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In Silico Analysis of nsSNPs in the Rb1 Gene for Predicting Pathogenicity and Disease Associations

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Abstract: Single nucleotide polymorphisms (SNPs) are changes at specific spots in DNA. These changes help identify genes linked to diseases or trace inherited conditions within families. Variations in the Rb1 gene can lead to retinoblastoma, which is cancer in one or both eyes, as well as other cancers like osteosarcoma, melanoma, leukemia, lung, and breast cancer. First, this study used the SNP database from NCBI to gather key data. It also analyzed how Rb1 is connected to other genes using GeneMANIA. Ten different tools were applied to screen for harmful SNPs, including SIFT, PolyPhen-2, I-Mutant 3.0, PROVEAN, SNAP2, PHD-SNP, PMut, and SNPs&GO. To estimate conserved amino acid regions, the Consurf Server was used, and Project HOPE was utilized to study the structural effects of mutant proteins. GeneMANIA showed that the Rb1 gene is strongly linked to 20 other genes, such as CCND1 and RBP2. The data obtained from the NCBI's dbSNP indicated that the total number of SNPs within the Rb1 gene region is 36,358. Of these, 345 were found in the 3' UTR, 65 in the 5' UTR and 34,543 in the intron regions. There were 844 coding SNPs including 199 synonymous, 450 non synonmous which consists of 425 missense, five nonsense, and 20 frameshift mutations. The remaining SNPs were of other types. This study focused on the 425 missense SNPs for research. From these, 17 mutations (D332G, R445Q, E492V, P515T, W516G, V531G, E533K, E539K, M558R, W563G, L657Q, A658T, R661Q, D697H, D697E, P796L, and R798W) were predicted to cause harmful effects on the structure and function of the Rb1 protein.

Keywords: Rb1Gene; nsSNPs; GeneMANIA; Insilicoanalysis; Coding; Mutation; Retinoblastoma.

1. Introduction

This research work is split into two parts. The first part will be retrieving data from the database and emphasizing Rb1, which stands for retinoblastoma. Called sporadic or genetic, this is much more aggressive if it is allowed to be left without treatment. The current work investigates the in-silico method of identifying the genetic disorder utilizing a variety of computer methods. On chromosome 13, the tumor suppressor gene Rb1 regulates cell division and proliferation. Until the cell is properly divided, it stops the cycle of cell division. Before the cell divides, Rb1 gets phosphorylated to pRb, eventually leading to the inactivation of the retinoblastoma protein [1]. This process makes the cell's entry into the cell cycle possible, possibly leading to a mutation in this gene [2]. While, chronic activation by Rb1 leads to a gradual decrease in the essential factors of DNA replication, with all targeted proteins showing suppressed DNA replication activity after more than 72 to 96 h of continuous activation [3]. This can sometimes lead to maladies where DNA replication gets suppressed in cellular biology [4]. Rb1 is part of the "pocket protein family," which contains retinoblastoma protein (RB), retinoblastoma-like protein 1, and retinoblastoma-like protein 2. The total count comes to at least 100 proteins these three homologous members can bind to. Hence, Rb1 is a multitasking protein with multiple phosphorylation and binding sites, especially with the E2F family [7]. Almost everything agrees because the human genome is composed of 3.2 billion nucleotides distributed in 24 linear molecules [8]. One further scope of research is the RB1 gene of chromosome 13, having approximately 114 million base pairs, accounting for 3.5%-4.0% of the total genome in cells. These nucleotide differences, known as "single-nucleotide polymorphisms (SNPs)," happen roughly once per

1,000 nucleotides, or at a rate of 1% of all nucleotides. As a result, there are over 100 million SNPs worldwide and 4-5 million SNPs within the human genome [9]. These changes can occur between genes in DNA [10]. SNPs can act individually to cause phenotypic changes or, at other times, act together to contribute to diseases like osteoporosis [11]. Researches show that more than half of the genetic alterations connected to diseases are linked to non-synonymous SNPs (nsSNPs) [12]. The severity of each mutation varies; some merely have minor consequences [13]. The function of the RB1 gene is impacted by hundreds of thousands of mutations [14]. One in every 16,000–18,000 live newborns is affected by retinoblastoma, which causes 9,000 new cases globally each year [15]. Leukocoria, or "amaurotic cat's eye reflex," crossed eyes, aberrant pupil appearance, strabismus, or inability to concentrate both eyes in the same direction, are common symptoms [16]. Other symptoms include Iris color changes, edema, redness, and blurred vision [17]. Two types of retinoblastoma are identified: unilateral, affecting one eye and usually detected at 24 months of age, and bilateral, affecting both eyes and detected at 12 months [18]. Retinoblastoma can cause visual loss in children, and in more severe situations, it may need the removal of the affected eye or eyes [19].

Retinoblastoma comes in two flavors: hereditary and non-hereditary [20]. Each offspring of a person with hereditary retinoblastoma has a 50% chance of receiving the mutant gene due to autosomal dominant inheritance [21]. This type, if inherited, frequently causes numerous tumors in both eyes [22]. Conversely, non-hereditary retinoblastoma is not inherited and does not affect future generations. The RB1 gene is present in two normal copies at birth in affected individuals, but mutations that cause cancer develop in early childhood [23]. However, these alterations are not transferable to progeny [24]. Non-hereditary retinoblastoma usually results in one tumor in one eye. While it can be difficult to determine if retinoblastoma is hereditary or non-hereditary [25], genetic testing can provide clarity [26]. Hereditary or germinal retinoblastoma, caused by genetic mutations in RB1, spreads to all body cells and is more dangerous as it often leads to cancer in both eyes or other parts of the body [27].

Mutations in the Rb1 gene are also the primary cause of pineoblastoma [28], a type of cancer that occurs in the pineal gland of the brain [29]. These genetic mutations are also linked to breast cancer, lung cancer, and osteosarcoma, a form of bone cancer [30]. Furthermore, Rb1 mutations cause sarcomas, or soft tissue malignancies [31], which usually appear in people between the ages of 10 and 20 [32], particularly in retinoblastoma survivors [33]. Melanoma, a severe type of skin cancer that often begins in the skin but can also, in rare instances, grow in the mouth, intestines, or eyes [35], is another condition that is exacerbated by Rb1 mutations. Approximately 25% of melanomas originate from simple moles [36]. Somatic mutations in the Rb1 gene have also been associated with leukemias, cancers of blood-forming cells [37]. This study aims to offer personalized medical treatment based on a detailed analysis of an individual's genome. While it can be difficult to identify functional SNPs in certain genes using conventional laboratory instruments and procedures, advances in in-silico approaches now allow for identifying these SNPs without requiring much lab work [38]. This study uses a variety of computational methods and tools to investigate genetic differences in SNPs and their possible effects on the composition and functionality of the Rb1 gene.

2. Materials and Methods

2.1. Data Gathering

All the required SNP particulars of the human Rb1 gene, i.e., "rsids, Protein accession Number=NP_000312.2, mRNA accession Number=NM_000498.3, and residue changes" have been extracted From National Center for Biotechnology Information (NCBI), which is SNP's Public database i.e. dbSNP https://www.ncbi.nlm.nih.gov/). Out of 844 coding SNPs, 425 missense SNPs were taken Into account for further analysis using different bioinformatics tools. Although a division of Coding SNPs at different regions is shown in Figure 1.

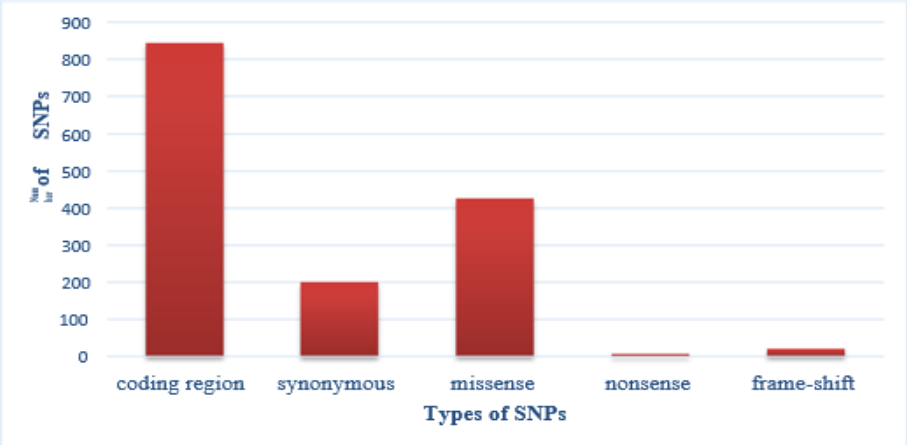


Figure 1. Graphical Portray regarding the division of coding SNPs for Rb1 Gene(as per NCBI)

2.2. Scanning the association of Rb1 with other Genes using GeneMANIA

A program called GeneMANIA comprises 597,392,988 relationships of 163,599 genes that provide information about each gene's function, including pathways, co-localization, genetic interactions, physical interactions, and protein domain similarity [39]. An integrated plugin in GeneMANIA makes use of a large database that contains information from several different organisms. GeneMANIA can be accessed via this website: https://genemania.org.

