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IN SILICO ANALYSIS OF CODING NON-SYNONYMOUS SINGLE NUCLEOTIDE POLYMORPHISMS (SNPs) OF HUMAN *NQO1* GENE AND THEIR IMPACT ON BENZENE INDUCED HAEMATOTOXICITY

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ABSTRACT
Occupational exposure to benzene can cause blood disorders including aplastic anemia and acute myelogenous leukemia. Significant decreases in the number of white blood cells and platelets have been reported in workers exposed to benzene. NQO1 protein is an enzyme that has attracted considerable attention because of its ability to detoxify a number of natural and synthetic compounds. It plays a necessary role in the protection of benzene workers against benzene toxicity by catalyzing two and four electron reduction of benzoquinone. In this study we utilized computational analyses tools to identify functionally important coding non-synonymous (ns) SNPs in human *NQO1*gene. nsSNPs were analyzed by different bioinformatics tools to predict their functional effects. 12 SNPS, out of 159 nsSNPs were found to be deleterious with double positive prediction by SIFT software and polyphen-2 server. Two out of these 12 deleterious SNPs were classified as high risk SNPs with common positive prediction by PROVEAN, PhD-SNP and SNPs&GO algorithmic tools. I- mutant detected an alteration in protein stability due to these two SNPs which further potentiates their functional impact. Analysis of these SNPs by Project Hope indicated that the two SNPS were found to be expressed in conserved regions, so variations in these regions may lead to potential functional changes. Bioinformatics algorithmic tools that were used in this study failed to classify the SNP (rs1800566) within the NQO1 gene at position 609 in exon6 (C- T) (the most reported SNP within NQO1 gene) as pathological SNP, this limitation draw an attention to improve the prediction capacity of these tools. In conclusion, our results suggest that the application of computational tools like SIFT, PolyPhen-2, PROVEAN, PhD-SNP, SNPs&GO, I-Mutant and Project Hope may provide an alternative approach for selecting target SNPs. Our results showed that the amino acid residue substitutions which had the greatest impact on the function of the NQO1 protein were R119P (rs11555215) and L7R (rs368942932). Failures to classify the most reported SNP, with its pathological effects, as pathological SNP by the used bioinformatics tools highlighted a clear limitation and draw an attention to improve the prediction capacity of these tools.

KEYWORDS: In silico analysis, Single nucleotide polymorphism (SNP), *NQO1* gene, Benzene induced haematotoxicity.

INTRODUCTION
Benzene, an aromatic hydrocarbon and a component of crude oil and gasoline, is produced at high levels and is widely used as an intermediate in the manufacture of plastics, resins, dyes, etc. Occupational exposure to benzene occurs through solvent exposures in the chemical industry, in petroleum refineries, oil pipelines, on ships and tankers, auto repair shops and bus garages.[1] Benzene constitutes approximately 1% of gasoline by weight in the United States and Western Europe and more in other nations.[2,3] Exposure is elevated in areas of heavy motor vehicle traffic and around gasoline filling stations.[2] The main route of exposure is inhalation[4], although dermal absorption is also possible.[5] Experimental studies indicate that approximately 50% of inhaled benzene is absorbed into the body.[6] Workers in areas where gasoline loading and unloading takes place, such as high-volume storage terminals, delivery stations, car repair stations, and gasoline stations, have the highest potential for exposure.[7] A characteristic effect of chronic benzene exposure can cause blood disorders including aplastic anemia and acute myelogenousleukemia.Significant decreases in the number of white blood cells and

platelets have been reported in workers exposed to benzene.[8] Although these toxic effects are related to metabolism of benzene in the liver, the particular metabolite (s) that damage bone marrow cells and the mode of toxic action are subject of debate.[9,10] There are numerous enzymes system that are involved in the metabolism of benzene such as Microsomal epoxide hydrolase(EPHX), various glutathione S- transferase (GSTS) and NAD(P)H quinoneoxidoreductase(NQO1). It has been speculated that polymorphic gene of the above enzymes predispose some individuals to benzene toxicity through this metabolism.[9,11-13]

NQO1 protein, is an enzyme that has attracted considerable attention because of its ability to detoxify a number of natural and synthetic compounds and, conversely, to activate certain anticancer agents.[14,15] It plays necessary role in the protection of benzene workers against benzene toxicity by catalyzing two and four electron reduction of benzoquinone. NQO1 enzyme is controlled by *NQO1*gene which is located in the long arm of chromosome 16 (16q22.1), it expands approximately 20 kb with 5 introns and 6 exons.[16] Failure to induce functional NQO1enzyme (mutant alleles) makes the cells more susceptible to the effects of benzene metabolites which may lead to increased risk of benzene poising.[17] This study aimed to determine the codingnon-synonymous single nucleotide polymorphisms(nsSNPs) within human *NQO1* gene that may increase the risk of benzene induced haematotoxicity, through their impact on the functional alteration of NQO1 protein, using computational algorithmic tools.

