In Silico Analysis of Single Nucleotide Polymorphisms (SNPs) of Three Isoforms of Human Nitric Oxide Synthase (nNOS, iNOS, eNOS) Genes

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ABSTRACT

The three isoforms of Nitric Oxide Synthase (NOS) synthesize free radical nitric oxide (NO), which has numerous protein targets in human body.Several vital processes are regulated and/or mediated by NO in nervous, immune and cardiovascular systems. Hence, alteration on NO level leads to pathological conditions in particular cells or tissues. Prior to conduction population genetic research, listing and identifying the most deleterious SNPs that may have strong relation with a particular disease is crucial. Hence, the aim of this study was to determine the functional non-synonymous Single Nucleotide Polymorphisms (nsSNPs) with emphasis on the exon regions for the neuronal, Inducible and Endothelial Nitric Oxide (nNOS, iNOS, eNOS) genes. Data from dbSNP database were functionally and structurally analyzed using different bioinformatics softwares. In the exon region, 222 SNP (from total 5293), 203 SNPs (from 1441) and 195 SNP (from 782) in nNOS, iNOS and eNOS, respectively were analyzed. Results of SIFT and PolyPhen predicted six SNPs in nNOS, iNOS, and seven SNPs in eNOS genes as damaging.

Keywords: nitric oxide synthase (NOS), nitric oxide (NO), single nucleotide polymorphisms (SNPs), bioinformatics softwares.

Classification: J.m

Language: English

London Journals Press

LJP Copyright ID: 975735
Print ISSN: 2514-863X
Online ISSN: 2514-8648

London Journal of Research in Computer Science and Technology

Volume 19 | Issue 2 | Compilation 1.0

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ABSTRACT

The three isoforms of Nitric Oxide Synthase (NOS) synthesize free radical nitric oxide (NO), which has numerous protein targets in human body.Several vital processes are regulated and/or mediated by NO in nervous, immune and cardiovascular systems. Hence, alteration on NO level leads to pathological conditions in particular cells or tissues. Prior to conduction population genetic research, listing and identifying the most deleterious SNPs that may have strong relation with a particular disease is crucial. Hence, the aim of this study was to determine the functional non-synonymous Single Nucleotide Polymorphisms (nsSNPs) with emphasis on the exon regions for the neuronal, Inducible and Endothelial Nitric Oxide (nNOS, iNOS, eNOS) genes. Data from dbSNP database were functionally and structurally analyzed using different bioinformatics softwares. In the exon region, 222 SNP (from total 5293), 203 SNPs (from 1441) and 195 SNP (from 782) in nNOS, iNOS and eNOS, respectively were analyzed. Results of SIFT and PolyPhen predicted six SNPs in nNOS, iNOS, and seven SNPs in eNOS genes as damaging. Whereas, I-mutant server showed decrease stability of proteins encoded by them. Then CPH modeler 3.2 and Chimera software version 1.2 showed structure of these proteins. Further, Proscan version 1.7 server, Promotor 2.0 prediction server and TSSG prediction program identified cis regulatory elements in the above genes. Interestingly, most deleterious SNPs found in this study have not reported yet, especially in eNOS.

Keywords: nitric oxide synthase (NOS), nitric oxide (NO), single nucleotide polymorphisms (SNPs), bioinformatics softwares.

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I. INTRODUCTION

Single Nucleotide Polymorphisms (SNPs) represent the most frequent form of polymorphism in the human genome.[1,2]. Nitric oxide synthases (NOSs) synthesize the metastable free radical nitric oxide (NO), it is an unorthodox messenger molecule, has numerous targets enzymes and proteins [3,4]. There are three isoforms of NOS: endothelial (eNOS), neuronal (nNOS), and inducible (iNOS)[5]. Neuronal NOS (nNOS) gene located within chr12, reference Sequence: NG_011991.2. It is calcium-dependent and produces low level of NO as a cell signaling molecule. It constitutively expressed in specific neurons of the brain. It has been identified by immunohistochemistry in: spinal cord, sympathetic ganglia, peripheral nitrergic nerves, epithelial cells of various organs, kidney (macula densa cells), adrenal glands, islet cells of pancreatic, and vascular smooth muscle. The major source of nNOS in mammalians, is the

