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Identification of Prognostic Genes Associated with Asthma in Pakistan

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ABSTRACT

Asthma is a severe bronchial inflammatory disorder with high prevalence. In the current study, a list of significantly asthma-associated genes was created to identify asthma-associated pathways and ranking of genes based on protein interactions and centrality-lethality hypothesis representing that knockdown of influential node and edge leads toward the development of the disease. This ranking allows identification of the influential protein for the targeted drug discovery and therapies. An extensive study of published articles was conducted to enlist asthma-associated genes reported significant (P-value < 0.05) in the Pakistani population. Pathway Connector (http://bioinformatics.cing.ac.cy/PathwayConnector) was used to analyze the pathways associated with genes as well as novel pathways through complimentary network analysis. Protein-protein interaction studies were conducted using String (https://string-db.org/) and genes were ranked on a centrality score basis using Cytoscape (https://cytoscape.org/index.html). *IL4, IL13, IL10, IL27, ADAM33, TBXA2R, FCER1β, ORMDL3, IKZF3, LRRC3C, GSDMA, GSTP1, GC, STAT6, CD14* and *ACE* are asthma-associated genes, significantly reported in the Pakistani population. Pathway-connector provided a network of forty-six pathways and their complimentary-analysis provided thirteen new pathways including (hsa04658) Th1/Th2 and (hsa04659) Th17 cell differentiation pathways having *STAT6, IL4,* and *IL13* genes having contribution in asthma. A network of appropriately matched proteins was provided by the string that was further utilized for centrality-measurement which ranked the thirteen most persuasive genes from top to bottom as *IL10, IL4, ORMDL3, IL13, MS4A2, ADAM33, STAT6, GSDMA, IKZF3, LRRC3C, IL27, CD14,* and *ACE*. This study enlightens the significantly associated asthma genes in the Pakistani population, figures out influential asthma linked pathways, and ranking of influential genes predicting their role in targeted drug discovery and therapies.

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FN, MUG, QUA and QAZ performed the all analyses. MUG, FN and MFS designed the study. FN prepared the first draft of the article. QUA and QAZ reviewed the article. MUG and MFS critically analyzed the write-up and finalized it for submission.

Key words
Asthma, Bronchial inflammation, Centrality analysis, Drug discovery, Therapeutics

INTRODUCTION

Asthma is a severe obstruction of airway passage and chronic inflammatory disorder, characterized by intense infiltration of lymphocytes and eosinophil, mucus hyper-production, and bronchial hyper-responsiveness (Meng and Rosenwasser, 2010). Symptomatically, asthma is recognized by wheezing, cough, and imbalanced breathing that range from mild to severe conditions that prove fatal for the patients. Asthma-associated phenotypes and clinical expression is regulated by the gene-environment interaction (Toskala and Kennedy, 2015). Improved management and assessment of asthma may reduce the prevalence rate but clinically no cure is available yet (Beasley *et al*., 2015).

It's a challenging task to discern expanding and multisource information related to complex disorders like asthma. Multiple tools of system-bioinformatics contributed to the synergistic study of multifactorial entities by exploiting information from different fields and supplying data relevant to the drug discovery by specifically targeting the biologically important components (Oulas *et al*., 2019).

Proteins are the crucial biological entities, protein-protein, and protein-environment interactions, which greatly impact biological studies. These interactions play a significant role in providing a better understanding of biological processes and help in analyzing their clinical and therapeutic role in complex disorders (Nibbe *et al*., 2011; Szklarczyk *et al*., 2018). Biological network studies emphasized the identification of the biologically significant nodes and edges in the biological networks.

Centrality analysis is used as an effective tool

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for identifying significant elements of the network (Mallik, 2018). Centrality-analysis includes different centrality measurements to rank the biological entities. In this study, the focused measurements include degree centrality, closeness centrality, and betweenness centrality. Degree-centrality focuses on the significant nodes that have interaction with different nodes. Degree centrality follows the Centrality Lethality hypothesis that focuses on the significance of the particular node in a biological network. Knocking down the nodes with high degree-centrality depicts the lethal effect and ultimately plays a major role in the disease susceptibility (Jeong *et al*., 2001). Betweenness-centrality calculates the shortest path between the nodes. The shortest path provides an understanding of the flow of information between nodes. Closeness-centrality measures the shortness of the shortest paths for a particular node from other nodes (Malik, 2018).

