Computational Analysis of Apoptosis Regulatory Genes: p53 and Bcl-2

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Abstract: Apoptosis is an important process of the cell cycle stages. It helps in the maintenance of cell homeostasis by controlling the cell life span. This process is regulated by majorly two categories of proteins i.e. pro-apoptotic and anti-apoptotic. p53 and Bcl-2 are the two major proteins that play a key role in the regulation of apoptosis. So it would be beneficial to study the regulation of these proteins at genetics level. By considering that the SNPs has always great impact on the expression of the genes, we performed the computational analysis of the SNPs of p53 and Bcl-2 genes. In the present study we found 298 and 44 ns SNPs of p53 and Bcl-2 as damaging respectively.

Keywords: Single nucleotide polymorphism, Apoptosis, p53, Bcl-2

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Introduction

Apoptosis is a form of cell death in which a programmed sequence of events leads to the elimination of cells without releasing harmful substances into the surrounding area. It is a normal physiological process that plays a crucial role in developing and maintaining the health of the body by eliminating damaged DNA, superfluous or unwanted cells, and when halted by genetic mutation may result in uncontrolled cell growth and tumor formation (Karam *et al.*, 2009). Between 50 and 70 billion cells die each day due to apoptosis in the average human adult (Rastogi et al., 2009). The process of apoptosis is regulated by generally two categories of genes

i.e. pro-apoptotic (Bcl-2, Bcl-xl and Mcl) and anti-apoptotic (Bax, p53, and cyt-c). Among these genes p53 and Bcl-2 has an important role in the regulation of apoptosis. p53 has a major role in various diseases such as cancers, autoimmune diseases and neurodegenerative diseases. This gene is considered to be the most frequent associated gene in every forms of human cancer (Wanger et al., 2015). The normal functioning of p53 is a potent barrier to cancer. Tumor-associated mutations in TP53, typically single nucleotide substitutions in the coding sequence, are hallmark of most human cancers and cause dramatic defects in p53 function (Matlashewski *et al.,* 2009). P53 gene expression stimulates a wide network of signals that act through extrinsic pathways and intrinsic pathways. This gene is most important and the master regulator gene of the genome (Matlashewski *et al.,* 1984; Bensaad *et al.,* 2006). The traditional view describing p53 activation in response to cellular stress comprises three basic steps: stabilization of p53, sequence specific DNA binding, and transcriptional activation of target genes (Green et al., 1991;Yee and Vousden., 2005). p53 stabilization is primarily achieved through events that disrupt its interaction with Mdm2, a negative regulator that mediates an ubiquitin-mediated degradation of p53. Further the above described anti-apoptotic genes, Bcl-2 is the most common apoptosis repressor. This is also one of the major regulators of programmed cell death and its morphological equivalent “apoptosis” is the Bcl-2 gene (Wyllie et al., 1980). The Bcl-2 gene was first discovered because of its involvement in t (14; 18) chromosomal translocations found in the majority of follicular B-cell lymphomas hence named as Bcl-2. This chromosomal translocation leads to misregulation of the normal Bcl-2 expression pattern to contribute to cancer (Tsujimoto et al., 1984; Nunez et al., 1989). The discovery of BCL-2 established a new paradigm in cancer biology, namely that apoptosis defects give cells selective survival superiority (Tsujimoto *et al*., 1984). BCL-2 gene expression has been positively associated with cancer cell differentiation and inversely with disease progression. There are various association studies reported of p53 and Bcl-2 gene. Due to the importance of these association studies in the regulation of apoptosis, we decided to evaluate the damaging/deleterious status of the SNPs of the targeted genes.

Materials and Methods

Prediction for deleterious/ damaging SNPs

Single nucleotide polymorphism (SNPs) consists of human genetic variations, occurring with an average density of ~1/1000 nucleotides of a genotype. SNPs are either neutral allelic

variants or missense variants. The SNPs that are causing missense residual changes at amino acid level are likely to affect the structure and function of protein. Such effect of a residual change at the level of single nucleotide variation can be predicted by different in silico tools. These include SIFT (Sorting of Intolerance From Tolerance) and I-Mutant.

