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LIFE SCIENCE AND BIOMEDICINE

NOVEL-RESULT

Identifying the function of methylated genes in Alzheimer's disease to determine epigenetic signatures: a comprehensive bioinformatics analysis

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Abstract

Gene methylation is one means of controlling tissue gene expression, but it is unknown what pathways influencing Alzheimer's disease (AD) are controlled this way. We compared normal and AD brain tissue data for gene expression (mRNAs) and gene methylation profiling. We identified methylated differentially expressed genes (MDEGs). Protein-protein interaction (PPI) of the MDEGs showed 18 hypermethylated low-expressed genes (Hyper-LGs) involved in cell signaling and metabolism; also 10 hypomethylated highly expressed (Hypo-HGs) were involved in regulation of transcription and development. Molecular pathways enriched in Hyper-LGs included neuroactive ligand-receptor interaction pathways. Hypo-HGs were notably enriched in pathways including hippo signaling. PPI analysis also identified both Hyper-LGs and Hypo-HGs, as hub proteins. Our analysis of AD datasets identified Hyper-LGs, Hypo-HGs, and transcription factors linked to these genes. These pathways, which may participate in Alzheimer's disease development, may be affected by treatments that influence gene methylation patterns.

Keywords: Alzheimer's disease; epigenetics; biomarkers; methylated differentially expressed genes.

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disease characterized by the accumulation of amyloid plaques and neurofibrillary tangles in neuron cells and which is manifested by the gradual development of dementia symptoms, including profound impairment of cognitive abilities (Rahman et al., 2020). The pathobiology of the AD is complex with genetic and epigenetic events are involved in the disease pathogenesis (Stoccoro & Coppede, 2018). While the hallmarks of the disease include the accumulation of amyloid plaques and neurofibrillary tangles in the brain (Dunckley et al., 2006), how these are related to AD development and, indeed, what are the key underlying mechanisms of AD is uncertain. A number of gene expression profiling studies have been performed comparing neuronal tissues of AD and control patients, including microarray gene expression analysis and array or sequencing-based analyses of bisulfite converted DNA to detect differences in gene methylation levels (Chouliaras et al., 2013;

Coppieters et al., 2014). The latter identifies and quantifies a key (though not the sole) epigenetic control of gene expression since a methylated gene promoter generally has blocked transcription. However, a combined approach to integrate gene expression and gene methylation data could uncover epigenetic signatures in AD.

Epigenetic mechanisms, including DNA methylation and histone modifications, play a crucial role in the development of the AD (Sanchez-Mut & Graff, 2015). Therefore, identification of methylated-differentially expressed genes (MDEGs) and discovering pathways may be useful for the clarification of how these and other mechanisms associated with AD may be controlled. Earlier studies have identified gene signatures in AD (Rahman et al., 2020; Semick et al., 2019). These studies provided gene signatures focused on either gene expression or methylation profiling. To provide an in-depth understanding of the biological mechanisms of AD, a conjoint analysis of gene expression and gene methylation analysis is considered.

1.1 Objective

In this study, we performed bioinformatic analysis of gene expression (mRNAs) and DNA methylation data from AD-affected neural tissues to identify differentially expressed genes (DEGs) and differentially methylated genes (DMGs), respectively. We aimed to identify overlapping methylated differentially expressed genes (MDEGs) to provide novel insights in AD pathogenesis. Our workflow of the analysis is summarized in Fig. 1.

