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ANALYSING SEX ASSOCIATED GENES ON ALZHEIMER DISEASE TRANSCRIPTOME TO DISCOVER GENDER BIASNESS AND THERAPEUTICS

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ABSTRACT

Alzheimer Disease (AD) is a brain disorder with no cure. Poorly understood AD etiology and mechanism behind it's progression are the prime causes of hindrance in discovery of its treatment. It is more prevalent in women and disease progression in male and female is different. This gender biasness of disease is well-known but unexplained. In current study sex-associated genes (X, Y, Mitochondria encoded and sex-biased genes) were analysed through RNA-Seq analysis of AD samples. Total 164 sex associated genes were found to be differentially expressed (DGE) in AD samples and majority of them were down-regulated (X, Y and sex-biased genes). Repressed activity of Y chromosome genes in AD samples justify it's importance and probably explains susceptibility of females in AD. Mitochondrial genes were found to be skewed towards up-regulation in all AD samples. The majority of DGE was in sync with the literature available and remaining others were lesser studied. Six Tc factor were also identified to be DGE. Some Tc factor and their target genes were found to have significant role in AD associated process. These results justify current approach and warrants to explore the role of all DGE genes in AD through further experimentation and lead to discovery of therapeutics.

Keywords: Alzheimer, transcription factor, RNA-Seq, Differential expression, sex-associated, women

INTRODUCTION

Alzheimer Disease (AD) is the leading cause of dementia with no cure. With the increasing number of people suffering from AD globally, there exists a dire need to identify and design therapeutics and provide assured treatment. According to World Alzheimer report 2015, over 46 million people live with dementia worldwide and is estimated to increase to 131.5 million by 2050 [1]. It is a complex neurodegenerative disorder which affects all sorts of cognitive functions like memory, thinking and reasoning. The exact cause and molecular mechanism altered in AD are not understood which is the prime cause of hindrance in development of the promising drugs and treatment [2]. The two identifying pathological features of AD include presence of amyloidβ (Aβ) and neurofibrillary tangles of tau protein [3].

Age and gender has been listed as the biggest risk factor for developing AD [4]. Almost two-thirds of American seniors living with AD are women [5]. A possible gender difference in risk of AD is further supported by recent evidence suggesting that brain's so called cognitive reserve is reduced in women [6]. The two prime differences, sex hormones and chromosomal differences, play significant role in sexual dimorphism of human brain affecting neurovascular development, neurogenesis and synaptogenesis [7]. Women are at significantly greater risk of developing AD is indicated by a meta-analysis of 13 population studies from across United States, Europe, and Asia [8]. Women and men are differently affected by AD and sex has been observed to play a significant role in pathology and disease progression [9]. At Alzheimer's Association International Conference 2015 many studies have put forward new evidence supporting that women's brains are more vulnerable than men's brains to AD [10].

Estrogen has been known to contrast the neurodegenerative processes that characterizes AD [11]. Estrogen replacement therapy in humans has been found to have neurologic benefits which include reversal of estrogen deficiency-induced memory dysfunction and reduced risk of Alzheimer's disease. *In vitro* analyses indicate that 17 β estradiol (E2) enhances neuronal survival after oxidative stress, excitotoxic insults, and β amyloid exposure ([12-14]. Estrogen has also been found to play neuroprotective role by preventing mitochondrial damage [15].

Since maternal inheritance of AD is speculated as mitochondria are maternally inherited there are numerous studies implicating role of mitochondrial

dysfunction in AD etiology. In AD brain it is observed that due to oxidative stress there is increased production of reactive oxygen species happens [16]. The cells lacking mitochondria are reported to be protected against Aβ toxicity, implicating role of mitochondria in AD pathology [17]. In order to understand AD etiology and pathogenesis broad interplay of various factors, genetic, hormonal and environmental, need to be understood. The role of sex and gender differences in the onset and course of AD remains ill-defined and demands further attention. Despite recent advances in the understanding of clinical aspects of sex differences in AD, the underlying mechanisms, for instance, how sex modifies AD risk and why the female brain is more susceptible to AD, are not clear. Gender differences in AD and in neurodegenerative processes appears to offer great promise for the future development of better strategies of intervention for patients.

