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Computational Detection of Deleterious Single Nucleotide Polymorphisms in Human Adenomatous Polyposis Coli Gene the Gate-Keeper of Colorectal Carcinoma

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ABSTRACT

Colorectal cancer (CRC) is one of the most diffuse cancers worldwide; evidences showed that Adenomatous Polyposis Coli (*APC*) is a multifunctional tumor suppressor gene that regulates and controls many biological functions; mutations in this gene has been reported in many cases of CRC. SNPs contribute to gene mutations and expression variations justifying phenotypic variations among population and hence such SNPs would be potential biological targets for identification and analysis; therefore this work focused on analysis of SNPs in the coding regions of *APC* gene found as non-synonymous variants (nsSNP) and those in the 3'un-translated region (3'UTR) affecting miRNA binding using computational methods. 333 nsSNPs were analyzed by tools that concerning structural and functional aberrations and measure degrees and scores of alterations; and then the protein variants were subjected to structural modeling to highlight the impact of amino acid substitution upon protein phenotype. Analysis with Sift and Polyphen resulted in 15 damaging nsSNPs out of a total and marked 5 amino acid substitutions (E142G, R99W, R24N, L680S and W157T) with probably high deleterious scores, while analysis of 51 3'UTR SNPs by its special tool PolymiRTS resulted in no single nucleotide variant at that region could disturb the conserved sites of miRNA. It has been found that the use of such computational analysis tools was highly valuable and critical to highlight life threatening mutations and early sounds for *APC* gene aberrations causing truncated products with adverse outcomes.

Keywords: Colorectal Carcinoma; *APC* nsSNPs; Single nucleotide polymorphism (SNP); Protein modeling, 3'UTR SNPs.

INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer worldwide and the fourth most common cause of death [1], the estimated new cases in 2014 are 136,830 (8.2%) while deaths are 50.310 (8.6%) [2]. Most colorectal cancers develop slowly over several years and before it develops, a growth of tissue or tumor usually begins as a non-cancerous *polyp* on the inner lining of the colon or rectum. The opportunity of changing into a cancer depends upon type of polyp; adenomatous polyps (adenomas*)* are polyps that can change into cancer, thus they considered as pre-cancerous lesions[3], neither hyperplastic polyps or inflammatory polyps are considered pre-cancerous [4].

Colorectal cancer may occur due to many documented factors such as age, diet, physical activity, obesity, heavy alcohol consumption, cigarette smoking and personal history of adenomatous polyps [5]. Inherited genetic risk also plays a major role in this cancer and the most common inherited conditions are familial adenomatous polyposis (FAP) and hereditary non polyposis colorectal cancer (HNPCC) or Lynch syndrome; HNPCC is associated with mutations in genes involved in DNA mismatch repair (MMR), namely the *MLH1*, *MSH2*, *MSH6*, *PMS1* and *PMS2* genes [6], whereas FAP is caused by germ-line mutations in the tumor suppressor gene *APC* which stands for adenomatous polyposis coli, it was discovered in 1991[7,8]. It is located on chromosome 5 (5q21-q22) containing 15 exons [9]. Exon 15 comprises more than 75% of the coding region of *APC* and is the common target for germ line and somatic mutations [10], it encodes a multi-domain protein that plays a major role in tumor suppression by antagonizing WNT signaling pathway; as the inappropriate activation of this pathway through mutation of *APC* gene contributes to cancer progression [11]. The most common type of mutation either non-sense or frame shift mutation commonly occurring in the mutation cluster region (MCR) of *APC* gene; that contains β-catenin binding sites (the 20 amino acid repeats) hence mutation in the gene causes loss of domains required for β-catenin binding to *APC* to initiate its subsequent degradation, the integral role in tumor suppressor activity of *APC* gene [12, 13]. The risk underlie accumulation of β-catenin in cytosol as a consequence of *APC* inactivation is that stabilization of β-catenin leads to activation of transcription factor boxes and target genes involved in carcinogenesis like Cyclin D1 and c-MYC [14]. Germ line mutation of *APC* results in familial adenomatous polyposis (FAP); the autosomal dominant inherited disease characterized by multiple intestinal polyps may reach to thousands of polyps usually progress to colorectal cancer [15]. Twenty to Fifty percent of classical FAP patients remain without detectable *APC* germ-line mutation (*APC* mutation negative patient) or attenuated *APC*; furthermore those patients display considerable phenotypic variations, fewer number of polyps (<100) and fewer extra-colonic manifestations such as upper gastrointestinal polyp, desmoids and osteomas [16].