SIFT (Sorting of Intolerance From Tolerance)

SIFT predicts whether an amino acid substitution affects protein function. SIFT prediction is based on the degree of conservation of amino acid residues in sequence alignments derived from closely related sequences, collected through PSI-BLAST. SIFT can be applied to naturally occurring nonsynonymous polymorphisms or laboratory-induced missense mutations. It is a sequence homology based tool that sorts intolerant from tolerant amino acid substitutions and predicts whether an amino acid substitution in a protein will have a phenotypic effect. SIFT is based on the premise that protein evolution is correlated with protein function. Positions important for function should be conserved in an alignment of the protein family, whereas unimportant positions should appear diverse in an alignment.

Working of SIFT

SIFT takes a query sequence and uses multiple alignment information to predict tolerated and deleterious substitutions for every position of the query sequence. SIFT is a multistep procedure that (1) searches for similar sequences, (2) chooses closely related sequences that may share similar function to the query sequence , (3) obtains the alignment of these chosen sequences, and (4) calculates normalized probabilities for all possible substitutions from the alignment. Positions with normalized probabilities less than 0.05 are predicted to be deleterious, those greater than or equal to 0.05 are predicted to be tolerated. The SIFT prediction for substitution were given as shown in table

Table 2.1: Showing the SIFT prediction and substitution

OUTPUT	DESCRIPTION
SIFT Score	Ranges from 0 to 1. The amino acid substitution is predicted damaging if the score is <0.05 and tolerated if score is >/=0.05
Median info	Ranges from 0 to 4.32, ideally the number would be between 2.75 and 3.5. This is used to measure the diversity of the sequence used for prediction. A warning will occur if this is greater than 3.25 because this indicates that the prediction was based on closely related sequence.

Seq at position	This is the number of sequences that have an amino acid at the position of prediction.

I-MUTANT

I-Mutant2.0 is a support vector machine (SVM)-based tool for the automatic prediction of protein stability changes upon single point mutations. I-Mutant2.0 predictions are performed either from the protein structure or, more importantly, from the protein sequence. I-Mutant2.0 can be used both as a classifier for predicting the sign of the protein stability change upon mutation , and as a regression estimator for predicting the related Delta G (δG) values. Acting as a classifier, I-Mutant2.0 correctly predicts (with a cross-validation procedure) 80% or 77% of the dataset, depending on the usage of structural or sequence information, respectively. When predicting DeltaG values associated with mutations, the correlation of predicted with expected/experimental values is 0.71 (with a standard error of 1.30 kcal/mol) and 0.62 (with a standard error of 1.45 kcal/mol) when structural or sequence information are respectively adopted. The web interface allows the selection of a predictive mode that depends on the availability of the protein structure and/or sequence. In the latter case, the web server requires only pasting of a protein sequence in a raw format.

