Kristu Jayanti Journal of Core and Applied Biology (KJCAB)

Volume 1, Issue 1 (July 2021), Page 28-32

A Review on the Methods for Identification of Mutations in the Tumour Suppressor Gene Retinoblastoma *RB1*

Kabilan G Mariappan[1], Manikandan Kathirvel[2*] and Kannaki Pasupathi[3]

[1]Department of Biotechnology, Alagappa University, Karaikudi, Tamilnadu, India

[2]Department of Life Sciences, Kristu Jayanti College (Autonomous), Bengaluru, Karnataka, India

[3]Department of Biotechnology, MGR University, Chennai, Tamilnadu, India

ABSTRACT

Retinoblastoma is the most common intraocular cancer of childhood. *RB1* is the gene responsible for causing retinoblastoma, spans more than 180 kilobases (kb) located on chromosome 13q14, which consist of 27 exons. Retinoblastoma in children may either be hereditary or non-hereditary. Mutations in *RB1* gene are mostly point mutations of non-sense or missense type but could also be of frameshift type. These mutations can be identified from both blood and tumour samples by Sanger sequencing and other molecular identification techniques such as Multiplex Ligation-dependent Probe Amplification (MLPA). 'Fragile' codons are codons which gets point mutated to form stop codons so that the resulting protein will be incomplete or immature. In *RB1*, fragile codons get mutated predominantly and lead to the truncation of *RB1* protein. The frequent mutations that predominantly occur in the arginine (CGA) codon, wherein changes in the single nucleotide results in the stop (UGA) codon, than any other fragile codon. The present paper reviews the role of RB1 mutations in retinoblastoma and the methods to identify it. We also make an attempt to identify the fragile codons in the RB genome based on the NCBI reference sequence NM_000321.2

Article History | Received: 01. 12. 2020; Revised: 10. 05. 2021; Accepted: 10. 06. 2021; Published Online: 26. 07. 2021

1. INTRODUCTION

Retinoblastoma (RB) is the most common primary cancer of eye in children, but rare compared to other cancers. RB is a pediatric ocular tumour which is caused by mutations in the retinoblastoma tumour suppressor gene (*RB1*). RB originates from sensory retina and is the common intraocular malignancy of childhood in children younger than 15 years. RB is the tumour of the retina that occurs either as an inherited or as a sporadic form *(Abraham et al., 2019)*. When the eyes are developing, they have progenitors (immature) cells called retinoblasts. *RB1* mutation can lead to uncontrolled growth in retinoblasts which forms a tumour called retinoblastoma. Leukocoria is the most clinically characterized feature of RB cases. Other clinical symptoms include squint, red eye, and reductionin vision. If RB is undiagnosed during leukocoria it may lead to more visible and permanent defect in eyes. In India, the number of incidence is about 1 in 15,000 live births. The number of enucleations and visual sequels due to retinoblastoma is high *(Gargallo et al., 2018)*. RB can be both unilateral and bilateral *i.e.,* tumour in single eye or tumour in both eyes respectively. There is another type called trilateral RB in which retinoblastoma combined with a histologically similar brain tumour which is most commonly found in the pineal gland *(De jong et al., 2014)*. Untreated retinoblastoma is fatal butit is curable if it is diagnosed at an earlier stage. Children with family positive history can be screened for mutation of *RB1* gene for the detection of *RB1* mutation *(Abramson et al., 2003)*. Improvements in the diagnosis and management of retinoblastoma are improving mortality which is associated to this condition in the developed nations *(Villegas et al., 2013)*.

2. KNUDSON'S HYPOTHESIS

According to Knudson's hypothesis, both the alleles of *RB1* have to be inactivated for the initiation of RB tumour suppressor gene located on chromosome 13q14. Tumour may be induced in children as the child carries

* Author for correspondence : Manikandan Kathirvel | manikandan@kristujayanti.com

mutation in both the alleles of *RB1* gene. Hereditary RB will have 2 mutations, one in germ cells and other in somatic cells. In non-hereditary, both the mutations will occur in somatic cells *(Knudson, 1971)*. Both unilateral and bilateral patients show two-hit confirming Knudson's two-hit hypothesis *(Gaikwad et al., 2015)*. One hit occurs in germline whereas other hit occurs in same allele of precursor cell which leads to tumour.

