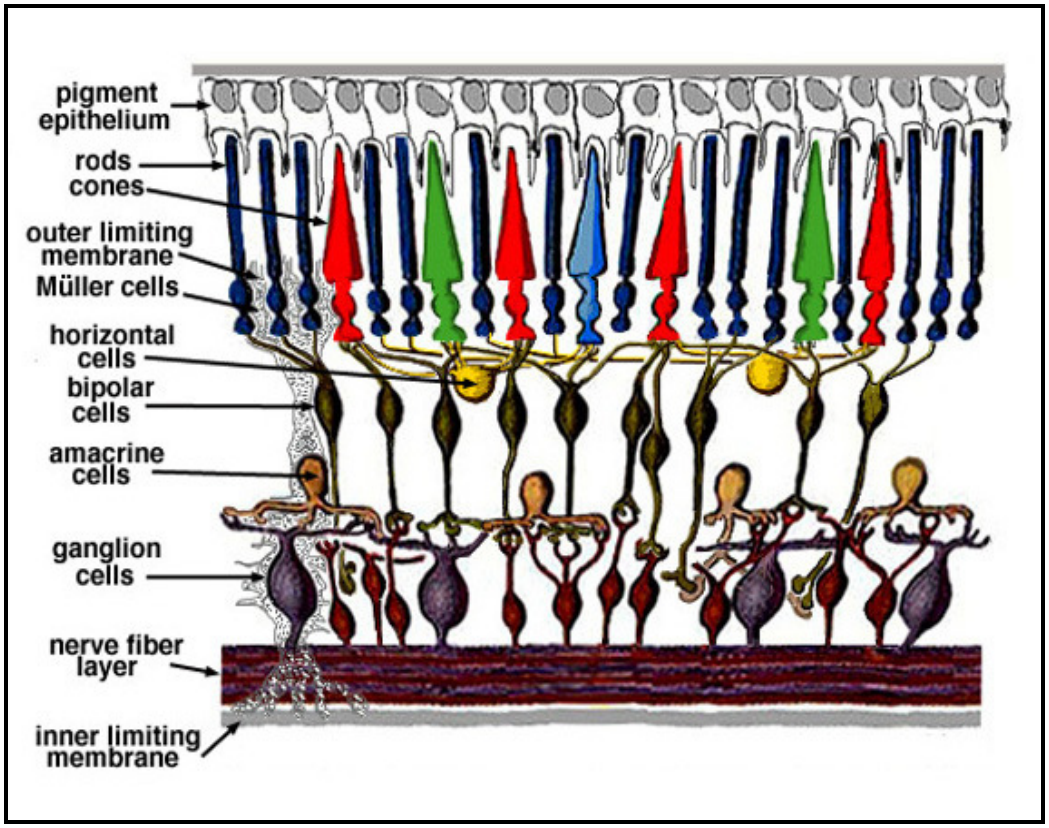

Figure 1.2 : Schematic diagram of retinal layers. Ganglion cells located innermost closest to the lens of eye while photoreceptor, which contains rods, and cones lie outer most of retina. (webvision.med.utah.edu/sretina.html)

1.1.1 Clinical features of retinoblastoma

1.1.1.1 Presenting signs and symptoms of retinoblastoma

The most common presenting sign of RB is leukocoria or white reflex behind the pupil (Abramson *et al.*, 1998), sometimes called cat eye's reflex, which becomes obvious on exposure to certain light condition such as flash photography. The second most common sign is strabismus or also called crossed-eye. Strabismus develops as a result of interruption of the central fixation by the tumor. In most of the RB cases with sign of strabismus, half of the patients have crossed-in eye (esotropia) and half have crossed-out eye (exotropia) (Abramson *et al.*, 1998). Although not all children with strabismus have retinoblastoma, fundus eye examination is essential to exclude the possibility of this disease. At advanced presentation, patients may present with sign of intraocular inflammation, with or without secondary glaucoma (Jerry and Carol, 2004).

This tumor can also extend to other parts of the body. Extraocular extension may cause proptosis and involvement of adjacent structure such as the brain. Extraocular extension usually occurs relatively late. Two-thirds of extraocular extension cases occur by spreading of the tumor through optic nerve to the brain. The tumors also can grow through the sclera or spread via haematogeneous metastasis. Once the tumor reaches the brain or causing haematogenous spread, the cure is almost impossible (Schipper *et al.*, 1985). Other signs of RB are severe pain in the affected eye, redness or irritation, and poor vision and blindness of the eyes.

