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Impact of Tobacco on Proinflammatory IL1α Protein Expression in Smokers and Chewing Tobacco Users: A Novel Approach towards Protein-Ligand Incorporated Docking Analysis

Poulami Majumder

Department of Biotechnology, Maulana Abul Kalam Azad University of Technology, Kolkata 700 064, West Bengal, India
E-mail: plm89.majumder@gmail.com

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ABSTRACT Smoking and chewing tobacco cause acute and chronic inflammation in host cells. This inflammation is linked to the pro-inflammatory signal of interleukin 1 alpha (IL1α) protein. A total of 350 individuals including smokers chewing tobacco users and healthy controls were taken to investigate the genotypic association of IL1α -889C/T polymorphism. The major tobacco components were also screened for docking analysis between them and the subsequent IL1α protein. All statistical analysis were done by using SPSS v16.0. Docking analysis manifests patchdock analysis followed by ligplot analysis. The outcomes of the study revealed the significant association of IL1α -889C/T gene promoter polymorphism with both smokers and chewing tobacco users. In silico study analyze the detrimental tobacco components in respect of their docked profile which are responsible for elevated proinflammatory response in host cells. This study concludes the bad effect of tobacco in tobacco users at its genotypic level and as well as its molecular level.

INTRODUCTION

Tobacco consumption is a common phenomenon throughout the world. Tobacco is considered as one of the most important risk factors in development of chronic inflammatory diseases including chronic obstructive pulmonary disease (COPD), chronic periodontitis (CP), asthma, cardiovascular and oral diseases (Offenbacher 1996; Bernzweig et al. 1998; Pauletto et al. 2000; Mannino and Buist 2007; Hylkema et al. 2007; Furrukh 2013). The detrimental effect of tobacco may lead to develop cancer such as lung cancer, oral cancer (Wald et al. 1996). In a report published by WHO (2019) is that the mortality rate due tobacco consumption is exponentially increasing and it is assumed to be more than 5 million (www.who.int/news-room/fact-sheets/detail/tobacco). Most of the people consume tobacco through smoking cigarettes, cigar, pipes, *bidi*, *hookahs* etc. and through chewing tobacco, *gutkha, gurakkhu*, snuff etc. (Thun et al. 2013). The major components of tobacco are nicotine and nicotine derived derivatives such as nicotine derived nitrosamine ketone (NNK), nitrosonornicotine etc. (Kapp 2005). The toxic effects of these components of tobacco causing chronic inflammation on host tissues which cumulatively increase the risk of those earlier mentioned inflammatory diseases (Lee et al. 2012). Studies showed that the pro-inflammatory interleukin 1 alpha (IL1α) might contribute to inflammatory diseases (Seymour and Gemmell 2001; Sakellari et al. 2003). The pro-inflammatory regulation mediated by interleukin 1 alpha increases the inflammatory response. This IL1α secretion is intrigued by the activated macrophages such as neutrophils, endothelial cells, epithelial cells (Tettamanti et al. 2017). It plays a pivotal role in regulation of immune responses. The unregulated signaling of IL1α causes detrimental effects which may results acute or chronic inflammation. The interleukin 1 alpha protein expression is being directed by the IL1α gene. The variant in this particular gene might causes disruption in proper protein signaling at host cells (Hanada and Yoshimura 2002). The mutation in promoter region may distort the normal processes of gene activation by unregulated the level of mRNA and thus the protein of the same (Meisel et al. 2004). The exposure to the tobacco through smoking and chewing tobacco triggers the risk of inflammatory diseases. So, there must be an important relation is present between the tobacco consumption and IL1α protein expression (Dinarello 2017). Studies show the direct

connection between tobacco consumption and elevated interleukin level in different inflammatory diseases such as COPD, CP, asthma, lung cancer etc. (Chung 2001; Rawlinson et al. 2003; Mannino and Buist 2007; Struch et al. 2008; Mantovani et al. 2017; Majumder et al. 2018; Majumder et al. 2019a). A recent study showed the detrimental effect of tobacco on IL1α protein expression (Majumder et al. 2019b). The major components of tobacco such as nicotine and other nicotine derivatives are the prime regulators for disrupted protein regulation and enhance the risk of the earlier mentioned inflammatory diseases. Researches show that the chronic exposure towards tobacco as well as nicotine and its derivatives affect the release of IL1α (Wang et al. 2017). So, the tobacco exposure can be marked as a valid risk factor towards inflammatory diseases though the direct connection between them is still debatable.