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skeletal muscle.nNOS has been implicated in modulating learning, memory, and neurogenesis. Disturbance in NO signaling , contribute to a variety of neurodegenerative pathologies such as excitotoxicity following stroke, multiple sclerosis, Alzheimer's, and Parkinson's diseases.Also Freudenberg mentioned in his review , some variants of nNOS associated with development of Psychiatric diseases such as Major depression, bipolar disorders, Autistic Spectrum disorder (ASD), Obsessive Compulsive disorder(OCD), Anxiety disorders, and Schizophrenia (Freudenberg et, al 2015[6-10]. In addition there are two researches done in 2012, and 2013 showed that here is significant association between some SNPs in nNOS and ischemic stroke [11,12]. Inducible (iNOS) gene located in: chr17, reference Sequence NG_011470.1. It encode calcium-independent enzyme that produces large amounts of NO.It identified basically in macrophages and express virtually in any cell or tissue that can be cytotoxic on parasitic microorganisms as well as on some tumor cells [13].Also can be expressed in cells when induced by bacterial lipopolysaccharide, cytokines, and other inducing agents . So, non-immune cells can be induced and affect the adjacent cells, for example ,Cytokine-activated endothelial cells, have been shown to lyse tumor cells, and induced hepatocytes kill malaria sporozoites. Also, it involved in non-specific allograft rejection[7,14-17]. Moreover excessive release of NO by iNOS plays a major role in septic shock, it lower the blood pressure predominantly due to it is effect in the vascular wall[18]. Interestingly, genetic variations in iNOS are involved in resistance to malaria as mentioned in research done by Maria de Jesus Trovoada, and her colleges in 2014 [19].

Endothelial NOS (eNOS) gene located in chr7, reference sequence NG_011992.1(NCBI). It encodes calcium-dependent NOS that produce low levels of NO, mostly it expressed in endothelial cells, and has been detected in cardiac myocytes, platelets, certain neurons of the brain, in syncytio-trophoblasts of the human placenta and in LLC-PK1 kidney tubular epithelial cells [7,17]. Physiological functions of eNOS include: vasodilation, inhibition of platelet aggregation and adhesion, inhibition of leucocyte adhesion,vascular inflammation, and Control of vascular smooth muscle proliferation. Furthermore, NO has been shown to inhibit DNA synthesis, mitogenesis, and proliferation of vascular smooth muscle cells [20,21], and has a role in Stimulation of angiogenesis postnatal, also had been found to be critical for collateral formation and angiogenesis post-ischemia [22]. One of the meta-analysis revealed that eNOS 4b/polymorphisms could be a risk factor for coronary artery disease, particularly in African populations [23]. Other research conclude that, (A-->G) change in eNOS is one of the most important variant associated with susceptibility to essential hypertension [24].Moreover, NOS is known to decrease the bioavailability of NO in sickle patients and play a role in different phenotypic presentation in patients, so every deleterious SNPs may affect the severity and outcome of the disease in sickle patients.

As Goldstein DB said in his paper, much attention of researches focused to date has focused on three polymorphisms in the eNOS gene which are: (-786T>C (rs2070744), intron 4 27-base-pair repeat, and Glu298Asp (rs1799983)), and this limits the study of eNOS to an isolated"candidate polymorphism" rather than a "candidate gene [25]. Therefore , identifying other pathological variants is required to carry out other genetic researches -the Same approach has been used to investigate the effect of nsSNP of HLA–*DRB1* and HLA-*DQB1* genes[26], so this study was done to identify the most deleterious SNPs in each isoform, and to determine the effect of them in structure and function and stability of proteins, that may contribute with other molecules to cause diseases.