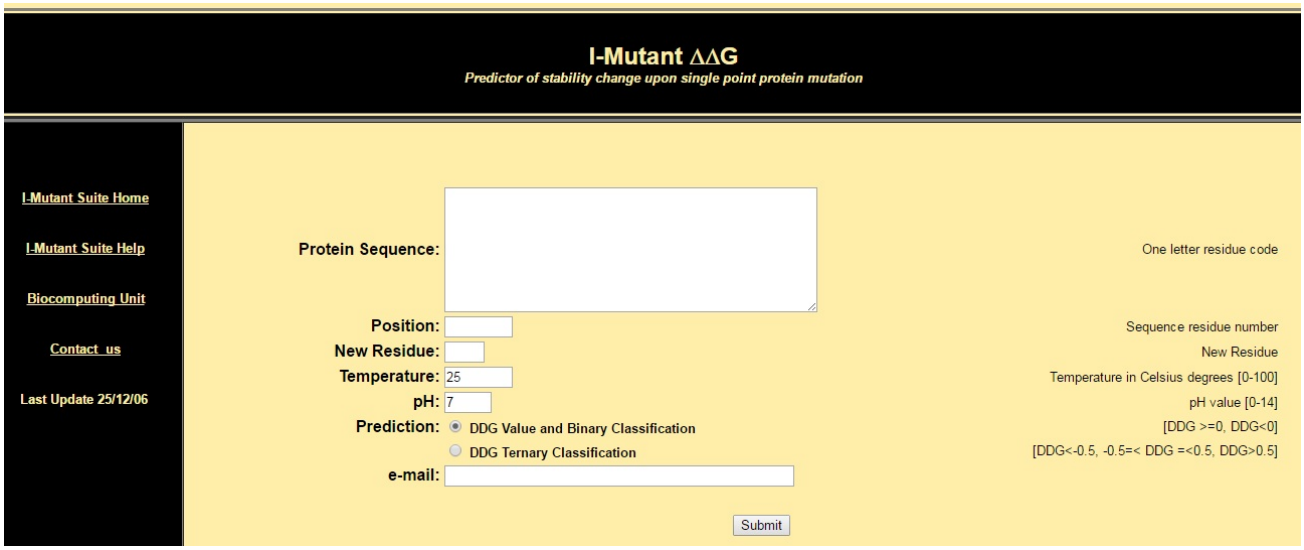

Figure 2.1: This picture is showing a snapshot of input format of I-Mutant.

Results and Discussion

SNP's distribution over p53 and Bcl-2 gene

SNPs are the most common type of genetic variations in any population, they occur when a single nucleotide in the genome is altered (Nachman et al., 2016). Even though many SNPs have no effect on biological functions of the cells but some can predispose the genotype to certain diseases and influence their immunological response to drugs, hence can be considered as biomarkers for disease susceptibility. In the present study the p53 and Bcl-2 genes have following SNPs distribution respectively.

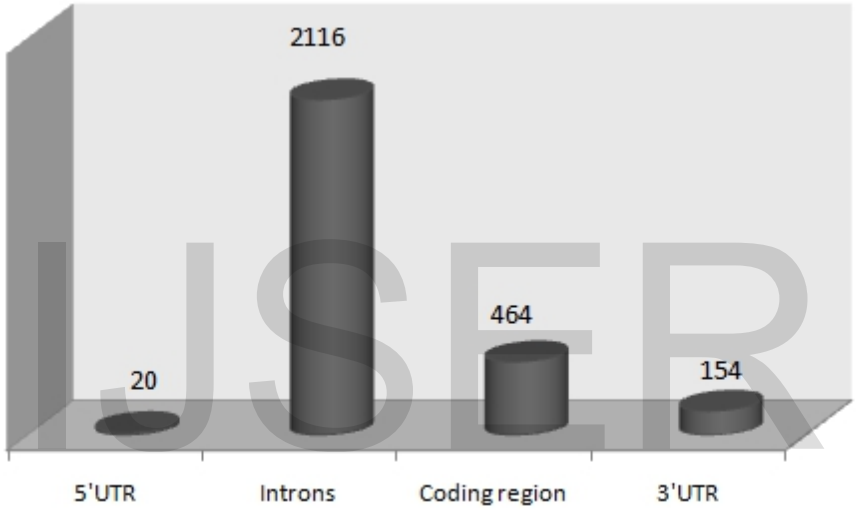

Figure 3.1: SNP distribution in p53 gene based on dbSNP.

The 5'UTR region has an important role in the regulation of expression of targeted gene. Because this is the promoter region where the polymerase and transcription factor binds. Hence the SNPs present at this region may influence/alter the binding affinity of polymerase and transcription factors which can further affect the gene expression.