The current study aims to explore novel and unexplored gene targets associated with human sex chromosomes, mitochondria and genes known to be sex biased in order to answer the hypothesis that AD affects men and women differently. Further the expression of sex chromosome associated, mitochondria and sex biased genes in AD samples from different region of brain was studied. Genes found to be differentially expressed in AD samples might be helpful in understanding and identifying novel therapeutic targets for AD.

MATERIAL AND METHODS

Data Collection

X and Y chromosomes and Mitochondrially encoded genes were listed as X, Y and M genes respectively (Table S1, S2, S3 Supplementary data). From a recent study genes differentially expressed in sex were tabulated as sex-biased genes [18] (Table S4 Supplementary data). These all were named as sex-associated genes. Further sequences and coordinates of these genes were extracted from National Centre for Biotechnology Information (NCBI). AD whole transcriptome data (SRA027308.2) reported in literature [19] was retrieved from Sequence Read Archive (SRA) of NCBI (Table S5 Supplementary data) and taken for expression analysis.

Expression Analysis Of Sex Associated Genes In AD And Control Samples

Whole transcriptome analysis pipeline (Fig. 1) was used to analyse the expression of sex associated genes in RNA Seq data (Table S5 Supplementary data) [20]. Galaxy platform [21] was used for RNA-Seq and further differential gene expression analysis.

Fastq Manipulation And Quality Check

FASTQ Groomer version 1.0.4 was used to convert RNA-Seq reads into fastqsanger format and FastQC was used to read quality reports (Galaxy Tool Version 0.65) [22]. The results were used to determine whether or not the data can be further processed.

Mapping Of RNA-Seq Reads & Assembly Of Transcripts

Tophat Gapped-read mapper for RNA-Seq data version 2.1.0 was used to map RNA-Seq reads by aligning them to reference human genome (Hg38). First reads located within the exons (non junction) were mapped to the reference genome. The mapping results were then analysed to identify splice junctions between exons. Mapped files were processed by Cufflink (Galaxy Version 2.2.1.0) [23]. The software assembles transcript to the reference genome (Hg38) and examines the abundance of transcripts. The abundance was calculated with respect to Fragments per Kilobase of exon model per million Mapped reads (FPKM) estimates for RNA-Seq data.

Differential Expression Analysis of Genes In AD Samples

All assembled transcripts were compared to the reference annotation using Cufflinks. The Cufflinks assembled transcripts were sent to Cuff compare along with a reference. His classified each transcript as known or novel. Then Cuffdiff algorithm was used to calculate differential gene expression (DGE) in the samples. It was used to perform three pair wise comparisons of expression between normal and diseased samples from temporal, frontal and total brain regions. Relative abundance of transcripts was calculated as FPKM value. Self developed Perl script was used to find DGE of sex associated genes in the RNA-Seq analysis output. The p-value cut-off was kept at < 0.05. Genes, up-regulated and down-regulated in the samples taken for the study were tabulated.

Functional Analysis Of DGE

For the functional enrichment of DGE in the study functional clustering was done. Gene Ontology term enrichment analysis on the basis of Molecular Function functional annotation category was done using panther (Protein Analysis THrough Evolutionary Relationships) analysis tools [24]. List of names of DGE genes were provided to the database, and searched according to *Homo sapiens* as target organism. Functionally classified results were obtained and analyzed with in-house developed PERL scripts.

Transcription Factor Analysis

The genes observed to be DGE in current study were explored to find if any of them encodes transcription factor (Tc factor). The genomics option in GeneCards

database [25] was selected and searched for transcription factor function and target genes. Database TRRUST [26] was also searched for finding target genes of Tc factor.