Many reports have found the development of second nonocular tumor amongst RB patients. Abramson *et al.* (1976) reported that most of the second tumor is oesteosarcoma with range of incidence between 50% to 80%. Ninety seven percent of those with second tumor are patients with both eyes affected with tumor (bilateral RB).

Tumors in retinal cells can invade and damage nearby tissue and organs. Tumor cells can travel to other parts of the body, growing and replacing normal cells and tissues. This process is called metastasis. In most neglected or untreated RB cases, the tumor can spread to extraocular parts, either through optic nerve (Magramm *et al.*, 1989) or sclera (Rootman *et al.*, 1978). Metastatic RB is usually found at regional lymph nodes, central nervous system (CNS), bone and bone marrow (Finger *et al.*, 2002; Finger *et al.*, 1999; Gunduz *et al.*, 2006) as well as the ovaries (Darius *et al.,* 2002). It has been reported that highest risk of developing metastatic RB occur among delayed diagnosed RB.

1.1.1.2 Laterality of retinoblastoma

RB can be unilateral or bilateral. Unilateral RB refers to one eye affected with the tumor, while bilateral RB refers to both eyes affected with this tumor. Most of RB patients presence with unilateral form, while less than 50% of RB cases are bilateral (Moll *et al.*, 1997; Kayembe, 1986; Choy *et al.*, 2002). In very rare cases (3%) patients are found with trilateral RB. Trilateral RB associated of bilateral RB with pinealoblastoma, the tumor of the brain. The commonest cause of death in RB is due to the intracranial extension (Hungerford *et al.,* 1987; Melamud *et al.,* 2006).

1.1.2 Inheritance characteristic of retinoblastoma

RB can occur by transmission of the genetic changes from parents to their children (hereditary RB) or sporadically (non-hereditary RB). Hereditary and non-hereditary RB differs in many aspects including pattern of laterality, age of presentation and family history background

Previous studies estimated around 40% of RB cases are germinal. Patients with germinal RB contained mutation in RB1 gene in all cells including the reproductive cells (sperm or ovum). They usually have a positive family history and have a high possibility to transmit the mutations to their offspring. Hereditary RB patients may inherit a copy of one abnormal allele of RB1 gene from one of the parent (Sorsby, 1972) or alteration at one RB1 allele occur in sperm or ovum just after fertilization. The transmission of the altered allele or mutations in the RB1 gene occurs via autosomal dominant inheritance, with almost complete penetrance (85% to 95%) (Draper *et al.*, 1992). Autosomal dominant inheritance means the mutation in the gene is located in one of the autosomes (chromosome 1 to 22). Therefore, both males and females have equal chance to develop this tumor. In this pattern of inheritance, single defective allele is sufficient for the individual to be affected by the disease.

Developing of RB require mutation in both alleles of RB1 gene. Individual with hereditary RB inherited the first mutated allele from their parents and mutation of the second allele occurs somatically in the retinal cells in their lifetime (Knudson, 1971) **(Figure 1.3)**. Parents who are carrying single defective copy of RB1 gene have higher chance of transmitting the gene, up to 50%. In some cases, mutations only

present in reproductive cells (either ovum or sperm) of the parents with RB, but without any mutation in somatic cells. This is known as gonadal mosaicism. If both parents carrying one copy of mutant alleles of RB1 gene, the possibility of transferring the mutated gene to their children become higher. Seventy-five percent (75%) of their children have risks of being affected with this disease and 25% to be more severe (Lohmann, 1999).