2.3. The anticipation of Damaging and Tolerated nsSNPs and functional effects by SIFT

The "Sorting Intolerant from Tolerant (SIFT)" method is being used, and it predicts the physical and chemical properties of amino acids as well as whether or not the substitution of an amino acid impacts protein function based on sequence homology [40]. Every piece of information above came from NCBI (https://www.ncbi.nlm.nih.gov/). By using the tolerance Index (TI) score, we were able to predict by SIFT the harmful and tolerable effects of non-synonymous SNPs discovered in the coding area. We can predict acceptable and harmful amino acid substitutions based on this score. Tolerance thresholds vary from 0.0 to 1.0, whereas harmful thresholds are less than 0.05 [41]. By selecting "Median Conservation Sequence Score 3.00" and utilizing various databases, including Swiss-Prot and TrEMBL, SIFT analysis was able to identify homologous sequences based on algorithms. The following URL will take you to Sift: https://sift.bii.a-star.edu.sg

2.4. The anticipation of functional impacts of nsSNPs using PolyPhen-2

The software PolyPhen-2 (Phenotyping Polymorphism) automatically forecasts the potential effects of replacement of amino acids on the structure and functionality of human proteins [42]. In order to calculate position-specific independent counts "PSIC" scores for both types of amino acids, Polyphen-2 finds "3D protein structures" and "contact information" of amino acids. The PSIC score difference is then calculated. The functional effect is directly correlated with the score difference, meaning that each functional effect would increase with a larger "PSIC" score [43]. The polyphen-2 scores fall between 0.0 and 1.0.

- It is expected that variants scoring between 0.0 and 0.15 are innocuous [44].
- Variants with scores between 0.15 and 1.0 are predicted to be potentially harmful [45].
- It is predicted that variants with scores between 0.85 and 1.0 will cause harm [46].

The URL for Polyphen-2 is http://genetics.bwh.harvard.edu/pph2

2.5. The anticipation of protein Stability using I-Mutant 3.0

I-Mutant 3.0 predicts fluctuations in protein stability via neural networks and Support Vector Machine (SVM) algorithms. Analysis indicates that I-mutant outperforms other tools [47]. “Distance-Scaled, Finite Ideal Gas Reference (DFIRE),” “FoldX,” and “Prediction of Protein Mutant Stability Changes (PoPMuSiC).” [48]. In I-Mutant 3.0, the wild type free energy was subtracted from the mutant accessible point to obtain the "free energy change (ΔΔG)." A positive sign of (ΔΔG) indicates the best stability of the protein. The negative sign of (ΔΔG) signifies reduced protein stability [49]. In summary, if (ΔΔG) > 0, it indicates enhanced protein stability, but if (ΔΔG) < 0, it signifies reduced protein stability [50].

I-Mutant 3.0 is located at http://gpcr2.biocomp.unibo.it/cgi/predictors/I-Mutant3.0/I-Mutant3.0.cgi

2.6. Identification of functional nsSNPs using PROVEAN

PROVEAN ("Protein Variation Effect Analyzer") is a rapid computational method that finds functionally significant nsSNPs, predicting if amino acid substitutions impact the protein's biological activity. Scores below -2.5 indicate detrimental SNPs, while scores above -2.5 are classified as neutral SNPs [52]. PROVEAN can be accessed at http://PROVEAN.jcvi.org/seq_submit.php

2.7. Validation to anticipate the functional impacts of sequence variants using SNAP2

SNAP2 serves as a bioinformatics tool designed to predict the functional consequences of sequence variants or amino acid mutations [53]. A trained classifier that utilizes the concept of machine learning is referred to as a neural network [54]. It is essential for distinguishing between affected and neural nsSNPs by considering straightforward data [55]. The visual representation exhibits a certain level of technicality, as illustrated in Figure 2.

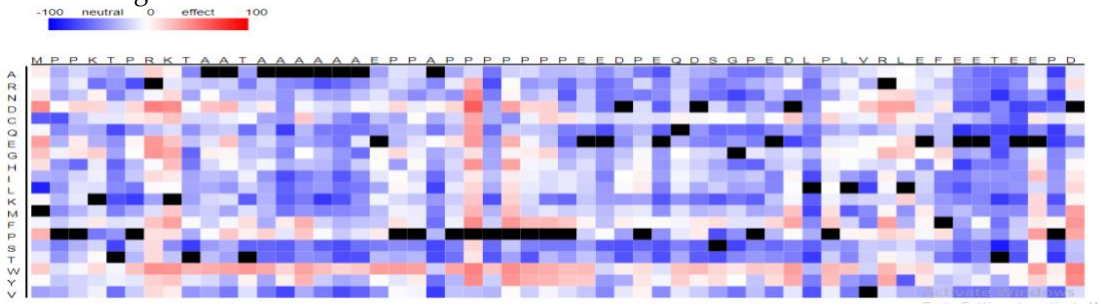


Figure 2. Heatmap Visual Representation

This visual representation has the following criteria;

- **Dark red** tiny blocks indicate score > 50 means a Strong Signal yet affected.
- **White** tiny blocks indicate -50<score<50 means weak signal.
- **Green** tiny blocks mean score <-50 means strong signal yet neutral.

SNAP2 is available at **https://www.rostlab.org/services/snap/**

2.8. Predicting diseased mutations Using PHD-SNP

PhD-SNP, which stands for "Predictor of human deleterious single nucleotide polymorphism," is a bioinformatics software developed using the Support Vector Machine (SVM) methodology [56]. This software is employed to differentiate between diseased and neutral amino acid substitutions among the deleterious nsSNPs identified by the aforementioned tools, including SIFT, Polyphen-2, I-Mutant 3.0, PROVEAN, and SNAP2 [57]. The main reason for utilizing this tool is to ensure accurate results through verification [32].

"PhD-SNP" can be accessed at http://snps.biofold.org/phd-snp/phd-snp.html

2.9. Predicting diseased mutations Using PMut

A bioinformatics web server is utilized to identify disease-causing mutations. It lacks objectivity. Neural Networks are utilized for information processing [58], yielding straight forward predictions regarding neutral or disease mutations, along with associated prediction scores. PMut can be accessed at http://mmb.irbbarcelona.org/PMut

2.10. Predicting diseased mutations Using SNPs&GO

It is a bioinformatics technique utilizing a support vector machine (SVM) to identify mutations that cause disease. Upon input submission, the program will analyze the data and deliver results in the form of neural or disease mutations, along with the Reliability Index (RI). If the RI value displayed by the tool exceeds 5, it signifies that this mutation is associated with a disease-related protein [59]. SNPs&GO can be accessed at http://snps.biofold.org/snps-and-go/snps-and-go.html

2.11. Evolutionary conservation analysis of nsSNPs:

The Consurf Server is a bioinformatics tool utilized to assess the evolutionary conservation score of amino acid positions essential for the function and structure of the Rb1 gene [60]. Evolutionary relationships assess consurf scores. The conservation score scale is shown in the results using a diagram and table derived from UniRef-90. The precision of the conservation score has markedly enhanced due to the empirical Bayesian method, given that no sequences for computation are fewer than [61]. The Consurf Server can be accessed via https://consurf.tau.ac.il.