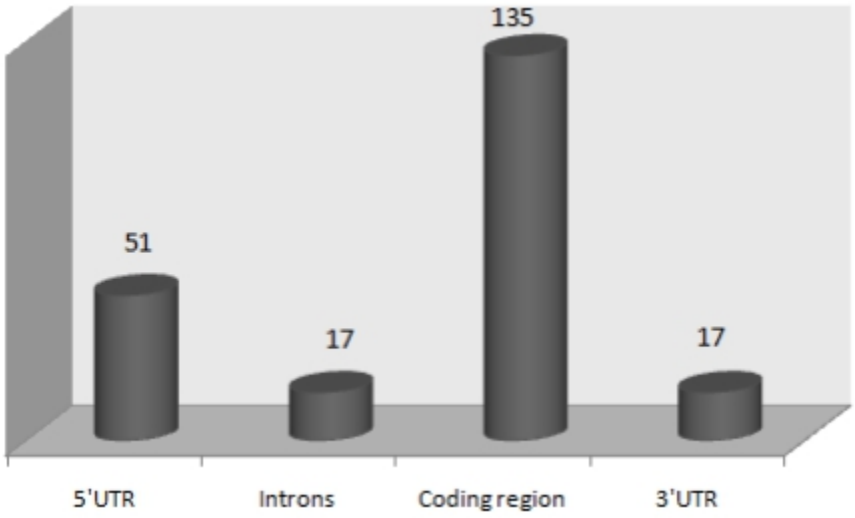

Figure3.2: SNP distribution in Bcl-2 gene based on dbSNP

The 3' UTR is the site for polyadenylation, which is the key determinant for the stability of mRNA. Intronic region may also play an important role in the regulation of gene expression. Because SNPs present at intronic region may alter/affect the binding of spliceosomal proteins on acceptor site and donor site of intron. SNPs present at coding region do not necessarily change the amino acid sequence of the protein that is produced, due to degeneracy of the genetic code.

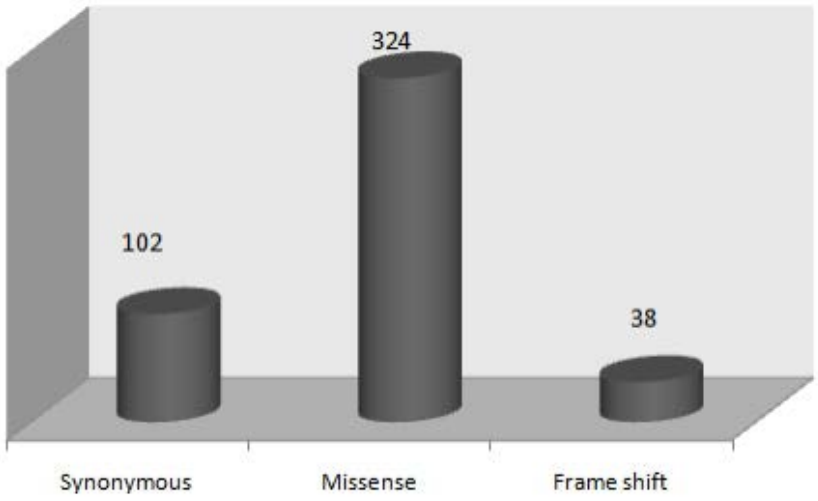

Figure 3.3: SNP distribution in coding region of p53 gene.

The SNPs in the coding region are of three types i.e. synonymous, non-synonymous and frame shift. Synonymous SNPs do not affect the protein sequence while non synonymous and frame shift change the amino acid sequence of the protein.

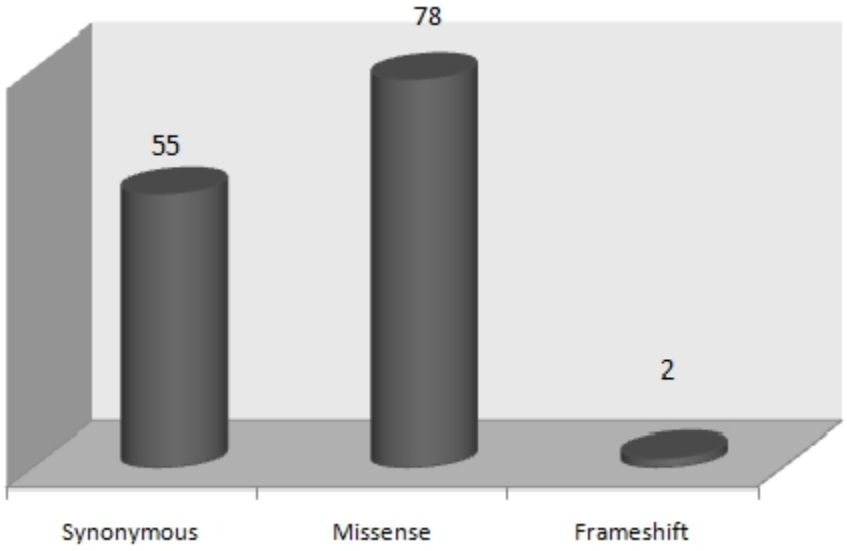

Figure 3.4: SNP distribution in coding region of Bcl-2gene

Evaluation of deleterious status SNPs of p53 and Bcl-2 by SIFT and I-Mutant

The SNPs of p53 and Bcl-2 genes were predicted to be damaging with the help of SIFT and I-Mutant as shown in table3.1 and table3.2. This indicates that damaging/deleterious SNPs evaluated by these tools may cause an amino acid distribution in the corresponding protein product that can affect the phenotypic effect of the p53 and Bcl-2 genes.