The toxic compounds of tobacco act as ligands and bind to the protein residues pockets during the interaction. The binding characterization is important to predict the association between them. Studies investigates the etiology of inflammation triggered by oral exposure in many ways. To make in-depth idea on interaction between tobacco components and IL1aprotein residues, an insight is needed on their binding characterization. Molecular docking is one of the most important tools to predict the ligand-protein binding mode (Meng et al. 2011). This tool generates 3-D model of protein-ligand binding complex and showing the relative orientation of these two interacting partners. This firm complex may contribute in the signal transduction. In this study the researcher has used a novel approach to show the pro-inflammatory IL1α protein expression affected by the smoking and chewing tobacco as well.

Objective

- To analyze the association between the tobacco consumption through smoking and chewing tobacco and the IL1α -889C/T gene polymorphism.
- To get more insight into ligand-protein binding complex.
- To analyze the binding state of the complex and effect on IL1aprotein regulation.
- To investigate and discuss the possible impact of tobacco consumption in smokers and chewing tobacco users.

MATERIAL AND METHODS

This study was conducted in two ways, one was in vitro study and another was in silico study.

In Vitro Study

A total of 350 study subjects were included in this study. The inclusion and exclusion criteria were being made for the study subjects. The inclusion criteria for smokers was that the individual should have smoked ≥10 cigarettes/ *bidi*/ cigar etc. for last five years. The individual taking chewing tobacco such as *gutkha, gurakkhu* etc. for last five years were considered as chewing tobacco users (Page and Deck 1997). The individuals with other addictions like alcohol or drugs were excluded. The number of smokers were 137, chewing tobacco users were 153. A total of 213 was non-smoker, 197 was non-user of chewing tobacco. The age range of the study subjects were 21 to 60 years. This study was gender unbiased. Genotyping of IL1α -889C/T was done by PCR with the assistance of a pair of primers, that is, 5'TCTGGGCCTCAA GTG ATTGT3'and 5'ATGCCCAAGGTGTGTCTTCT3'. The PCR condition was: 1 cycle 95°C for 5 min; 30 cycles (95°C for 30 sec, 58°C for 45 sec, 72°C for 30 sec); final extension at 72°C for 7 min. This whole process was followed by sequencing by ABI Prism 3500 DNA genetic analyzer. Chi-square and logistic regression analysis were done to analyze the association between IL1a-889C/T polymorphisms smoker and chewing tobacco users. The statistical significant was set at 5 percent level of significance. All statistical analysis was done in SPSS v16.0.

In silico Study

In silico study involves computer aided modelling, docking and simulation. The X-ray crystallography structure of IL1α was obtained from protein database in PDB format (https://www.rcsb.org). The IL1α protein conformation was analyzed by Ramachandran plot in Procheck software tool (https://www.ebi.ac.uk/thornton-

