Network and pathway enrichment analysis of Attention Deficit/Hyperactivity Disorder candidate genes

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ABSTRACT

Background: Attention deficit/hyperactivity disorder (ADHD) is a well-known multigenic neurodevelopment disorder. It is a psychiatric disease which mainly affects the children and adolescence. Globally, 3%–5% of children are suffering from this mental disorder.

Aims and Objectives: This disease is characterized by hyperactivity, impulsiveness and inattentiveness. Suffering individuals are also observed with sleep related problems. Though, its polygenic, to study the complexity of these genes, we used a purely network approach. Firstly, we collected all the candidate genes involved in ADHD through a literature survey.

Materials and Methods: We investigated these genes using STRING 10 and Cytoscape v 3.3.0 for protein protein interaction network. Accordingly, we attempted to identify the hub genes based on definite parameters like betweenness centrality, clustering coefficient and node degree using Network analyzer. Likewise, the key transcriptional regulators were acknowledged by means of MatInspector program. Finally, the enrichment analysis was executed using ClueGO.

Results: As a result, dopamine receptor D2, brain derived neurotrophic factor, HTRF1A, and dopamine receptor D4 were recognized as hub genes among the reported ADHD genes. While, 17 transcription factors (TFs) were conveyed as the key TFs for these hub genes.

Conclusion: Functional enrichment analysis revealed regulation of dopamine and behavioral fear response pathways. These pathways have been assumed to play a central role in the ADHD within the selected candidate genes.

Key words: Attention-deficit/hyperactivity disorder, candidate genes, functional enrichment, protein-protein interaction, transcription factors

INTRODUCTION

Attention-deficit/hyperactivity disorder (ADHD) is the most common psychiatric disorder in children, whereas it is chronic and undiagnosed in adults. Adult ADHD is recently payed attention by the scientists as before it...
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was not included as the serious disorder. Basically ADHD is a neurodevelopmental disorder with high prevalence in children especially in US.[4] About 3%–5% in children worldwide are affected, with a high male-to-female ratio.[5] ADHD is a childhood onset disorder as has a drastic effect on the child’s growth, functioning, productivity and quality of life ahead.[6-7] There are some features which are observed in the suffering patients, i.e., impairing inattention, frequently inattentive and careless towards works and can be distracted by external stimuli very easily.[8,9] Hyperactivity, usually have restless feeling and act as driven by motor, impulsiveness, they can’t wait or burst out.[8,9] Diagnostic and Statistical Manual of Mental Disorders-IV states that, diagnosis of ADHD can be done on the basis of two different symptoms or either by both, inattentiveness and hyperactivity or impulsivity.[10] Scientist have studied this disorder in a different research field of neuropsychology, neurophysiology, and neuroimaging to understand the deep aspect of the disease relative to the inattentiveness.[10]

The neuropsychological studies were done on animal model to recognize the behavioral response or cognitive response of affected individuals such as controlling, arousal and activation.[11,12] While other model suggests that attention is constituted of three fundamentals i.e., automatic orienting, voluntary orienting and vigilance or alerting.[13,14] Now a day’s theories start, including the conclusion of intraindividual variability (IVI) in individuals with ADHD[15,16] which includes the following tests such as attention classrooms,[17] speed, variability, and timing output.[18] The results of the test show that the affected ADHD people had low IVI in reaction time when any reward is considered.[19]

Other then neuropsychological studies, many researches were also done on the animal model. This work explains about the connection between dopamine release in various part of the brain, such as the prefrontal cortex, nucleus accumbens and striatum with the behavior of ADHD.[19] Researcher paid attention towards the dysfunction of the locus coeruleus-norepinephrine system.[20] Many experiments were performed such as electroencephalogram (EEG), it was used to measure the electrical impulses of the brain externally.[21] It showed the abnormal activity of brain networks, which is somehow related to the ADHD.[22] Through this experiment they calculated alpha and delta waves.[23] Alpha waves are involved in brain processing, whereas delta waves are responsible for brain maturation and attention task as well.[22,24] Increase theta waves results in decreased alpha waves.[25] Other experiments were done by magnetoencephalography which clear outs the spatial and temporal of brain activity.[26]

There is a great contribution of neuroimaging studies in understanding inattentiveness character of ADHD patients. Magnetic resonance imaging (MRI) mainly focuses on the brain structure, diffusion tensor imaging (DTI) and functional MRI (FMRI), where structure shows the anatomical composition of the brain, which is followed by the study of disparity in white matter trace through DTI and thermodynamic activation by FMRI.[10] These findings suggest that prefrontal region is responsible for the attention or if get damaged can produce distractibility and poor attentiveness.[27,28] One more factor is involved in the process, i.e., cortico-striato-thalmo-cortical loop, which is a part of thalamus have great impact on ADHD children as this loop regulate the cognitive and emotional activities of brain.[29] FMRI studies have revealed many unidentified areas which are involved in attention related problems. In the recent work of FMRI it describes about the connectivity and networking of the thalamus in the hemisphere. Which directly indicates that failure in a mode of networking in the brain can produce a great defect in the functioning of the thalamus as it responsible in transporting sensory information.[29]