Table 3.1: List of nsSNPs (tolerated/deleterious) that were analyzed by computational methods I-Mutant 2.0 and SIFT.

S.no.	rs number	Base change	Type of mutation	Amino acid change	I-Mutant DDG (Kcal/mol)	SIFT damaging SNP's
1.	rs80184930	T>C	Rev	S >P	- 0.50*	Damaging
2.	rs17881470	T>G	Fwd	S>A	-0.68*	Damaging

3.	**rs17882252**	**G>C/A**	**Fwd**	**E>Q/K**	**-0.38***	**Damaging**
4.	**rs112431538**	**G>A**	**Rev**	**E>K**	**-1.13***	**Damaging**
5.	**rs28934574**	**C>T**	**Fwd**	**R>W**	**-0.01***	**Damaging**
6.	**rs17849781**	**C>G**	**Rev**	**P>A**	**-1.11***	**Damaging**
7.	**rs28934576**	**G>TTCCAA**	**Fwd**	**R>LLPPHH**	**-0.38***	**Damaging**
8.	**rs121913343**	**C>T**	**Fwd**	**R>C**	**-0.62***	**Damaging**
9.	**rs55832599**	**C>T**	**Rev**	**R>W**	**-2.16***	**Damaging**
10.	**rs72661119**	**A>G**	**Fwd**	**N>D**	**-0.42***	**Damaging**
11.	**rs28934577**	**T>GGAA**	**Fwd**	**L>RRQQ**	**-2.36***	**Damaging**
12.	**rs28934571**	**G>T**	**Fwd**	**R>S**	**-1.30***	**Damaging**
13.	**rs11540652**	**G>T,C,A**	**Fwd**	**R>L,P,Q**	**-2.25***	**Damaging**
14.	**rs28934575**	**G>A**	**Fwd**	**G>S**	**-1.42***	**Damaging**
15.	**rs28934573**	**C>TTGG**	**Fwd**	**S>FFCC**	**-3.67***	**Damaging**
16.	**rs35163653**	**G>A**	**Fwd**	**V>M**	**-2.00***	**Damaging**
17.	**rs72661117**	**G>A**	**Fwd**	**D>N**	**-0.51***	**Damaging**
18.	**rs28934578**	**G>AA**	**Fwd**	**R>HH**	**-2.24***	**Damaging**
19.	**rs28934874**	**C>TA**	**Fwd**	**P>ST**	**-0.90***	**Damaging**
20.	**rs28934875**	**G>C**	**Fwd**	**A>P**	**-1.04***	**Damaging**
21.	**rs28934873**	**T>C**	**Fwd**	**M>T**	**-1.91***	**Damaging**
22.	**rs11540654**	**G>AAAA**	**Fwd**	**R>H**	**-0.21***	**Damaging**

*** indicate the value of DDG in negative which means there is decrease in stability of the protein.**

A total number of nsSNPs of p53 gene were evaluated by I-Mutant (SNP damage prediction tools). Out of which 298 (91.9%) of nsSNPs were predicted to affect the stability of p53 protein.

SIFT predicted 22(6.07%) of nsSNPs as damaging. Further the total number of nsSNPs were predicted to be deleterious by all two prediction softwares were 22(6.07%) as shown in table 3.1.