Reports suggested that *APC* mutations are not exclusively predispose to colon cancer but also associated with several other diseases like Medulloblastoma (MDB), Hereditary desmoid disease, Mismatch repair cancer syndrome (MMRCS), Gastric cancer (GASC) and Hepatocellular carcinoma (HCC) this may be justified by location of mutation in *APC* gene causing variable gene features, symptoms and variable phenotypic outcomes; that is the retinal lesions develop when 3' mutations involved; while 5' mutations predispose to attenuated FAP (AFAP) (<100 polyps) [17,18,19]. These mutations may result from DNA sequence variations at the level of single base nucleotide among the individuals of species giving rise to polymorphisms (SNPs); occurring once every several hundred base pairs throughout the genome as their minute variations contribute to gene expression variation and eventually to phenotypic variation in populations [20]; specially the non-synonymous single nucleotide polymorphism (nsSNP) occurring in the coding regions of a gene may cause an amino acid substitution in the corresponding protein product and hence affecting the phenotype; thus nsSNPS represent potential targets in human inherited diseases [21], in addition to micro-RNA (miRNA) which are small nucleotide regulatory stretches that regulates and modulates gene expression by binding to the complementary sites at 3'untranslated regions (3'UTR) of a targeted messenger RNA (mRNA) [22]. It has been suggested that a single miRNA may bind to hundreds of mRNA targets thus disturbance in their quantities result in diverse functional outcomes; as their increased or decreased levels reported in a variety of cancers. 3'UTR SNPs may affect phenotypes by altering normal miRNA binding or introducing new binding sites result in altered protein products that may associate carcinogenesis; this suggests that 3'UTR SNPs could be valuable biomarker for cancer prognosis [23].

Many reports propose that SNPs in the *APC* gene could predispose to colorectal adenomatous phenotype and underlie the risk of colonic adenoma/carcinoma sequence [24]; therefore this work focus on detection and analysis of functionally deleterious SNPs of *APC* gene prioritizing non-synonymous (nsSNP) and 3`UTR SNPs for their potentiality in cancer predisposition and prognosis using computational tools to highlight mutations underlying phenotypic variations (classical or atypical forms of a disorder) and to predict the structural and functional consequences of these polymorphisms.

MATERIALS AND METHODS

The critical step in this work was to select SNPs for analysis by computational softwares; the selection was prioritizing SNPs in the coding region (exonal SNPs) that are non-synonymous (nsSNP) and SNPs at un-translated region at 3'ends (3'UTR) to predict the effect on miRNA binding on these regions that may greatly associated with tumor progression [25]. The SNPs and the related ensembles protein (ESNP) were obtained from the SNPs database (dbSNPs) for computational analysis from http://www.ncbi.nlm.nih.gov/snp/ and Uniprot database http://www.uniprot.org for related protein sequences.

Predicting damaging amino acid substitutions using SIFT (v5.1):

SIFT is an online computational tool to detect a harmful non-synonymous single-base nucleotide polymorphism (nsSNP); the genetic mutation that causes a single amino acid substitution (AAS) in a protein sequence subsequently altering the carrier's phenotype and health status. The software traces AAS and Sorting Intolerant From Tolerant (SIFT) and predicts whether these substitutions affect protein function by using

sequence homology, SIFT predicts the effects of all possible substitutions at each position in the protein sequence [21]. Furthermore, the algorithm performs a comprehensive search in protein repositories to find the tolerance of each candidate compared to the conserved counterparts [26]. Non-synonymous reference SNPs identity (rsSNPs ID) were downloaded from online dbSNPs of NCBI, and then submitted to SIFT. Results are expressed as damaging (In-tolerated) or benign (Tolerated) depending on cutoff value 0.05; as values below or equal to 0.0 _ 0.04 predicted to be damaging or intolerant while (0.05_1) is benign or tolerated, then the damaging SNPs were re-analyzed by Polyphen software which predicts the effect of mutations on both structural and functional sides. SIFT is available as online tool at http://sift.jcvi.org.