In the present study, genes significantly associated with asthma specifically reported in the Pakistani population were selected from literature and subjected to the pathway as well as protein interaction studies. Asthma-associated proteins were ranked based on centrality score to identify therapeutic targets. These studies will help researchers to design future research related to therapeutics by identifying influential genes.

MATERIALS AND METHODS

Selection of the genes

Genes that were significantly associated with asthma in the Pakistani population, were selected by extensive study of literature reported in the Pakistani population through Google Scholar, PubMed, Science Direct, BioMed Central, and other web sources related to the subject. The P-value <0.05 was significant selection criteria for the genes.

Enrichment analysis

Pathway-based study of asthma-associated genes allows a comprehensive and better understanding of molecular mechanisms linked to complex disorders. Pathway Connector, an online web source, was used to find the pathways associated with selected genes (Minadakis *et al*., 2018). Complimentary networks were created from the enrichment results by selecting KEGG (Kanehisa and Goto, 2000) as the default browser. The complimentary network was constructed by selecting the top-10 enriched pathway and edge-betweenness algorithm along with assigning weight to edges.

Protein-protein interaction study

In this analysis, our selected genes were converted into appropriately matched proteins and protein-protein interactions between the selected genes set were analyzed using STRING (Szklarczyk *et al*., 2018). All the source interactive channels were selected for data retrieval including Textmining (scientific abstract extracts), Experiment (lab experiment based), Database (from organized databases), Co-expression (gene's expression experimentation and correlation), Neighborhood (identification of repetitive genes nearby), Gene-fusion (fusion score obtained per species), and Co-occurrence (depicts the protein data phylogenetically across species) (Gazouli *et al*., 2019; Szklarczyk *et al*., 2018). These interactive sources were used to construct interactions using an interaction score of 0.400 without increasing the interaction shells and applying the display specification of hiding disconnected nodes.

Centrality based ranking

Protein interactions obtained from string analysis provide the score from one protein node to the other node. To find the significant protein nodes, protein interactions were subjected to CYTOSCAPE, freely available software for systems (Shannon *et al*., 2003). Degree, betweenness, and closeness centralities were considered to identify significant proteins. Selected centralities were assigned a weighted function approach based on the literature study; as 0.2 factor for the degree-centrality, 0.3 factor to closeness-centrality, and 0.5 factor to the betweenness-centrality, to signify the protein networks (Gazouli *et al*., 2019).

RESULTS AND DISCUSSION

Gene related the asthma

Table I enlists 16 genes that are significantly associated with asthma in Pakistan. As asthma is a polygenic condition, this study focused on genes related to asthma. This study includes *FCER1, ORMDL3, ADAM33, TBXA2R, GSTP1, GC, STAT6, ACE, CD14, IKZF3, LRRC3C, GSDMA, IL4, IL13, IL10,* and *IL27* genes with P-values (0.05). We identified *IL-10, IL-4, IL-13,* and *IL-27* to be strongly linked to asthma. Micheal *et al*. (2011,2013) found a strong connection between IL-4 and IL-13 and atopic and non-atopic asthma in Pakistan. *IL-13* also has a role in increased mucus production, inflammation, and asthma (Micheal *et al*., 2013). Saba *et al* (2013) identified IL-10 as a key interleukin for T-cell immunity modulation. Reduced *IL-10* expression has been linked to asthma exacerbation (Saba *et al*., 2013, 2017). The T-allele of variations of IL-27 revealed a substantial correlation with COPD (Shahid *et al*., 2019). One of the main susceptibility genes for asthma, defined by loss in lung function, bronchial hyperresponsiveness, and airway remodeling, is ADM33 (Davies *et al*., 2016).

Table I. Sixteen significantly asthma associated genes selected from review of literature.