Table 3.2: List of nsSNPs (tolerated/deleterious) that were analyzed by computational method I-Mutant 2.0

Serial no.	rs number	Base change	Type of mutation	Amino acid change	IMutant DDG(K cal/mol)
1.	rs377753316	G>A	Rev	S>N*	-2.32
2.	rs557000269	G>T	Rev	V>L*	-0.26
3.	rs752310933	C>G	Rev	L>V*	-1.34
4.	rs751464195	C>T	Rev	P>S*	-0.30
5.	rs201085318	T>C	Rev	M>T*	-1.08
6.	rs755252289	G>A	Rev	G>E*	-1.31
7.	rS777784952	C>T	Rev	R>C*	-2.54
8.	rs747504890	G>A	Rev	G>R*	-1.40
9.	rs762635201	C>T	Rev	S>F*	-0.76
10.	rs763718170	T>C	Rev	S>P*	-0.13
11.	rs761696930	G>A	Rev	D>N*	-1.84
12.	rs750253286	C>T	Rev	A>V*	-0.01
13.	rs752942153	A>G	Rev	T>A*	-1.74
14.	rs565014924	T>C	Rev	V>A*	-0.29
15.	rs748978916	A>G	Rev	S>G*	-0.66
16.	rs761382113	C>T	Rev	P>S*	-0.41
17.	rs760559670	C>G	Rev	A>G*	-0.47
18.	rs753603356	C>T	Rev	P>S*	-1.06

19.	rs746401408	A>G	Rev	R>G*	-1.59
20.	rs756650851	C>T	Rev	A>V*	-1.64
21.	rs780606244	G>A	Rev	A>T*	-2.97
22.	rs768259110	C>T	Rev	P>S*	-1.99
23.	rs774056251	C>T	Rev	R>W*	-0.74
24.	rs772554403	G>A	Rev	A>T*	-0.49
25.	rs766277782	C>G	Rev	T>R*	-1.92
26.	rs759325300	C>T	Rev	H>Y*	-1.65
27.	rs749745319	C>G	Rev	P>A*	-2.53
28.	rs755382644	C>G	Rev	A>G*	-0.60
29.	rs779096658	G>T	Rev	A>S*	-1.00
30.	rs1800477	G>A	Fwd	A>T*	-1.33
31.	rs540701354	G>T	Rev	D>H*	-1.71
32.	rs767612613	G>A	Rev	G>R*	-0.10
33.	rs546449806	C>G	Rev	A>G*	-2.11
34.	rs760840889	G>C	Rev	A>P*	-1.37
35.	rs766508625	G>C	Rev	D>H*	-0.27
36.	rs754418219	G>A	Rev	W>-	-0.98
37.	rs779372254	G>A	Rev	G>S*	-2.30
38.	rs758123306	T>G	Rev	H>Q*	-2.03
39.	rs777401949	C>A	Rev	H>N*	-2.76
40.	rs780634396	A>G	Rev	K>E*	-0.89
41.	rs776360417	G>A	Rev	M>I*	-0.07
42.	rs745851862	G>C	Rev	V>L*	-0.39

43.	**rs775404824**	**G>A**	**Rev**	**G>E***	**-1.20**
44.	**rs768498963**	**G>T**	**Rev**	**A>S***	**-1.36**

*indicate the value of DDG is negative which means that there is decrease in the stability of the proteins.

None of the SNPs were found to be damaging with the help of SIFT. Out of 78 nsSNPs, 44 (56.41%) were predicted to affect the stability of Bcl-2 protein.

Drugs targeting p53 and Bcl-2 genes

There are various drugs which are used to target the p53 gene. Some drugs target the genes involved in p53 pathway so indirectly increase the expression of p53. Some drugs are given below along with their target, mechanism of action and their pharmacological action.

Table 3.3: Drugs targeting p53 gene

Drug	Target	Mechanism of action	Pharmacological effect
Cisplatin	**DNA**	**Inhibits DNA synthesis, chemotherapy drug, Platinum, Potent pro-apoptotic anticancer agent; activates caspase-3.**	**Inhibits cell cycle by inducing the overexpression of p53 gene.**
Fluorouracil	**Thymidylate synthase, DNA and RNA**	**RNA processing inhibitor and thymidylate synthase inhibitor**	**Induces p53 gene expression.**
Docetaxel	**Apoptosis regulator Bcl-2**	**Microtubulin disassembly inhibitor, Tubulin and VEGF inhibitor, Taxanes, Microtubule stabilizer**	**It induces the expression of p53 by phosphorylation of p53 protein. P53 status is crucial determinant of docetaxel sensitivity.**
Paclitaxel	**Apoptosis regulator Bcl-2**	**Tubulin and Bcl-2 inhibitor, Taxanes**	**Arrests cell cycle at G-phase via p53 dependent and independent pathway.**