Prediction of functional modification using Polyphen-2 (Polymorphism Phenotyping v2):
It is a software tool to predict possible impact of an amino acid substitution on both structure and function of a human protein by analysis of multiple sequence alignment and protein 3D structure, in addition it calculates position-specific independent count scores (PSIC) for each of two variants, and then calculates the PSIC scores difference between two variants. The higher a PSIC score difference, the higher the functional impact a particular amino acid substitution is likely to have. Prediction outcomes could be classified as probably damaging, possibly damaging or benign according to the value of PSIC as it ranges from (0_1); values closer to zero considered benign while values closer to 1 considered probably damaging and also it can be indicated by a vertical black marker inside a color gradient bar, where green is benign and red is damaging [27]. nsSNPs that predicted to be intolerant by Sift has been submitted to Polyphen as protein sequence in FASTA format that obtained from UniproktB/Expasy after submitting the relevant ensemble protein (ESNP) there, then we entered position of mutation, native amino acid and the new substituent for both structural and functional predictions.
PolyPhen version 2.2.2 is available at http://genetics.bwh.harvard.edu/pph2/index.shtml

Protein Modeling:
a- Chimera 1.8:
Investigating 3D (three-dimensional) structure of proteins is helpful in predicting the effect of SNPs on the structural level and in displaying the degrees of alteration. UCSF Chimera is highly extensible software for interactive visualization and analysis of molecular structures; Chimera (version 1.8) software was used to scan the 3D structure of specific protein and then modifies the original or native amino acid with the candidate to display the impact that can be produced. However some proteins has no readily available 3D structure in database, hence using Chimera would help in predicting the possible 3D structure for proteins under query. It has been innovated by using already available models in database repositories by attaching their protein data bank identity (PDB ID) into chimera for such 3D would be suitable template for innovating a new 3D structure harboring the candidate amino acid; this applied for SNP (rs137854569) causing AAS (S280Q) designed by PDB ID: 1JPP. Chimera (version 1.8) is available at: http://www.cgl.ucsf.edu/chimera/.

b- Project Hope (version 0.4.1):
Project hope is a new online web-server to search protein 3D structures by collecting structural information from a series of sources, including calculations on the 3D protein structure, sequence annotations in UniproktB and predictions from DAS-servers. HOPE works online where one can submit a sequence and mutation only for those that predicted to be damaging by both SIFT and Polyphen (Double Positive) servers. Protein sequences were submitted to project hope server in order to analyze the structural and conformational variations that have resulted from single amino acid substitution corresponding to single nucleotide substitution. Project Hope is available at: http://www.cmbi.ru.nl/hope.

PolymiRTS data base (version 3.0) for Polymorphism in microRNA Target Site:
PolymiRTS database was designed specifically for the analysis of non-coding SNPs namely 3'UTR, it aims to identify single-nucleotide polymorphisms (SNPs) that affect miRNA targets in human and mouse. We used this computational server in order to determine 3'UTR SNPs in *APC g*ene that may alter miRNA binding on target sites resulting in diverse functional consequences. All SNPs located in that region were selected and submitted to PolymiRTS (v3.0), available at: http://compbio.uthsc.edu/miRSNP/.

RESULTS AND DISCUSSION
Adenomatous Polyposis Coli gene was investigated in dbSNP/NCBI. *APC* gene containing a total of 4448 SNPs; of which 333 were non-synonymous SNPs (nsSNPs) and 51 were in the non-coding region (3'un-translated region) and 11 SNPs at 5'UTR. Only nsSNPs and 3'UTR SNPs were selected for computational analysis.

Predictions by SIFT and Polyphen:
Predictions of deleterious nsSNPs was performed by SIFT and Polyphen softwares; out of 333 snSNPs only 15 (4.5%) were predicted to be damaging by both servers. First, we submitted batch nsSNPs (rs SNPs) to Sift server; then the resultant damaging nsSNPs were submitted to Polyphen as query sequences in FASTA Format, it traced 7 probably damaging nsSNPs out of which 5 nsSNPs were marked with the most highest deleterious scores (E142G , R99W , R24N , L680S and W157T), the other 8 nsSNPs were scored as possibly damaging. Results are shown in the table below:

Table 1. Prediction of nsSNPs by SIFT and Polyphen

Gene Name	*SNP ID*	*Chromosome Location*	*Nucleotide Change*	*Amino acid change*	*Polyphen-2 results*	*PSIC Score*	*SIFT result*	*Tolerance Index*
APC	rs143145868	Chr5:112767219	G/T	G84V	Possibly damaging	0.594	Damaging	0.02
	rs77907679	Chr5:112819306	A/G	E142G	Probably damaging	1.000	Damaging	0.03
	rs141576417	Chr5:112780865	C/G	Q203E	Probably damaging	0.951	Damaging	0.02
	rs137854578	Chr5:112839777	A/T	S1112C	Possibly damaging	0.479	Damaging	0.02
	rs76306073	Chr5:112841115	G/T	D1558Y	Possibly damaging	0.910	Damaging	0.01
	rs139196838	Chr5:112767263	C/T	R99W	Probably damaging	1.000	Damaging	0.02
	rs150882838	Chr5:112842787	C/T	S2115F	Probably damaging	0.995	Damaging	0.01
	rs145945630	Chr5:112754960	C/T	R24N	Probably damaging	1.000	Damaging	-1
	rs137854583	Chr5:112780880	C/T	Q208T	Possibly damaging	0.723	Damaging	-1
	rs137854582	Chr5:112837687	A/T	L680S	Probably damaging	1.000	Damaging	-1
	rs137854572	Chr5:112828001	C/T	Q523S	Possibly damaging	0.821	Damaging	-1
	rs137854569	Chr5:112815499	C/G	S280Q	Possibly damaging	0.542	Damaging	-1
	rs121913462	Chr5:112839510	G/T	E1306I	Possibly damaging	0.842	Damaging	-1
	rs137854576	Chr5:112775676	A/G	W157T	Probably damaging	1.000	Damaging	-1
	s137854577	Chr5:112780901	C/T	Q215T	Possibly damaging	0.825	Damaging	-1

PolyPhen-2 result: POROBABLY DAMAGING (more confident prediction) / POSSIBLY DAMAGING (less confident prediction), PSIC SD: Position-Specific Independent Counts, Tolerance Index: Ranges from 0 to 1

Analysis of 3'UTR SNPs by Polymirts (v3.0):

The selected 3'UTR SNPs were 51; all submitted online to PolymiRTS server and resulted in no single pathogenic variant that could cause altered miRNA binding to the 3'UTR and hence no obvious consequences on protein truncation could be expected; despite that we could not excluded possible effects on transcription and splicing efficiency on these regions. This seems to agree with the findings of Karl *et al*. in 2001 [28].

Amino Acid Substitution effects on protein structure:

Project Hope revealed the 3D structure for the truncated proteins with its new candidates, in addition, it described the reaction and physiochemical properties of these candidates. Here we present the results upon each candidate and discus the conformational variations and interactions with the neighboring amino acids:

C/G mutation (rs141576417) caused conversion of Glutamine into a Glutamic acid at position *203* (Q203E). The wild-type residue was neutral while the mutant residue is negatively charged; this difference in charge between the native and mutant amino acid may cause repulsion between the mutant residue and neighboring residues. *(Fig 1*)

A/G mutation (rs77907679) led to conversion of Glutamic acid into a Glycine at position *142* (E142G). The mutant residue is neutral and smaller than the

negatively charged native residue. The native residue forms a hydrogen bond with Arginine on position 222. The size difference between native and mutant residue makes that the new residue not in the correct position to make the same hydrogen bond as the original wild residue did. The difference in hydrophobicity will also affect hydrogen-bond formation. (*Fig 2*)

C/T mutation (rs145945630) resulted in change of *Arginine to* Aspargine at position 24 (R24N); the mutant residue is smaller than the native residue and is neutral. The native residue forms hydrogen bond with glutamic acid and aspargine at positions 71 & 20 respectively thereby changes in size and charge for mutant residue cause loss of interaction and conformation. (*Fig 3*)

C/T mutation (rs137854583) caused change of Glutamine to Threonine at position 208 (*Fig 4*) while the dSNP: (rs137854582) of *(A/T)* mutation led to the mutant Serine instead of native Leucine at position 680 (*Fig 5*); both nsSNPs cause frank structural variations as well as dSNP:rs137854572 (C/T) that changed Glutamine to Serine at 523 (*Fig 6*); moreover the later mutant residue fails to make hydrogen bond with Leucine at 515 as was the case with native residue.