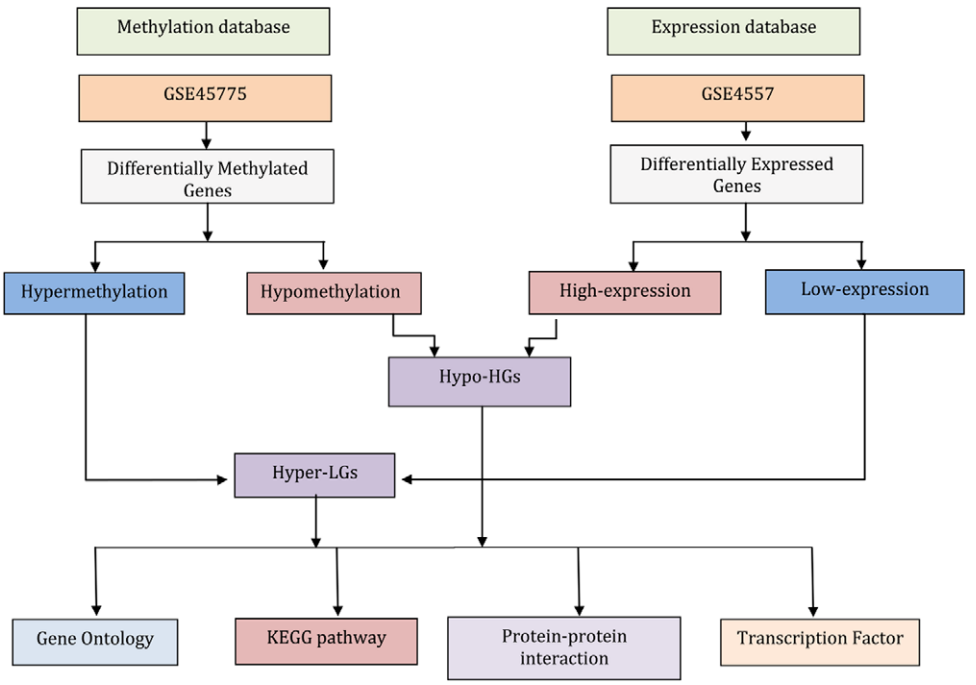

Figure 1. Flowchart describing the data analysis processes in this work. The gene expression data for neurons from post-mortem brain tissue from Alzheimer's disease (AD) patients and matched controls were used to identify differentially expressed genes (DEGs). Similarly, genome-wide DNA methylation data of AD compared matched controls were subjected to identify differentially methylated genes (DMGs). Comparing highly expressed genes with hypomethylation genes (i.e., genes that have high expression levels because of a lack of suppression by methylation) identified the genes termed here Hypo-HGs. Similarly, low-expression genes with hypermethylation (suggesting low expression levels due to suppression methylation) identified the genes termed here Hyper-LGs. Then, we annotated Hypo-HGs and Hyper-LGs using Gene Ontology (GO) and KEGG pathway to identify GO and pathway. The protein-protein interaction (PPI) networks of the Hypo-HGs and Hyper-LGs were also investigated to identify hub genes for these networks. Gene-transcription (TF) factor analysis was also performed to detect potential key regulators of the activities of these genes.

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2. Methods

2.1 Acquisition of transcriptomic and DNA methylation datasets

We utilized mRNA gene expression profiling data (GSE4757) and DNA methylation profiling data (GSE45775) from studies of AD and control samples of brain tissue. These datasets were obtained from the NCBI-GEO database. The GSE4757 mRNA profiling datasets contained 20 samples that consisted of 10 AD tissue samples and 10 non-AD control tissue samples. Samples were obtained from the same patient and the same brain region. Selected neurons containing neurofibrillary tangles and normal neurons from the entorhinal cortex of 10 mid-stage AD cases via laser capture microdissection were used for gene expression dataset. The methylation microarray data from GSE45775 dataset contained 20 samples that included 15 AD tissues and 5 control samples which consisted of DNA methylation profiling of normal hippocampus and different Alzheimer Braak stages hippocampus samples. The entorhinal cortex is an area of the brain located in the medial temporal lobe which has a central role in neuronal networks that underlie memory functions. Similarly, the hippocampus also plays a key role in memory and knowledge acquisition. It was recently determined that the entorhinal cortex could be a new player for memory formation that works in parallel to the hippocampus (O'Neill et al., 2017). Although data obtained from the two different brain regions were integrated in the present study, both of these brain regions are thought to participate in memory functions.