Cisplatin is platinum containing drug. Relationship between cisplatin sensitivity and p53 status is unclear. In cisplatin-based chemotherapy, overexpression of p53 was found. Cisplatin inhibits cellular proliferation by p53 dependent apoptosis and p53 independent cell cycle arrest (Zamble et al., 1998). Paclitaxel inhibit growth of cells by inhibiting microtubule depolymerization. This leads to cel cycle arrest at G1 and G2/M phases. Paclitaxel act via both p53 dependent and p53 independent apoptotic pathways. Fluorouracil is widely used in treatment of gastrointestinal, breast and lung cancer. It induces p53 gene expression and accumulation, followed by induction of cell growth inhibition. Similarly, there are various drugs which target Bcl-2 gene.

Table 3.4: Showing different drugs targeting Bcl-2 gene

Name	Target	Mechanism of action	Pharmacological action
Docetaxel	Apoptosis regulator Bcl-2	Microtubulin disassembly inhibitor, Tubulin and VEGF inhibitor, Taxanes, Microtubules stabilizer	Block the function of Bcl-2 by binding Bcl-2 protein.
Paclitaxel	Apoptosis regulator Bcl-2	Tubulin and Bcl2 inhibitor, Taxanes	Inhibit the function of Bcl-2.
Carboplatin	DNA	Antitumor agents that forms platinum-DNA adducts, Platinum	Lack of Bcl-2 target suppression.
Cisplatin	DNA	Inhibits DNA synthesis, chemotherapy	Over expression of Bcl-2 inhibits apoptosis induced

		drug, Platinum, Potent pro-apoptotic anticancer agent; activates caspase-3.	**by cisplatin.**

Docetaxel is a chemotherapy drug to treat breast cancer. BCL-2 expression in breast cancer may serve as a predictive biomarker for responsiveness to ABT-737 (inhibitor of Bcl-2 family) combined with docetaxel chemotherapy. Docetaxel induces programmed cell death (apoptosis) in cancer cells by binding to an apoptosis stopping protein called Bcl-2 (B-cell leukemia 2) and thus arresting its function. Paclitaxel is a mitotic inhibitor used in cancer chemotherapy. Bcl-2 over expression leads to the prevention of chemotherapy (paclitaxel)-induced expression of FasL and blocks paclitaxel-induced apoptosis. Carboplatin is an antineoplastic in the class of alkylating agents and is used to treat various forms of cancer. Cisplatin is one of the most powerful chemotherapy drugs for treatment of cancers of testis, ovary, head, neck, lung and other solid tumors. Over expression of Bcl-2 in bladder cancer cells inhibits apoptosis induced by cisplatin drug (http://www.genecards.org/).

Subcellular location of p53 and Bcl-2 genes

The cells of eukaryotic organisms are elaborately subdivided into functionally-distinct membrane-bound compartments, which are known as their subcellular location within the cell. The below diagrams show the subcellular location of p53 and Bcl-2 genes and the confidence number given to each organelle is according to the differential amount of gene expression present in them. The highest confidence number depicts the largest amount of gene expression and the lowest confidence number depicts the least amount of gene expression in the organelle.

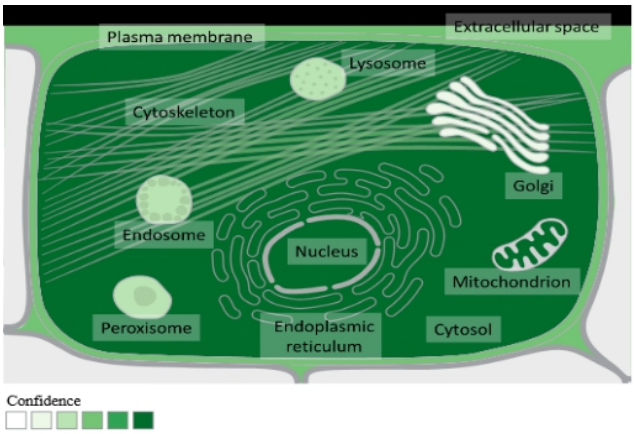

Figure 3.5: The differential abundance of p53 expresssion in various organelles and the intensity of colour depict the relative abundance of p53 in different organelles.