3. *RB1* GENE

RB1 was the first identified prototypical tumour suppressor gene. The basic estimated test for RB is the presence of mutation in *RB1* *(Skalet et al., 2018)*. It is located on the chromosome 13q14 and spans around 180 Kb with 27 exons. In *RB1* all the 27 exons contribute to the 2.7 Kb open reading frame coding for 110 KDa retinoblastoma proteins. This protein involves in regulation of negative gene expression by interfering in cell cycle checkpoint between G1 and entry into the S phase. pRB protein maintains the cell growth and controls the cell from diving in uncontrolled manner. pRB also interacts with other proteins to influence cell survival, apoptosis and cell differentiation. Molecular genetic diagnostics for retinoblastoma are a prerequisite for accurate risk prediction and effective management *(Joseph B et al., 2006)*. RB is primarily caused by biallelic inactivation of *RB1* gene. Mutation in *RB1* gene is mostly point mutation that is either nonsense mutation or missense mutation. The spectrum of *RB1* mutations also includes frame shift mutation and deletion/ duplications where they can be identified using Sanger sequencing/ Multiplex ligation-dependent probe amplification (MLPA). These mutations lead to truncation of protein and immature protein production. A rapid screening strategy was developed by prioritizing the order of exons, based on the frequency of nonsense mutations to find mutation in *RB1* *(Thirumalairaj K et al., 2015).*

4. HEREDITARY RETINOBLASTOMA

India has the highest number of retinoblastoma (RB) patients among the developing countries owing to its increasing population. Among the patients with RB, about 40% have the heritable form of the disease *(Thirumalairaj et al., 2015).* The abnormalities in *RB1* gene are congenital and it is present in all cells ofthe body, this is known as germ line retinoblastoma. Early diagnosis and identification of carriers of heritable *RB1* mutations can improve disease outcome and management *(Tomar et al., 2017).* Children with hereditary retinoblastoma may have multifocal tumour in the same eye or it can occur in both eyes. It also has high risk of forming other cancers. Children with hereditary retinoblastoma can pass the *RB1* mutation on to their offspring. A small number of children with this form of retinoblastoma will develop another tumour in pineal gland of brain, this is also known as trilateral retinoblastoma.

5. NON-HEREDITARY RETINOBLASTOMA

It is also known as sporadic retinoblastoma. In this *RB1* mutation occurs during the development of the organ in the child and their family history will be negative. About 60% of child with RB is non-hereditary retinoblastoma. Children with non-hereditary retinoblastoma will have tumour mostly in one eye *i.e.,* unilateral retinoblastoma. Usually this non-hereditary retinoblastoma will not be passed on to next generation. Unilateral RB also occurs due to mutation in *mycn* gene, In the absence of mutation in *RB1* *(Rushlow et al., 2013)*. This type of retinoblastoma often found in the later age of the child compared to those children with hereditary retinoblastoma.

6. FRAGILE CODONS

Fragile codons are those that can be mutated to a stop codon at single step *(Casci T et al., 2011)*. Stop codons are UAA, UAG and UGA. Fragile codons become stop codon by point mutation (non-sense mutation) *i.e.,* single nucleotide change. This fragile codon mutation in *RB1* leads to truncation of pRB protein so that there will be dysregulation in cell cycle and the cells undergo uncontrolled differentiation. Mutations of fragile codon occur either by transition or transversion of point mutation (Figure 1).