A/G mutation (rs137854576) caused substitution of amino acid from Tryptophan to Threonine (W157T); the wild-type and mutant amino acids differ in size. The mutant residue is smaller than the wild-type residue and less hydrophobic. The mutation might cause loss of hydrophobic interactions with other molecules on the surface of the protein. (*Fig 7*)

C/T mutation (rs137854577) resulted in Glutamine to Threonine substitution (Q215T); the mutant residue is smaller and more hydrophobic than the native residue. This also will cause a possible loss of external interactions. (*Fig 8*)

C/G mutation (rs137854569) that caused position 280 to be changed from small sized Serine into the larger sized Glutamine seemed to affect the hydrophobicity of that position *(Fig9*); this also reported with G/T mutation that causes Glutamic acid1306 Isoluecine conversion.

G/T mutation of (rs143145868) led to change of Glycine to Valine at position *84* (G84V). The wild-type and mutant amino acids differ in sizes; the mutant residue is bigger and this might lead to bumps. The glycine residue is the most flexible residue. It is possible that the residue is needed at this position to make a special backbone conformation or to facilitate movement of the protein. The mutation introduces a less flexible residue thereby disturbing this conformation or movement.

A/T mutation (rs137854578) caused conversion of Serine into a Cysteine at position *1112* (S1112C).The hydrophobicity of the wild-type and mutant residue differs. The mutation introduces a more hydrophobic residue at this position. This can result in loss of hydrogen bonds and/or disturb correct folding.

G/T mutation (rs76306073) resulted in change of Aspartic acid into a Tyrosine at position *1558* (D1558Y).The neutral mutant residue is bigger and more hydrophobic than the negatively charged native residue.

C/T mutation (rs139196838) leads to conversion of Arginine into Tryptophan at position *99* (R99W). The mutant residue is bigger and more hydrophobic than the native residue this can result in loss of hydrogen bonds and/or disturb correct folding. The wild residue was positively charged while the mutant residue is neutral.

C/T mutation (rs150882838**)** results into conversion of Serine into a Phenylalanine at position *2115* (S2115F). These amino acids differ in their properties; the mutant residue is located near a highly conserved position thus may be greatly damaging.

Unfortunately searching for 3D models and prediction of altered configuration for nsSNPs: rs137854578 (S1112C), rs76306073 (D1558Y), rs150882838 (S2115F), rs139196838 (R99W), rs143145868 (G84V) and rs121913462 (E1306 I) was failed as we could not find the targeted positions in protein sequences that fetched to chimera by protein PDB IDs.

3D Models of nsSNPS by Project hope and Chimera:
Eight proteins three dimensional structures were successfully designed by project hope, providing that protein backbone displayed in grey color, native (wild) residues with green color and mutant residues with red color. Also schematic structures were attached to each 3D structure, to represent the variations in the side chains among amino acid substitutions. Protein backbone is colored red while unique side chains of each amino acid colored black. All shown in figures below.

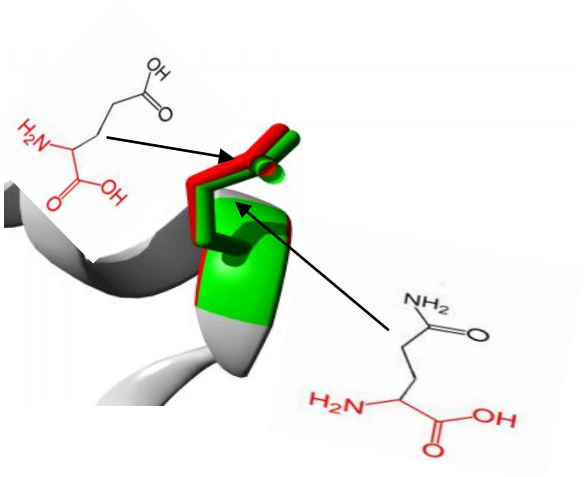


Figure 1. SNP ID: rs141576417 Protein position 203 changed from Glutamine to Glutamic acid.