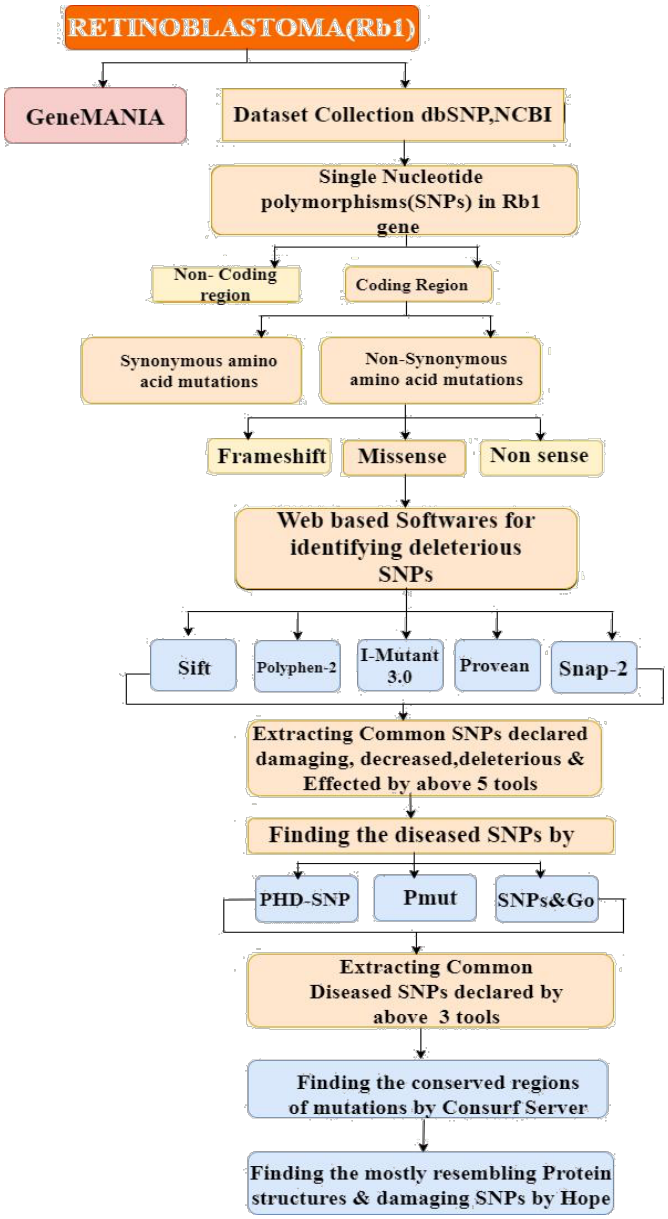


Figure 3. Workflow of in silico methods used in this Study

2.12. Predicting the impact of nsSNPs on 3D Protein Structure

Project Hope is an accessible web-based bioinformatics tool utilized for the collection of 3D protein structures by doing calculations on 3D protein coordinates, utilizing sequence annotations from the UniProt database, and obtaining estimations through DAS services. "Project Hope" can be accessed at (http://www.cmbi.ru.nl/hope/). Figure 3 on the subsequent page illustrates the comprehensive technique employed in this investigation.

3. Results

According to the GeneMANIA software, Rb1 is associated with 20 other genes. Among these 20, CCND1 is strongly associated with Rb1, the sub-component of "holoenzyme". Different parameters i.e., physical interactions, genetic interactions, and co-expression of the Rb1 gene with Other genes are shown in Table 1 and Figure 4.

Table 1. Gene Description Rank Using GeneMANIA

Sr #	Genes	Description	Rank
1.	RB1	RB transcriptional corepressor 1	N/A
2.	CCND1	cyclin D1	1

3.	RBP2	retinol-binding protein 2	2
4.	ATF7	activating transcription factor 7	3
5.	CBX4	chromo box 4	4
6.	TAF1	TATA-box binding protein associated factor 1	5
7.	GTF3C2	general transcription factor IIIC subunit 2	6
8.	ID2	inhibitor of DNA binding 2, HLH protein	7
9.	ELF1	E74, like ETS transcription factor 1	8
10.	CDK4	cyclin-dependent kinase 4	9
11.	PAX3	paired box 3	10
12.	BRF1	BRF1, RNA polymerase III transcription initiation factor 90 kDa subunit	11
13.	RBL1	RB transcriptional corepressor like 1	12
14.	RBL2	RB transcriptional corepressor like 2	13
15.	PURA	purine-rich element binding protein A	14
16.	PPP2R3B	protein phosphatase 2 regulatory subunit B"beta [Source:HGNC Symbol; Acc: HGNC:13417]	15
17.	SUV39H1	suppressor of variegation 3-9 homolog 1	16
18.	IRF3	interferon regulatory factor 3	17
19.	PAX5	paired box 5	18
20.	DNMT1	DNA (cytosine-5-)-methyltransferase 1	19
21.	GTF2H1	general transcription factor IIH subunit 1	20

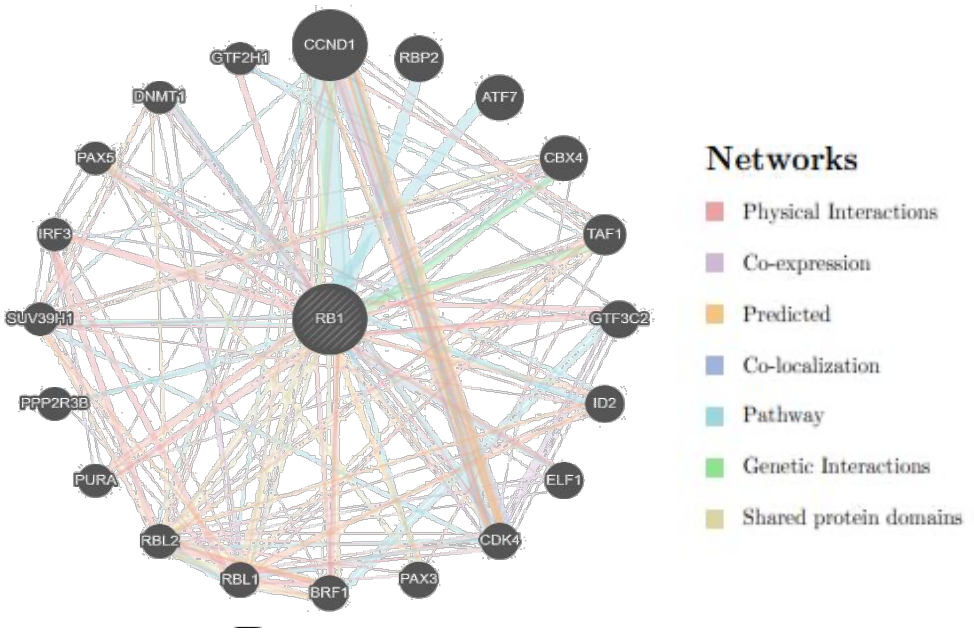


Figure 4. Results of GeneMANIA for Rb1 Gene

Table 2. Summary

Sr #	Tools	Results
1.	Sift	Total SNPs=425 Damaging=139 Tolerated=276 N/A=7 Not secured=3
2.	Polyphen-2	Total SNPs=425 Possibly damaging=93 Probably Damaging=156 Benign=176
3.	I-Mutant3.0	Total SNPs=425 Decreased =325

		Increased=100
4.	PROVEN	Total SNPs=425 Deleterious=89 Neutral=336
5.	SNAP2	Total SNPs=425 Effect=200 Normal=225
6.	PHD-SNP	Total Common damaging SNPs=40 Diseased=26 Neutral=14
7.	Pmut	Total Common damaging SNPs=40 Diseased=24 Neutral=16
8.	SNP& GO	Total Common damaging SNPs=40 Diseased=40 Neutral=0
9.	Consurf Server	Total Common damaging SNPs=21 Conserved=19 Non-Conserved=2
10.	HOPE	Total Common damaging SNPs=21 Damaging=17 Non –Damaging=04

3.1. SIFT Results

Out of all coding SNPs, only missense SNPs were taken into account, as these nsSNPs directly affect the structure and function of the protein. First, to find out whether the mutations affect the part of the protein, a total of 425 missenses were submitted into SIFT. Out of 425, 139 were declared as damaging nsSNPs.

3.2. Polyphen-2 Results

Polyphen-2 is used to analyze the possible effect of amino acid substitution on a protein's structure and function. When 425 nsSNPs were submitted into polyphen-2,93 were declared as Possibly damaging,156 as Probably Damaging, and 176 were Benign.

3.3. Mutant 3.0 Results:

I-Mutant 3.0 is used to automatically analyze changes in Protein stability upon mutations on specific points. I-Mutant predicted 325nsSNPs as decreased stability when all 425 nsSNPs were submitted into I-Mutant 3.0.

3.4. PROVEAN Results

PROVEAN Server to further forecast whether amino acid substitution affects the biological function of the protein or not. PROVEAN declared 89 nsSNPs as deleterious when all 425 nsSNPs were submitted to it.

3.5. SNAP2 Results

After this, SNAP2 was used to determine the impact of mutations on the protein's function. SNAP2 declared 200 nsSNPs as affected out of 425.

3.6. Results of Comparison Method

To go for the best, the comparison method is used to compare the results of all the above tools. Table 3 shows the 40 common deleterious nsSNPs declared by SIFT Polyphen-2, I-Mutant 3.0, PROVEAN, and SNAP2.

4. Results of PhD-SNP, PMut, and SNPs&GO

To analyze whether these above 40 Common damaging SNPs are disease-causing or neutral, we will check them utilizing several tools, i.e., PhD-SNP, PMut, and SNPs&GO. "S2 Appendix" shows the results of "PHD-SNP, PMut, and SNPs&GO" when these 40 joined deleterious nsSNPs (declared by Sift, Polyphen-2, I-Mutant 3.0, PROVEAN and SNAP2) were submitted to them. Figure 5 shows that All three tools, i.e., PhD-SNP, PMut, and SNPs&GO display the result in the form of neutral and disease-causing mutations.