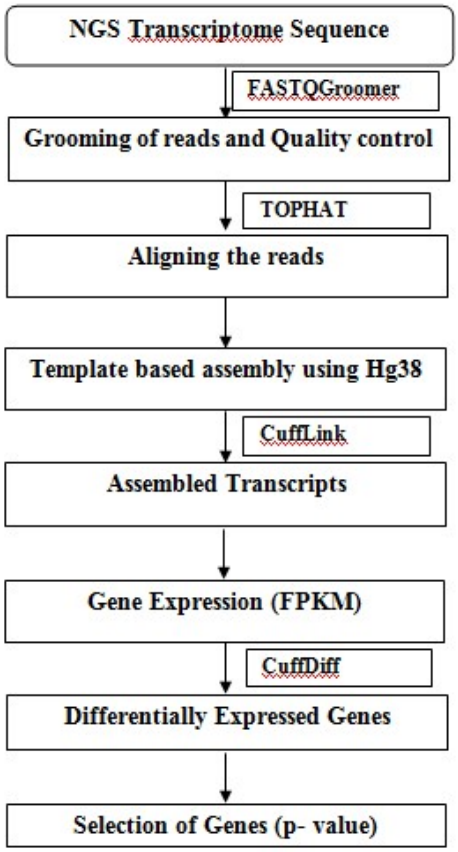

Fig. 1 Whole transcriptome analysis pipeline

RESULTS AND DISCUSSION

110 years after the discovery of AD, scientists and researchers stand bare-handed as far as therapeutics and treatment of disease is concerned. No cure on horizon, millions of people suffering from the disease and numerous more affected every day poses a grave threat to researchers in 21st century. Gender and sex have been linked with AD innumerable times in past. The exact molecular mechanisms of how gender influences AD progression are yet to be explored [27]. Since no study examines exclusively sex related genes with respect to AD this is an effort to explore this association which would be helpful in understanding, preventing and designing therapeutics for AD.

Total 2409 genes encoded by X chromosome, 529 genes encoded by Y chromosome, 37 genes encoded by

mitochondrial genome and 159 genes known to be differentially expressed in male and female [18] were listed as X chromosome associated, Y chromosome associated, mitochondrial and sex biased genes respectively (Table S1, S2, S3, S4 Supplementary data) has been taken for the study.

These genes were taken for whole transcriptome analysis (Fig. 1) and more than 95% mapping percentage was achieved. The quality of mapping of reads was comparable to earlier studies (Table 1) [28]. It indicates the reliability and quality of assembly.

On the basis of p-value significance ($p<0.05$) the range of DGE observed for X chromosome genes from -2.5 to 3.69 in frontal and -3.2 to infinity in temporal lobe and from -6.13 to 3.2 in whole brain of AD samples. X chromosomes genes down-regulated in two AD samples are tabulated in Table 2. It was observed that DGE of sex-associated genes were skewed more towards down-regulation (149/164 genes) in AD brain. In Y chromosome none of the genes were observed to be significantly up-regulated in any of the AD samples taken for the study. The range of Y chromosomes genes down-regulated in the AD samples is from -3.06 to -1.52 (Table S12 and S13 Supplementary Data).

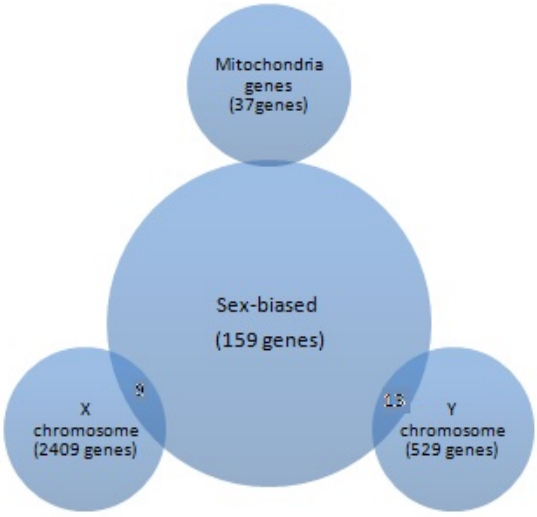

Fig. 2 Venn diagram representing no. of X, Y, Mitochondria and sex-biased genes taken for the study. Intersection of X chromosome with sex-biased study shows 9 common genes whereas intersection of Y chromosome with sex-biased shows 13 common genes.