srv/software/PROCHECK/). The Ramachandran plot statistics for IL1α protein conformation has been described in Table 1. The component of tobacco used in this study were nicotine, NNK, N-nitrosoanabasine, N-nitosodimethylamine and N-nitrosonornicotine. They were used as ligand compounds. The 3D model was designed in molecular network tool (https://www.mn-am.com) by using their SMILES (Simplified Molecular Input Line Entry Specification). All ligands undergo the screening to check the bioactivity with the targeted protein by using "Lipinski rule of five" (Lipinski et al. 2001; Lipinski 2004). The further processing was done in Molinspiration chemoinformatics software (Molinspiration Galaxy 3D Structure Generator v2016.01 beta) for ligand properties (http://www. molinspiration. com) and their properties were obeyed the "Lipinski rule of five" as all of the ligands possess less than 5 H-bond donors and less than 10 H-bond acceptors with molecular weight ranging from 74-207dalton. The coefficient log P range was 0.1-1.5 which is less than 5. The IL1α protein and the earlier mentioned ligands were docked by using patchdock in PDB format with default RMSD (root mean square deviation) value of 4.0. The patchdock analysis comprised of binding affinity energy, preferred interaction area, rotational and translational angles etc. The interaction was viewed using PyMOL v.1.7.4.5. (https://pymol.org/edu). The further analysis regarding the interaction, binding strength and possible effect of the binding complex is evaluated by PDBsum (www.ebi.ac.uk/pdbsum) and PLIP (http://plip.biotec.tu-dresden.de).

Table 1: Ramachandran Plot Statistics of IL1α protein conformation

Ramachandran plot statistics	
a. Residues in most favored region	74 (68.2%)
b. Residues in additional allowed region	34 (30.9%)
c. Residues in generously allowed region	1 (0.9%)
d. Residues in disallowed region	0
Number of non-glycine and non- proline residue	110 [a+b+c+d] (100%)
Number of end residues	2
Number of glycine residues	8
Number of proline residues	6
Total number of residues	126

*Data were collected from Procheck (https://www.ebi.ac.uk/thornton-srv/software/PROCHECK/)

RESULTS

IL1α-889C/T Genotypic Distribution

In Table 2 the genotypic distribution of IL1α -889C/T gene polymorphism has been discussed. 2X2 contingency Chi-square had been performed for this section and odds ratio, 95

Table 2: Genotypic distribution of IL1α-899C/T gene polymorphisms in both smoker and chewing tobacco (ChT) user group

IL1α-899C/T gene polymorphism	*Smoker N=137 (%)*	*Non-smoker N=213 (%)*	*OR (95% CI), P*	*AOR (95% CI), p*	*ChT user N=153*	*ChT non-user N=197*	*OR (95% CI),P*	*AOR (95% CI), p*
CC	31 (22.6)	102 (47.9)			44 (28.7)	110 (55.8)		
CT	72 (52.6)	85 (39.9)	2.79 (1.67-4.64)*, <0.0001*		60 (39.3)	73 (37.1)	2.05 (1.26-3.34), 0.003*	4.96
T T	34 (24.8)	26 (12.2)	4.3 (2.24-8.24) <0.001*	(5.62-24.24), <0.0001*	49 (32.0)	14 (7.1)	8.78 (4.39-17.43), <0.0001*	(2.67-9.2), <0.0001*

Abbreviations: OR= Odds ratio, CI= confidence interval, P= Fisher's exact test probability value, AOR=Odds Ratio
*Significant at 5% level of significance

percent confidence interval and Fischer exact tests (Chi square p values) were tabulated here. The frequency of CC genotype in smoker is 22.6 percent and in non-smoker is 47.9 percent. The frequency of CT in smoker is 52.6 percent that is higher than non-smoker (39.9%). The frequency of variant genotype TT in smoker is 24.8 percent which is double of the TT frequency in non-smoker (12.2%). The variant genotype is significantly positively associated in smoker group (OR= 4.3, 95%CI=2.24-8.24, p<0.0001). Like-wise smoker group, chewing tobacco users have significantly higher CT and TT genotypic distribution than chewing tobacco non user (OR=2.05, 95%CI= 1.26-3.34, p=0.003 and OR=8.75, 95%CI=4.39- 17.43, p<0.0001 respectively). Logistic regression analysis has been performed in both smoker and chewing tobacco user group. The adjusted odds ratio in smoker group is 11.67 (95%CI=5.62-24.24, p<0.0001) and in chewing tobacco user is 4.96 (95% CI=2.67-9.2, p<0.0001) after adjusting age and gender of the studied groups. All statistical analysis had been done at 5 percent level of significance.