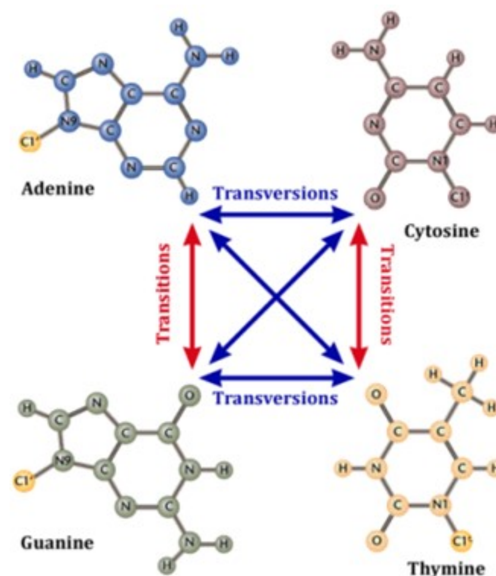

Figure 1: Point mutations Transitions and Transversions *(Adapted from Steven M. Carr, 2014)*

7. MUTATION ANALYSIS BY DNA SEQUENCING

Sanger sequencing is the method which is preferred for the identification of mutation in DNA from both blood and tumour of the children. DNA will be amplified using PCR to get maximum number of exon copies and that will be taken to sequencing. Sanger sequencing also known as the chain termination method, is a technique for sequencing the DNA based upon the selective incorporation of chain terminating dideoxynucleotides (ddNTPs) by DNA polymerase. It was developed by Frederick Sanger *et al.,* in 1977. Using advanced automated Sanger sequencing method makes the sequencing process easier than the conventional method. Dye-termination sequencing utilizes labeling of the chain terminator ddNTPs, which permits sequencing in a single reaction, rather than 4 reactions as in the labeled-primer method. In dye-terminator sequencing, each of the 4 dideoxy nucleotide chain terminators is labeled with fluorescent dyes, each of which emit light at different wavelengths. Owing to its greater expediency and speed, dye-terminator sequencing is now the mainstay in automated sequencing. Its limitations include dye effects due to differences in the incorporation of the dye labeled chain terminators into the DNA fragment, resulting in unequal peak heights and shapes in the electronic DNA sequencing trace chromatogram.

8. SCREENING FOR MUTATIONS IN *RB1* GENE

RB1 mutation can be analyzed by sequencing patients DNA which can be isolated from either blood samples or tumour samples. In case of hereditary RB, children will have mutation in tumour and also in blood and for non-hereditary, RB mutation will be present only in tumour of the children. By performing DNA sequencing point mutation like nonsense and missense mutation can be identified and thenature of the mutation can also be studied.

Mutation in exon region can lead to truncation of protein as exon is the coding regions of gene. In *RB1,* there are about 27 exons to screen in entire gene. There is a stepwise strategy for the detection of mutation based on their frequency of getting mutation *(Thirumalairaj et al., 2015)*. According to Thirumalairaj *et al.,* there are total of 3 sets of exons and the four step strategy used that helps in the easy and quick diagnosis of mutation.

9. MUTATION IDENTIFICATION USING MLPA METHOD

Multiplex Ligation-dependent Probe Amplification (MLPA) can be used to identify large deletions and duplications of nucleotide sequence, presence of DNA methylation, single nucleotide polymorphism (SNPs) and point mutation. As MLPA helps in detection of large fragment of nucleotide DNA sequence it was widely used in molecular diagnosis laboratory as gold standard method for diagnosis. MLPA assay usually helps in diagnosis of mutation only in RB cells. MLPA is a probe based method for diagnosis which uses about 40 different probes as multiplex PCR assay. The steps involved in MLPA assay are:

1. Denaturation of DNA
2. Probe hybridization
3. Ligation and amplification of DNA sequence
4. Electrophoresis for separation of amplified products
5. Data analysis *(Stuppia L et al., 2012)*.

Both homozygous or hemizygous deletions in gene can be detected with clear peak for target gene.