II. MATERIAL AND METHODS

Softberry (http://www.softberry.com/),and is the most The data of SNPs of the 3 isoform of human NOS genes was collected from National Center for

Biological Information (NCBI) web site. The information of the SNP (i.e protein accession number and SNP ID) of these isoform was retrieved from NCBI dbSNP. (http://www.ncbi.nlm.nih.gov/snp/) and Swiss Prot databases (http://expasy.org/),

2.1 In silico analysis of the functional impact of coding nsSNPs using

(SIFT) software (Sorting Tolerant From Intolerant),(http://blocks.fhcrc.org/sift/SIFT.html) is an online bioinformatics tool used to predict whether an amino acid substitution will affect the protein function or not.[27]. The main underlying principle of this program is that it generates alignments with a large number of homologous sequences, and assigns scores to each residue ranging from zero to one. Scores close to zero indicate evolutionary conservation of the genes and intolerance to substitution, while scores close to one indicate tolerance to substitution only [28]. *(PolyPhen) software:* Is an online bioinformatics program (http://genetics.Bwh.harvard.edu/pph2/), automatically predict the consequence of an amino acid change on the structural and functional protein level. The program search for protein 3D structures, do multiple alignments of homologous sequences and amino acid contact information in several protein structure databases, then calculate position-specific independent count scores (PSIC) for each of two variants, and then computes the PSIC scores difference between two variants. The higher PSIC score difference, indicate that the functional impact of particular amino acid substitution is likely to occur [29, 30].

2.2 Identification of cis regulatory elements using

1. PROSCAN version 1.7 Web Promoter Scan Service

It predicts promoter regions based on homologies with putative eukaryotic Pol II promoter sequences. The site is serviced and maintained by Dr. Dan Prestridge at the Advanced Biosciences Computing Center, University of Minnesota. (http://bimas.dcrt.nih.gov/molbio/proscan/)

2 Promoter 2.0 Prediction Server

It predicts transcription start sites of vertebrate Pol II promoters in DNA sequences. It has been developed as a frequently updated database of simulated transcription factors that interact with sequences in promoter regions. It builds on principles that are common to neural networks and genetic algorithms. The site is serviced and maintained by Steen Knudsen at The Center for Biological Sequence Analysis at the Technical University of Denmark. (http:/www.cbs.Dtu.dk/services/Promoter/)

3 TSSG

This is the tool that used in Recognition of human Pol II promoter regions and transcription start sites, located in accurate mammalian cis element prediction program. Also has the fewest false positive predictions[31].

2.3 Prediction of the protein stability using

1) I-Mutant server

To support the results and to predict the stability of deleterious SNPs, I-Mutant2.0 online software has been used (http://folding.biofold. org/i-mutant/i-mutant2.0.html). It is a web server using for automatic prediction of protein stability changes upon single-site mutations, the result is a sign of DDG (the free energy change), positive sign (+) indicates increase of stability, negative sing (-) indicates decrease of stability.

2.4 Modeling of the deleterious SNPs using

1) CPH model 3.2 server

It has been used to predict the 3D structure for those proteins with an unknown 3D structure model. It is a protein homology modeling server, where the template recognition is based on profile to profile alignment, guided by secondary structure and exposure predictions. http://www.cbs.dtu.dk/services/CPHmodels/.

2) Chimera software version 1.8.1

This is a homology modeling software that has been used to generate the mutated models of each of the selected PDB (protein database) entries. This software is used to browse respectively locate the 3D structure of the specific protein and then alter the native amino acid with a mutated one to then look for structural effect that may produce. The outcome is then a graphic model depicting the mutation [32].

2.5 Analysis of the deleterious SNPs using project hope

It is online software (http://www.Cmbi.ru.nl/hope) has been used to build an automatic mutant analysis server that analyzes the structural and functional effects of point mutations, it is easy to understand and deal with and provide attractive results for the researchers [33]. It evaluate the effect of the mutation on the following features: Contacts made by the mutated residue, structural domains in which the residue is located, modifications on this residue and known variants for this residue.