No	Gene ID	Gene position on chromosome	SNP ID	Functional consequence	Asthma phenotype	Genotype/ allele	Or (95%CI)	P-value	Association in Pakistani population
1	FCER1B	11q12.1	rs2583476	Intron variant	Asthma	TT	1.99 (1.02–3.89	0.033	Significant (Saba *et al*., 2018), (gender based, highly associated in males)
2	ORMDL3	17q21.1	rs11650680	Intergenic variant	Asthma	CT	1.86 (1.09–3.17)	0.01	Significant (Saba *et al*., 2018) (gender based, highly associated in females)
			rs12603332	Intron variant	Urban Asthma	C allele	0.482	0.0046	Significant (Saba *et al*., 2013)
								0.026 Association with male gender	
3	ADAM33	20p13	rs2280091	Missense variant	Asthma	G allele	0.69, (0.50–0.97)	0.03	'G allele' protective factor (Saba *et al*., 2013, 2018)
						G allele	0.837 (0.624-1.122)	0.234	Associated with asthma (Ghani *et al*., 2019)
			rs2787094	Non-coding transcript variant	Asthma	CC, C allele	2.08 (1.45-2.99)	<0.0001	Significant (Raja-Kaukab *et al*., 2014)
			rs528557	Synonymous variant	Asthma		1.439 (0.945 - 2.192)	0.0189	Significant (Sabar *et al*., 2016)
4	TBXA2R	19p13.3	rs1131882	Synonymous variant	Asthma	A-allele	0.73, (0.52–1.01)	0.05	'A allele' protective factor (Saba *et al*., 2013, 2018)
5	GSTP1 enzyme	11q13.2	rs1695	Missense variant	Asthma	AA(Ile/Ile)	2.27 (1.339 - 3.854)	0.003	Significant (Al-Arifa Jahan, 2016), Genetic polymorphism study
6	GC (vitamin D-binding protein)	4q13.3	rs4588	Missense variant	Asthma	GC1S/GC1S 0.52	(0.203- 1.335)	0.250	Less frequent with asthma more with control (Al-Arifa Jahan, 2016)
			rs7041	Missense variant	Asthma	GC2/GC2	3.14 (1.78- 5.535)	<0.001	Significant (Al-Arifa and Jahan, 2016), Genetic polymorphism studies
7	STAT6	12q13.3	rs4559	Non-coding transcript variant	non-atopic asthma	T/T		0.029	Significant
			rs324011	Intron variant	non-atopic asthma	G/G		0.00077	Significant
8.	Angiotensin I-converting enzyme (ACE)	17q23.3	rs4646994		Asthma	II	3.38 (2.35–4.84)	<0.0001	"I allele" significantly associated with asthma (Saba *et al*., 2016) polymorphism study
						I/D	0.43 (0.33–0.57)	≤0.0001	"D allele" Less significant, protective effect is more common (Saba *et al*., 2016) polymorphism study

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No	Gene ID	Gene position on chromosome	SNP ID	Functional consequence	Asthma phenotype	Genotype/ allele	Or (95%CI)	P-value	Association in Pakistani population
9.	CD14	5q31.3	rs2569190	Intron variant	Allergic rhinitis	CT (C159T), T allele 0.50 (0.28-0.87)	0.01		Significant (Micheal *et al*., 2011)
			rs2569191	Upstream variant	Atopic asthma	GG (A1145G), G allele	0.53 (0.29-0.98	0.02	Significant (Micheal *et al*., 2011)
10.	IL-4	5q31.1	rs2243250	Upstream variant	Atopic asthma	TT	1.36 (0.92-2.01)	0.04	significant (Micheal *et al*., 2013)
			rs2227284	Intron variant	Atopic asthma	GG	2.21 (1.46-3.37)	<.001	significant (Micheal *et al*., 2013)
11.	IL13	5q31.1	rs1881457	Intron variant	Atopic asthma	C allele	2.40 (1.41-4.09)	0.01	Significant (Shazia *et al*., 2013)
			rs1800925	Intron variant	Asthma	T allele	1.45 (1.04–2.02)	0.03	Significant (Ghani *et al*., 2017)
12.	*IL10*	1q32.1	rs1800896	Upstream variant	Asthma	G allele	1.38 (1.01–1.88)	0.04	Significant (Saba *et al*., 2013)
13.	IKZF3	17q12-q21.1	rs3816470	Intron variant	Asthma	G allele	3.082 (1.755-5.41)	8.89x10^-5	Significant (Shahid *et al*., 2015)
14.	LRRC3C	17q21.1	rs6503525	Intron variant	Asthma	C allele	1.43 (0.918-2.226)	Dominant model 0.03114 Family history 0.01877	Significant for asthma in family history (Shahid *et al*., 2015)
15.	GSDMA	17q21.1	rs3859192	Intron variant	Asthma	C allele	2.705 (1.297-5.64)	0.03987	Significant for asthma in family history (Shahid *et al*., 2015)
16.	IL27	16p13	rs153109	Intron variant	COPD				Significant (Shahid *et al*., 2019)