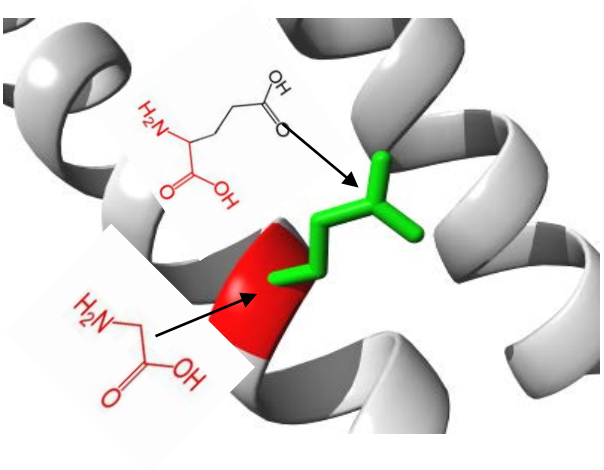


Figure 2. SNP ID: rs77907679 Protein position 142 changed from Glutamic acid to Glycine.

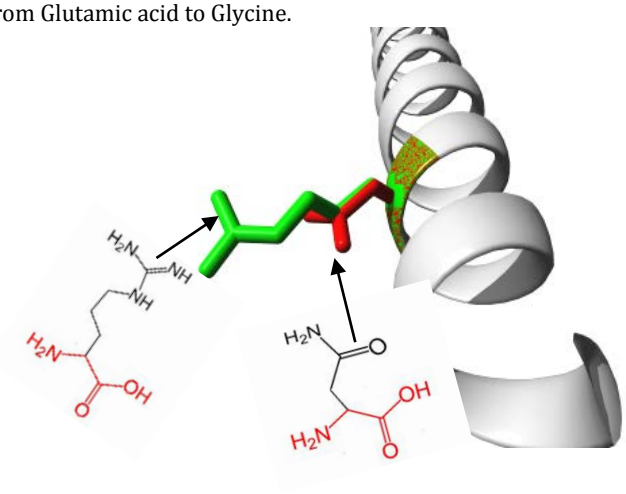


Figure 3. SNP ID: rs145945630 Protein position 24 changed from Arginine to Aspargine.

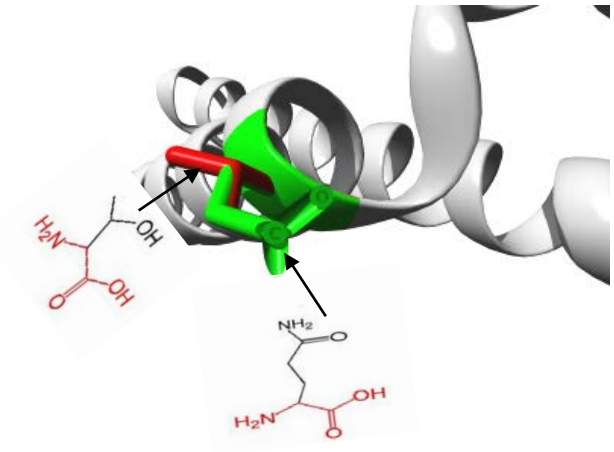


Figure 4. SNP ID: rs137854583 Protein position 208 changed from Glutamine to Threonine.

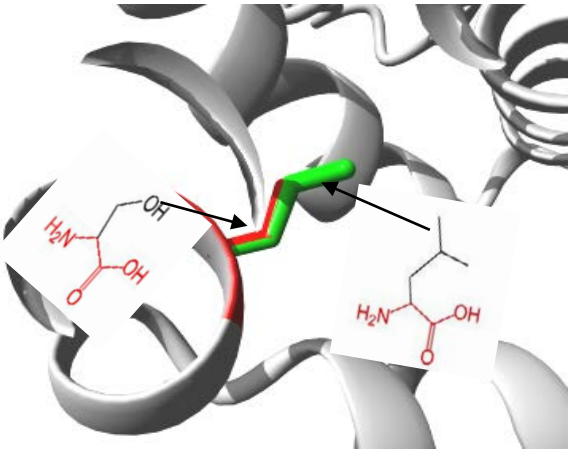


Figure 5. SNP ID: rs137854582 Protein position 680 changed from Leucine to Serine.