9. OTHER METHOD FOR DIAGNOSIS

1. Quantitative Multiplex PCR
2. AS4-PCR
3. Promoter Methylation assay
4. Chromosomal Micro assay
5. Next Generation Sequencing *(Richter S et al., 2003)*

10. IDENTIFICATION OF FRAGILE CODONS USING REFERENCE SEQUENCE

There are several exons which are already reported for fragile codon mutation but it was not completely reported in *RB1* gene. There are several hotspots in *RB1* in which non-sense mutations occur more often, but the entire hotspot regions were unknown till now. Fragile codons were identified from the reference sequence retrieved from National Center for Biotechnology Information (NCBI) (Ref Seq NM_000321.2 and Genbank accession number L11910.1). These hotspot regions were identified by changing nucleotide sequence in every codon. The mutation in susceptible codons identification helps in recognition and easy detection of fragile codons. The study suggests that arginine (CGA) has the higher frequency of mutation compared to other codons in the entire coding region. cDNA sequence was retrieved from NCBI and codons were identified by splitting 3 nucleotide from cDNA sequence. By altering every nucleotide in individual codons, fragile codons were identified. The total possibilities of getting mutation in fragile codons were highlighted with unique color for identification (Figure 2). The total number of amino acid which can be mutated to stop codon in entire *RB1* coding region is listed in Table 1**.**

```
1    ATG CCG CCC AAA ACC CCC CGA AAA ACG GCC GCC ACC GCC GCC GCT
16   GCC GCC GCG GAA CCC CCG GCA CCG CCG CCG CCG CCC CCT CCT GAG
31   GAG GAC CCA GAG CAG GAC AGC GGC CCG GAG GAC CTG CCT CTC GTC
46   AGG CTT GAG TTT GAA GAA ACA GAA GAA CCT GAT TTT ACT GCA TTA TGT
62   CAG AAA TTA AAG ATA CCA GAT CAT GTC AGA GAG AGA GCT TGG TTA ACT
78   TGG GAG AAA GTT TCA TCT GTG GAT GGA GTA TTG GGA GGT TAT ATT CAA
94   AAG AAA AAG GAA CTG TGG GGA ATC TGT ATC TTT ATT GCA GCA GTT GAC
110  CTA GAT GAG ATG TCG TTC ACT TTT ACT GAG CTA CAG AAA AAC ATA GAA
126  ATC AGT GTC CAT AAA TTC TTT AAC TTA CTA AAA GAA ATT GAT ACC AGT
142  ACC AAA GTT GAT AAT GCT ATG TCA AGA CTG TTG AAG AAG TAT GAT GTA
158  TTG TTT GCA CTC TTC AGC AAA TTG GAA AGG ACA TGT GAA CTT ATA TAT
174  TTG ACA CAA CCC AGC AGT TCG ATA TCT ACT GAA ATA AAT TCT GCA TTG
190  GTG CTA AAA GTT TCT TGG ATC ACA TTT TTA TTA GCT AAA GGG GAA GTA
206  TTA CAA ATG GAA GAT GAT CTG GTG ATT TCA TTT CAG TTA ATG CTA TGT
222  GTC CTT GAC TAT TTT ATT AAA CTC TCA CCT CCC ATG TTG CTC AAA GAA
238  CCA TAT AAA ACA GCT GTT ATA CCC ATT AAT GGT TCA CCT CGA ACA CCC
254  AGG CGA GGT CAG AAC AGG AGT GCA CGG ATA GCA AAA CAA CTA GAA
