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An Integrated Analysis to Predict Functionally Altered Genetic Variants in *FTO* with Potential Cancer Risk

BanerjeeShuvam, DharShrinjana and BhattacharjeePritha*

Environmental Epigenomics Lab, Department of Environmental Science, University of Calcutta, 35, Ballygunge Circular Rd, Ballygunge, Kolkata, West Bengal 700019
Email:777.pritha@gmail.com

ABSTRACT

The Fat Mass and Obesity associated protein (*FTO*), belonging to AlkB-related Fe(II) and 2-oxoglutarate-dependent dioxygenase family is responsible for demethylation of nucleic acids.Studies revealed significant association of *FTO* with adiposity, developmental anomalies and cancer, but precisemechanisms remain elusive. Our objective is to predict novel genetic variants in *FTO* with functional implications through *in-silico* approaches.From a total of 86,197 SNPs reported in the NCBI, 348 were missense variants, of which 28 were reported to be associated with disease outcomes. Integrated in-silico analyses identified 10 variants of which 4 were reported and 6 are novel. Among 10 variants, 60% show major alteration in secondary RNA structure. Protein structures of these variants were also altered due to physico-chemical properties of amino acids, ligand binding sites and change in enzyme active site. Hence, further studies are required for validation of the predicted SNPs.

KEYWORDS: *FTO*, In-silico, SNP annotation, Protein Secondary Structure, I-tasser

*Corresponding author

PrithaBhattacharjee*

Environmental Epigenomics Lab,

Department of Environmental Science,

University of Calcutta, 35, Ballygunge Circular Rd,

Ballygunge, Kolkata, West Bengal 700019

Email:777.pritha@gmail.com

1 INTRODUCTION

TheFat-Mass & Obesity associated (*FTO*) protein coding gene with molecular weight of 50kDa consists of 9 exons spanning more than 400kb on chromosome 16, encodes the enzymebelonging to the AlkB-related non-haem iron- and 2-oxoglutarate-dependent dioxygenase family and localizes to the nucleus[1-4]. These homologues of AlkB protein has special role in responding against alkylation damage of DNA via oxidative demethylation. *In vitro* studies implicit that *FTO* may function as a nucleic acid demethylase (1) preferably for 3-methylthymidine (3-meT) in single-stranded DNA and 3-methyluracil (3-meU) in single-stranded RNA[5].In addition to this, it acts as a transcriptional coactivatorby enhancing the binding potential of the CCAAT/enhancer binding proteins (*C/EBP*s) from unmethylated as well as methylation-inhibited gene promoters[6].

Evidence from epidemiological and functional studies suggests that *FTO*confers an increased risk of obesity through subtle changes in food intake and preference as well as non-adiposity traits, such as cardio-metabolic traits, type 2 diabetes mellitus and osteoarthritis[7]. Although precise function of *FTO* gene is still unknown, several genome-wide association scan (GWAS) have identified a cluster of single nucleotide polymorphisms (SNPs) in intron 1 of *FTO* gene was the first obesity susceptibility locus risk in human[4,7,8]. In fact, a number of studies also revealed the association of *FTO* with cancer risk especially breast cancer[9-11], colon cancer[12], thyroid cancer[13], myeloid leukemia[14], prostate cancer[15], endometrial cancer and pancreatic cancer[16]. *FTO* gene is highly polymorphic in different ethnic population[13]. Till date it has been observed that variants rs9939609, rs17817449, rs8050136, rs1477196, rs6499640, rs16953002, rs11075995 and rs1121980 are associated with the risk of developing cancer[17]. There has been also a report suggesting a novel association between single nucleotide polymorphism in intron 8 and melanoma[18].

FTO escalates cancer incidences independent of obesity by stimulating different intracellular signalling pathways involved in carcinogenesis such as signal transducer and activator of transcription 3 (*STAT3*), phosphoinositide 3-kinase/ protein kinase B (*PI3K/Akt*), *cyclin D1* and matrix metalloproteinases (*MMP*s)[12].Upregulation of *FTO* gene inducing m6A RNA demethylation[1] promotes self-renewal and tumorigenesis of Glioblastoma Stem Cells[19].The demethylase activity of *FTO* for m6A in cells actually inhibits the all-trans-retinoic acid which induces the expression of *ASB2* and *RARA*; thereby, induce the growth inhibition and chromatin condensation for oncogenic cells which in turn promotes leukemic oncogene-mediated cell transformation and leukemogenesis[14]. This study mainly deals with the Single Nucleotide Polymorphisms (SNPs) of *FTO* gene, present in the coding region. As introns get spliced out during post-transcriptional modification, there are very few chances of any polymorphism, though introns can play major roles if alternative splicing occurs due to mutation at regulatory sites.Although few missense SNPs of *FTO*are there which have been

reported in recent past, however the mechanisms of those associations need to be unveiled. So, we are focusing on missense SNPs as these have a negative impact on structure and function of a protein. Thus, our primary objective is to identify the novel genetic variants with potential disease risk and their functional implications.