Patchdock Analysis

In Figure 1 the docking orientation of the protein-ligand interaction has been featured in three-dimensional (3D) model. The mesh like structure indicates IL1α protein structure while stick like structures signify ligands of interest. In Table 3 the patchdock profile of IL1α-ligands interactions are tabulated. The highest complementary binding of IL1α protein is showing with nicotine is 3274 in accordance of the complementary score. This score is based on the geometric shape of protein-ligand complex. This complex is getting firm with strong H bonding and free energy. The interaction between IL1α-NNK is occupying the largest approximate interface area which is 388.7. The smallest area is occupied by IL1α-N-nitrosodimethylamine complex, that is, 200.5. ACE or the atomic contact energy predicts the strength of binding affinity of that particular ligand to IL1α protein. According to the pathchdock interaction profile the strongest binding affinity is formed by N-nitrosonornicotine with this protein, that is,

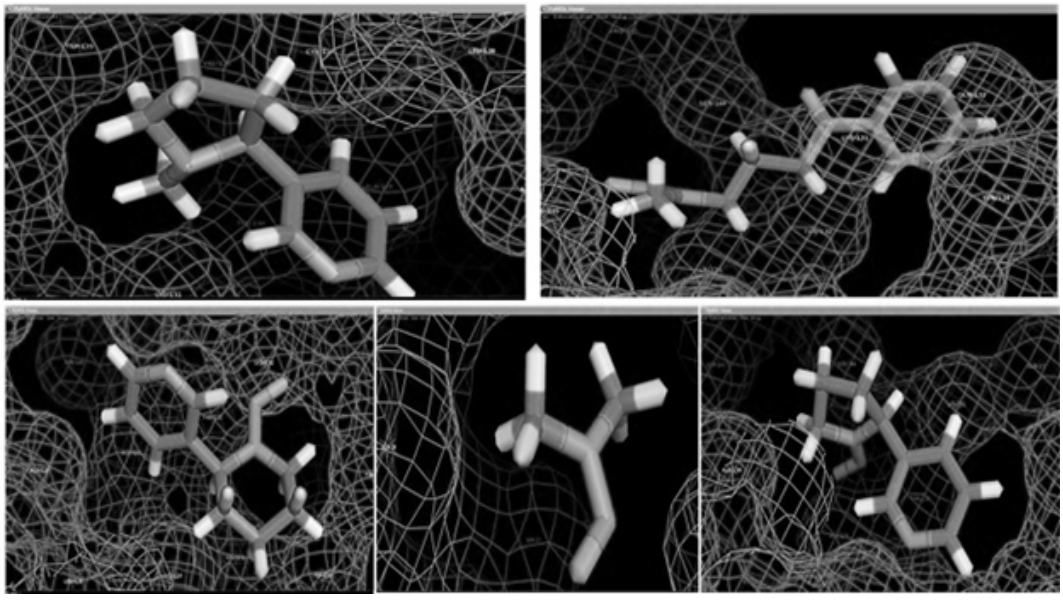

Fig. 1. Three-dimensional (3D) orientation of the docked complex; IL1α patchdock profile; the mesh like structure indicate IL1α protein structure and stick like structure signify ligands. (From left to right): IL1α-Nicotine, IL1α-NNK, IL1α-N-nitrosoanabasine, IL1α-N-nitrosodimethylamine and IL1α-N-nitrosonornicotine

Table 3: Patchdock profile of IL1α

Ligand	*Score*	*Area*	*ACE*	*Rotational parameter*	*Translational parameter*
Nicotine	3274	293.20	-68.64	1.48 -0.62 -2.63	6.62 80.65 31.03
NNK	3049	388.70	-68.10	-3.03 0.50 -2.44	7.19 81.13 27.69
N-nitrosoanabasine	2684	308.30	-70.76	1.04 -0.38 -2.74	6.94 81.15 30.07
N-nitrosodimethylamine	1812	200.50	-30.89	2.85-1.17 0.06	20.66 77.01 23.78
N-nitrosonornicotine	2554	280.00	-74.29	-0.41-0.46 -2.88	6.51 80.28 30.53

-74.29Kcal/mol. The lowest ACE generated in N-nitrosodimethylamine ligand (-30.89Kcal/mol). Though the other three ligands are almost showing similar strong binding energy (-68.64 Kcal/mol for nicotine, -68.10Kcal/mol for NNK, -70.76Kcal/mol for N-nitrosoanabasine). These ligands are binding to the studied protein at different residue pockets at different angles. Those angles are represented as rotational and translational angles during the compatible interaction of ligands with the protein.