III. RESULTS

Table 1: Shows numbers of SNPs in regions of nNOS, iNOS ,and eNOS genes (based on the dbSNP database)

		Number of SNPs	
sNPS	nNOS	iNOS	eNOS
Exons	222	203	195
5UTR	19	29	2
3UTR	145	43	8
Introns and other	4907	1166	477

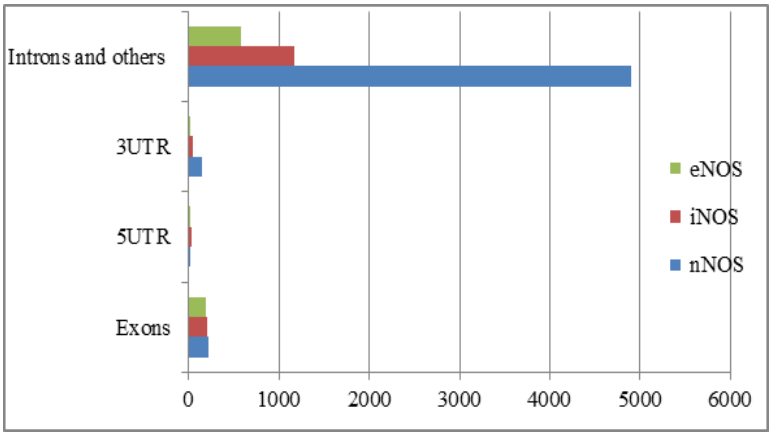

Figure 1: Bar chart represents the distribution of 3′ UTR, 5′ UTR, Exons and intronic and other SNPs for nNOS, iNOS and eNOS genes (based on the dbSNP database)

Table 2: Shows List of deleterious nsSNPs of iNOS that were analyzed using SIFT and Polyphen software's, And also result of I-Mutant server

Rs id nNOS	Position	Sift Score	Prediction	Poly2 Score	Predioction in PolyPhen	Stability
rs 56308341	R23H	0.01	DAMAGINE	1	Probably damaging	Decrease
rs 76839820	TI05M	0.01	DAMAGINE	1	Probably damaging	Decrease
rs 78402290	R19c	0.01	DAMAGINE	1	Probably damaging	Decrease
rs80348085	R48S	0.01	DAMAGINE	1	Probably damaging	Decrease
rs 78422671	R1129H	0.02	DAMAGINE	0.903	Probably damaging	Decrease
rs 55922940	L216P	0.01	DAMAGINE	0.814	Possible damaging	Decrease
Rs id iNOS	Position	Sift Score	Prediction in Sift Score	Poly2 Score	Predioction in PolyPhen	Stability
rs 28944201	R1009C	0.02	DAMAGINE	0.984	Probably damaging	Decrease
rs 406104261	R506W	0.02	DAMAGINE	0.996	Probably damaging	Decrease
rs 112588673	R452Q	0.01	DAMAGINE	0.99	Probably damaging	Decrease
rs 143835443	L720F	0.05	DAMAGINE	0.97	Probably damaging	Decrease
rs 150704221	R750H	0.01	DAMAGINE	0.821	Possible damaging	Decrease
145383683	V1037l	0.02	DAMAGINE	0.456	Possible damaging	Decrease
Rs id eNOS	Position	Sift Score	Prediction in Sift Score	Poly2 Score	Predioction in PolyPhen	Stability
rs 146141837	R1172C	0.01	DAMAGINE	1	Probably damaging	Decrease
rs 14178 7079	R530Q	0.01	DAMAGINE	0.984	Probably damaging	Decrease
rs 143324164	R128W	0.01	DAMAGINE	0.993	Probably damaging	Decrease
rs 145711802	F172L	0.03	DAMAGINE	0.937	Possible damaging	Decrease
rs 141456642	E156K	0.03	DAMAGINE	0.935	Possible damaging	Decrease
rs 145000830	Q411H	0.02	DAMAGINE	0.591	Possible damaging	Decrease
rs 3918232	V827M	0.04	DAMAGINE	0.476	Possible damaging	Decrease