Sr#	Rsid's	AA Change	Sift Prediction	Score	Polyphen-2 Prediction	Score	I-Mutant 3.0 Prediction	DDG value	PROVEAN Prediction	Score	SNAP2 Prediction	Score
1.	rs983885759	L120Q	Damaging	0	Probably Damaging	1	Decrease	-1.44	Deleterious	-3.968	Effect	69
2.	rs749495284	F131L	Damaging	0	Probably Damaging	0.999	Decrease	-1.09	Deleterious	-3.786	Effect	67
3.	rs766849295	Y155C	Damaging	0	Probably Damaging	1	Decrease	-0.72	Deleterious	-5.529	Effect	53
4.	rs1034616967	D156Y	Damaging	0.03	Probably Damaging	0.93	Decrease	-0.56	Deleterious	-2.689	Effect	40
5.	rs141366046	V190G	Damaging	0.01	Probably Damaging	0.695	Decrease	-2.74	Deleterious	-2.575	Effect	69
6.	rs1267000381	V213M	Damaging	0	Probably Damaging	1	Decrease	-0.28	Deleterious	-2.6	Effect	79
7.	rs1273219762	C283Y	Damaging	0.01	Probably Damaging	0.949	Decrease	-1.79	Deleterious	-4.455	Effect	61
8.	rs765678030	E323G	Damaging	0.01	Probably Damaging	0.969	Decrease	-1.63	Deleterious	-3.883	Effect	50
9.	rs868847993	Y325H	Damaging	0	Probably Damaging	0.605	Decrease	-0.82	Deleterious	-3.158	Effect	74
10.	rs763377384	D330N	Damaging	0.03	Probably Damaging	0.605	Decrease	-2.08	Deleterious	-3.695	Effect	67
11.	rs763184576	D332G	Damaging	0.03	Probably Damaging	0.999	Decrease	-1.05	Deleterious	-5.239	Effect	73
12.	rs748635133	G423E	Damaging	0.05	Probably Damaging	0.998	Decrease	-1	Deleterious	-4.381	Effect	69
13.	rs747509282	R445Q	Damaging	0	Probably Damaging	1	Decrease	-0.4	Deleterious	-2.985	Effect	69
14.	rs759079385	R451C	Damaging	0.01	Probably Damaging	1	Decrease	-1.02	Deleterious	-3.618	Effect	24
15.	rs771480219	E492V	Damaging	0	Probably Damaging	1	Decrease	-1.62	Deleterious	-6.529	Effect	79
16.	rs1158433317	L512S	Damaging	0.05	Probably Damaging	0.977	Decrease	-0.79	Deleterious	-2.526	Effect	67
17.	rs866664638	P515T	Damaging	0	Probably Damaging	1	Decrease	-0.46	Deleterious	-7.467	Effect	66
18.	rs138201027	W516G	Damaging	0	Probably Damaging	1	Decrease	-1.62	Deleterious	-10	Effect	81
19.	rs1331702695	F526V	Damaging	0.01	Probably Damaging	0.999	Decrease	-0.62	Deleterious	-5.333	Effect	67
20.	rs143324585	V531G	Damaging	0	Probably Damaging	1	Decrease	-1.93	Deleterious	-6.533	Effect	76
21.	rs1237070816	E533K	Damaging	0	Probably Damaging	1	Decrease	-2.21	Deleterious	-3.733	Effect	88
22.	rs148379933	E539K	Damaging	0.01	Probably Damaging	1	Decrease	-2.35	Deleterious	-3.3	Effect	79
23.	rs139494954	M558R	Damaging	0	Probably Damaging	0.935	Decrease	-1.61	Deleterious	-4.5	Effect	46
24.	rs143400770	L561P	Damaging	0.01	Probably Damaging	1	Decrease	-0.02	Deleterious	-5.332	Effect	75

25.	**rs139500527**	**W563G**	**Damaging**	**0**	**Probably Damaging**	**1**	**Decrease**	**-2.32**	**Deleterious**	**-12.133**	**Effect**	**88**
26.	rs772068738	P595L	Damaging	0.02	Probably Damaging	0.813	Decrease	-0.49	Deleterious	-3.915	Effect	35
27.	rs775051210	S608C	Damaging	0.01	Probably Damaging	1	Decrease	-2.04	Deleterious	-3.279	Effect	26
28.	rs562956970	L657Q	Damaging	0	Probably Damaging	0.939	Decrease	-1.05	Deleterious	-5.1	Effect	69
29.	rs202119986	A658T	Damaging	0	Probably Damaging	1	Decrease	-2	Deleterious	-3.389	Effect	50
30.	rs750578651	R661Q	Damaging	0	Probably Damaging	1	Decrease	-0.93	Deleterious	-3.581	Effect	84
31.	rs1172128543	H686R	Damaging	0.01	Probably Damaging	1	Decrease	-1.32	Deleterious	-4.85	Effect	72
32.	rs775195256	E693G	Damaging	0.01	Probably Damaging	1	Decrease	-0.73	Deleterious	-3.942	Effect	67
33.	rs1358369644	D697H	Damaging	0.02	Probably Damaging	0.998	Decrease	-1.11	Deleterious	-4.5	Effect	72
34.	rs3092903	D697E	Damaging	0.04	Probably Damaging	1	Decrease	-0.72	Deleterious	-2.796	Effect	16
35.	rs1363146373	Y771C	Damaging	0.01	Probably Damaging	0.950	Decrease	-1.81	Deleterious	-4.943	Effect	59
36.	rs754507551	P786S	Damaging	0.03	Probably Damaging	0.995	Decrease	-0.75	Deleterious	-2.688	Effect	14
37.	rs1467492987	P789H	Damaging	0	Probably Damaging	0.997	Decrease	-0.66	Deleterious	-3.802	Effect	39
38.	rs1158706854	P796L	Damaging	0	Probably Damaging	0.485	Decrease	0	Deleterious	-5.221	Effect	19
39.	rs187912365	R798W	Damaging	0	Probably Damaging	1	Decrease	-1.21	Deleterious	-3.986	Effect	75
40.	rs1394610552	G836D	Damaging	0.05	Probably Damaging	1	Decrease	-1.36	Deleterious	-3.289	Effect	46

Figure 5. Table of 40 COMMON nsSNPs DECLARED DAMAGED BY SIFT, Polyphen-2,I-Mutant3.0, PROVEAN & SNAP2

- PHD-SNP predicts 26 out of 40 mutations as Diseased and 14 as neutral mutations.
- PMut predicts 24 out of 40 as diseased and 16 as neutral mutations.
- SNPs&GO predicts 40 out of 40 mutations have disease effects.

That 21 nsSNPs which are bold, are the common diseased mutations declared by all the above three tools.

Table 3. LIST OF nsSNPs predicted as Diseased by PHD-SNP , PMut and SNPs&GO

Sr #	RSID's	AA Change	PHD-SNP Prediction	RI	PMut Prediction	Score	SNPs&GO Prediction	RI
1.	rs983885759	L120Q	Disease	4	Disease	0.52	Disease	8
2.	rs749495284	F131L	Disease	4	Disease	0.79	Disease	9
3.	rs766849295	Y155C	Neutral	1	Neutral	0.49	Disease	9
4.	rs1034616967	D156Y	Neutral	7	Neutral	0.16	Disease	7
5.	rs141366046	V190G	Neutral	0	Disease	0.62	Disease	9
6.	rs1267000381	V213M	Disease	3	Neutral	0.43	Disease	9
7.	rs1273219762	C283Y	Disease	5	Disease	0.63	Disease	9
8.	rs765678030	E323G	Neutral	2	Neutral	0.48	Disease	7

9.	rs868847993	Y325H	Neutral	5	Disease	0.62	Disease	8
10.	rs763377384	D330N	Neutral	5	Neutral	0.14	Disease	7
11.	rs763184576	D332G	Disease	7	Disease	0.82	Disease	9
12.	rs748635133	G423E	Disease	3	Neutral	0.21	Disease	8
13.	rs747509282	R445Q	Disease	7	Disease	0.62	Disease	9
14.	rs759079385	R451C	Disease	4	Neutral	0.24	Disease	9
15.	rs771480219	E492V	Disease	7	Disease	0.82	Disease	10
16.	rs1158433317	L512S	Neutral	2	Neutral	0.34	Disease	9
17.	rs866664638	P515T	Disease	6	Disease	0.81	Disease	9
18.	rs138201027	W516G	Disease	9	Disease	0.7	Disease	10
19.	rs1331702695	F526V	Disease	9	Neutral	0.48	Disease	9
20.	rs143324585	V531G	Disease	8	Disease	0.77	Disease	10
21.	rs1237070816	E533K	Disease	8	Disease	0.82	Disease	10
22.	rs148379933	E539K	Disease	5	Disease	0.66	Disease	10
23.	rs139494954	M558R	Disease	7	Disease	0.62	Disease	6
24.	rs143400770	L561P	Disease	8	Disease	0.7	Disease	10
25.	rs139500527	W563G	Disease	9	Disease	0.82	Disease	9
26.	rs772068738	P595L	Neutral	4	Neutral	0.43	Disease	7
27.	rs775051210	S608C	Neutral	3	Neutral	0.47	Disease	8
28.	rs562956970	L657Q	Disease	9	Disease	0.73	Disease	10
29.	rs202119986	A658T	Disease	6	Disease	0.53	Disease	9
30.	rs750578651	R661Q	Disease	9	Disease	0.84	Disease	10
31.	rs1172128543	H686R	Neutral	1	Neutral	0.26	Disease	8
32.	rs775195256	E693G	Disease	3	Neutral	0.32	Disease	9
33.	rs1358369644	D697H	Disease	3	Disease	0.63	Disease	9
34.	rs3092903	D697E	Disease	1	Disease	0.56	Disease	8
35.	rs1363146373	Y771C	Neutral	0	Disease	0.6	Disease	9
36.	rs754507551	P786S	Neutral	3	Neutral	0.3	Disease	8