Ligplot Analysis

The patchdock interaction analysis is being followed by the ligplot analysis (Fig. 2). This is an illustration of ligand binding site at different amino acid residue pockets in two-dimensional

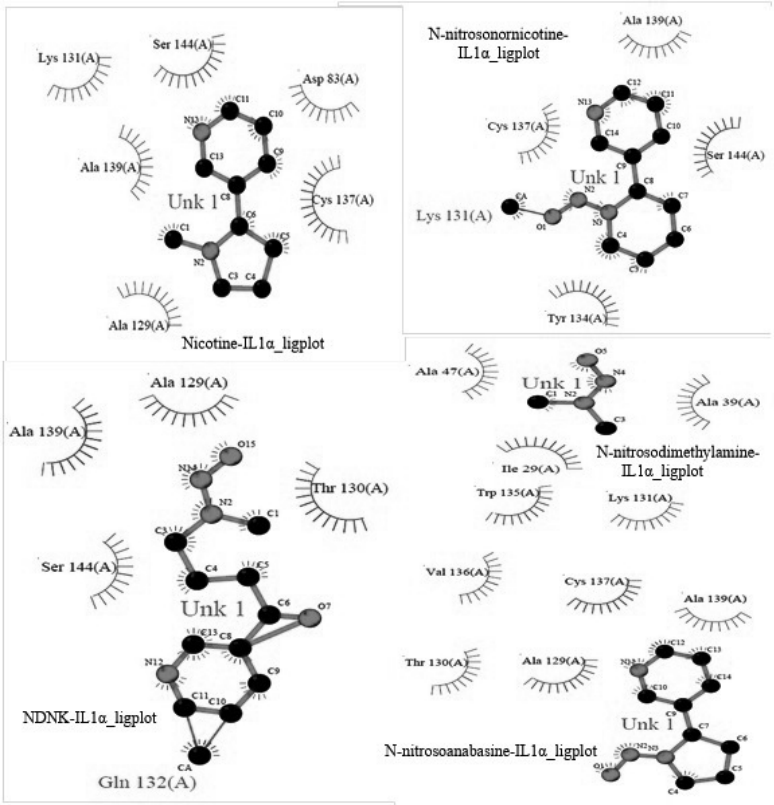

Fig. 2. Ligplot analysis of IL1α and ligands. The lines of the ligand are indicating hydrogen bonds while hydrophobic interactions are represented by an arc with small spokes radiating towards the interacting ligand atoms

(2D) orientation. The nature of binding is being mentioned in different textured lines and dots. The five studied ligands bind to various adjacent amino acid residues during the interaction. In Table 4 the interacting residues of IL1α are charted. Nicotine interact with IL1α at specific positions like lysine 131, serine 144, aspartic acid 83, cysteine 137, alanine 129 and 139. NNK interact with IL1α at the positions such as serine 144, alanine 129 and 139, threonine 130. The remaining ligands and their interacting amino acid residues are also described in the same way (see Table 4). These ligplots help to envisage the nature of protein regulation caused by those interactions.