Table 1 RNA-Seq reads mapping percentage to reference genome (Hg38)

S. No.	SRR ID	Tissue type	Input Reads	Mapped reads	% mapping
1	SRR085474	Frontal Normal	15772947	15276636	96.9
2	SRR085726	Frontal Diseased	15228832	15276636	96.2
3	SRR085471	Temporal Normal	15256752	14809336	97.1
4	SRR085473	Temporal Diseased	14227702	13560187	95.3
5	SRR087416	Whole Diseased	14720816	14253449	96.8
6	SRR05725	Whole Normal	13442077	12972477	96.5

Table 2 X chromosome genes down-regulated in two AD samples

S.no.	Gene	Log2(Fold change)		
		Frontal lobe	Temporal lobe	Whole brain
1	BTF3P8	-1.3	-2.09	NA
2	ARHGEF9-IT1	-1.3	-2.09	NA
3	IDSP1	-1.6	-2.24	NA
4	LINC00893	-1.6	-2.24	NA
5	IDS2	-1.6	-2.24	NA
6	KLHL34	NA	-2.45	-1.22
7	YY2	-1.58	NA	-2.6
8	ZNF449	-1.69	NA	-2.34
9	HEPH	-1.55	NA	-1.89
10	HS6ST2-AS1	-2.2	NA	-2.17
11	AMOT	-1.45	NA	-1.6
12	MIR4329	-1.45	NA	-1.6
13	HNRNPA1P25	-1.32	NA	-1.69
14	LOC101928903	-2.22	NA	-2.17
15	LOC105373311	-1.98	-1.18	-1.69

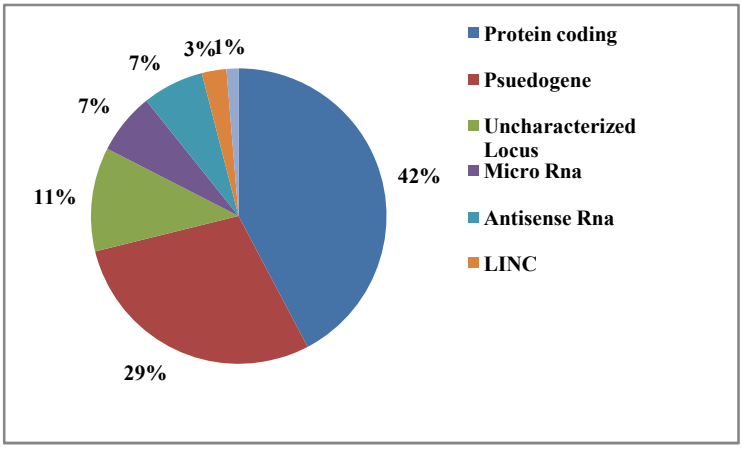

Fig. 3 Pie chart representing type of Differentially Expressed Genes

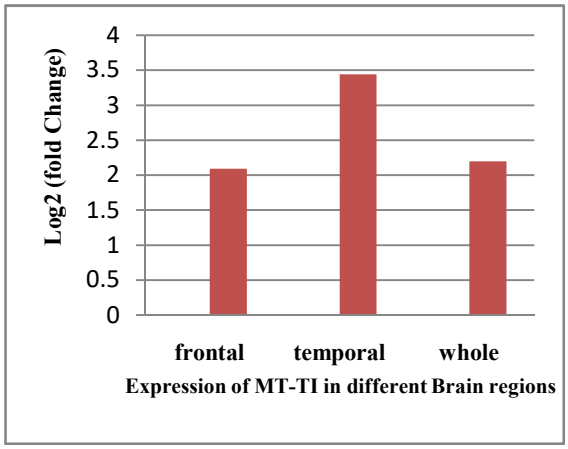

Fig. 4 Expression of MT-TI gene in different Brain Regions

DGE of sex-associated genes in RNA-Seq data of AD samples (Table S5 Supplementary data) were analysed. Total 164 sex-associated genes were found to be differentially expressed ($p < 0.05$) in AD (Table S6-S21 Supplementary Data). On the basis of p-value significance ($p<0.05$) in X chromosome, Y chromosome, mitochondrial genome and sex-biased genes 119, 8, 0 and 22 genes were observed

to be down-regulated and 8, 0, 4 and 3 genes were up-regulated respectively (Table S6-S21 Supplementary data). In frontal lobe, temporal lobe and whole brain 62, 25 and 62 genes were down-regulated respectively. In frontal lobe, temporal lobe, whole brain 8, 3 and 4 genes were found to be up-regulated respectively (Table S6-S21 Supplementary data).