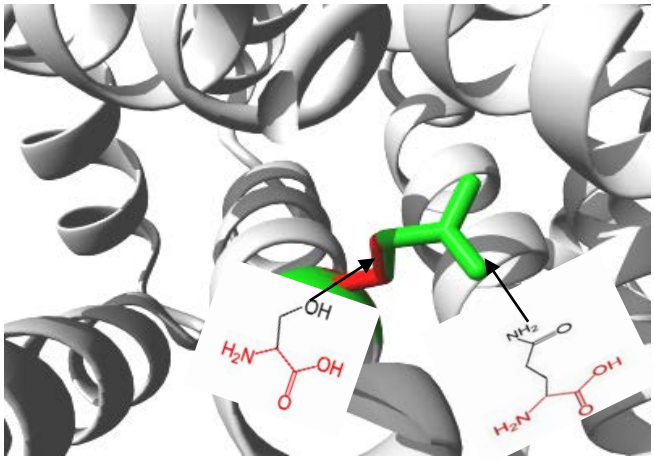


Figure 6. SNP ID:rs137854572 Protein position 523 changed from Glutamine to Serine.

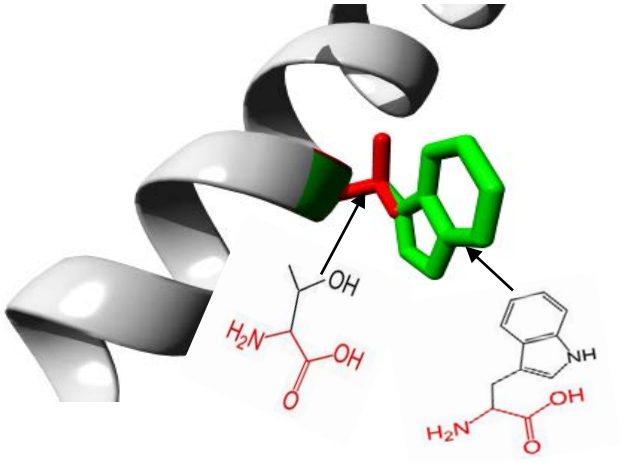


Figure 7. SNP ID: rs137854576 Protein position 157 changed from Tryptophan to Threonine.

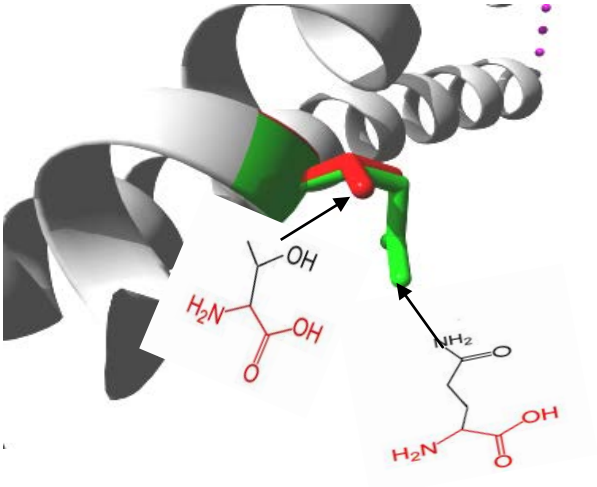
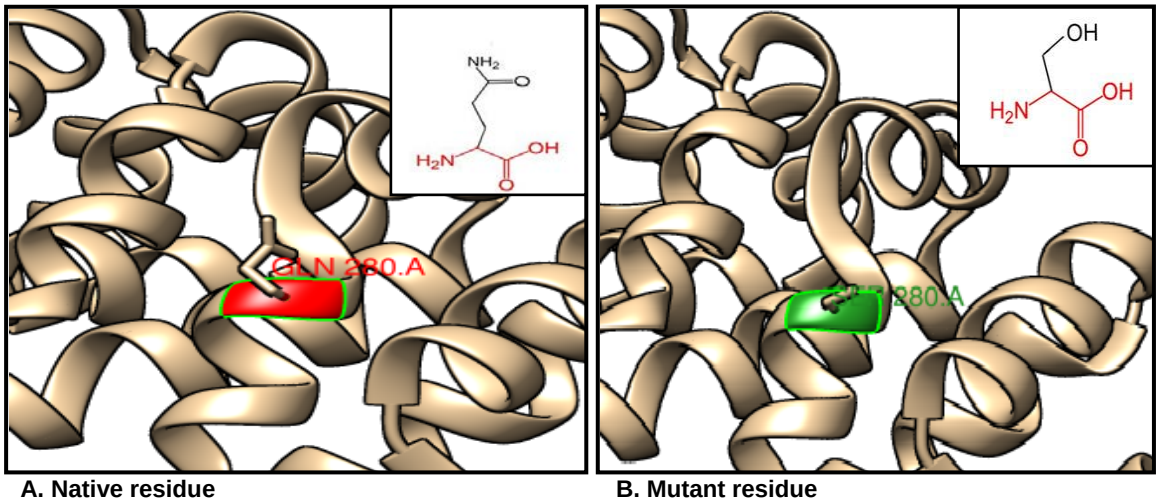