Table 3: Shows results of proscan version 1.7 and tssg of n*NOS*gene

No	Strand	Promoter Location	No.	Stand	Promoter Location
1	Forward	5317 to 5567	16	Forward	140126 to140376
2	Forward	5591 to 5841	17	Forward	145087 to 145337
3	Forward	15511 to 15761	18	Forward	149576 to 149826
4	Forward	22122 to 22372	19	Forward	154991 to 155241
5	Forward	34103 to 34335	20	Reverse	157321 to 157071
6	Forward	35351 to 35601	21	Reverse	142595 to142345
7	Forward	37430 to 37680	22	Reverse	118101 to 117851
8	Forward	40608to 40858	23	Reverse	98357 to 98107
9	Forward	42576 to 42826	24	Reverse	89682 to 89432
10	Forward	43442to 43692	25	Reverse	56773 to 56523
11	Forward	72314 to 72564	26	Reverse	35798 to 35548
12	Forward	106229 to 106479	27	Reverse	18620 to 18370
13	Forward	114649 to 114899	28	Reverse	5845 to 5595
14	Forward	128185 to 128435	29	Reverse	5411 to 5161
15	Forward	131052 to 131302			

Table 4: Shows result of proscan version 1.7 and tssg of *eNOS and iNOS* genes

eNOS Promoter		iNOS Promoter		
No	Strand	Location	Stand	Location
1	Forward	2376 to 2626	Forward	2869 to 3119
2	Forward	18192 to 18442	Forward	6509 to 6759
3	Forward	22274 to 22524	Forward	16614 to 16864
4	Reverse	22746 to 22496	Forward	37132 to 37382
5	Reverse	18539 to 18289	Reverse	37784 to 37534

6	Reverse	11775 to 11525	Reverse	25471 to 25271
7	Reverse	9846 to 9596	Reverse	3137 to 6887
8	Reverse	2873 to 2623		

Table 5: Shows results of promotor 2.0 Prediction Server of nNOS, INOS , and eNOS genes

Rs id nNOS	Position	Sift Score	Prediction	Poly2 Score	Predioction in PolyPhen	Stability
rs 56308341	R23H	0.01	DAMAGINE	1	Probably damaging	Decrease
rs 76839820	TI05M	0.01	DAMAGINE	1	Probably damaging	Decrease
rs 78402290	R19c	0.01	DAMAGINE	1	Probably damaging	Decrease
rs80348085	R48S	0.01	DAMAGINE	1	Probably damaging	Decrease
rs 78422671	R1129H	0.02	DAMAGINE	0.903	Probably damaging	Decrease
rs 55922940	L216P	0.01	DAMAGINE	0.814	Possible damaging	Decrease
rs id inos	Position	Sift Score	Prediction in Sift Score	Poly2 Score	Predioction in PolyPhen	Stability
rs 28944201	R1009C	0.02	DAMAGINE	0.984	Probably damaging	Decrease
rs 406104261	R506W	0.02	DAMAGINE	0.996	Probably damaging	Decrease
rs 112588673	R452Q	0.01	DAMAGINE	0.99	Probably damaging	Decrease
rs 143835443	L720F	0.05	DAMAGINE	0.97	Probably damaging	Decrease
rs 150704221	R750H	0.01	DAMAGINE	0.821	Possible damaging	Decrease
145383683	V1037l	0.02	DAMAGINE	0.456	Possible damaging	Decrease
rs id enos	Position	Sift Score	Prediction in Sift Score	Poly2 Score	Predioction in PolyPhen	Stability
rs 146141837	R1172C	0.01	DAMAGINE	1	Probably damaging	Decrease

rs 14178 7079	R530Q	0.01	DAMAGINE	0.984	Probably damaging	Decrease
rs 143324164	R128W	0.01	DAMAGINE	0.993	Probably damaging	Decrease
rs 145711802	F172L	0.03	DAMAGINE	0.937	Possible damaging	Decrease
rs 141456642	E156K	0.03	DAMAGINE	0.935	Possible damaging	Decrease
rs 145000830	Q411H	0.02	DAMAGINE	0.591	Possible damaging	Decrease
rs 3918232	V827M	0.04	DAMAGINE	0.476	Possible damaging	Decrease