Among the DGE only 65 genes were protein coding genes and rest (99 genes) were non-protein coding genes (non-pcg). The non-pcg includes psuedogene, anti sense RNA, intronic transcript, uncharacterized locus, Long intergenic non protein coding RNA and micro RNA (Fig. 3)

Four mitochondrial genes were found to be up-regulated in all the AD samples (Table S15-S17 Supplementary Data) and none were down-regulated ($p<0.05$). *MT-TI* gene (mitochondrially encoded tRNA-isoleucine) was found to be up-regulated in all three diseased samples (Fig. 4) (Table S14-S16 Supplementary Data).

According to literature increased level of mitochondrial gene expression and oxidative damage in a transgenic mutant mouse model of AD have been demonstrated [42] which justifies current results. *MT-TI* (Mitochondrially Encoded TRNA Isoleucine) is the non coding RNA gene which is observed to be up- regulated in all the three brain samples, frontal lobe, temporal lobe and whole brain (Fig. 4). It is involved in biosynthesis of isoleucine amino acid. Furthermore, an isoleucine codon initiates ND2 synthesis (subunit 2 of NADH coenzyme Q reductase) which is the complex I in electron transport chain. Complex I is involved in production of superoxide which leads to oxidative stress one of the causes of AD [43]. Hence this gene is proposed as a drug target for AD. Alteration in expression of *MT-TI* is found to be associated with various disorders like juvenile parkinsonism, leigh syndrome [44]. None of the studies have ever reported differential expression of *MT-TI* gene in AD samples. This gene can be used as a drug target since reduction in oxidative stress may have therapeutic effect in AD.

Sex biased genes which were differentially expressed range from -3.36 to 1.8 in frontal lobe, -4.5 to none in temporal lobe and -4.2 to infinity in whole brain AD sample , 22 were down-regulated and three were up-regulated (Table S17-S21 Supplementary Data). Out of these twenty genes were encoded by autosomes and two genes (*NLGN4Y* and *PCDH11Y*) were encoded by Y chromosome. Sex biased *P2RY12*, *NR3C2*, *MEGF10* and *SDC10* genes were also found to down-regulated in more than one AD sample (Table 3).

Table 3 sex-biased genes down-regulated in two AD samples

	Gene	Log2(Fold change)		
S.no.		Frontal lobe	Temporal lobe	Whole brain
1	P2RY12	-3.36101	-4.53705	NA
2	NR3C2	-1.72471	NA	-1.66281
3	MEGF10	NA	-2.55887	-1.43005
4	SDC2	NA	-2.77229	-2.56542

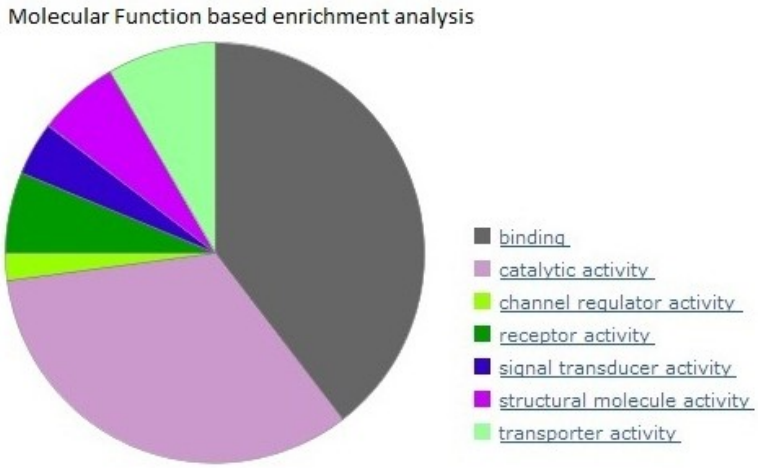

Fig. 5 Pie chart classifying DGE sex-associated genes on the basis of Molecular Function

Functional annotation of DGE on the basis of molecular Function (Fig. 5) classifies them into seven different classes. Binding and catalytic activity are found to be the most enriched class (Table S23 Supplementary Data).