2 METHODOLOGY

2.1 ***Preliminary sorting of FTO variants:*** We have investigated all variants of *FTO* gene in Human (*Homo sapiens*) from National Center for Biotechnology Information (NCBI)dbSNP Build 151 (https://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?geneId=79068, accessed on June 2018) and only missense variants were selected.A detailed literature survey on "*FTO* gene, obesity and cancer" was done on the basis of available data of global population andreported literatures corresponding to the missense variants up to 2018 were also enlisted. Literatures relating to missense variants of *FTO* causing disease outcome were extracted by searching keywords like "*FTO* gene", "Obesity", "Cancer", "Risk allele", "Polymorphisms", "Disease outcome", "Missense mutation", "Mechanisms" in different permutation-combination and also by the rs_ID number of the *FTO* variants. Variations were sorted based on their association with obesity and cancer risk, whether reported/not.

2.2*Short listing based on SNP annotation tools:* SNP annotation is a process to predict the effect of an individual SNP in functional alteration.SIFT, Mutation Taster were used for initial screening, also can be considered asdouble positive prediction. In addition to these, Polyphene2 and MetaSNP were used to know whether a particular variant might cause functional alteration and disease outcome (Supp. Table.1).

2.3 ***Secondary structure of RNA sequence generation:*** We further generated the Secondary RNA structures usinga web-based tool for RNA secondary Structure Generation "RNAstructure" (https://rna.urmc.rochester.edu/RNAstructureWeb/Servers/Predict1/Predict1.html, version 6.0.1) of those finally shortlisted variants to verify how the subtle amino acidalterations might lead to major structural alteration and finally resulting in disease outcome (Figure1).

2.4*Functional analysis using I-tasser:* To confirm the functional alteration because of structural changes corresponding the single nucleotide polymorphisms along with determining structure and function of protein molecules, *I-TASSER* (*Iterative Threading Assembly Refinement*) was performed(https://zhanglab.ccmb.med.umich.edu/*I-TASSER*/, accessed on May-August, 2018).

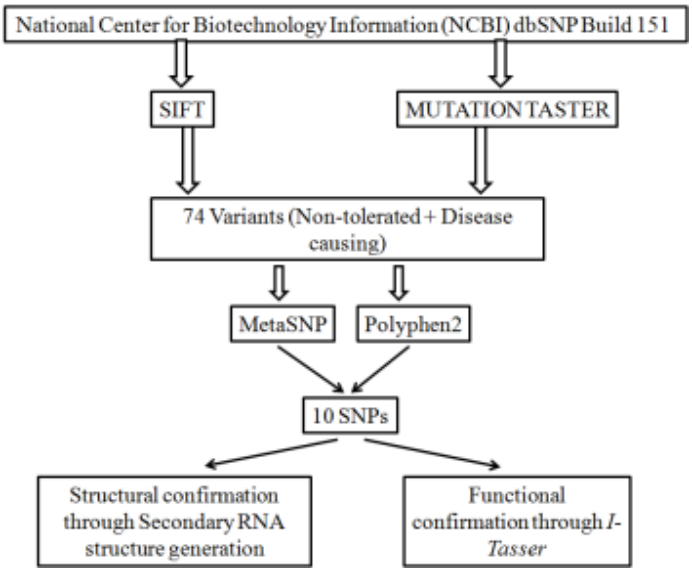

Figure1.Web based tools used in this study

3 RESULT

3.1*Results of web-based short listing:* All the 348 missense SNPs were tested using SIFT and Mutation Taster.Out of 348 missense variants, SIFT and Mutation Taster selected74 variants as both "Not Tolerated" and "Disease causing", among them 6 were reported already.Combined analysis of 4 web-based tools, identified 10 variants of which 4 were reported and 6 are novel(Table1).