37.	rs1467492987	P789H	Neutral	0	Neutral	0.5	Disease	9
38.	rs1158706854	P796L	Disease	3	Disease	0.81	Disease	10
39.	rs187912365	R798W	Disease	3	Disease	0.67	Disease	9
40.	rs1394610552	G836D	Neutral	2	Neutral	0.5	Disease	9

4.1. Results of Consurf Server

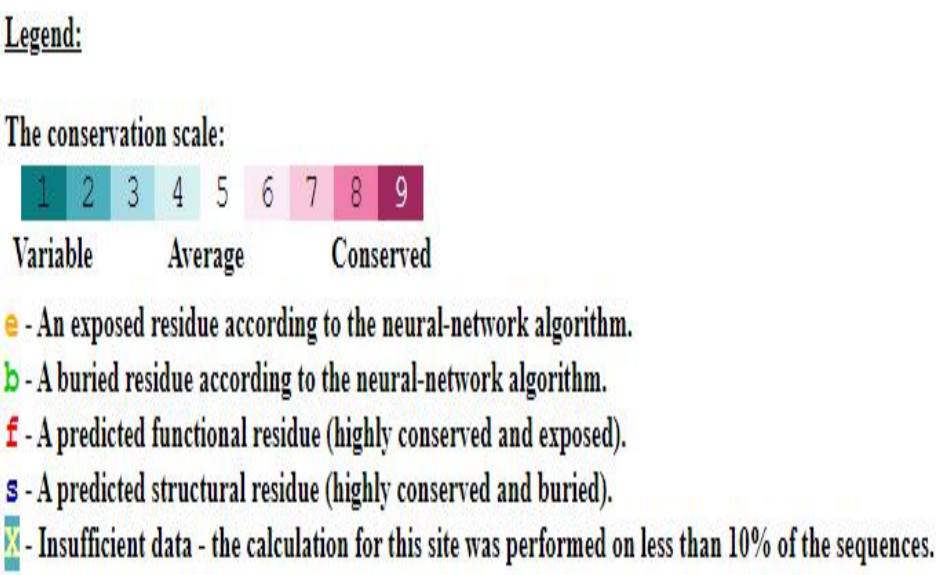
The role of evolutionary information is much more vital as it is used in finding out those mutations. Which might give rise to detrimental effects on human health. For this, all that common diseased. Mutations declared by PHD-SNP, PMut and SNPs&GO were submitted into the consurf web server in order to estimate their evolutionary conservation scores because diseased nsSNPs located at Conserved regions are incredibly high risk diseased to the protein's structure and function than nsSNPs located in non-conserved regions. Evolutionary scores of above 21 diseased nsSNPs are being calculated from the consurf Server as Shown in Figure 5, and then their results were displayed in "**S3 Appendix.**" The following Legend scale describe the conversation scale used in Figure 5.

In consurf output, color shows the evolutionary conservation. Range from 1-4 shows variability in Conservation is indicated by blue. Range from 5-6, shows average protection and It is indicated by white. Range from 7-9 shows conservation and is characterized by purple. The residue (e) Means exposed, (b) means buried,(f) means functional, i.e., highly conserved and exposed, (s) means Structured, i.e., highly conserved and buried, and (x) means insufficient data. The black bold Downward arrows represent.

L120Q,F131L,C283Y,D332G,R445Q,E492V,P515T,W516G,V531G,E533K,E539K,M558RL561P ,W563G,L657Q,A658T,R661Q,D697H,D697E,P796L and R798W amino acid mutations of SNPs i.e. rs983885759,rs749495284,rs1273219762,rs763184576,rs747509282,rs771480219,rs866664638,rs1 38201027,rs143324585,rs1237070816,rs148379933,rs139494954,rs143400770,rs139500527,rs562 956970,rs202119986,rs750578651,rs1358369644,rs3092903,rs1158706854 and rs187912365 respectively.)

From Table 5, Residue (e) shows exposed, (b) shows buried,(f) shows functional i.e. highly conserved and exposed, (s) shows structured, i.e., highly conserved and buried. Conservation scores from 1-4 means variable, 5-6 means intermediate, and 7-9 mean highly. Conserved scores.

From Figure 5 and "S3 Appendix", results can be concluded that out of 21 highly-risk diseased SNPs, 19 are located on conserved regions(Score:7-9) while remaining two mutations i.e. L120Q, L561P Having scored 4 and 5, respectively, are located in non-conserved regions. Appendix S3, the The conservation score of conserved mutations is shown in bold. This means that these 19 conserved mutations are much more damaging to the function and structure of the Rb1 protein.



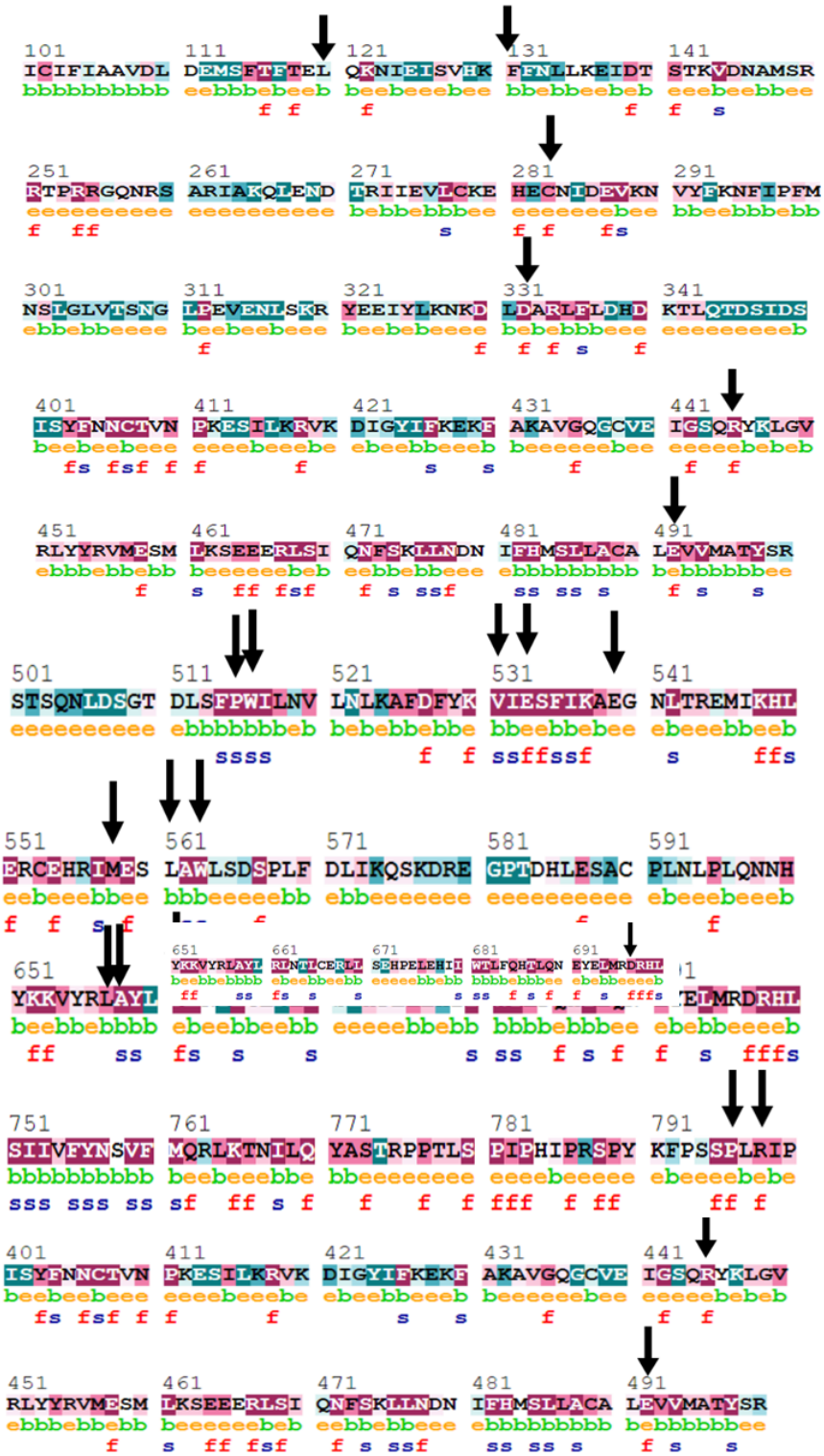


Figure 6. The output of Consurf by the uniRef90 Protein database

Table 4. Conservation Profile of High Risk nsSNPs in Rb1 by UniRef-90

SR #	Rsid's	Residue & Position	Conservation Scores	Function
1.	rs983885759	L120Q	4	B
2.	rs749495284	F131L	**7**	B