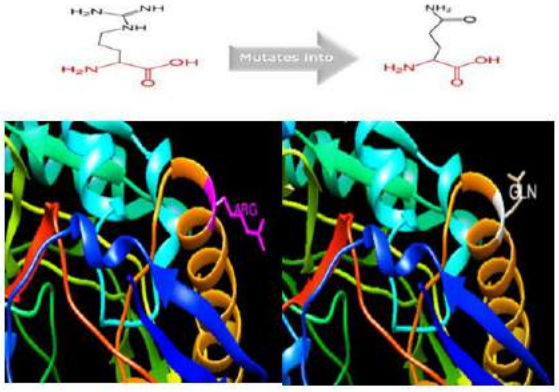

Figure 2: Shows Structure model (cartoon shape) of wild type amino acid Arginine (R)of *i*NOS in "stick" (magenta color) (left), and mutant type Glutamine (Q) in "stick" (grey color) at position 452 using chimera software

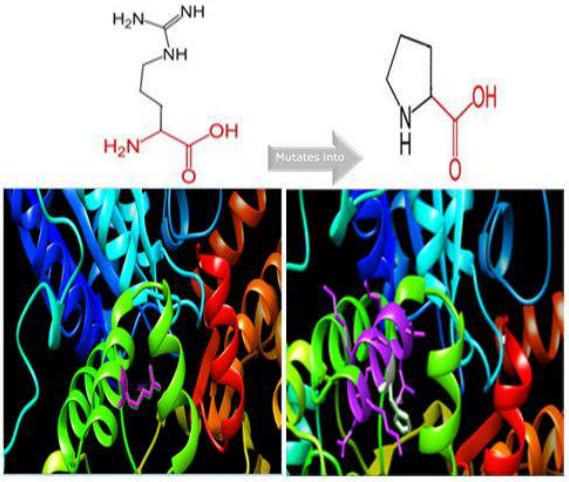

Figure 3: Shows Structure model (cartoon shape) of wild type amino acid (R) in "stick" (Magenta color) (left), and mutant type (H) in "stick" (Grey color) of nNOS gene (right) at position 1129 using chimera software

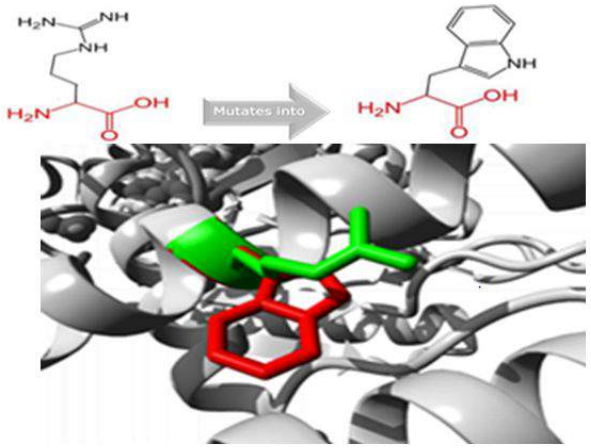

Figure 4: Shows wild amino acid (R) and mutant one (w) in position128 in eNOS gene and Close-up of the mutation using project hope. The protein is colored grey, the side chains of both the wild-type and the mutant residue are shown and colored green and red respectively