2.2 Data processing and identification of differentially expressed genes

We employed GEO2R web-utility to identify DEGs and differentially methylated genes (DMGs) by comparing AD samples compared to control. The microarray datasets were processed and normalized in GEO2R. A *p-value* < 0.05 and |t| > 2 was considered as the cut-off criteria to identify the DEGs and DMGs. We identified overlapping MDEGs between the GSE4757 and GSE45775 datasets. The mutually common genes between down-regulated and hypermethylation genes were termed as hypermethylated-lowly expressed genes (Hyper-LGs). Similarly, the common genes between upregulated and hypomethylation genes were regarded as hypomethylated-highly expressed genes (Hypo-HGs).

2.3 Functional and pathway enrichment analysis

We performed functional annotation of the identified MDEGs via Enrichr (Kuleshov et al., 2016) to detect Gene ontology (GO) terms and KEGG pathways. p-value < 0.05 was considered as statistically significant for enrichment analysis.

2.4 Protein interactome analysis

We utilized the STRING database (Szklarczyk et al., 2017) to study the protein-protein interaction (PPI) network for Hyper-LGs and Hypo-HGs the via NetworkAnalyst (Xia et al., 2015). The hubs were selected based on degree >20 to identify a high number of interacting hub proteins in the PPI networks.

2.4.1 Transcription factor analysis

We have analyzed and identified the regulatory transcription factors (TFs) that interact with MDEGs, suggesting these TFs may regulate the identified MDEGs utilizing the TRNASFAC and JASPAR databases via Enrichr (Kuleshov et al., 2016). A p-value < 0.05 was considered to designate the statistically significant TFs.

3. Results

3.1 Methylated differentially expressed genes in AD

We analyzed the gene expression and methylation data to identify DEGs or DMGs. We identified overlapping genes, termed here 18 Hyper-LGs, by matching down-regulated DEGs with the

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hypermethylated DMGs; 10 Hypo-HGs were identified by comparing and up-regulated DEGs and hypomethylated DMGs.

To clarify the biological significance of the identified MDEGs, GO enrichment analysis was performed (Table S1). With regard to Hyper-LGs, enriched biological processes (BP) included notably positive regulation of potassium ion transport, and regulation of glucose metabolic process. The enriched GO terms for Hypo-HGs were enriched in BP included positive regulation of transcription.

3.2 Molecular pathways from epigenetic perspective

The Hyper-LGs demonstrated enrichment in pathways of nitrogen metabolism, nicotine addiction, neuroactive ligand-receptor interaction, amyotrophic lateral sclerosis (ALS). Hypo-HGs were significantly involved in hippo signaling pathway, cGMP-PKG signaling pathway, alcoholism, TGF-beta signaling pathway (Table 1:).

3.3 Protein-protein Interaction to identify hub proteins

We analyzed the PPI of MDEGs. The Hyper-LGs PPI network had 208 nodes and 209 edges (Fig. 2), while the Hypo-HGs network consisted of 542 nodes and 574 edges (Fig. 3). Thetopological analysis showed hub genes for both the Hyper-LGs and Hypo-HGs networks. Hub proteins (TOMM22, TBX5, ANK2, GRIA2, COPS7B, RORA) were detected as Hyper-LGs, while hub proteins (BMP2, GATA4, HDAC11, GGA2, CREB3, RASSF1) were identified for Hypo-HGs.

3.3.1 Transcription factors of methylated-differentially expressed genes

The generation of gene products can be regulated at both transcriptional and post-transcriptional levels. The TFs directly regulate the expression (i.e., transcription) of DEGs, thus we sought to detect the TFs that may regulate the MDEGs. Table 2: showed the TFs that regulate the MDEGs.