269  AAT GAT ACA AGA ATT ATT GAA GTT CTC TGT AAA GAA CAT GAA TGT AAT
285  ATA GAT GAG GTG AAA AAT GTT TAT TTC AAA AAT TTT ATA CCT TTT ATG
301  AAT TCT CTT GGA CTT GTA ACA TCT AAT GGA CTT CCA GAG GTT GAA AAT
317  CTT TCT AAA CGA TAC GAA GAA ATT TAT CTT AAA AAT AAA GAT CTA GAT
333  GCA AGA TTA TTT TTG GAT CAT GAT AAA ACT CTT CAG ACT GAT TCT ATA
349  GAC AGT TTT GAA ACA CAG AGA ACA CCA CGA AAA AGT AAC CTT GAT GAA
365  GAG GTG AAT GTA ATT CCT CCA CAC ACT CCA GTT AGG ACT GTT ATG AAC
381  ACT ATC CAA CAA TTA ATG ATG ATT TTA AAT TCA GCA AGT GAT CAA CCT
397  TCA GAA AAT CTG ATT TCC TAT TTT AAC AAC TGC ACA GTG AAT CCA AAA
413  GAA AGT ATA CTG AAA AGA GTG AAG GAT ATA GGA TAC ATC TTT AAA GAG
429  AAA TTT GCT AAA GCT GTG GGA CAG GGT TGT GTC GAA ATT GGA TCA CAG
445  CGA TAC AAA CTT GGA GTT CGC TTG TAT TAC CGA GTA ATG GAA TCC ATG
461  CTT AAA TCA GAA GAA GAA CGA TTA TCC ATT CAA AAT TTT AGC AAA CTT
477  CTG AAT GAC AAC ATT TTT CAT ATG TCT TTA TTG GCG TGC GCT CTT GAG
493  GTT GTA ATG GCC ACA TAT AGC AGA AGT ACA TCT CAG AAT CTT GAT TCT
509  GGA ACA GAT TTG TCT TTC CCA TGG ATT CTG AAT GTG CTT AAT TTA AAA
525  GCC TTT GAT TTT TAC AAA GTG ATC GAA AGT TTT ATC AAA GCA GAA GGC
541  AAC TTG ACA AGA GAA ATG ATA AAA CAT TTA GAA CGA TGT GAA CAT CGA
557  ATC ATG GAA TCC CTT GCA TGG CTC TCA GAT TCA CCT TTA TTT GAT CTT
573  ATT AAA CAA TCA AAG GAC CGA GAA GGA CCA ACT GAT CAC CTT GAA TCT
589  GCT TGT CCT CTT AAT CTT CCT CTC CAG AAT AAT CAC ACT GCA GCA GAT
605  ATG TAT CTT TCT CCT GTA AGA TCT CCA AAG AAA AAA GGT TCA ACT ACG
621  CGT GTA AAT TCT ACT GCA AAT GCA GAG ACA CAA GCA ACC TCA GCC TTC
637  CAG ACC CAG AAG CCA TTG AAA TCT ACC TCT CTT TCA CTG TTT TAT AAA
653  AAA GTG TAT CGG CTA GCC TAT CTC CGG CTA AAT ACA CTT TGT GAA CGC
669  CTT CTG TCT GAG CAC CCA GAA TTA GAA CAT ATC ATC TGG ACC CTT TTC
685  CAG CAC ACC CTG CAG AAT GAG TAT GAA CTC ATG AGA GAC AGG CAT TTG
701  GAC CAA ATT ATG ATG TGT TCC ATG TAT GGC ATA TGC AAA GTG AAG AAT
717  ATA GAC CTT AAA TTC AAA ATC ATT GTA ACA GCA TAC AAG GAT CTT CCT
733  CAT GCT GTT CAG GAG ACA TTC AAA CGT GTT TTG ATC AAA GAA GAG GAG
749  TAT GAT TCT ATT ATA GTA TTC TAT AAC TCG GTC TTC ATG CAG AGA CTG
765  AAA ACA AAT ATT TTG CAG TAT GCT TCC ACC AGG CCC CCT ACC TTG TCA
781  CCA ATA CCT CAC ATT CCT CGA AGC CCT TAC AAG TTT CCT AGT TCA CCC
797  TTA CGG ATT CCT GGA GGG AAC ATC TAT ATT TCA CCC CTG AAG AGT CCA
813  TAT AAA ATT TCA GAA GGT CTG CCA ACA CCA ACA AAA ATG ACT CCA AGA
829  TCA AGA ATC TTA GTA TCA ATT GGT GAA TCA TTC GGG ACT TCT GAG AAG
845  TTC CAG AAA ATA AAT CAG ATG GTA TGT AAC AGC GAC CGT GTG CTC AAA
861  AGA AGT GCT GAA GGA AGC AAC CCT CCT AAA CCA CTG AAA AAA CTA CGC