Figure 8. SNP ID: rs137854577 Protein position 215, changed from Glutamine to Threonine.

Patients with mutations in *APC* gene may develop variable disorders and tumors as well, SNPs variations in most cases result in critical amino acid variations that causes aberration of protein structure and function. Regarding diseases and tumors associated with *APC* gene mutations; we find that SNP of rsID (rs139196838) resulted in change of Arginine into Tryptophan at position 99(R99W) reported to be damaging and thereby pathogenic in our hands; thus may predispose to CRC. This has been strongly suggested by *Karl et al*. who observed same mutation in a female of 64 years old with family history of colorectal carcinoma [28], in addition dbSNP (rs137854583) of Q208T mutation resulted in frank structural change that may cause severe damage to protein function, enforced by the findings of *Dhaliwal* and *Su,* they reported the same mutation in patient with multiple intra-peritoneal desmoid tumors and hepatocellular carcinoma [29,30]. Analysis on dbSNP (rs137854569) showed that there is a change in amino acid from Serine to Glutamine (S280Q), this disagreed with the findings of *Nishisho* who reported that the mutation was from Serine to Threonine (S280T) in a patient with polyposis and mandibular osteoma [8].

Chimera Models:



A. Native residue **B. Mutant residue**

Figure 9. SNP ID: rs137854569, A represents the native residue serine with small atom at position 280 while structure B represents mutant glutamine residue of larger sized atom, designed by PDB ID: 1JPP

Our investigations on dbSNP: rs137854572 presented a mutation caused by C/T transition which changed amino acid from Glutamine to Serine (Q523S) opposed the findings of Fodde in affected members of a Dutch family with FAP1 as his reported mutation for the same SNP was from Glutamine to Threonine on a totally different position (Q541T) [31]. Also this *in silico* analysis found that the amino acid substitution at 1112 (Serine to Cysteine) resulted from dSNP (rs137854578) is different only at position from that reported by Oda et al. who found that serine changed into cysteine but at position 1395 (S1395C) for the same SNP [32]. In addition investigations upon mutations occurring at positions 157 and 215 due to deleterious SNPs (rs137854576 and rs137854577) respectively; were aided by findings of Spirio et al.[33] and Hamilton et al.[34]. Spirio observed W157T mutation in family members diagnosed with attenuated familial adenomatous polyposis (AFAP) in addition to another 3 asymptomatic individuals; while Hamilton reported Q215T mutation in family members with familial adenomatous polyposis (FAP), moreover one member developed anaplastic astrocytoma as an extra-colonic manifestation of FAP. Also in this *in silico* study we detected eight deleterious nsSNPs (rs76306 073/D1558Y, rs150882838/S2115F, rs143145868/G 84V, rs121913462/E1306I, rs77907679/E142G, rs141 576417/Q203E, rs145945630/R24N and rs13785458 2/L680S) that seemed to be new single pathological variants

CONCLUSION

This work presented multiple damaging SNPs that affect *APC* gene and subsequently cause alteration and truncation of the gene product; the key factor for these aberrations was the presence of multiple pathological

variants in the coding regions (nsSNPs) rather than those in the 3'UTR regions. This raises up the great demand for thoroughly investigating nsSNPs and highly considering their risks in *APC* gene truncations; on the other hand no frank conclusion could be made upon pathogenicity of the 3'UTR variants. Also we found that not all reported mutations of *APC* gene related directly to colorectal cancer but related to other health problems and disorders that may later unveil for CRC as early extra-colonic manifestations. Hence use of such computational analysis methods is critical to highlight variable mutations and early sounds for life threatening aberrations of *APC* gene in suspected family members.

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