4. Discussion

The development and progression of AD is the result of complex interplay of epigenetics and genetics mechanisms at multistage. Epigenetic perturbation, especially of DNA methylation, contributes immensely to the pathobiology of AD (Stoccoro & Coppede, 2018; Kawalia et al., 2017). The identification of potential biomarkers for AD will not only improve the understanding of how the pathogenesis of AD is controlled but may also open new avenues of treatment strategies. We identified 18 Hyper-LGs and 10 Hypo-HGs as key gene signature in AD. The enrichment and PPI analysis provided significant pathways and methylated hub genes which may provide novel insights into the pathogenesis of AD. The pathway analysis of Hyper LGs showed enriched ALS pathways (Rusina et al., 2007) in ALS patients may be accompanied by cognitive impairment and existence of neurofibrillary tangles and plaques affecting neurons, (Rusina et al., 2007) suggesting the importance of the identified pathways in AD pathogenesis (Ravetti et al., 2010). Our analysis also showed pathways enriched by Hypo-HGs. Among the pathways, the pre-activation of hippo signaling pathway is associated with neurodegenerative diseases including AD (Mueller et al., 2018). We obtained "alcoholism pathway" enriched by the MDEGs, which probably plays roles in AD pathogenesis because the alcohol has been found to be involved in neuroinflammation in dementia, suggesting an additional mechanism in neurodegenerative disease. Our analysis identified "TGF-beta signaling pathway" as involved in AD. Increasing evidence suggests that dysregulation of TGF-beta signaling pathway play critical roles in AD (von Bernhardi et al., 2015). In brief, in order to identify new therapeutic targets, exploration of signaling pathways and biomarkers involved in MDEGs will provide new understanding about AD pathogenesis.

We studied the PPI based on the proteins encoded by the MDEGs. Among the hubs, TOMM22 serve as the main receptor for accumulation of amyloid β (Aβ) peptides in AD (Hu et al., 2018). Previous

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Table 1. Pathway analysis of methylated-differentially expressed genes related to Alzheimer's disease patient samples.

Category		Term	Adj. *p*-value	Genes
Hyper-LGs	KEGG	Nitrogen metabolism	0.015	CA12
		Circadian rhythm	0.027	RORA
		Pentose and glucuronate interconversions	0.030	KL
		Nicotine addiction	0.035	GRIA2
		Neuroactive ligand-receptor interaction	0.036	GRIA2;ADRA2A
		Endocrine and other factor-regulated calcium reabsorption	0.042	KL
		Cocaine addiction	0.043	GRIA2
		Amyotrophic lateral sclerosis (ALS)	0.044	GRIA2
Hypo-HGs	KEGG	Hippo signaling pathway	0.002	BMP2;RASSF1
		cGMP-PKG signaling pathway	0.002	CREB3;GATA4
		Alcoholism	0.003	CREB3;HDAC11
		Viral carcinogenesis	0.004	CREB3;HDAC11
		RNA polymerase	0.015	POLR3E
		Bladder cancer	0.020	RASSF1
		Vasopressin-regulated water reabsorption	0.021	CREB3
		Cocaine addiction	0.024	CREB3
		Pathways in cancer	0.027	BMP2;RASSF1
		Basal cell carcinoma	0.031	BMP2
		Cytosolic DNA-sensing pathway	0.031	POLR3E
		Cortisol synthesis and secretion	0.032	CREB3
		Non-small cell lung cancer	0.032	RASSF1
		Amphetamine addiction	0.033	CREB3
		Thyroid hormone synthesis	0.036	CREB3
		Leishmaniasis	0.036	CYBA
		Insulin secretion	0.042	CREB3
		TGF-beta signaling pathway	0.044	BMP2
		Prostate cancer	0.047	CREB3
		Aldosterone synthesis and secretion	0.047	CREB3
		Melanogenesis	0.049	CREB3
		Longevity regulating pathway	0.049	CREB3

studies showed that the gene *ANK2* is involved in AD (Higham et al., 2018). Our analysis also detected *RORA* as a hub which is distinctively overexpressed in the hippocampus of AD brain (Acquaah-Mensah et al., 2015). With regard to Hypo-HGs, we detected seven hub proteins, including BMP3, GATA4, HDAC11 and CREB, which have been previously described in brain functions and neurodegenerative diseases. Among these hubs we noted BMP2, the nuclear form of BMP2 has previously been shown to play a role in hippocampus memory formation (Cordner et al., 2017). Transcription factor GATA4 has been shown to be significantly differentially expressed in AD compared to controls (Garranzo-Asensio et al., 2018). It should also be noted that HDAC inhibitors are known to be a potential drug target in AD