Table 4: IL1α-ligands interacting amino acid residues

Ligands	*Interacting residues*
Nicotine	Lys131, Ser144, Asp83, Cys137, Ala129, Ala139
NNK	Ser144, Ala129, Ala139, Thr130
N-Nitrosoanabasine	Cys137, Ala129, Ala139, Lys131, Trp135, Val136, Thr130
N-nitrosodimethylamine	Ala 47, Ala39, Ile29
N-nitrosonornicotine	Ser144, Ala139, Cys137, Tyr134

DISCUSSION

Tobacco consumption is the most preventable cause of inflammatory diseases worldwide. About 80 percent of the total population are intaking tobacco through smoking cigarette, bidi, cigar and chewing tobacco for example, *gutkha, gurakkhu* etc. (www.who.int/news-room/fact-sheets/detail/tobacco). The mortality rate through tobacco consumption is rapidly increasing in India as well as other developing and underdeveloped nations (www.who.int/countries/). It may not directly cause death but can trigger the actions of associating of gene polymorphisms and related signaling anomalies of the linked protein. Such anomaly in the signaling process of the protein causes inflammation at the host cells. Thus, resulting into the increased severity of the inflammatory diseases leading to COPD, CP, lung-diseases and cancer.

In this study, the researcher found promising outcomes to validate the proposed objectives as follows:

- IL1α -889C/T gene polymorphism is significantly associated in both smoker and chewing tobacco user group.
- The docked interaction between nicotine and IL1α protein possess highest complementary score while NNK occupies the largest approximate interface area.
- N-nitrosonornicotine ligand binding affinity to IL1α protein is higher than the other four ligands.
- The most common amino acid residual pockets found in ligplot analysis are serine, alanine, lysine, cysteine.

The association between IL1α gene and tobacco is an important segment for the etiology behind inflammatory response. IL1α gene regulates IL1α protein expression and helps in immune response regulation by activating the tumor necrosis factor. Presence of IL1α -889C/T variant causes fourfold increased level of IL-1α protein and elevates the inflammatory signal (Faist et al. 1998). The researcher found a significant association IL1α gene polymorphism in smokers and chewing tobacco users. It infers that tobacco consumption is linked to IL1α -889C/T polymorphism and it is assumed to cause the inflammatory diseases. In a recent study a synergic interaction between smoking and patients with oral inflammation has been found including the association with IL1α -889C/T gene polymorphism. Their study concluded that the reduction of smoking as well as tobacco consumption decrease the severity of host cell inflammation (Chantarangsu et al. 2016). Because tobacco consumption induces the intracellular signaling of epithelial cells that lead to inflammatory gene activation that is, interleukin1α as well. Another approach of this study is in insilico mode, the other way to investigate the effect of tobacco on IL1α protein expression at its molecular level. Five major components (nicotine, NNK, N-nitrosoanabasine, N-nitrosodimethylamine, N-nitrosonornicotine) of tobacco are act as ligand towards IL1α protein. Before dock these ligands and protein, knowledge about protein conformation is needed. In Ramachandran plot statistics of IL1α protein shows 68.2 percent of amino acid residues are located in the most favorable region. 30.9 percent residues are in the additional favorable region. So, 99.1 percent residues are present in favored region while