Table1. Polyphen2 and MetaSNP score of the shortlisted variantsafter initial screening by SIFT and Mutation Taster

SNP_ID	Residue Change	Base Alter	MetaSNP Score	MetaSNP Prediction	Polyphen-2 Score	Polyphen-2 Prediction
rs121918214	Arg316Gln	G>A	0.761	Disease	0.999	Probably Damaging
rs139577103	Arg96His	G>A	0.771	Disease	1.000	Probably Damaging
rs16952624	Ala405Val	C>T	0.060	Neutral	0.025	Benign
rs79206939	Ala134Thr	G>A	0.097	Neutral	0.105	Benign
rs764576608	Asp81Gly	A>G	0.757	Disease	1.000	Probably Damaging
rs1203776934	Gly103Asp	G>A	0.781	Disease	1.000	Probably Damaging
rs1179039850	Val228Leu	G>T	0.686	Disease	0.997	Probably Damaging
rs771907956	His231Arg	A>G	0.750	Disease	1.000	Probably Damaging
rs757311078	Arg96Cys	C>T	0.776	Disease	1.000	Probably Damaging
rs1284248706	Ala341Asp	A>C	0.718	Disease	0.997	Probably Damaging

The minimum and maximum score for both Polyphene2 and Meta SNP ranges from 0 and 1.We found only 2 "Tolerated" variants, i.e. Ala405Val and Ala134Thr which however were

predicted as "Benign". Apart from these two, 8 variants found to be "Not tolerated", "Probably damaging" as well as having "Disease causing" potential.

3.2 *RNA Secondary Structure Analysis:* All these 10 single nucleotide polymorphisms were predicted to be the most deleterious and secondary RNA structures demonstrate the structural changes of the corresponding variants (Table2).

Table2. Secondary RNA structure alteration of the predicted variants

SNP_ID	Residue Change	Base Alter	Wild type codon	Mutant codon
rs121918214	Arg316Gln	G>A	CGU/CGC/CGA/CGG/AGA/AGG	CAA/CAG
rs139577103	Arg96His	G>A	CGU/CGC/CGA/CGG/AGA/AGG	CAC
rs16952624	Ala405Val	C>T	GCT/GCC/GCA/GCG	GTT/GTC/GTA/GTG
rs79206939	Ala134Thr	G>A	GCT/GCC/GCA/GCG	ACT/ACC/ACA/ACG
rs764576608	Asp81Gly	A>G	GAT/GAC	GGT/GGC
rs1203776934	Gly103Asp	G>A	GGT/GGC	GAT/GAC
rs1179039850	Val228Leu	G>T	GTA/GTG	TTA/TTG
rs771907956	His231Arg	A>G	CAT/CAC	CGT/CGC
rs757311078	Arg96Cys	C>T	CGU/CGC/CGA/CGG/AGA/AGG	TGT/TGC
rs1284248706	Ala341Asp	A>C	GCT/GCC	GAT/GAC

We found that 60% of the predicted variants cause major structural alteration and 40% with minor or no alterations. Among the 4 reported variants, only 2 variants show minor RNA structure changes whereas 4 variants (Figure2) among the novel 6 polymorphisms showing major structural alteration (Figure3).

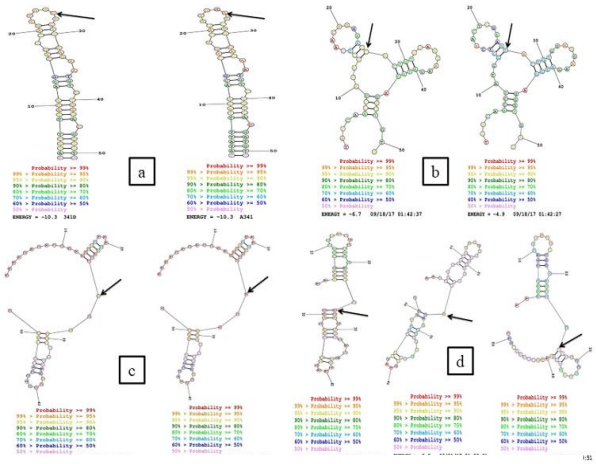

Figure2. Representation of null or minor RNA structure alterations in (A) A341D, (B) A405V, (C) A134T and (D) R96C