ADHD not only affects the stimuli problems, but it has an adverse effect on sleep also. Many people who are suffering from ADHD are followed by a sleep disorder such as Narcolepsy, Rhythmic Movement Disorder, Delayed Sleep Phase Syndrome, and Obstructive Sleep Apnea.[30] Studies say that about 18.9% of ADHD affected people are suffering from Narcolepsy. These individuals have drowsiness and have a loss of skin shade during emotional challenges.[31] Due to drowsiness automatically there is a loss of attention. While Rhythmic movement is related to the movement of the body like head or body banging before the sleep.[30] Delayed sleep phase syndrome is mainly observed in adolescence and the onset can be seen as insomnia.[32]

Though, ADHD is considered as heritable, despite of this some other finding tries to investigate the other genetic causes of disorder by focusing on the neurodevelopment profile.[33,34] Recent studies are going on to search out the relation between the copy number variations (CNVs) and ADHD, i.e., how CNVs is linked with this disorder after all it is a neurodevelopment misbalance disorder.[35] CNVs refer to large and rare chromosomal excised and duplication[36] which can prove to be a major cause of genetic variation which is somehow related to the neurological defects.[36,37] The limited population of UK with affected individuals was examined for CNVs in which various single nucleotide polymorphisms (SNPs) were genotyped and judge against the control one.[35] According to researcher large number of CNVs was found in the people who were suffering from intellectual related problems.[35] Whereas, no precise chromosome was involved in such cases[37] i.e., the experiment couldn’t identify the proper area of aberration involved and sometimes even it does not relate with the disorder.[35,37] Along with it, many evidences show that CNVs is greatly responsible for neurodevelopment diseases such as schizophrenia, and autism.[36,38]
Till now, we discussed about the ADHD disease itself, but the genetic basis of the disease is vaster because this disorder is considered as polygenic in nature. It is needed to understand this multiple gene associated with the disorder in more deep to fight against the disease. Therefore, studies were done to find out the ADHD genes which are explained by various previous reports. The study clarifies about responsible gene and its association. Other information was given by the core data which explains about the SNPs, pathway analysis. Furthermore the genetic factors (transcription factor [TF]) CNV, Variable number tandem repeat (VNTR), and microsatellite, and pathways involved. Large numbers of powerful bioinformatics tools are available to find out the involved genes and how it affects the various biological pathways. We aimed to find out the networking of ADHD genes and analyzed the hub gene based on the largest connection with the other genes. Secondly, the TFs were retrieved which are helpful in regulating these genes. And finally, the enrichment pathways were analyzed for these hub genes.

The concept of network has been announced in systems biology as it exactly tells the inner workings of several multifaceted biological systems. Networking of candidate genes is widely studied for creating gene regulatory networks and listing drug targets by employing the data from GWAS or gene expression. Network approach mainly emphasizes on the interrelationship between the different constituents of protein-protein interaction (PPI) network and the identification of the perceptive molecules linked with the disease. PPI delivers both local as well as the global vision towards the interacting components taking part in similar biological events which can be achieved by biological trials or computational methods. Recently, PPI approach has been promising for drug designing. This study involves the PPI network of the candidate genes important for ADHD genes. For network construction, all candidate genes were taken into account that are involved in ADHD disease. Hence, the present study computes the following work: (a) identifying candidate genes responsible for ADHD genes and generating a network with the help of computational tools, (b) finding hub genes, (c) detecting main transcriptional regulators, (d) generating transcriptional factors, and (e) implementing enrichment analysis to credit the function of hub genes. The transcriptional factors helps in regulating the gene. TFs also controls the gene expression, and ensure the correct gene is expressed at the right time and right place (Lodish et al., 2000).

This study highlights the key candidate genes as well as transcription regulators and shows its expression with the help of bioinformatics tools. The study also tells the potential mechanism that is involved in ADHD with the help of genes and pathways present in the network. The outline of the work is shown in Figure 1 and it can also be applied to other diseases.

**Figure 1: Graphical abstract**

**MATERIALS AND METHODS**

**Collection of candidate gene**

Genes for this study were retrieved from ADHD gene database (http://adhd.psych.ac.cn/). We have found 232 genes to be involved in ADHD.