MATERIALS AND METHODS

The Information of nsSNPs within *NQO1*gene (SNP ID, protein accession number, position and residue change) was retrieved from NCBI dbSNP database. The FASTA format of the protein sequence and its isoforms (three isoforms) was obtained from Uniprot KB at Expassy database.

nsSNPs were analyzed by different bioinformatics tools to predict their functional effects. These algorithmic programs included: SIFT-Sorting Intolerant From Tolerant, PolyPhen-2-PolymorphismPhenotyping v2,PhD-SNP -Predictor of human -Deleterious Single Nucleotide Polymorphisms, SNPs & GO,PROVEAN-Protein Variation, I-mutant and project Hope.

Deleterious nsSNPs of *NQO1* gene were initially predicted by SIFT program and PolyPhen-2 server. SNPs, with double positive prediction by the both software (SIFT/polyphen-2), were considered pathological and were summited for further analysis with PROVEAN, PhD-SNP and SNPs&GO algorithmic tools. SNPs, with common positive prediction by all of these algorithmic tools, were classified as high risk SNPs, and all additional investigations were held for only these SNPs. The stability alterations of NQO1protein with the high risk SNPs were analyzed by I-Mutant server to predict their RI and free energy change values, and were analyzed by Project Hope software to determine the effect of these mutations on the protein structure.

SIFT software: "Sorting Intolerant from Tolerant". This is a sequences homology-based tool that presumes that important amino acids will be conserved in the protein family. Hence, changes at well-conserved positions tend to be predicted as deleterious. The cutoff value in the SIFT program is a tolerance index of ≥0.05. The higher the tolerance index, the less functional impact a particular amino acid substitution is likely to have.[18,19]

The server PolyPhen-2(Polymorphism Phenotyping v2) has been used to analyze the structural damage due to coding nsSNPs which can affect protein functionality. The server is able to calculate a score on the basis of the characterization of the substitution site to a known protein three-dimensional structure. A PSIC score has been calculate for each variant of each site and the difference between them reported. The higher the PSIC score difference is, the higher is the functional impact a particular amino acid substitution is likely to have.[20-22]

PROVEAN (Protein Variation Effect Analyzer) is a software tool which predicts whether an amino acid substitution or indel has an impact on the biological function of a protein. PROVEAN is useful for filtering sequence variants to identify nonsynonymous or indel variants that are predicted to be functionally important. The performance of PROVEAN is comparable to popular tools such as SIFT or PolyPhen-2. A fast computation approach to obtain pairwise sequence alignment scores enabled the generation of precomputed PROVEAN predictions for 20 single AA substitutions and a single AA deletion at every amino acid position of all protein sequences in human and mouse.[23]

SNPs & GO web server was used to predict the human disease related mutations. This server was mainly based on support vector machines which can corroborates all the information regarding variations from the existing databases. It annotates variations as deleterious based on information derived from Gene Ontology (GO) Predictor with overall accuracy of 82%. The server also implements PhD-SNP method that take in input different subsets of SNPs&GO's input features. The PhD-SNP method takes in input the first 45 elements vector encoding for the sequence and profile information. Selecting the option "All methods" the prediction of PhD-SNP is calculated and included in the output.[24,25]

I-Mutant Suite is a suite of support vector machine (SVM)-based predictors of protein stability changes according to Gibbs free energy change, enthalpy change, heat capacity change, and transition temperature. I-Mutant predictor can evaluate the stability change upon single site mutation starting from the protein structure or from the protein sequence. The output result of the

predicted free energy change (DDG) classifies the prediction into one of three classes: largely unstable (DDG < −0.5 kcal/mol), largely stable (DDG>0.5kcal/mol), or neutral (-0.5≤ DDG≤0.5 kcal/mol).[26-28]

Project Hope software is developed at the Centre for Molecular and Biomolecular Informatics CMBI at Radboud University in Nijmegen. It is an online web service where the user can submit a sequence and mutation. This software collects structural information from a series of sources, including calculations on the 3D protein structure, sequence annotations in UniProt and predictions from DAS-servers. It combines this information to give analyze the effect of a certain mutation on the protein structure and will show the effect of that mutation in such a way that even those without a bioinformatics background can understand it.[29]