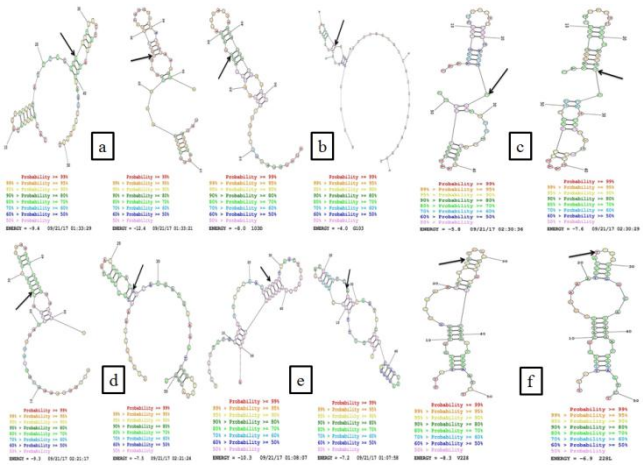

Figure3. Representation of major RNA structure alterations in (A) D81G, (B) G103D, (C) R96H, (D) H231R, (E) R316Q and (F) V228L

3.3 *Protein structure generation & functional validation:* To identify whether the variants alter protein secondary structure, ligand binding site or active site, we analyzed the variants through *I-tasser*. *I-tasser* demonstrates the alteration of functionality of the predicted single nucleotide variants (Figure4) based on protein secondary structure, ligand binding sites and Enzyme commission number with their active sites (Table3).

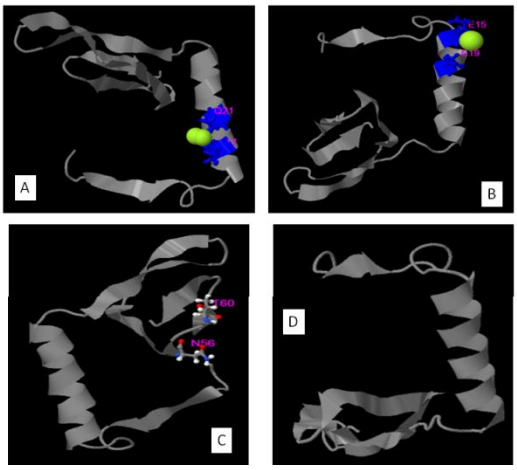

Figure4. *I-tasser*results showing the alteration in ligand binding site of the (A) Wild type and (B) Mutant type and also the enzyme active site for (C) Wild typeand (D) Mutant type

Table3. Functional alteration of the predicted variants based on protein structure, ligand binding sites and enzyme regulation

SNPs	Property of Amino acid			Property of protein					
	Code	Physical	Chemical	Ligand Binding site			Enzyme regulation		
				Name	Site	C-score	EC No.	Site	C-score
D81G	Asp	Polar; -ve charge; Hydrophilic	Acidic R-group & their amides	OXY	17,21	0.17	2.7.11.1	56, 60	0.246
				CLA	24,27	0.14	3.4.24. 57	36	0.244
	Gly	Nonpolar; Neutral; Hydrophilic	Aliphatic R-group	FE	15, 19	0.18	-	-	-
R96C	Arg	Polar; +ve charge; Hydrophilic	Basic R-group	A4F	15,21,23, 26,38,39	0.16	-	-	-
	Cys	Nonpolar; Neutral; Hydrophilic	S-containing group	No change		0.12	-	-	-
R96H	His	Polar; +ve charge; Hydrophilic	Basic	RHN	36, 46, 48, 49	0.12	-	-	-
				k-mer	36	0.11			
				A4F	25, 31, 33, 36, 48, 49	0.11			
R316Q	Arg	Polar; +ve charge; Hydrophilic	Basic R-group	OGA	15,27,29, 36,38,42	0.17	-	-	-
				FE	21,25	0.11			
	Gln	Polar; Neutral; Hydrophilic	Neutral (Amides of acidic amino acids R-group)	OGA	15,27,29, 36,38,42	0.19	-	-	-
H231R	His	Polar; +ve charge; Hydrophilic	Basic	FE2	36,38	0.31	-	-	-
				MD6	8,10,36,38,49	0.23			
	Arg	Polar; +ve charge; Hydrophilic	Basic R-group	NKG	8,10,33,36,38,39,49	0.29	-	-	-
				CA	36,38,39	0.29			
V228L	Val	Nonpolar; Hydrophobic	Aliphatic R-group	FE2	39,41	0.21	-	-	-
				MD6	11,13,39, 41,52	0.16			
				3I3MA00	11,24,36, 37,39,41	0.10			
	Leu	Nonpolar;	Aliphatic R-group	ZN	39,41	0.18	-	-	-