**Protein protein network construction through STRING search**

PPI is the physical contact between the two or more proteins which result from electrostatic or biochemical measures. The PPI network helps in the construction of interacted networks from a given query protein or genes. In this study, PPI network was constructed for candidate genes using STRING 10 (Search Tool for the Retrieval of Interacting Genes) database. The STRING database gives the known and the predicted protein interactions, including physical and functional relations which are mainly derived from high throughput experiments, genomic context, co-expression, neighborhood and literature mining.

**Network parameter analysis**

The PPI network created was visualized by Cytoscape version 3.3.0 software and analyzed with the help of Cytoscape plug-in “Network Analyzer.” To know the hub gene
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or the functional gene, the topological parameters were set in network analyzer for node degree, betweenness centrality (BC) and clustering coefficient (CC). For a given network, each gene is characterized as a node and the interactions between the genes are known as edges. Node degree is the sum of edges linked to it, therefore, a high degree signifies the hub genes owning chief biological functions. BC shows the importance of a node by the number of small paths passing through each node. The BC of the node is computed as:

\[ BC = \sum_{s \neq n \neq t} \left( \frac{\sigma_{st}(n)}{\sigma_{st}} \right) \]  

Where, \( n \) is node, \( s \) and \( t \) are nodes in the network other than node \( n \), \( \sigma_{st} \) denotes the number of shortest paths from \( s \) to \( t \), and \( \sigma_{st}(n) \) is the number of shortest paths from \( s \) to \( t \) of node \( n \).

Whereas, CC is the number of connected pairs or edges between the nodes. It is defined as follows:

\[ CC = 2e_n / (k_n^2 - 1) \]  

Where, \( k_n \) is the number of neighbors of node \( n \) and \( e_n \) is the number of connected pairs between all neighbors.

The value of BC and CC always lies between 0 and 1. Therefore, the decisive network was visualized centred on BC, CC and a node degree, and the hub genes were generated based on higher BC values, CC values, and the node degree with the cut off \( \geq 0.005 \), \( \geq 0.2 \) and \( >40 \) respectively.

Regulatory networking attention-deficit/hyperactivity disorder genes
Promoter analysis was carried out by using MatInspector program, a Genomatix software (GmbH, Michigan, U.S.A) suite to categorize TFs which regulate hub genes. MatInspector program operates the huge archive of matrix descriptions of TF binding sites to trace the matches in DNA sequences.

Functional enrichment analysis
Functional enrichment analysis was performed to comprehend the biological significance of the hub genes and their TFs in ADHD using Cytoscape plugin called “ClueGo” version 2.2.2. ClueGo assists the visualization of functional genes presented as a clustered network and graph using statistical analysis. Two sided hyper geometric test and correction method Bonferroni step-down was used as a statistical test for the enrichment analysis with a kappa score of 0.4.

RESULTS AND DISCUSSION
Network structure of hub genes
PPI network of ADHD genes was designed using STRING. The network gained from STRING was visualized in Cytoscape, and is shown in Figure 2.

Using Network analyzer, the Hub genes were selected with the help of topologies which is restricted to high node degree, higher BC and CC values as described in the methodology. These topology constraints are represented in as a graph and the hub genes with their predefined topologies are presented in Table 1.

Among 232 ADHD genes, dopamine receptor D2 (DRD2), brain-derived neurotrophic factor (BDNF), dopamine receptor D4 (DRD4), 5-hydroxytryptamine (serotonin) receptor 2A (HTR1A), are regarded as the hub genes (key nodes) exhibiting the utmost connectivity in the network comprising which were categorized under the standard of minimum 40 networks which also describes 1140 edges and 232 nodes. Among these hub genes, DRD2 regarded as a super hub gene as it has the highest node degree which means DRD2 has a maximum number of interacting neighbors or genes. DRD2 and DRD4 are dopamine genes and are highly involved in the dopamine pathway these genes can be easily influenced by the environmental condition therefore the polymorphism is widely studied.

### Table 1: Selection of hub genes based on following parameters

| Gene     | BC     | CC      | Node degree |
|----------|--------|---------|-------------|
| DRD2     | 0.062016 | 0.350725 | 46          |
| BDNF     | 0.120169 | 0.261616 | 45          |
| DRD4     | 0.039625 | 0.412311 | 42          |
| HTR1A    | 0.02825  | 0.443902 | 42          |