STAT6 is linked to lung function and regulates Th2-immune response through IL4 and IL13 pathways (Micheal *et al*., 2013). STAT6 mutations are linked to non-atopic asthma in Pakistani women. ORMDL3 was linked to childhood asthma, whereas TBXA2R was linked to chronic airway inflammation in multiple genetic investigations (Saba *et al*., 2017). The TT-genotype of FCER1B rs2583476, the CT-genotype of rs11650680, and the C-allele of ORMDL3 rs12603332 (chr.17q21) have recently been linked to asthma in Pakistani women (Shahid *et al*., 2019). The promoter polymorphism of CD14 has been associated with atopic asthma in adult Pakistanis and has been demonstrated to regulate IgE levels (Ono *et al*., 2013). In Pakistan, Shahid *et al*. (2015) found a 17q21 gene connection with asthma. The G-allele of IKZF3 rs3816470 was linked to asthma in the study population. The C-allele of GSDMA polymorphism (chr17q21) variation rs3859192 has been linked to asthma. LRRC3C was shown to be relevant in asthma with a family history in variation rs6503525. Aside from these genes, polymorphisms in GSTP1, group-specific component (GC), and angiotensin I-converting enzyme (ACE) genes have been associated with asthma (Al-Arifa and Jahan, 2016; Saba *et al*., 2016).

Enrichment analysis

Figure 1 shows the direct network connection between the pathways associated with selected genes. Enrichment analysis results were used to construct a complementary network by providing top-10 enriched pathways as input (Table II). Complimentary network depicts new pathways Th1 and Th2 cell differentiation (hsa04658) and Th17-cell differentiation (hsa04659) (having common genes *STAT6, IL4,* and *IL13*) associated with the selected genes by retrieving data from defaulted KEGG database.

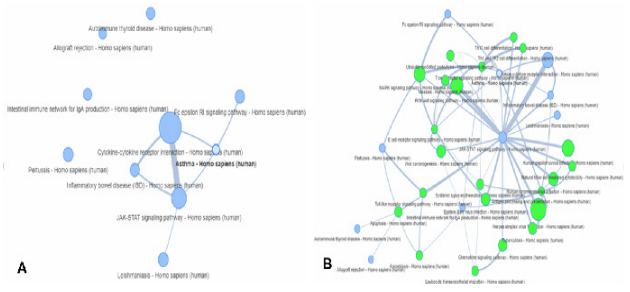

Fig. 1. (A) Direct interaction between pathways linked with input gene list; (B) Complimentary pathway network.

Enrichment analysis provided numerous pathways based on the genes putative functional involvement by scoring the pathways. However, as indicated in Table II, asthma is the top enrichment. The top ten pathways from the enrichment analysis were chosen for more research on asthma (Saba *et al*., 2018). Pathways including MAPK signaling, ubiquitin-mediated proteolysis, T-cell receptor pathways, Epstein-Bar virus infection, and viral carcinogenesis were introduced. The complementary route network has shown interaction with asthma (Fig. 1). Inflammatory reactions and immunological regulator T-cell differentiation are regulated by these pathways, and their alteration may help find effective therapies (Huang *et al*., 2017).