		Hydrophobic		NKG	11,13,36, 39,41,42, 52	0.14			
G103D	Gly	Nonpolar; Neutral; Hydrophilic	Aliphatic R-group	A4F	18,24,26, 29,41,42	0.14	5.3.4.1	37	0.181
				RHN	29,39,41, 42	0.14	2.5.1.10	2	0.178
				CA	38,45	0.11	2.5.1.1	1,5,33	0.172
	Asp	Polar; -ve charge; Hydrophilic	Acidic R-group & their amides	A4F	18,24,26, 29,41,42	0.16	-	-	-
A341D	Ala	Nonpolar; Hydrophobic	Aliphatic R-group	MG	13,14	0.14	-	-	-
				MG	11,12,13	0.11			
	Asp	Polar; -ve charge; Hydrophilic	Acidic R-group & their amides	CG	13,14,16	0.07	-	-	-
A134T	Ala	Nonpolar; Hydrophobic	Aliphatic R-group	CLA	40,41,43,44, 45,47,48	0.13	-	-	-
				Nuc.Acid	36,37,40,41, 44	0.09			
	Thr	Polar (uncharged); Hydrophillic	Non-aromatic hydroxyl	CLA	40,41,43,44, 47	0.12	1.13.11.12	3	0.236
				CLA	36,37,40,41, 44	0.11			
A405V	Ala	Nonpolar; Hydrophobic	Aliphatic R-group	BCL	31,34,35,38, 39,42	0.11	-	-	-
				3CS	38,39,41,42, 43,45	0.10			
	Val	Nonpolar; Hydrophobic	Aliphatic R-group	ZN	38,42	0.10	-	-	-
				PEPTIDE	38,41,42,45, 48,49	0.10			

4 DISCUSSION

The present study identified the novel genetic variants of *FTO* with potential disease association and explores the effects of predicted mutations at the proteomic level.A total of 86,197 SNPs reported in the NCBI dbSNP Build 151, of which 348 were missense variants. According to literatures in Pubmed, 28 missense variants were reported to be associated with several diseases.

Here in this study, out of 10 finally predicted single nucleotide variants, 4 were already reported to be associated with diseases, viz. Arg316Gln, Arg96His, Ala405Val and Ala134Thr.A very recent case study on a new born girl reported that Arg316Gln due to its loss-of-function[4] lead to rare growth retardation and developmental delay syndrome and also plays an important role in early development of human central nervous and cardiovascular systems[20]. Similarly, loss-of-function of Arg96His was found and reported to be associated with adiposity. In the same study, Ala405Val was found both in obese and lean group[21]. In East Asians population, Ala134Thr was reported to be associated ($p=1.3\times10^{-8}$, OR=4.3) withthiopurine induced leucopenia [22].

Literatures suggested that Arg316Gln is located at conserved 2-OG and Fe-binding site that involved in 2-OG coordination, by forming stabilizing salt bridges with the carboxylates of this co-substrate. Thus, this mutation was completely inactive in the uncoupled reaction assay with 3-methylthymidine, i.e. unable to convert 2-OG to succinate in either the absence or presence of 3-methylthymidine[4,5,21]. Our findings illustrate that a significant structural alteration was found in secondary RNA as G>A change in "Arg" codon eventually converts into "Gln" codon. "Arg" is a positively polar, basic amino acid and essential for protein biosynthesis while "Gln" is polar without charge amino acid. *I-tasser* confirmed that N-oxalylglycine, Fe^{3+} ligand binding site present in wild type sequence but loss of active site found in mutant type, so this may be an explanation of enzyme inactivity.

In case of Arg96His which is located within putative "substrate recognition lid" of the protein, i.e. essential for binding of the primary substratebut is not involved in interactions with the co-substrate 2-OG (21). Crystal structure of *FTO* suggested that 3-meT along with NOG (N-oxalylglycine) forms H-bond with this residue, which is being broken as a result of mutation[5]. It has been reported that themolecular size of "Arg" is larger than "His" and also a charge difference takes place between wild type and that of mutant[23].Interestingly, despite of being similar molecular nature, G>A or G>C change in "Arg" convert into "His" showing major structural alteration in secondary RNA.Ligand binding alteration is found due to substitution, although active site shows null change.Though the alteration of secondary RNA structure forAla134Thr is null, G>A change in "Ala" convert into "Thr" may lead to some functional alteration of the proteinbecause "Ala" is a hydrophobic, non-polar molecule while "Thr" is hydrophilic, polar (uncharged) molecule. This variant occurs at double