BC – Betweenness centrality; CC – Clustering coefficient and node degree with cut off \( \geq 0.05 \), \( \geq 0.2 \), and \( >40 \) respectively were set as parameters in network analyzer for gene prioritization; BDNF – Brain-derived neurotrophic factor; DRD – Dopamine receptor; HTR1A – 5-hydroxytryptamine (serotonin) receptor 1A
in these genes. DRD4 gene is mainly expressed in the prefrontal cortex of brain. These particular areas of the brain have great importance in behavioral manner, i.e., cognitive action and if found to be affected can lead to severe emotional blockages. The other gene with more number of interactions is BDNF. BDNF gene comes under the neurotrophin family. It plays a significant role neurodevelopment pathway which involves the proliferation of the dopaminergic neuron cells. Evidences show that this gene is present in the piriform cortex hypothalamus and amygdala of rats, which somehow shows its association with the stimulus and response process. The fourth hub gene is serotonin dependent gene HTR1A. Serotonin dysfunction may lead to the aggressive attitude. HTR1A receptor shows affinity towards the endogenous neurotransmitter serotonin. These are available in large quantity in human nervous system such as, raphe nucleus septum hippocampus, and cerebral cortex. This receptor have some impact on dopamine secretion in above explained brain areas.

Hub genes are possibly considered as functionally significant when compared to other genes, as it has the largest number of connections. It has been confirmed that deletion of any of the hub gene creates a maximum frequency of disease phenotypes than the deletion of any normal gene which shows less interaction. This property progresses the understanding of hub or key genes linked to the diseases.

**Predicted key regulatory transcription factors**

Transcriptional regulators of these hub genes (DRD2, BDNF, DRD4, HTR1A) were identified using MatInspector program. A list was generated for each hub gene displaying common TFs. TFs found to be common in these hub genes are ZBED4.02 (Zinc finger, BED-type containing 4; polyG binding sites), ZBED4.01 (Zinc finger, BED-type containing 4; GC-box binding sites), E2F1.01 (E2F TF 1), WT1.0 (Wilms TumorSuppressor), EGR2.02 (Egr-2/Krox-20 early growth response gene product), CKB0X.01 Collagen krox protein (zinc finger protein 67-zfp67), KLF6.01 (Core promoter-binding protein with 3 Krueppel-type zinc fingers (KLF6, ZF9)), VMYB.05 (v-Myb, variant of AMV v-myb), MZF1.01 (Myeloid zinc finger protein), NM23.01 (NME/NM23 nucleoside diphosphate kinase 1 and 2), PLAG1.01 (Plasmodium adeno gene (LAG) 1, a developmentally regulated C2H2 zinc finger protein), SP1.03 (Stimulating protein 1, ubiquitous zinc finger transcription facto r), ZKSCAN3.01 (Zinc finger with KRAB and SCAN domains 3), ZBP89.01 (Zinc finger TF ZBP-89), ZNF202.01 (Zinc finger protein 202), ZNF263.01 (Zinc finger protein 263), ZKSCAN12 (zinc finger protein with KRAB and SCAN domains 12). This indicates that these TFs might play a crucial role in regulating these ADHD hub genes.

**Enrichment through ontology**

Functional enrichment analysis of ADHD genes was performed using the “ClueGo” software for the better understanding of pathways involved in the hub genes and their key transcriptional regulators. The pathways involved in these hub genes are “Dopamine metabolic pathways” and “behavioral fear response pathway” shown in Figures 3 and 4.

Many environmental factors are seen to incriminate the neurotransmitter dopamine which is supposed to be associated with ADHD. Mainly the genes which can alter the dopamine pathway are DRD4 and DRD2. The study suggests that on the brain, dopamine neurotransmitter affects the ADHD to a very great extent. It is experimented that the dopamine neurotransmitter deficit may face motivational issues. It also states that mesoaccumbens dopamine pathways (these are dopamine cells, which are present in the midbrain and have projection) which may be altered and results in ADHD. This study concludes that dopamine reward pathways as to be one of the reasons of inattentiveness in ADHD.

The other pathway which is prone by the affected genes is behavioral fear response. Many examinations were done to understand and relate the fear response pathway
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with ADHD. Research are still going on to understand the connection however, by physiological point of view inhibition response is very poor,[71,72] and considered as the key point for ADHD and inhibits the working procedure and operate the other process in between include amygdale, raphe nucleus, septohippocampal system, hypothalamus, locus ceruleus, and neurocircuitry incorporating the frontal cortex.[73-74] Faulty, BIS process unsuccessful to cooperate with normal, adaptive activation responses and normal stress action which comes out as a fearful reinforcement.[73]

CONCLUSION

In this study, we investigated ADHD interacting proteins to model the PPI network. Hub genes (DRD2, BDNF, DRD4, HTR1A) were identified along with their common TFs. Functional enrichment analysis revealed 'dopamine metabolic pathways' and "behavioral fear response pathway" to play a central role within the selected ADHD hub genes. Any mutation in these hub genes and TFs may alter the phenotype of gene or protein, which is already reported in previous reports.

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Conflicts of interest
There are no conflicts of interest.

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