These classes are diverse in nature hence this outcome states the multifactorial nature of the disease as the DGE genes possess varied functional properties.

It was observed that DGE *FOXO4*, *POU3F4*, *MBTPS2*, *YY2*, *LOC101928917* and *ZNF449* genes encode transcription factor (Tc Factor) (Table S22 Supplementary Data). Further target gene analysis of these Tc factor revealed that gene *FOXO4* regulates six target genes, genes *POU3F4* and *MBTPS2* have one target gene each and FOR genes *YY2*, *LOC101928917* and *ZNF449* no hits were obtained (Table S22 Supplementary Data). Transcription factors include proteins that initiate and regulate transcription of genes. *FOXO4* Tc factor plays role in regulation of insulin signalling pathway and oxidative stress [45]. *POU3F4* gene encodes POU-III class of neural transcription factors with glucagon as it's target gene. It has been observed in clinical studies that treatment with glucagon like peptide 1 (GLP1) there is increase in cognitive ability, blood glucose metabolism and synaptic function

[46]. These all processes are observed to be affected in AD hence could be the promising target for AD therapeutics [47].

MBTPS2 gene encodes intramembrane zinc metalloprotease, which is essential in development. This protein is involved in intramembrane proteolysis of sterol-regulatory element-binding proteins (SREBPs) and the ER stress response. ER stress is found to be generated by amyloid β in AD [48]. *YY2* encodes a multifunctional transcription factor that has Kruppel-like zinc fingers in its C-terminal region. It is found to be expressed in AD samples but it's role in disease progression is not clear [49]. *ZNF449* encodes a nuclear protein that likely functions as a transcription factor [37]. The protein includes an N-terminal SCAN domain, and seven C2H2-type zinc finger motifs. LOC101928917 (Heat Shock Transcription Factor, X-Linked-Like) is a Protein Coding gene and very less is known about it. Since it is known that heat shock factors are involved in protein folding and stress, which are the two prime changes happening in AD brain, it could be associated with AD. According to results, three transcription factors (*FOXO4, POU3F4* and *MBTPS2*) have been found to regulate target genes which are associated with AD previously. Further studies need to be done in order to explore the role of these Tc factors for better understanding and therapeutics for AD.

The DGE genes observed in current work should be the first priority of research in order to explore the proposed link between AD and gender. High number of DGE of sex associated genes and majority of them getting repressed in AD brain samples is in concurrence with published literature to support this study [50]. These genes should be further explored in different experimentation for their exact role in AD. Results obtained in current study are in sync with the earlier studies reporting significant role of sex and gender in AD pathogenesis. Hence these results support the idea behind the study that there exists a strong link between AD and gender which if inferred would aid in unveiling AD pathology and discover new therapeutic targets.

5. CONCLUSION

It is a first successful attempt to study all sex-associated genes together in AD context. Most of the DGE sex associated genes (X, Y and sex-biased) were found to be down-regulated in AD samples in current study. This justifies earlier reports of repressed transcriptional activity in AD brain. Reduced expression of Y chromosome encoded genes in AD signifies the relevance of Y chromosome in AD. Interestingly none of the Y

chromosome encoded genes were up-regulated in AD and its reduced transcriptional activity indicates the probable cause of women being more prone to AD. It is possible that increased expression of Y chromosome encoded genes lead to promising therapeutic targets. Increased expression of mitochondrial genes in AD is in concurrence with earlier reported literature. Transcription factors were identified to be differentially expressed out of which some regulates expression of genes which play significant role in AD hence it is suggested that all identified Tc factors should be explored in experimentation with respect to their association with AD. Majority of the genes were down-regulated which was in sync with the published literature. These genes might be helpful in understanding AD better and design novel therapeutics.

6. ACKNOWLEDGEMENT

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