The research indicated Th1 and Th2 differentiation as well as Th17 differentiation. Included in these pathways are our chosen gene list genes IL-13, IL-4, and STAT6. Because sequence variations influence the original functioning of genes, abnormalities due to sequence pathogenic variants affect the original functionality of genes (Cosmi *et al*., 2011). By beginning IgE class switching, Th2-cells have been shown to have an inflammatory function in asthma (Vroman *et al*., 2015). Th17 cells generate IL17A, which stimulates chemokines and neutrophils, causing airway inflammation. It is also implicated in asthmatic airway remodeling by Th17 cells (Cosmi *et al*., 2011; Newcomb and Peebles, 2013). Th17-cells are resistant to corticosteroid therapy; therefore, these pathways might be studied therapeutically.

Protein-protein interaction

The protein-protein interaction network was acquired from the STRING analysis. It provides a network that shows the interaction between the proteins associated with genes (Fig. 2).

Proteins are ranked according to their centrality score from highest toward lowest. Table III represents the combined score obtained from the sum of degree, betweenness and closeness centralities.

Protein-protein interactions studies emphasize the disease-associated genes, proteins, protein networks, and sub-networks (Sevimoglu and Arga, 2014). The protein-protein interaction studies help us find important proteins and their interactions with other proteins in the pathway network (Fig. 2).

The Centrality-Lethality theory states the relevance of the nodes and edges involved in networks. It means that destroying a node or edge causes sickness (Jeong *et al*., 2001; Mallik, 2018). Protein centrality ranking revealed important nodes. They ranked well among our protein list based on degree, betweenness, and closeness centralities.

IL10, the most important protein (1.716616), maybe a better therapeutic target soon. IL10 is a cytokine generated by Th2 cells that are linked to inflammatory and allergy reactions (Hawrylowicz and O'Garra, 2005).

Table II. Top 10 selected enriched pathways.

Pathway ID	Description	Combined score
hsa05310	Asthma - *Homo sapiens*	1596.004
hsa05321	Inflammatory bowel disease (IBD) - *Homo sapiens*	1194.842
hsa05330	Allograft rejection - *Homo sapiens*	512.3525
hsa04672	Intestinal immune network for IgA production - *Homo sapiens*	381.2278
hsa04630	Jak-STAT signaling pathway - *Homo sapiens*	378.7684
hsa05320	Autoimmune thyroid disease - *Homo sapiens*	335.9301
hsa04664	Fc epsilon RI signaling pathway - *Homo sapiens*	243.6045
hsa05140	Leishmaniasis - *Homo sapiens*	222.1044
hsa04614	Renin-angiotensin system - *Homo sapiens*	217.590029
hsa05133	Pertussis - *Homo sapiens*	214.39842

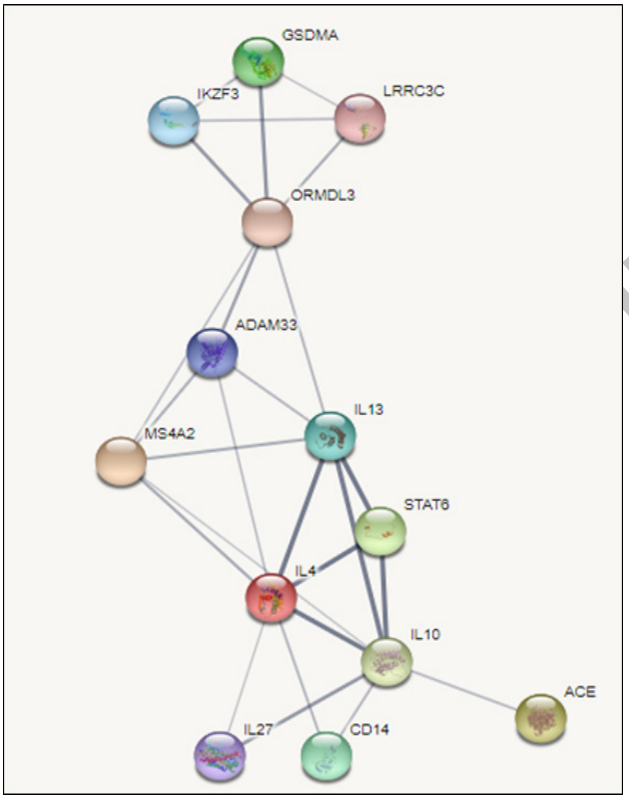

Fig. 2. Protein-protein interactions among the best matched proteins of the genes.