RESULTS

Uniprot KB at Expassy database was used to obtain the reference sequence of NQO1 protein. The enzyme apparently serves as a quinonereductase in connection with conjugation reactions of hydroquinons involved in detoxification pathways as well as in biosynthetic processes such as the vitamin K-dependent gamma-carboxylation of glutamate residues in prothrombin synthesis. The protein has three isoforms: P15559-1 isoform has been chosen as the 'canonical' sequence, it consists of 274 amino acids; P15559-2 isoform differs from the 'canonical' sequence in which the amino acids 140-173 are missing; P15559-3 isoform has amino acids 102-139been missing. The gene has 20 physical interactions with other genes (interactors) which they have similar functions and illustrated in (fig.1).

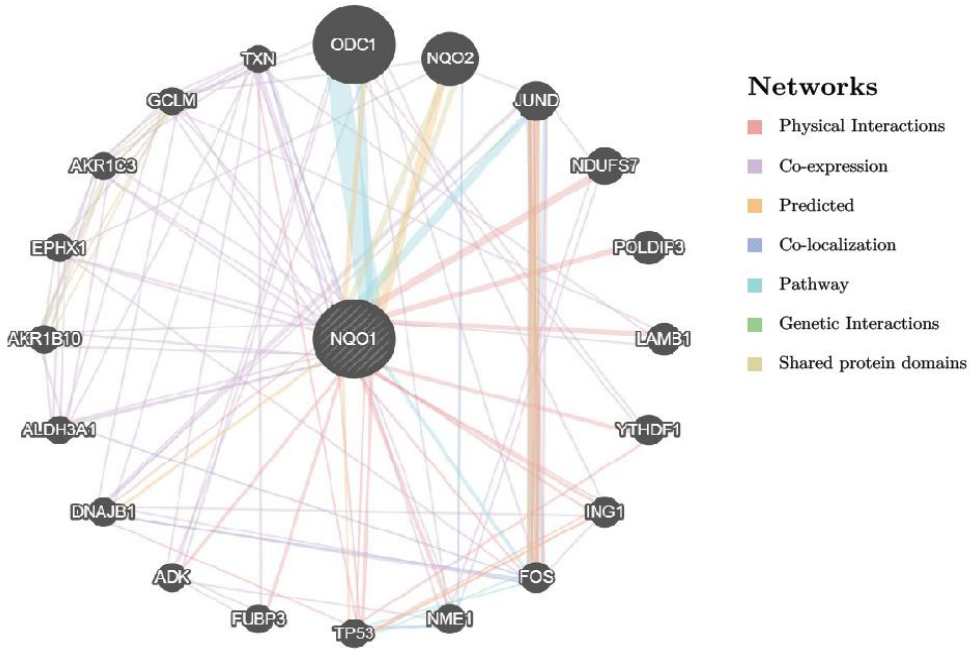

Fig. 1: Functional interaction between NQO1 and its related genes.

Table 1: Prediction result of SIFT and Polyphen-2 programs.

NO.	SNP	A. ACID CHANGE	SIFT		Polyphen-2	
			PREDICTION	SCORE	PREDICTION	SCORE
1	rs1131341	R139W	DELETERIOUS	0.002	PRO. DAMAGING	1.00
2	rs11555215	R119P	DELETERIOUS	0.004	PRO. DAMAGING	1.00
3	rs34447156	Q269H	DELETERIOUS	0	PRO. DAMAGING	1.00
4	rs45476693	I85T	DELETERIOUS	0.002	PRO. DAMAGING	1.00
5	rs114112422	R211C	DELETERIOUS	0	PRO. DAMAGING	1.00
6	rs114112422	R139C	DELETERIOUS	0	PRO. DAMAGING	1.00
7	rs114238154	M1T	DELETERIOUS	0.006	PRO. DAMAGING	0.61
8	rs144412546	K91Q	DELETERIOUS	0	PRO. DAMAGING	1.00
9	rs145498187	E71K	DELETERIOUS	0	PRO. DAMAGING	1.00
10	rs200972816	S40L	DELETERIOUS	0	PRO. DAMAGING	1.00
11	rs368942932	L7R	DELETERIOUS	0	PRO. DAMAGING	1.00
12	rs369179544	S82G	DELETERIOUS	0.008	PRO. DAMAGING	0.96

PolyPhen-2 result: POROBABLY DAMAGING (more confident prediction) / POSSIBLY DAMAGING (less confident prediction); PSIC SD: Position- Specific Independent Counts software if the score is ≥ 0.5; Tolerance Index: Ranges from 0 to 1; The amino acid substitution is predicted damaging if the score is ≤ 0.05 and tolerated if the score is > 0.05.