3.	rs1273219762	C283Y	**8**	F
4.	rs763184576	D332G	**9**	F
5.	rs747509282	R445Q	**9**	F
6.	rs771480219	E492V	**9**	F
7.	rs866664638	P515T	**9**	S
8.	rs138201027	W516G	**9**	S
9.	rs143324585	V531G	**9**	S
10.	rs1237070816	E533K	**9**	F
11.	rs148379933	E539K	**7**	E
12.	rs139494954	M558R	**8**	B
13.	rs143400770	L561P	5	B
14.	rs139500527	W563G	**9**	S
15.	rs562956970	L657Q	**8**	B
16.	rs202119986	A658T	**9**	B
17.	rs750578651	R661Q	**9**	F
18.	rs1358369644	D697H	**8**	F
19.	rs3092903	D697E	**8**	F
20.	rs1158706854	P796L	**9**	F
21.	rs187912365	R798W	**8**	F

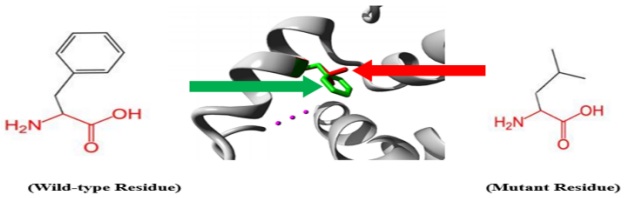
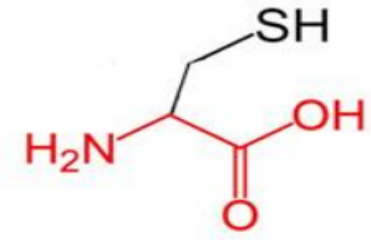
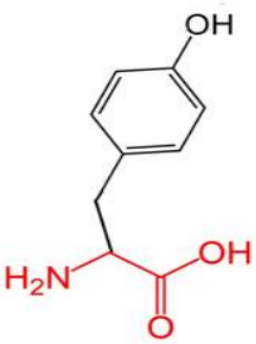
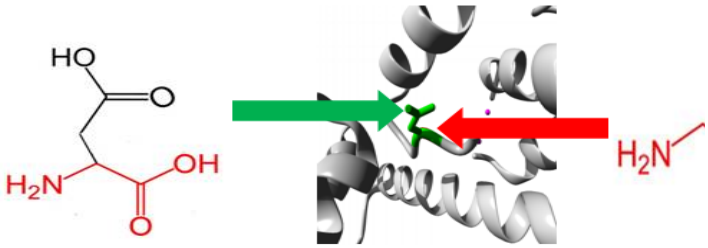
4.2. Results of Project Hope

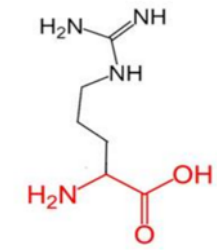
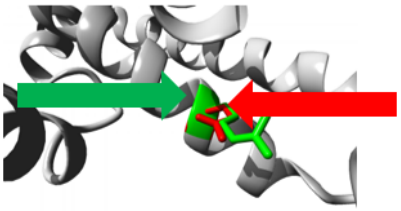
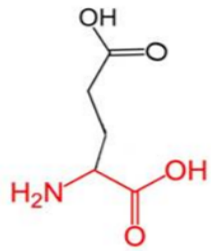
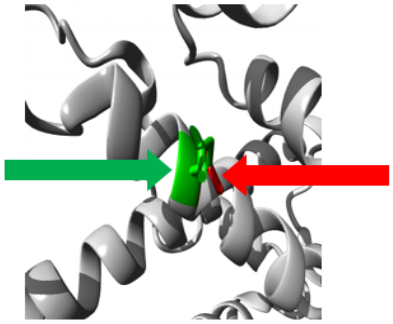
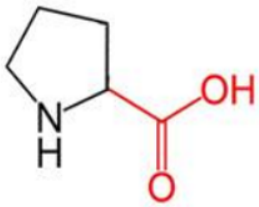
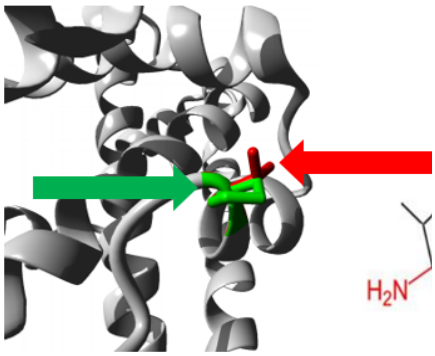
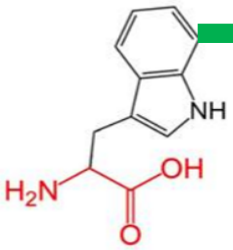
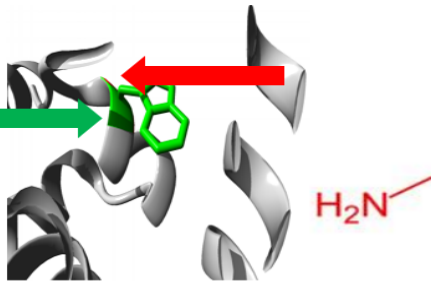
The 19 conserved mutations identified by the Consurf Server were submitted to Project HOPE to obtain the 3D protein structure and the physical properties of the amino acids. HOPE concluded that, in many cases, the size of the mutant residue was smaller than the wild-type residue. Observations were made in SNPs with rsIDs including rs749495284, rs763184576, rs747509282, rs771480219, rs138201027, rs143324585, rs139500527, and rs750578651, which impact positions F131L, D332G, R445Q, E492V, W516G, V531G, W563G, and R661Q, respectively. Conversely, in other SNPs such as rs1273219762, rs1237070816, rs148379933, rs139494954, rs562956970, rs202119986, rs1358369644, rs3092903, rs1158706854, and rs187912365 at positions C283Y, E533K, E539K, M558R, L657Q, A658T, D697H, D697E, P796L, and R798W, the size of the mutant residue exceeded that of the native residue.

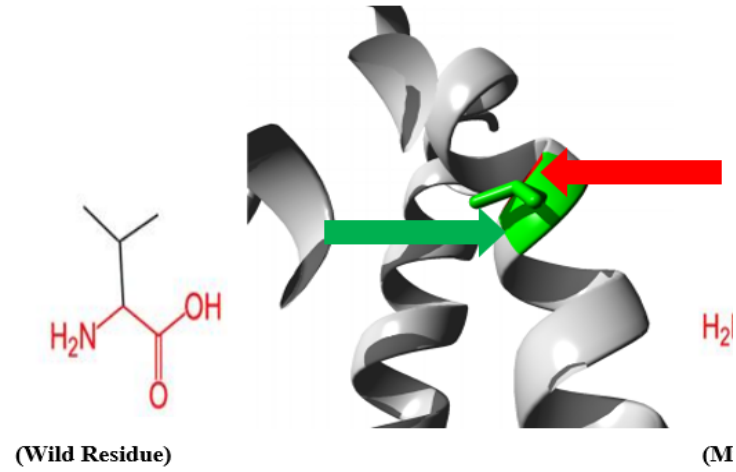
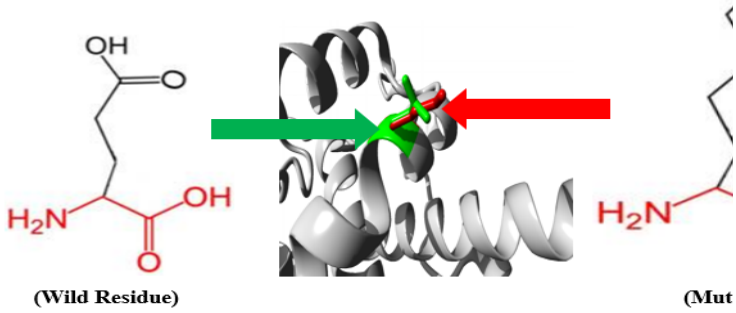
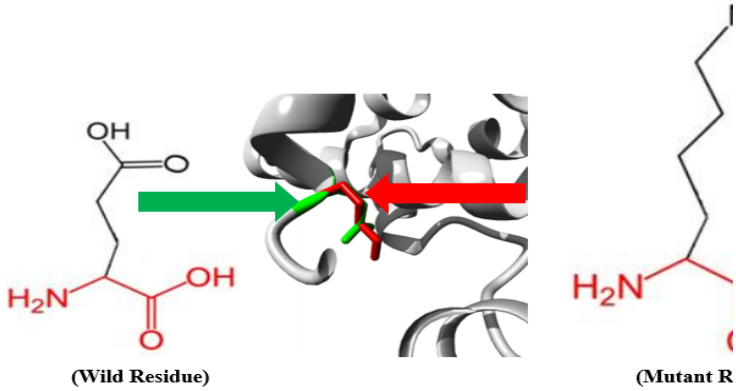
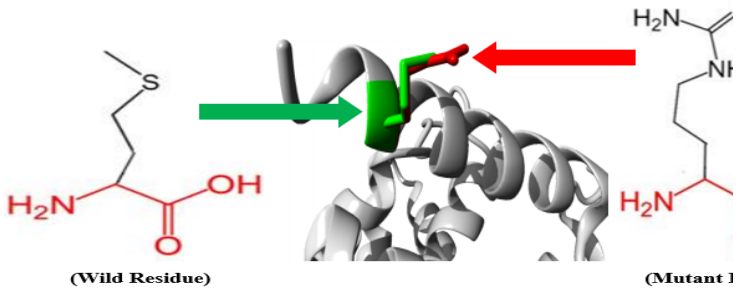
Regarding charge differences, in many SNPs, such as rs763184576, rs771480219, and rs1358369644 (positions D332G, E492V, and D697H), the wild-type residue was negatively charged, while the mutant residue was neutral. The wild-type residue had a positive charge for SNPs like rs750578651 and rs187912365 (positions R661Q and R798W), while the mutant residue was neutral. In some cases, such as rs1237070816 at position E533K, the wild-type residue was negatively charged, while the mutant had a positive charge. In SNP rs148379933 at position E539K, the wild-type residue had a negative charge, while the mutant residue had a positive charge. In SNP rs139494954 at position M558R, the wild-type residue had a neutral charge, but the mutant residue had a positive charge.