stranded β-helix in a "jellyroll fold" containing all the catalytic apparatus that is well conserved in all AlkBhomologues [5,21]. This study depicted theNuc.Acid (Nucleic acid) binding site at the target residue which due to mutation binds to CLA (Chlorophyll A molecule) and also gains an active site for Lipoxygenase enzyme. Another reported variantAla405Val which found to be located within COOH-terminal domain did not affect the enzyme activity because functions of this region remain unknown[5,21].Our study shows a null structural alteration in secondary RNA due to C>T transversion in "Ala" to "Val", and a minute change in protein structure. We assume that this structural alteration might be due to similar physical and chemical properties of both the amino acid. *I-Tasser* reported that this mutation affect ligand binding property.

Here we identified 6 novel single nucleotide polymorphisms with predicted functional alteration. Major structural alteration was found in Asp81Gly, Arg96Cys, Gly103Asp, His231Arg; a slight alteration in Val228Leu and no alteration found in Ala341Asp. In Asp81Gly, "Asp" is negatively charged polar molecule while "Gly" is neutral, non-polar molecule. Apart from RNA structure, we found molecular oxygen as ligand to be bound near the target residue in wild type protein where Fe^{3+} replaces O_2 in mutant type. Even in case of Asp81, two active sites for non-specific serine/threonine protein kinase and one site for glycoprotease are found while mutation cause loss of active site of the protein.A similar transition causes alteration from Histidine to Arginine at 231 position of the protein resulting major change in secondary RNA structure which leads to protein structure alteration as well. Crystal structure of *FTO* illustrates that this position is conserved in all phyllogenetic group and it binds strictly to Fe (II) molecule. But when Arginine substitutes this Histidine, loss of Fe (II) also occur.In case of Gly103Asp, alteration of amino acid from Glycine to Aspartic acid occurs due to G>A substitution which also cause significant change in secondary RNA structure as well as protein structure. Here also molecular change from neutral non-polar to negatively charge polar may affect solubility of the residue. This residue is located at conserved substrate binding site also form ligand binding complex with 2-OG and Mn^{2+}, which seems to be unaltered when mutation occur. In Gly103, one active site for protein disulfide-isomerase, one site for farnesyl-diphosphate synthase and three for dimethylallyltransferase; but no active site found when mutation occur.Another considerable change in secondary RNA structure occurs due to C>T transversion that alter Arginine to Cystein at 96 position of the protein. In this variant, location of the residue plays important role in protein function as describe in Arg96His. But in this case, molecular change from positively polar to neutral nonpolar causes an interruption of the H-bond as well as solubility of the residue. However, no change in ligand binding site and active site found due to mutation at this position of the protein.On the other hand, G>T transversion cause amino acid alteration from Valine to Leucine at 228 position

of the protein lead to a slight modification in secondary RNA structure which might be due to similar physico-chemical properties. This residue is similar to that of H231 thus due to mutation $Fe^{2+}$is unable to bind at the target amino acid.However, C>A change in Ala341Asp did not show any structural alteration but Alanine to Aspartic acid might cause functional changes of the protein.As this residue is located at C-terminal of the protein[5,21], according to literatures the function of this residue remains unknown.This study hypothesis that wild type residue is hydrophobic in nature while mutant is hydrophilic, thus solubility is extremely hindered. Even metal ligand binding site near the target residue is also replaced by amine compound, thus may alter the function of the protein.

5 CONCLUSION

In conclusion, our study suggests that the application of computational tools like SIFT, Mutation Taster, MetaSNP, PolyPhen2, Secondary RNA structure and *I-Tasser* may provide an alternative approach for identifying novel SNPs. In a total of 86,197 SNPs in FTO gene, 348 were found to be missense variants and among them 10 variants (Arg316Gln, Arg96His, Ala405Val and Ala134Thr, Asp81Gly, Arg96Cys, Gly103Asp, His231Arg, Val228Leu, Ala341Asp) were identified as potential disease causing SNPs through our integrated *in silico* analysis. An important part of this study is the explanation behind alteration of protein function due to single nucleotide polymorphisms. These variants could be considered as early biomarker of disease occurrences. Hence, further studies are required to validate the association of the novel SNPs in obesity and cancer risk.

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