IV. DISCUSSION

Nitric oxide synthase is an enzyme, with three isoform (i.e. nNOS, iNOS, eNOS) synthesis Nitric oxide (NO), as messenger molecule and neurotransmitter; it plays an important role in regulation and modulation of a lot of processes in the nervous, immune, and cardiovascular systems. From the database of single nucleotide polymorphisms in a national center of biotechnology (NCBI), 5293, 1441, and 782 SNPs in nNOS, iNOS, ande NOS respectively, were obtained and categorized as shown in figure 1, table1. So an effort was done to identify most deleterious SNPs that can affect the structure, function and stability of the proteins that encode these genes . SNPs was submitted to the SIFT as well as to the PolyPhen server, their prediction are six damaging SNPs in both nNOS and INOS ,and 7 damaging SNPs in eNOS as shown in tables 2. It has been reported in two studies done in 2002 and 2010 in Japanese population, SNP (C276T) and SNP (rs41279104) in nNOS are significantly associated with schizophrenia, with p value of allelic frequency = 0.000007. and p value of genotype = 0.0013 , allelic p =), respectively [34,35]. Also mentioned in two studies, one done by Manso, H. and his colleages , and other in Chinese population, SNPs in nNOS (rs2293050, rs2139733, rs7308402 and rs1483757) were significantly associated with susceptibility to stroke and ischemic stroke (rs7308402) respectively,[11,12]. rs1800780 in the eNOS associated with susceptibility to essential hypertension as reported in B. Yang et. al, 2013[24].

I- mutant server's result showed that, some deleterious SNPs decrease the stability of these proteins while the other increase it as shown in tables 2. To visualize the effect of these damaging SNPs, cph modeler sever, Chimera software and project hope has been used for probably damaging SNPs as shown in figure 2,3,4. Important point is that, only one 3D structure of nNOS (1129) , iNOS (452), and eNOS (128) were done from all other damaging SNPs, the problem is that, there is no pdb id for protein encoding these genes, in addition, there is no full structure done for these isoforms , then protein sequence were checked again for their 3D structural using pBLAST analysis and also protein data bank (pdb), they showed similarity is very weak association with proteins found in data bank http://www.rcsb.org/pdb/home/home.do, so, visualization of 3D structure of deleterious SNPs that found just in chain C -which extent from 755 to 1418- and chain A- from 83 to 505- and from 76 to 480 in nNOS, iNOS, and eNOS respectively has been appeared. Hap map project (http://hapmap.ncbi.nlm.nih.gov/) and 1000 genome project has been used to check the allelic frequency of these damaging SNPs that has been appeared in chains of the protein encoded genes, SNP (rs78422671) in nNOS which is missense transition mutation from G To A that change the amino acid sequence from Arginine (R**)** – hydrophilic- to Histidine (H)- hydrophilic- not found in Hap Map project, in 1000 genome project (MAF = 0.0002/1). SNP (rs112588673) in iNOS ,which is missense transition mutation from G to A, that convert the amino acid from Arginine (R)- hydrophilic - to

Glutamine (Q)- hydrophilic-, not found neither in HapMap project, nor in 1000 genome project. The last SNP (rs143324164) in eNOS ,which is missense transition mutation from C to T, that convert he amino acid from Arginine (R)- hydrophilic - to t tryptophan (W)

- Aromatic , hydrophobic -, it was not found in HapMap project, but was found in 1000 genome project with MAF = 0.0004/2 . Furthermore, cis regulatory elements in three genes (nNOS, iNOS, and eNOS) were determined, as shown in tables 4,5,6. Numbers of promotor start sites by Proscan version 1.7 server is equal to that came out by TSSG prediction program , but differ from the results when Promotor 2.0 prediction server was used , so this server less reliable than others two. Combination of clinical evidences, and in silico analyses strongly recommended to increase our knowledge and to understand the effect deleterious SNPs and their role in the pathogenesis of the NO related diseases. Hence, these most deleterious SNPs may constitute distinct genetic markers that may be used as powerful mutation-screening in disease epidemiological studies.

V. CONCLUSION

Bioinformatics field is a powerful field nowadays worldwide and used in all fields of life sciences. Six SNPs in both (nNOS, iNOS) ,and seven SNPs in eNOS genes as damaging., these deleterious SNPs will affect the bioavailability of nitric oxide, and thus will contribute in the pathogenesis of human immune, neuro and cardiovascular diseases.

IV. RECOMMENDATION

More researches are warranted to detect the deleterious SNPs that come out from this study. x-ray crystallography and NMR must be done for other chain of the Three isoforms of human NOS , in order to be able to do 3d structure of them and to visualize the mutation that may occur on them

Disclosure statement.

The authors declare that, there is no conflict of interest regarding the publication of this paper.

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