Two SNPS, with common positive prediction by all of these algorithmic tools, were detected and were classified as high risk SNPs (table 2). All additional investigations were held for only these two SNPs.

The stability alterations of *NQO1* protein with the 2 SNPs were analyzed by I-Mutant server to predict their RI and free energy change values. Result revealed that rs11555215 - R119PnsSNPs decreases the stability of *NQO1* protein, while rs368942932 - L7RnsSNPs increases the stability of *NQO1* protein (Table 3). These SNPs might cause maximum damage to the protein by affecting its stability.

Table 2: High risk nsSNPs identified by PROVEAN, PhD-SNP and SNPs&GO.

NO.	SNP	A. ACID CHANGE	PhD		SNP&GO-		PROVEAN	
			Prediction	RI	Prediction	RI	Prediction	SCORE
1	rs11555215	R119P	Disease	9	Disease	7	Deleterious	-6.15
2	rs368942932	L7R	Disease	7	Disease	0	Deleterious	-5.32

Table 3: Prediction result of I-Mutant software.

NO.	SNP	A. ACID CHANGE	i-mutant		
			Prediction	RI	DDG
1	rs11555215	R119P	Decrease	2	-1.8
2	rs368942932	L7R	Increase	5	0.2

RI: Reliability Index; DDG: free energy change value(DDG < 0: decrease stability, DDG >0 increase stability)

These two SNPs were submitted to the Project Hope software and they indicated pathological polymorphisms change in the amino acids. These two SNPS were found to be expressed in conserved regions.

The wild-type residue charge was positive, while the rs11555215 – R119P mutant residue charge is neutral. This difference will disturb the ionic interaction made by the original, wild-type residue. The mutant residue is smaller than the wild-type residue. The size difference between wild-type and mutant residue makes that the new residue is not in the correct position to make the same hydrogen bond as the original wild-type residue did. It is more hydrophobic than the wild-type residue. The difference in hydrophobicity will affect hydrogen bond formation (fig. 2,).

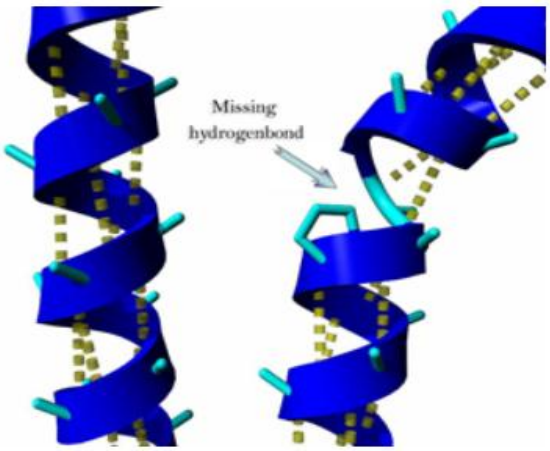

Fig. 2: The wild-type residue is located in an α-helix. Proline disrupts an α-helix when not located at one of the first 3 positions of that helix (rs11555215).

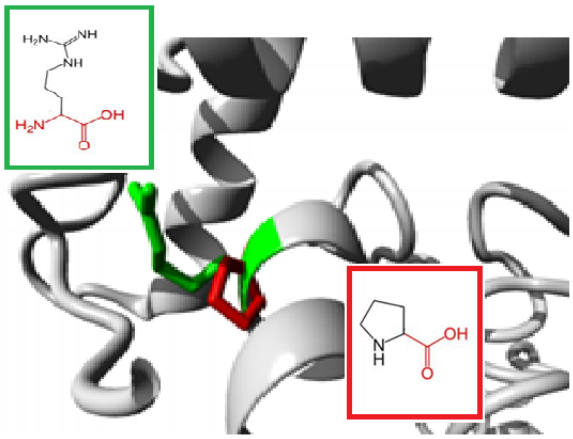

Fig. 3: The side chain of both the wild-type(Arginine) and the mutant residue (Proline) of rs11555215 is shown and coloured green and respectively.