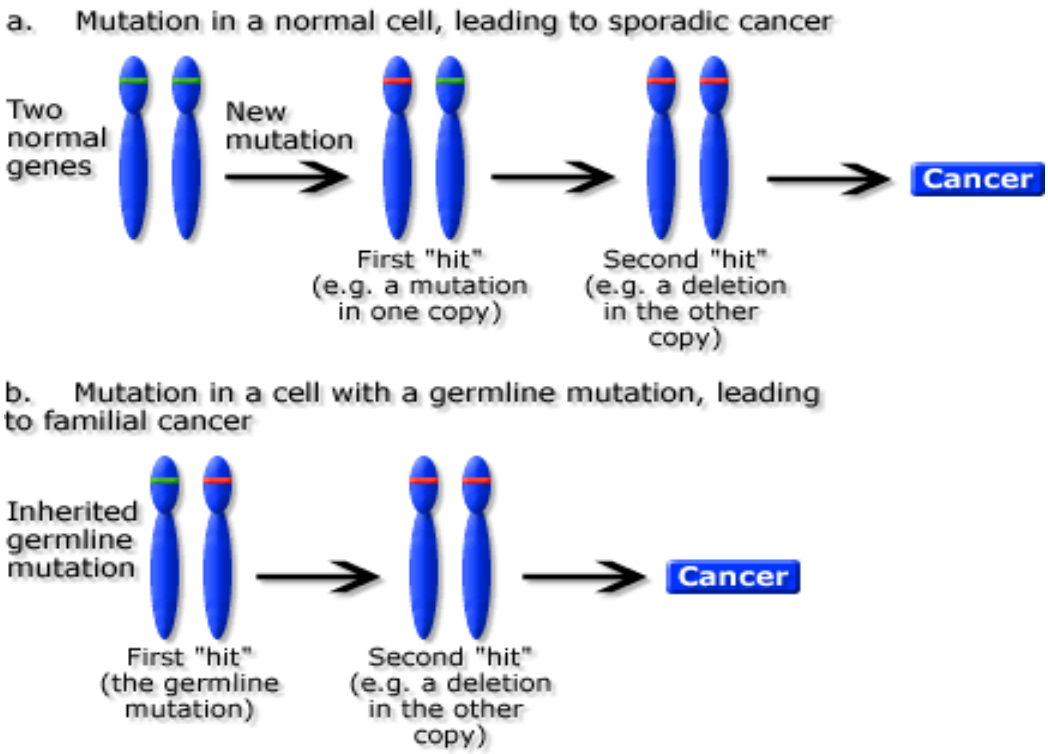

Figure 1.3: Mutational event among hereditary and non-hereditary (sporadic) RB. (a) In sporadic RB, both first and second mutations occur sporadically. (b) In hereditary RB, mutation in one allele is inherited from a parent and second mutation occur sporadically which will lead to tumor formation. (http://images2.clinicaltools.com/images/gene/cancer/knudson.gif)

Usually, hereditary RB patients develop this disease early in life (Knudson, 1971) and mostly affected both eyes. They are more likely to develop more than one independent tumor (multi focal) in one or both eyes. Germline mutations are also associated with increased risk of secondary tumor among the RB survivors. The most common secondary tumors are osteosarcoma (37.0%), other sarcoma (16.8%), melanomas (7.4%), brain tumor (4.5%), leukemia (2.4%), and non-Hodgkins lymphomas (1.6%) (Moll *et al.*, 2001). Although most of hereditary RB is bilateral, almost 17% of the hereditary RB present with unilateral RB (Lohmann *et al.,* 1997).

In all of RB cases, 60% occurred sporadically. Most sporadic RB affect only one eye and presented at an older age than hereditary RB (Lohmann *et al.*, 1997). In sporadic RB, mutation in both of RB1 alleles occurs sporadically and independently during somatic development of retinal cells. This may explain the late presentation of sporadic RB cases than hereditary RB because sporadic RB needs two mutational events while hereditary RB needs one more hit, as they already inherited one mutant allele.

The triggering factor for somatic mutation is still unclear. Orjuela *et al.* (2000) suggest that papillomavirus (HPV) might play a role in RB since they found the sequences of this virus in RB tumor tissues. The environment factor might influence the development of this disease. More cases of RB have been found in less developed compared to developed country.

1.1.3 Staging of retinoblastoma

Diagnosis of RB is done by several tests. It includes complete eye and funduscopic examination, where the pupil will be dilated so that the entire retina can be viewed and examined. In addition, the children also are examined through imaging studies such as computerized tomography (CT) scan and Magnetic Resonance Imaging (MRI). Imaging test provide detailed images of organs and structure within the eye. More over, confirmation of RB also can be done by molecular and cytogenetic screening tests (Chintagumpala *et al.,* 2007; Melamud *et al.,* 2006).

Children confirmed with RB are then staged according to the presentation of the tumor. Staging is a process of finding out the location, size and extension of tumors. The stage of cancer is one of the important factors in choosing the appropriate effective treatment.