877  TTT GAT ATT GAA GGA TCA GAT GAA GCA GAT GGA AGT AAA CAT CTC CCA
893  GGA GAG TCC AAA TTT CAG CAG AAA CTG GCA GAA ATG ACT TCT ACT CGA
909  ACA CGA ATG CAA AAG CAG AAA ATG AAT GAT AGC ATG GAT ACC TCA
924  AAC AAG GAA GAG AAA TGA
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Figure 2: The fragile codons identified in the NCBI reference sequence for *RB1* gene

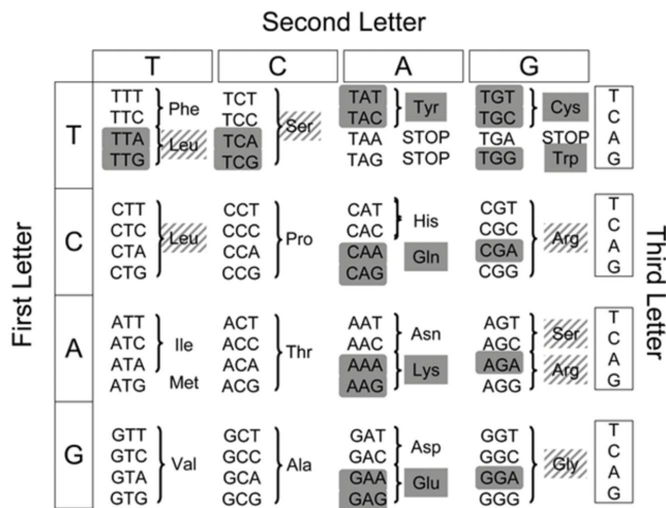

Figure 3: The Genetic code *(Adapted from Brian P. Cusack, 2011)*

Amino Acid	Probability of getting mutation
Arginine	29
Glycine	16
Glutamic acid	73
Tryptophan	7
Cysteine	15
Tyrosine	28
Serine	27
Leucine	27
Glutamine	35
Lysine	76
Total fragile codons in RB1	333

Table 1: Total number of amino acid which can be mutated to stop codon in entire *RB1* coding region.

11. CONCLUSION

RB is the most common cancer caused mostly for children's below age of 6. This cancer is caused by mutation occurred in tumour suppressor gene *RB1*. Mutation like point mutation *i.e.,* missense and nonsense are predominant which converts the coding region to stop codon and which in turn leads to truncation of pRB. For example, arginine (CGA) is the amino acid which can easily get mutated to a stop codon (TGA). These mutations can be identified by using DNA sequencing which is a gold standard for identification of mutations. The other mechanisms that result in mutations are insertion, deletion, duplication, etc. Lohmann database is an online free website which contains all the reported data about RB mutation from which fragile codons can be identified. There are other methods for the identification of other kind of mutations but point mutation are the most predominant in children. Hereditary RB gets transmitted through stem cells but non hereditary RB occurs during mitosis in zygote formation or else after birth due to any other kind of causative agents. Mutations in hereditary RB are usually present in both tumour and blood cells as it is passed from the parent cells but in non-hereditary RB mutations mostly won't be present in proband's blood cells. Hereditary and non-hereditary RB can be identified by screening proband's parent's blood. Next generation sequencing is the most advanced method for screening of mutation in proband's DNA but Next-Generation Sequencing (NGS) are highly cost when compared to the gold standard Sanger sequencing and Sanger sequencing helps in easy diagnosis of mutation compared to other method of diagnosis.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest in this research work

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