there is no residue present in white colored region, the disallowed or unfavorable region. In Table 1 the Ramachandran plot statistics has been showed where the number residues with their location is clearly interpreted. After protein conformation analysis the docking between protein and ligands have been done. In Figure 2 the ligand-protein complexes are illustrated in three-dimensional orientation. The highest complementary score is showing by nicotine ligand (3274) and the least complementary score is 1812 for N-nitrosodimethylamine interaction with protein. It means nicotine is the best geometric fit to the protein with higher atomic desolvation energy (Nataraj et al. 2017). The contact area between interacting partners is a pivotal parameter which defines the thermodynamics and energy exchange between them and it also regulates the rotational and translational parameters for adequate ligand binding (Swanson et al. 2004). NNK interaction shows the largest contact area, that is, 388.7 which means NNK interaction with studied protein is the most impactful one. N-nitrosodimethylamine occupies the smallest contact area in comparison to other four ligands. that is, 200.5. The best complementary binding affinity and intensity of the interaction has been prompted as atomic contact energy (ACE) (Chang et al. 2007). The binding affinity of N-nitrosonornicotine ligand is higher than the others though the others also possess a firm binding affinity. According to the patchdock profile, four ligands cohabit the effective interaction with IL1ainflammatory expression in respect of binding affinity score except N-nitrosodimethylamine (-30.89Kcal/mol). It signifies all ligands except N-nitrosodimethylamine binding affinity towards the IL1α can be assumed to produce higher impact on inflammation activity of interleukin by triggering the intracellular signaling (Lu et al. 2009). During smoking, nicotine and NNK are more consumed while N-nitrosonornicotine is more consumed through chewing tobacco (Gohar and Benjamin 2006). Hence, it is easy to understand that the cigarette smoking and chewing tobacco both are equally harmful and impactful to proinflammatory activity of IL1α protein. After ligplot analysis the adjacent amino acid residues are found to be interacted with the IL1α protein. Ligplot study is mainly focused on the interactions mediated by hydrogen bonds and by hydrophobic bonds (Patil et al. 2010). In case of nicotine ligplot profile, hydrophobic bonds have been found with alanine 129 and 139, cysteine 137, aspartate 83. NNK also shares hydrophobic bond with serine144, alanine129 and 139, threonine130. N-nitrosoanabasine makes hydrophobic bonds with cysteine137, alanine139, tryptophan135 and threonine130 and hydrogen bond with lysine131 of IL1α protein. N-nitrosonornicotine ligand shares hydrophobic bond with tyrosine134. These above-mentioned hydrogen bond and hydrophobic bond between the ligand and amino acid helps to stabilize the ligand with protein unit. This stabilized binding complex structure can easily alter the binding affinity and the mode of protein expression as well. The most common interactive amino acid residues which are found in almost all ligands interactions are serine, alanine129 and139, serine144, cysteine137. So, it can be assumed as the common interactive amino acid residues are more active site of the IL1α protein complex which play the pivotal role in altering the inflammatory response. Then it built an elevated level of inflammation as well as immune response in host tissue leads to different severe inflammatory diseases. N-nitrosodimethylamine is a very small ligand that's why the scope of interaction is very less in regards of patchdock and ligplot profile. Though the docking study faces difficulties as the molecular structures in software are not exact, containing experimental errors. In addition, molecules usually undergo conformational changes upon association, known as induced ût. Docking algorithms must be tolerant to those diûculties, making the docking task one of the most challenging problems in structural bioinformatics (Galal and Hessel 2018).

Henceforth, the cumulative analysis of this study reveals the significant association of tobacco consumption with *IL1α* gene polymorphisms in smoker and chewing tobacco user population. In addition, the molecular docking study reveals that interleukin protein regulation got disturbed or can be affected by the exposure of tobacco and its similar compounds, and if interleukin, the proinflammatory protein regulations got interrupted, severe inflammation may occur that leads the increasing risk of inflammatory diseases such as, COPD. This interruption

mostly leads by the binding of nicotine, NNK, N-nitrosoanabasine and N-nitrosonornicotine with IL1α protein residue.

CONCLUSION

This study reveals that significant association of IL1α -889C/T polymorphism in smoker and chewing tobacco user groups. This study is also showing the impact of nicotine and its derivatives on IL1α protein regulation in regards of their binding affinity, intensity and compatibility with the protein. In accordance of in-silico study, Nicotine, NNK, N-nitrosornicotine are considered to be the most impactful ligand consumed through tobacco may have most effective and influencing towards elevated pro-inflammatory response of IL1α protein.

RECOMMENDATIONS

This study recommends the analysis of the protein expression of IL1α in smokers and chewing tobacco users. Further study is needed in a comparatively large population for better understanding in this regard.

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ABBREVIATIONS

A: Adenine
C: Cytosine
G: Guanine
T: Thymine
CP: Chronic Periodontitis
COPD: Chronic Obstructive Pulmonary Disorder
DNA: Deoxyribonucleic Acid
IL1α: Interleukin 1 Alpha
NNK: Nicotine Derived Nitrosamine Ketone
PCR: Polymerase Chain Reaction
SPSS: Statistical Package for the Social Sciences

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