There are three main classifications used for RB. It is Reese-Ellsworth Classification, Tumor-Nodal-Metastasize (TNM) Staging and International Classification of Retinoblastoma (ICRB).

Reese and Ellsworth presented their suggestion on classification of RB at 67th Annual Meeting of American Academy of Ophthalmology (Reese and Ellsworth, 1963). This classification, named as RE Classification, is the earliest staging system that has been evaluated for RB in early 1960s.

TNM Classification is a general cancer staging system used for malignant tumors. It describes the extent of cancer in patient's body. TNM Classification has been developed by International Union Against Cancer (UICC) in order to provide a global standard staging system in classifying the spread of cancer in human body. TNM Classification are referred to T for tumor size, N for the involvement of regional lymph nodes and M for distant of metastasize.

The current staging system for RB is International Classification of Retinoblastoma (ICRB) (Murphree, 2005). This classification gives guidelines for clinicians to choose the most appropriate treatment and might assist the prediction of the focal treatment and chemo reduction method **(Table 1.1)**. The ICRB is more robust classification and was proposed for better prediction of successful treatment to preserve the globe and if possible maintaining the vision (Saleh, 2006).

Table 1.1 : International Classification of Retinoblastoma (ICRB)

Group	Features	Treatment
A	Small tumor: <3 mm Large tumor: <3 mm	Cryotherapy, laser photocoagulation or transpuppillary thermotherapy
B	Macular: <3 mm to foveola Juxtapapillary: <3 mm to disc Subretinal fluid: <3 mm from the margin Focal seeds	Chemotherapy, Brach therapy or cryotherapy
C	Subretinal seeds: <3 mm Vitreous seeds: <3 mm Both subretinal and vitreous seeds: <3 mm Diffused seeds	Chemoreduction, chemotherapy, cryotherapy or brachytherapy
D	Subretinal seeds: >3 mm Vitreous seed: >3 mm Both subretinal and vitreous seeds: >3 mm	Chemo reduction, thermotherapy or low dose of external beam radiotherapy
E	Extensive retinoblastoma occupying more than 50% or neovascular glaucoma or opaque media from hemorrhage in anterior chamber, vitreous or subretinal space	Chemoreduction, thermotherapy, low dose of external beam radiotherapy or enucleation

1.1.4 Incidence of retinoblastoma

Incidence of RB in the worldwide population range from 1 in 10,000 to 23,000 live births (Freedman and Goldberg, 1976; Sanders *et al.*, 1988). In the United State (US), the mean incidences of RB over 30 years time (1975 to 2004) are 11.8 per million children before 4 years old. This number was stable for within that period with low variability of range (10.9 to 12.8 per million live births) (Broaddus *et al.*, 2009). In other Northern America region such as Mexico, RB disease tends to be the second most common solid tumor among pediatrics patients after central nervous systems (CNS) tumor. Data from 16 Mexican centers from January 1997 to December 2002 showed 83.3 new cases per year among children below 15 years old (Leal-Leal *et al.*, 2004).

In European region, cumulative incidence of RB was found to be from 44.2 to 67.9 per million live births. Data from Automated Childhood Cancer Infromation Systems (ACCIS) contains information of RB patients from 60 pediatrics and general centers from 1978 to 1997. It covered most of European country including British Isle, East, North, South and West region. Highest incidence occurs in the first year of life compared to other below 14 years old children (MacCarthy *et al.*, 2006).

In the Bantu of South Africa, data of RB cases shows high incidence number, 1 in 10,000 live births. Higher cases were diagnosed among 2 to 3-year old children (Freedman and Goldberg, 1976). Numbers of RB in Africa are higher than other population such as Caucasoid population. More sadly, most of the cases end with death.

The incidence of RB in Japan and Singapore are quite similar. Both of this region reported 1 case in every 16 000 live births (Takano *et al.*, 1991; Tan *et al.*, 1997). 98% of cases were found among children under 5 years old (Tan *et al.*, 1997). In another Asian country such as Taiwan, the average annual incidence rate is 4.45 per million children under 10 years old and 8.58 per million children under 5 year old. The incidences of this disease have significantly increased from 1979 to 2003 (Chen *et al.*, 2009).

In Malaysia, the actual incidence of RB remains unclear. A report of RB cases among this population from 2001 to 2007 indicated that 105 cases have been reported to the medical centers with majority cases were diagnosed from 1 month to 14 years old children (Menon *et al.*, 2009).