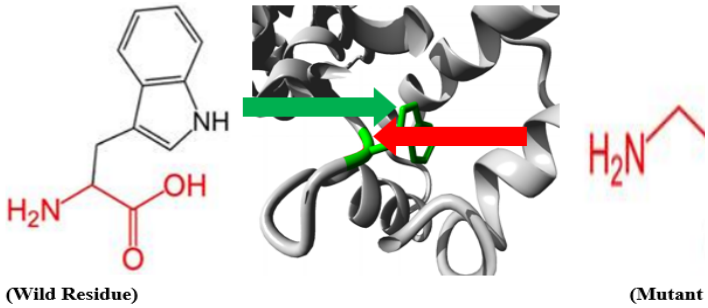
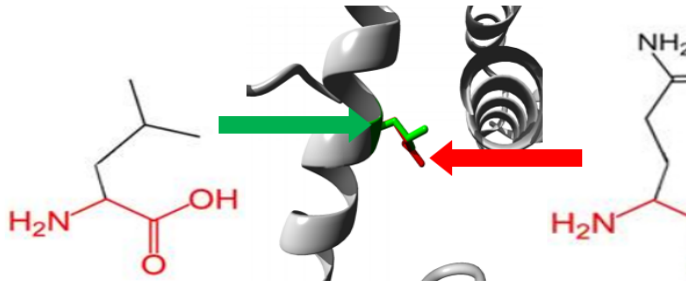
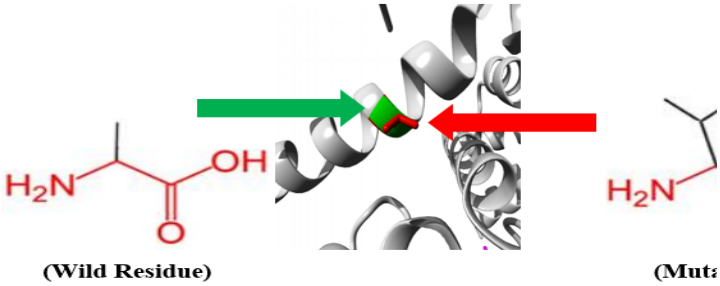
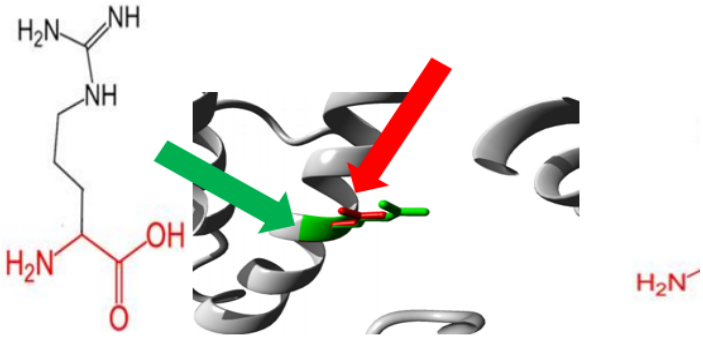
Regarding hydrophobicity, the wild-type residue was more hydrophobic than the mutant residue in several SNPs, including rs866664638, rs138201027, rs143324585, rs139494954, rs139500527, rs562956970, and rs202119986 at positions P515T, W516G, V531G, M558R, W563G, L657Q, and A658T. Conversely, the mutant residue had higher hydrophobicity than the wild-type residue in SNPs such as rs771480219 and rs187912365 at positions E492V and R798W. The protein structures of these mutations, along with the amino acid changes for each nsSNP, are shown in Table 6.

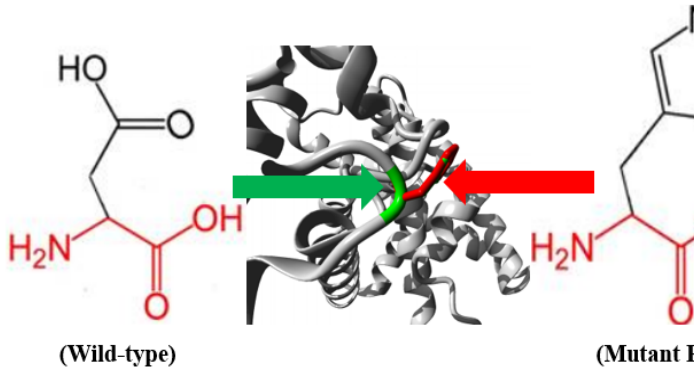
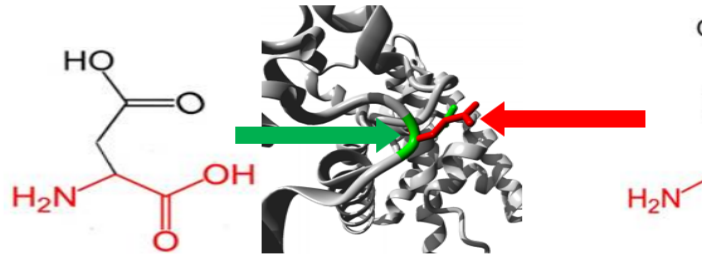
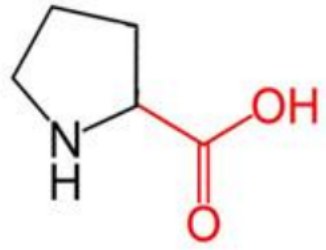
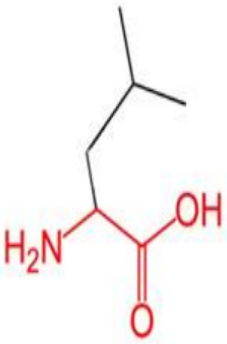
Table 5. Results of Project Hope