The wild-type residue charge was neutral, while the rs368942932 – L7Rmutant residue charge is positive, the mutant residue introduces a charge in a buried residue which can lead to protein folding problems. The mutant residue is bigger than the wild-type residue. The wild-type residue was buried in the core of the protein. The mutant residue is bigger and probably will not fit. It is more hydrophobic than the wild-type residue. The mutation will cause loss of hydrophobic interactions in the core of the protein.[30]

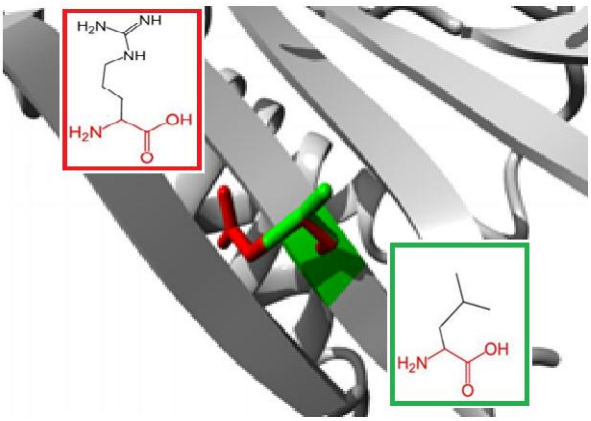

Fig. 4: The side chain of both the wild-type(Lucine) and the mutant residue(Arginine) ofrs368942932 is shown and coloured green and red respectively.

DISCUSSION

Computational analyses provide a major insight to predict SNPs which can potentially affect the structure and function of the encoded protein. NQO1 is a flavoenzyme that plays an important role in protection against endogenous and exogenous quinones by catalyzing two- or four-electron reductions of these substrates. Quinone compounds are present within our bodies and in our natural environment. The two- and four-electron reductions catalyzed by NQO1 are beneficial to the cell by preventing redox cycling, which leads to the generation of free radicals; therefore, NQO1 protects the cell from unwanted oxidative damage.[31-33]

In this study we utilized computational analyses tools to identify functionally important nsSNPs in human *NQO1*gene.nsSNPs were analyzed by different bioinformatics tools to predict their functional effects. 12 SNPS were found to be deleterious with double positive prediction by the both software (SIFT/polyphen-2). Two out of these 12 deleterious SNPs were classified as high risk SNPs with common positive prediction by PROVEAN, PhD-SNP and SNPs&GO algorithmic tools. Alteration of protein stability due to these SNPs further potentiates their functional impact. Analysis of these SNPs by Project Hope indicated that the two SNPS were found to be expressed in conserved regions, so variations in these regions may lead to potential functional changes.

No previously published data were retrieved concerning the effects of these SNPs (rs11555215 and rs368942932) on the function of NQO1 protein during our search in the PubMed-NCBI database. Therefore the validation of these nsSNPs in any disease is required to complement this finding.

Our analysis approach with the bioinformatics algorithmic tools failed to classified the SNP (rs1800566) within the *NQO1* gene at position 609 in exon6 (C- T) as pathological SNP, this SNP results in a proline to serine substitution at position 187 in the amino acid structure of the NQO1 protein, resulting in loss of enzyme activity.[34] This polymorphism has previously been associated with benzene-induced toxicity, and other medical diseases.[35-38]

We previously studied the influencing of NQO1 C609T polymorphism as a genetic risk modifier for haematotoxicity among 100 fuel station workers, chronically exposed to variable concentration of petroleum derivatives air pollutants during their work on the fuel filling station. We have demonstrated robust changes in TWBCs count, neutrophils count and platelets count among fuel station workers with homozygous (TT) mutant genotype.[39] We also studied the association of NQO1 C609T polymorphism with different haematological malignancies, in previous studies. Our results indicated a 2.9-fold increased risk of Acute lymphoid leukaemia for those carrying NQO1 mutant alleles[40], a 2.4-fold increased risk of Acute myeloid leukaemia for those carrying NQO1 mutant alleles[41] and a 3.1-fold increased risk of Polycythaemia Vera for those carrying NQO1 mutant alleles.[42] Failures to classify this SNP as pathological SNP by the used bioinformatics tools highlighted a clear limitation and draw an attention to improve the prediction capacity of these tools.

CONCLUSION

In conclusion, our results suggest that the application of computational tools like SIFT, PolyPhen-2, PROVEAN, PhD-SNP, SNPs&GO, I-Mutant and Project Hope may provide an alternative approach for selecting target SNPs. Our results showed that the amino acid residue substitutions which had the greatest impact on the function of the NQO1 protein were R119P (rs11555215) and L7R (rs368942932). bioinformatics algorithmic tools, that were used in this study, failed to classified the SNP (rs1800566) within the NQO1 gene at position 609 in exon6 (C- T) as pathological SNP, which is the most reported SNP within NQO1 gene, this limitation draw an attention to improve the prediction capacity of these tools.

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