Most of RB cases are equally distributed among male and female patients (Menon *et al.*, 2009; Leal-Leal *et al.*, 2004; Kayembe, 1986). Although Freedman and Goldberg (1976) found distribution of RB cases with a male preponderance with male to female ratio of 2:1, the differences are not statistically significant. In contrast, Devesa (1975) found higher number of bilateral cases among female children with a ratio of 9: 1. The gender difference to the progression of RB remains unclear.

In addition, higher mortality rate were found in blacks compared to whites. Incidence of RB in blacks and whites are 9.8 to 13.0 and 10.8 to 11.3 cases per million respectively. However, the differences of mortality rate between these two populations are not significant, thus excluding the contribution of ethnic race background in predisposition to RB tumor (Broaddus *et al.*, 2009).

1.2 RB1 gene

Retinoblastoma susceptibility gene or also known as RB1 gene is the first tumor suppressor gene and hereditary cancer gene to be cloned. In 1986, Friend *et al.* (1986) had isolated RB1 gene from long q arm of chromosome 13 at band 14.2 **(Figure 1.4)**. It consists of 27 exons ranging from 31-1873bp in size and 26 introns ranging from 80-70500bp. The whole gene is 180,388bp in size (Toguchida *et al.*, 1993). Promoter region of RB1 gene is located at 186-206bp upstream from initiation codon. It contains binding domain for various transcription factor protein such as RBF-1, Sp1, ATF and E2F (Ohtani-Fujita *et al.*, 1993). The transcription of DNA by RNA polymerase is initiated at promoter region. The efficiency of DNA transcription varies by interaction between short regulatory DNA sequences within the promoter region with transcription factor proteins. Transcription factor is a large family of proteins that regulate the expression of the gene either positively or negatively. Transcription factor can increase or decrease the rate of transcription through interaction with the complex of protein including RNA polymerase (Winter *et al.,* 2002). The transcript of RB1 gene then transcribed to 4.7kb mRNA (messenger RNA). It comprised of 2.7kb open reading frame that encoded for a protein that contained 928 amino acids with molecular mass 110kDa. This protein is known as retinoblastoma phosphoprotein, pRb.

The association of RB1 gene in developing tumor is not restricted to the RB only. Abnormalities of RB1 gene have also been found in other cancers such as osteosarcoma (Toguchida *et al.*, 1988; Miller *et al.*, 1996), breast cancer (Eyfjord and Thorlacius, 1992; Ceccarelli *et al.*, 1998), acute leukemia (Sauerbrey *et al.,* 1998;

Kornblau *et al.,* 1998; Ahuja *et al.*, 1991), bladder cancer (Grossman *et al.*, 1998; Niehans *et al.*, 1999), lung cancer (Otterson *et al.*, 1994; Harbour *et al.*, 1988) and other cancers (Sellers and Kaelin, 1997; Mutirangura *et al.*, 1999).

RB1 is a class of tumor suppressor gene. In this class of gene, both alleles need to be inactivated to allow the progression of tumor. Inactivation of one allele frequently occurs by point mutation and the second allele by deletion.

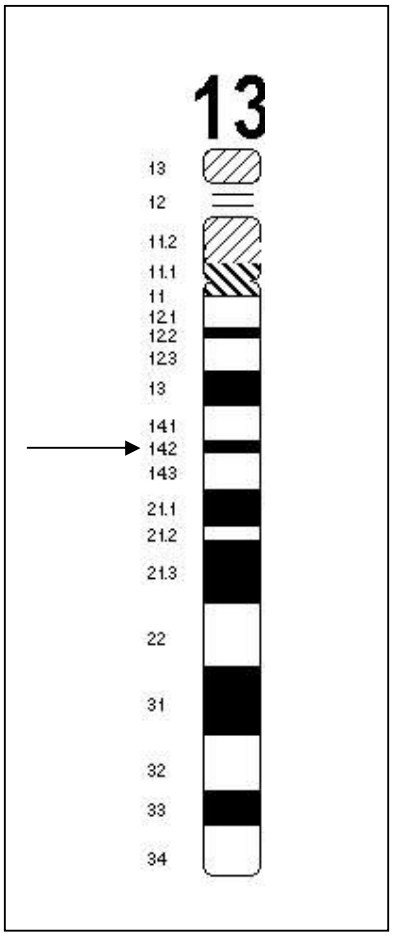

Figure 1.4 : Chromosome 13. RB1 gene located at long q arm at sub-band 14.2 (www.csmc.edu/csri/korenberg/int-bac-sts.html)