From the above figure, we found that mitochondrion, nucleus, endoplasmic reticulum and cytosol have maximum abundance of p53 gene. In mitochondrion, this is due to the intrinsic pathway of apoptosis (Mitochondrial pathway) which involves the mitochondrial events. p53 also acts as a transcription factor, that is why p53 is abundant in nucleus. As post-translational modifications of p53 gene occur in endoplasmic reticulum, so gene is present in abundance in ER also. It is also abundant in cytosol because it is the site for p53 protein translation.

Table 3.6: The confidence number which, represent the abundance of p53 gene expression in different organelles.

Compartment	Confidence
Mitochondrion	**5**
Nucleus	**5**
Endoplasmic Reticulum	**5**
Cytosol	**5**
Plasma Membrane	**3**
Extracellular	**3**
Cytoskeleton	**3**
Peroxisome	**2**
Lysosome	**2**
Endosome	**2**
Golgi Apparatus	**1**

As shown in above table, peroxisomes, lysosomes and endosomes also have lesser abundance of p53 gene. These are the sites for degradation of p53 protein. Golgi also specifically involved in post-translational modification of p53 gene, after ER, because it is the minor site for PTMs of p53 protein. In case of Bcl-2 gene, the cellular localization is shown in the diagram below.

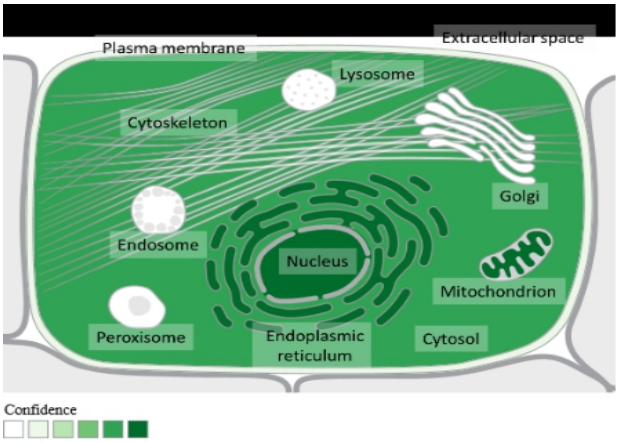

Figure 3.6: The above diagram showing the differential abundance of Bcl-2 expression in various organelles and the intensity of color depicts the relative abundance of Bcl-2 in different organelles.

We found that mitochondrion, nucleus and endoplasmic reticulum has maximum abundance of Bcl-2 gene.

Table 3.7: Showing confidence number which, represent the abundance Bcl-2 gene expression in different organelles

Compartment	Confidence
Mitochondrion	5
Nucleus	5
Endoplasmic Reticulum	5

Cytosol	**4**
Plasma membrane	**1**

In mitochondrion, this is due to the intrinsic pathway of apoptosis (Mitochondrial pathway) which involves the mitochondrial events. As post-translational modifications of Bcl-2 gene occur in endoplasmic reticulum, so gene is present in abundance in ER also. It is also abundant in nucleus because in nucleus, protein and mRNA expression is high. Cytosol and plasma membrane have minimum abundance of Bcl-2 gene. In cytosol, translation of Bcl-2 protein takes place.

Conclusion

In case of p53 gene, 298 (91.9%) of nsSNPs were predicted to affect the stability of p53 protein. SIFT predicted 22(6.07%) of nsSNPs as damaging. Out of 78 nsSNPs, the damaging status of Bcl-2 gene was found to be 44 (56.41%) and these were predicted to affect the stability of Bcl-2 protein. But the Impact of single amino acid change on protein stability is one of the most promising ways to find out the damaging effect of SNP on protein's conformational, structural and functional aspect. Hence the prediction of the phenotypic effect of nsSNPs using in silico methods may provide a greater understanding of genetic differences in susceptibility to disease like cancer, autoimmune diseases and neurodegenerative disorders.

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