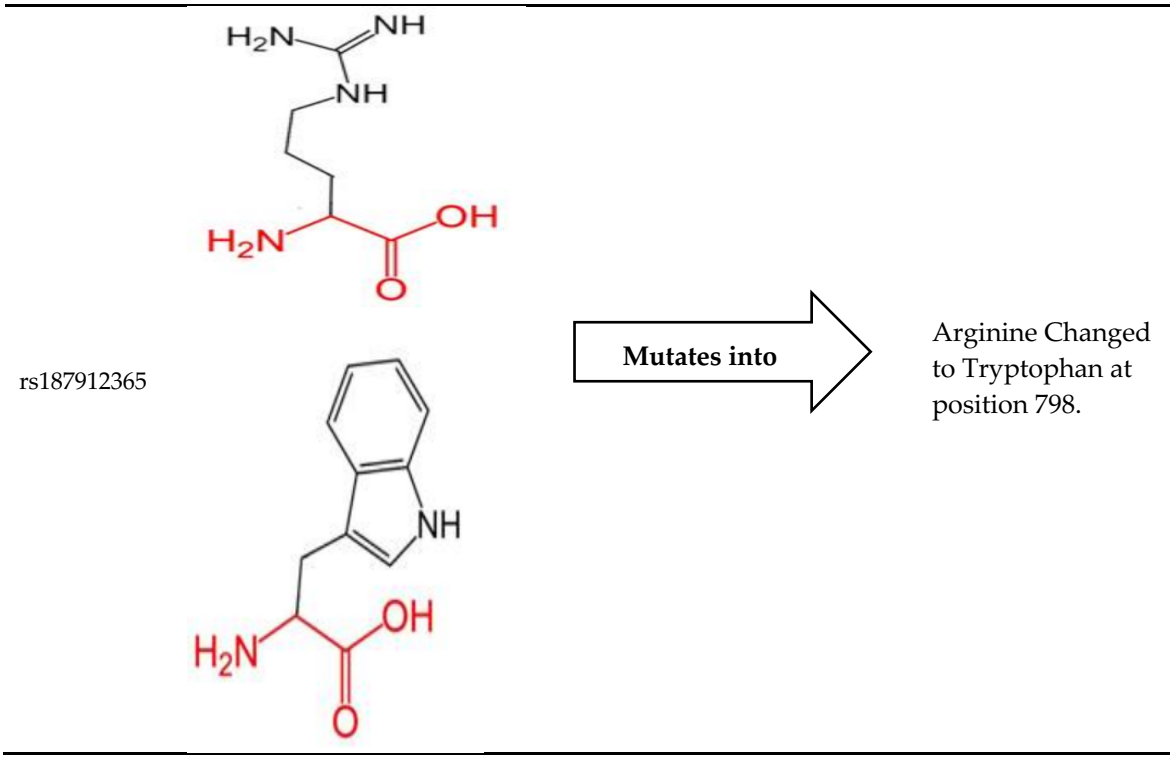
SNP ID	3D Structure	Amino Acid Change
rs749495284		Phenylalanine Changed to Leucine at position 131.
rs1273219762		Cysteine Changed to Tyrosine at position 283.
		However the 3-D structure or modelling template is being missed on hope for unknown reason but administrator is being apprised in this regard.
rs763184576		Aspartic acid Changed to Glycine at position 332.

rs747509282	 (Wild-type residue)		Arginine Changed to Glutamine at position 445.
rs771480219	 (Wild Residue)		Glutamic Acid Changed to Valine at position 492.
rs866664638	 (Wild Residue)		Proline Changed to Threonine at position 515.
rs138201027	 (Wild Residue)		Tryptophan Changed to Glycin at position 516.

rs143324585		Valine Changed to Glycine at position 531.
rs1237070816		Glutamic Acid changed to Lysine at position 533.
rs148379933		Glutamic Acid changed to Lysine at position 539.
rs139494954		Methionine Changed to Arginine at position 558.

rs139500527	 (Wild Residue) (Mutant	Tryptophan Changed to Glycine at position 563.
rs562956970	 (Wild Residue) (Mutant Re	Leucine Changed to Glutamine at position 657.
rs202119986	 (Wild Residue) (Muta	Alanine mutated to Threonine at position 658.
rs750578651	 (Wild-type Residue) (Mutan	Arginine Changed to Glutamine at position 661.

rs1358369644	 (Wild-type) (Mutant I	Aspartic Acid Changed to Histidine at position 697.
rs3092903	 (Wild Residue) (Muta	Aspartic acid to Glutamic acid at position 697.
rs1158706854	 **Mutates into** 	Proline Changed to Leucine at position 796. Protein structure can't be generated by hope because of lack of structural information.



Therefore, after giving protein structures of 19 mutations, Project Hope concluded that out of 19, 17 mutations i.e. D332G, R445Q, E492V, P515T, W516G, V531G, E533K, E539K, M558R, W563G,L657Q, A658T, R661Q, D697H, D697E, P796L and R798W are responsible for Rb1 protein Damage. Just two mutations, i.e., F131L and C283Y, are not the sources of protein damage.

5. Discussion

Due to technological advances, the number of recognized genomic variants (mostly single nucleotide polymorphisms (SNPs)) in the human genome is growing rapidly. In population genetics and molecular biology, it is of great interest to separate deleterious SNPs from quasi-neutral ones. Here, we depict an in-vitro analysis identifying functional SNPs within the Rb1 gene. The keyThe key novelty of this work is to identify more deleterious SNPs and perform structural analysis on them.

Rb1 is a tumor suppressor gene that regulates cell growth by inhibiting cells from undergoing uncontrolled division. However, mutations in this gene sometimes cause dramatic problems, such as retinoblastoma (eye cancer that affects very young children), some forms of breast cancer and melanoma, a type of skin cancer. These disorders are such a threat that knowing how these mutations come about is crucial.

This research systematically identifies functional SNPs in the Rb1 gene and investigates how these SNPs affect protein function and structure, ultimately leading to diseases. Information regarding the Rb1 SNP was acquired from the NCBI dbSNP database. 36,358 SNPs were identified, comprising 345 in the 3' UTR region, 65 in the 5' UTR, and the remainder in the intronic region.

Among these coding SNPs, 199 were synonymous and 450 non-synonymous (425 missense, five nonsense and 20 frameshift). The missense mutations were particularly interesting in this study because they more directly modulate Rb1 protein function and structure.

It employed bioinformatics tools (SIFT, PolyPhen-2, I-Mutant 3.0, PROVEAN and SNAP2) and the analysis showed 40 common deleterious SNPs. Of the 40 SNPs analyzed, PHD-SNP2, PMut and SNPs&GO determined that 21 mutations are disease-causing. The others were analyzed by Consurf Server for their conservation in their area (as the more harmful a mutation can be if it is conserved). Analysis indicated that 19 out of 21 mutations were within conserved regions. Project HOPE, in the follow-up work, to analyze the impact of these 19 mutations on protein structure and function. Protein structure analysis revealed that 17 out of the 19 mutations were highly deleterious, resulting in severe damage to protein. The three-dimensional structure of amino acids has been analyzed regarding hydrophobicity, charge, size and

flexibility. All tools employed confirmed that the impact of these 17 mutations on the structural and functional integrity of Rb1 was severe.

In short, these 17 mutations are very important for the diagnosis and therapy of both genetic diseases, such as retinoblastoma and its related disorders. This understanding serves as a foundation for advancing personalized medicine in these individuals. The renal development pathway is sufficiently delicate that any disruption in Rb1 protein function can significantly affect related disease pathways, highlighting the necessity for early detection and targeted intervention.

6. Conclusion

In this study, the in-silico analysis was performed using several bioinformatics tools to determine the deleterious mutations in the Rb1 gene associated with diseases like retinoblastoma. We studied 36,358 SNPs obtained from dbSNP, NCBI and of them, 844 were coding SNPs, 425 missense, 5 nonsense and 20 frameshifts. We employed a ten-step method utilizing the following bioinformatics tools to ensure high reliability and correctness of the results: SIFT, PolyPhen-2, I-Mutant 3.0, PROVEAN, SNAP2, PHD-SNP, PMut, SNPs&GO, Consurf, and Project HOPE. This methodology allowed us to identify and select 17 pertinent nsSNPs that directly influence the structure and function of the Rb1 protein. These results suggest that using multiple computational methods for SNP analysis is important, specifically for determining the pathogenicity and structural effect of the SNPs. The fact that SNPs are located in conserved regions and the results of the analysis with Consurf Server prove that SNPs can be considered as having a deleterious effect. The last assessment, made through Project HOPE, demonstrated that these mutations affect the physical characteristics of the Rb1 protein, including hydrophobicity, charge, size, and flexibility, in a pathogenic manner. This study identifies 17 pathogenic mutations (F131L, C283Y, D332G, R445Q, E492V, P515T, W516G, V531G, E533K, E539K, M558R, W563G, L657Q, A658T, R661Q, D697H, D697E, P796L, and R798W) which will be useful These findings not only help to elaborate the molecular basis of Rb1 gene mutation but also provide a direction for the personalized medicine and treatment. Since retinoblastoma is still a global concern, our study could be a significant source of information on genetic testing and treatment.

7. Abbreviations

The following table 7, defines all the abbreviations used in our study.

Table 3. Abbreviations

Abbreviation	Full Form
SNPs	Single Nucleotide Polymorphisms
Rb1	Retinoblastoma 1 gene
NCBI	National Center for Biotechnology Information
UTR	Untranslated Region
nsSNP	Non-synonymous Single Nucleotide Polymorphisms
SIFT	Sorting Intolerant From Tolerant
PROVEAN	Protein Variation Effect Analyzer
SNAP2	Screening for Non-Acceptable Polymorphisms
PHD-SNP	Predictor of Human Deleterious Single Nucleotide Polymorphism
PMut	Pathogenic Mutations Prediction
SNPs&GO	Single Nucleotide Polymorphisms and Gene Ontology
HOPE	Have (y)Our Protein Explained
SVM	Support Vector Machine
GeneMANIA	Gene Multiple Association Network Integration Algorithm
I-Mutant 3.0	A software predicting protein stability upon mutation
dbSNP	Database for Single Nucleotide Polymorphisms
Consurf	A server that estimates evolutionary conservation scores
3D	Three-Dimensional

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