1.2.1 Retinoblastoma protein, pRb

Since RB1 gene was identified as tumor suppressor gene, its encoded protein, pRb emerged as key regulator for cell cyle (Friend *et al.,* 1986). Inactivation of pRb had been targeted by researchers in many studies of human cancer (Sherr, 1996; Weinberg, 1995). This phosphoprotein was found to bind with various endogenous nuclear proteins such as E2F transcription factor and tumor viral oncoprotein such as E1A adenovirus and human papillomavirus E7 (Whyte *et al.,* 1988; Huang *et al.,* 1993). pRb protein normally play as negative regulator in the cell cycle (Kaelin, 1999). In another study, pRb was found to control the chromatin structure during G1 to S phase transition in cell cycle process (Isaac *et al.,* 2006).

Basically, pRb consist of four domains; N terminus, small pocket consists of A and B domain and C terminus **(Figure1.5)**. Combination of small pocket A and B and part of C terminus make a complex which is known as large pocket domain (Harbour, 1998). Pocket domain A and B play a critical role for pRb protein as transcription repressor by serving binding site for other proteins (Chow and Dean, 1996; Chellapan *et al.,* 1991). In addition, frequent mutations have been identified within this region that is associated with non-functioning of pRb (Braggio *et al.,* 2004; Lohmann, 1999). The other two N and C terminus were less studied, but it was believed to play specific role for pRb protein. Although no clear function was identified for N-terminus of pRb protein, some studies found interaction of pRb-binding protein through this residue (Inoue *et al.,* 1995). In a study by Xu *et al.* (1994), they suggested that N-terminal region of pRb protein may play a role in regulation of cell growth or differentiation-mediated by pRb protein. The C-terminus

is part of large pocket domain. It contains some domains that might be important for protein-protein interaction (Shew *et al.,* 1990). One of the proteins that were found to bind to C-terminus domain is c-Abl tyrosin kinase, which latter might potentially regulate the transcriptional activity in S-phase of cell cyle (Welch and Wang, 1993).

1.2.1.1 Pocket A and B domain of pRb protein

The small pocket A and B pocket domain is the most conserved region in pRb protein. Domain A consists of amino acid 379 through 572 while domain B consists of amino acids 646 through 772. Both of these domains are separated by a nonconserved spacer region **(Figure 1.5)** (Harbour, 1998). Small pocket domains are sufficient and necessary for interaction with viral tumor protein such as simian virus 40 large T antigens and adenovirus E1A (Kaelin *et al.,* 1990). Both of domains A and B are required for transcriptional repression, but not the nonconserved separater region. Neither A nor B domain play the repression activity alone, but need both. One pocket domain is able to recruit the other domain to form repressor motif at the promoter, thus blocked the transcription (Chow and Dean, 1996). Mutations affect either A or B domain blocked the repression activity, signifying the importance and sufficiency of pocket domain for transcriptional repression (Dick and Dyson, 2002; Chow and Dean, 1996). Together with part of C-terminus domain, pocket A and B have ability to interact with E2F transcription factor and repress the transcription-mediated by this protein (Chow and Dean, 1996).