Previously, investigations on animal models found therapeutic targets of IL10 to treat allergic diseases (Ichinose and Barnes, 2004). In humans, IL10 regulates T lymphocytes through antigen-presenting cells (APCs). These data suggest that Th2 cell-mediated treatments might be an IL10 therapeutic target and help cure airway inflammation (Coomes *et al*., 2016).

Table III. List of ranked genes their combined centrality scores.

Gene		Combined score
1.	*IL10*	1.716616
2.	*IL4*	1.669394
3.	*ORMDL3*	1.575974
4.	*IL13*	1.506313
5.	*MS4A2 (FCER1B)*	1.259171
6.	*ADAM33*	0.993651
7.	*STAT6*	0.75
8.	*GSDMA*	0.72
9.	*IKZF3*	0.72
10.	*LRRC3C*	0.72
11.	*IL27*	0.524138
12.	*CD14*	0.524138
13.	*ACE*	0.316129

IL4 regulates IgE production by B-cells and Th-cell differentiation. Previously, medicines targeting IL4R, such as Dupilumab, have shown promising results in Phase-III studies. The combination or sequential therapy targeting IL4 and IL13 for improvement in asthma may be effective for the Pakistani population.

ORMDL3 of chr.17q21 is a therapeutic target for bronchial reactivity. This asthma-prone gene has been linked to sphingolipid production, ER Ca2+ signaling, and unfolded protein activation. This ER regulation method required inflammation. These regulatory points are currently being studied and may have therapeutic value (Ono *et al*., 2013).

The IL13 was ranked as a significant node in earlier research on asthma genetics in diverse populations

(Ichinose and Barnes, 2004; Rael and Lockey, 2011). Brikizumab and tralokinumab were powerful interleukin-directed medications in clinical studies, although their role varied by community. Since IL4 and IL13 are connected, pharmacological ineffectiveness may be owing to their dependency, which requires more study (Dunn and Wechsler, 2015; Ichinose and Barnes, 2004). The MS4A2 -chain (FCER1) has been linked to the release of proinflammatory cytokines. FCER1 is being studied therapeutically in mice to treat asthma (Pavón-Romero *et al*., 2018).

ADAM33 has been shown to promote the proliferation of airway/bronchial smooth muscles through regulating VEGF (Pei *et al*., 2016). This smooth muscle proliferation causes airway remodeling, which causes Th2-inflammation and asthma (Davies *et al*., 2016). This VEGF regulatory point may be a useful asthma therapy target (Pei *et al*., 2016). STAT6 regulates JAK/STAT pathways triggered by IL4 and IL13. STAT6 is involved in IgE manufacturing and eventually Th2-cell inflammations, which is important in asthma development in animal models. Understanding STAT6's function in asthma etiology may lead to new treatments for severe asthmatics (Antczak *et al*., 2016).

Ranking studies include GSDMA, IKZF3, and LRRC3C on 17q21. The therapeutic and diagnostic function of 17q21 haploblock variations needs more study due to their variable involvement in pathogenic cell types. Identifying harmful cell types and disease-associated pathways may be an important therapeutic target (Schmiedel *et al*., 2016). IL27 is another interleukin that has been studied therapeutically. The TLR7/8 agonist resiquimod (R848) has been shown to suppress IL27 in asthma and additional research may help uncover new treatment targets (Jirmo *et al*., 2016).

Previous research linked CD14 to atopic illnesses by influencing T-cell maturation and hence IgE levels. The therapeutic function of CD14 in atopic diseases is largely unknown (Agarwal *et al*., 2016). The involvement of the Renin-angiotensin system makes ACE phenotyping a therapeutic target (RAS). ACE inhibitors were used to treat asthma in several recent research, although this medication is now under question owing to its greater dosage required. RAS research may help identify treatment targets for lung illnesses (Tan *et al*., 2018).

CONCLUSION

The study's main goal was to illustrate the prospects of focused medication development. Having no prior research on the ranking of asthma-related proteins in the Pakistani population, it revealed the important gene candidates for asthma therapy. Recent research shows that interleukins are the most important asthma therapeutic targets in Pakistan. This study may help doctors to determine target precise targets for asthma therapy.

Statement of conflict of interest

The authors have declared no conflict of interest.

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