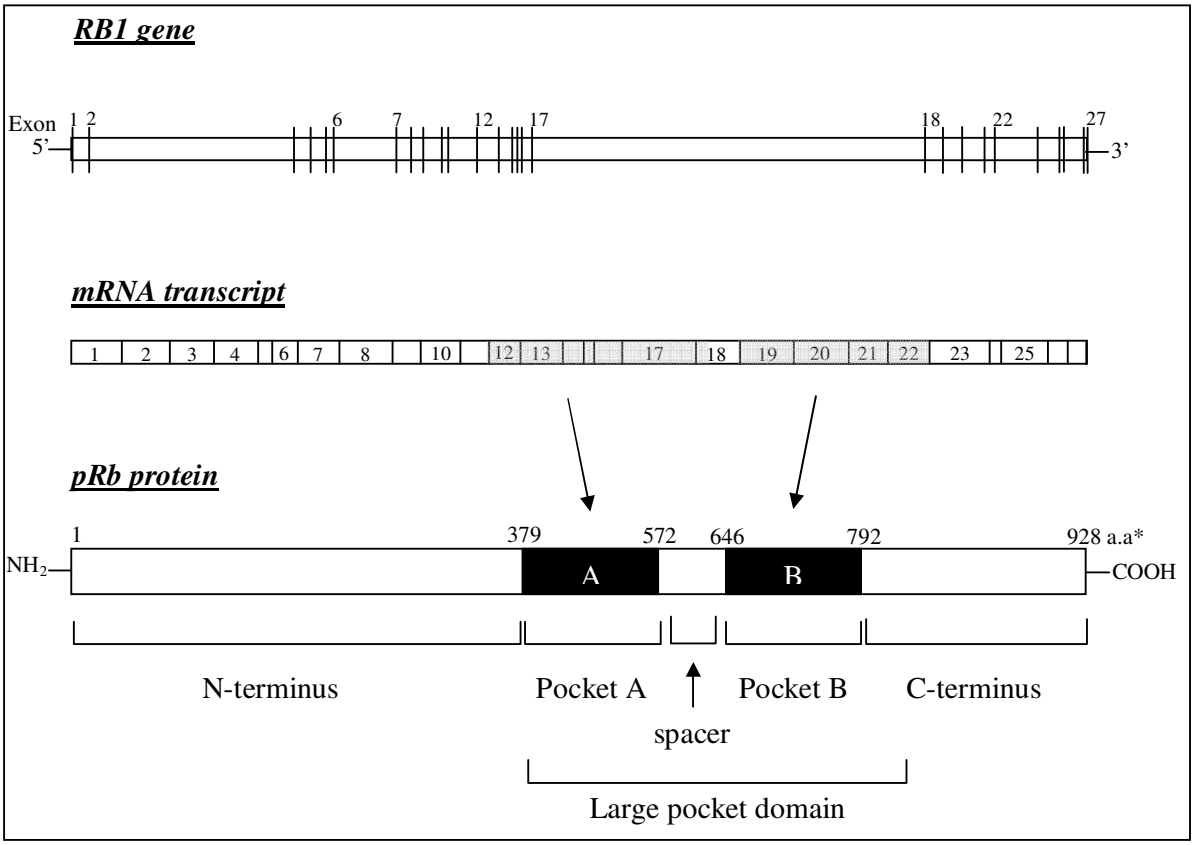

Figure 1.5 : Structure of RB1 gene, mRNA transcript and pRb protein. Coloured boxes in mRNA transcript indicated the exons encoding for pocket A and B domain. *a.a refer to amino acids

The small pocket A and B of pRb binds to various nuclear proteins through two types of binding; binding involving LXCXE (L = leusine, C = cysteinc, E = glutamic acid, x = any amino acid) or non-LXCXE sequence–containing protein. Interaction involving LXCXE motif, a peptide sequence occur between pRb and several tumor viral proteins such as simian virus 40 large T antigen, adenovirus E1A and human papillomavirus E7 as well as endogenous protein such as HDAC1 and 2. Certain nuclear protein which lacks of LXCXE motif such as transcription factor E2F and C/EBP interact with pRb protein via non-LXCXE binding. Domain B carry LXCXE motif, while domain A play a role to activate the small pocket complexes (Audo and Sahel, 2003).

1.2.2 pRb protein and cell cycle

pRb regulates the tumor progression by preventing the cell cycle activity at G_1 phase from entering S phase. In mammalian cells, the cell cycle are divided to 4 major division or phases; S phase, where the DNA are synthesized and replicated, M phase or mitotic phase where the chromosomes are divided into two nuclei in the mitosis process and 2 ‘gaps’; G_1 and G_2 phases. In the G_1 and G_2 phase, the cells are prepared for upcoming process in S and M phase respectively. In the resting cells, the cycle will stop by entering the G_0 phase after mitotic phase.

The role of pRb in cell cycle is basically related to their ability to bind with several nuclear proteins that can modulate their activities. Among these, the most studied nuclear protein that binds to pRb is transcription factor, E2F. The E2F-binding site have been found in several promoter sequence of growth promoting cellular genes

such as cyclin A, DNA polymerase alpha, cyclin D1, cdc2, c-myb, c-myc, tymidine kinase and E2F itself (Horowitz, 1993). Binding to pRb confiscate the transcription factor, which then blocked the E2F-mediated growth stimulation (Chellappan *et al.*, 1991; Hiebert *et al.*, 1992).

Activation and inactivation of pRb are modulated by phosphorylation events which are controlled by complexes of cyclin-dependent kinases (CDKs) (Sherr, 1996; Wong and Weber, 2007). The pRb is active under hypophosphorylation and inactivated under hyperphosphorylation. The pRb inhibits the cell growth by binding to and repressing the activity of regulatory proteins including E2F transcription factor which is necessary for G_1 to S phase transition. In the resting cells, pRb remains active until the cells are ready to divide. Phosphorylation of pRb in G_1 phase by complexes of cyclin-dependent kinase (CDKs) and its cyclin partner inactives the pRb protein and reduce its ability to bind to E2F protein, thus allowing the progression of the cell. Phosphorylation of pRb is controlled by at least two distinct cyclin kinase complexes, D-type cyclins with cdk4/6 which then completed by cyclin E-cdk2 complexes (Lundberg and Weinberg, 1998). There are two distinct mechanisms of transcriptional repression by pRb protein. Transcriptional activity can be repress either by direct interaction between E2F transcriptional factor to the pRb binding site or by recruitment of chromatin-remodelling enzyme by pRb-E2F complexes (Poznic, 2009). Binding of E2F to pRb protein directly blocks the transcription-mediated E2F protein (Helin *et al.,* 1993). Modifications of chromatin structure occur through histone acetylation (Felsenfeld, 1992). Complexes of repressor recruit histone deacetylases (HDACs) and form association with the complexes. This association inhibits the transcription by removing the acetyl groups

from histone core which then tighten the DNA and nucleosomes (Hasig *et al.,* 1997; Siddiqui *et al.,* 2003).

1.2.3 Knudson two hits hypothesis

In 1953, Carl O. Nordling, a scientist from Finland proposed the multi numbered of mutated genes in cancerous cells (Nordling, 1953). He also suggested that the accumulation of mutations might increase with age and cell proliferation rate, while the cell proliferation increased by exposure of the tissues to the mutatgens. His theory of multimutations in cancerous cells was supported by Ashley (1969), who suggested that cancer arises from 3 to 7 mutations depend on the different types of cancer.

This theory then was formulated by a cancer geneticist, Alfred Knudson. In 1971, Knudson accidently observed different graph pattern of age on onset between two types of RB cases while studying the epidemiological data of RB patients. In his observation, he had found significant age of onset between hereditary and sporadic RB children. Hereditary cases more often diagnosed earlier than sporadic cases. In addition, most of hereditary disease have tumor in both eyes, suggesting underlying predisposition. In contrast, sporadic or non-hereditary RB have delay onset and most often occur unilaterally. Knudson hypothesized that multi mutations are necessary to initiate the tumor. In inherited RB, the first mutation is transmitted from parents via germ line cells and second mutations occur somatically, which later lead to tumor arises. In sporadic cases, two mutations have to take place before the tumor can

develop. This observation explains the differences of age of onset between inherited and sporadic RB.

1.3 Spectrum of mutations in RB1 gene

Mutations are defined as any changes or alteration of DNA sequences as a result of chemical or physical agent actions or rare errors in DNA replication (Winter *et al.,* 2002). Type of mutation can be classified according to their effect on DNA structure, function, inheritance or protein sequence. It can occur in a small or large scale. Small length mutation involved alteration of single nucleotide such as single nucleotide substitution, deletion or insertion, while larger scale mutation includes amplification or gene duplication, deletion part of chromosomes or loss of heterozygosity as a result of loss of one allele (Winter *et al.,* 2002).

Commonly mutations are classified based on their impact to the protein sequence. The types of mutation are missense, nonsense, frameshift and silent muatation. All types of mutation alter the protein sequence except silent mutation. Missense mutation usually occur at first and second nucleotide of codon (sequence of three nucleotide that encoded for an amino acid) sequence which later changed the amino acid and protein sequence as well. Nonsense mutations change the codon to termination codon, thus affecting the translation of mRNA transcript to end prematurely and produced shortened version of protein that lack part of its carboxyl-terminal domain. These mutations give deleterious effect and always appear with mutant phenotype. Frameshift mutation occurs when extra base has been inserted or deleted from DNA sequences. If the insertion or deletion is not in triplet (sequence of