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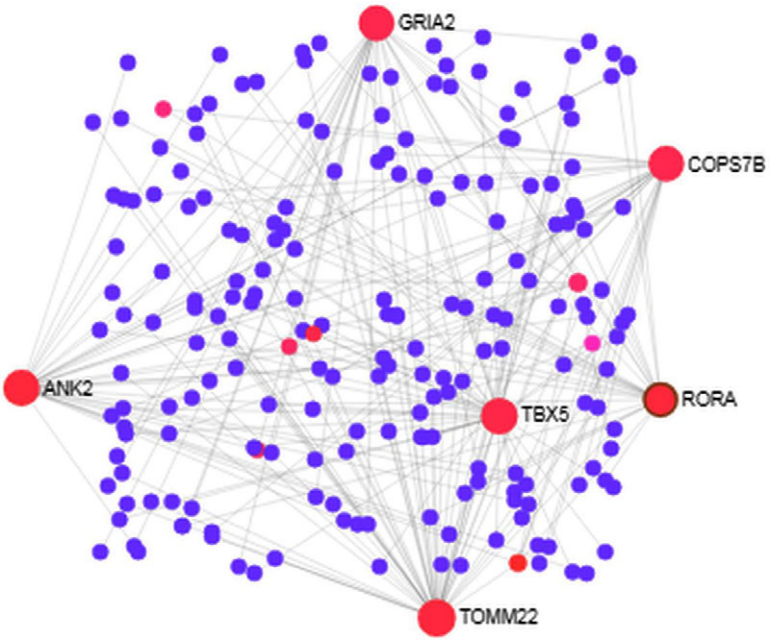

Figure 2. Protein-protein interaction analysis of the Hyper-LGs in Alzheimer's disease. The proteins are represented in nodes (blue and red). Red nodes are marked as hub nodes. The larger node size indicates their degree in topological analysis. Edges in gray show the interactions among interacting proteins in the network.

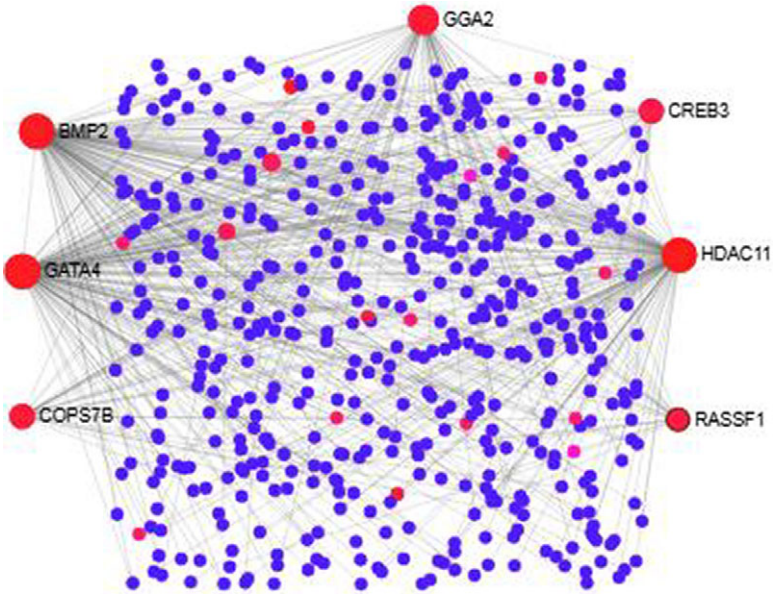

Figure 3. Protein-protein interaction analysis of the Hypo-HGs in Alzheimer's disease. The proteins are represented in nodes (blue and red). Red nodes are marked as hub nodes. The larger node size indicates their degree in topological analysis. Edges in gray show the interactions among interacting proteins in the network.

(Yang et al., 2017) so HDAC11 could be a novel drug target for AD. CREB signaling has been a known link to neurodegenerative disorders and plays many important roles in brain cell functions (Saura & Valero, 2011).

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Table 2. Transcription factors of methylated-differentially expressed genes related to Alzheimer's disease patient samples.

Category	Term	Adj. *p*-value	Genes
Hyper-LGs	EIF4EBP1	0.015	ANK2;TOMM22
	XBP1	0.016	ANK2;R3HDM1;TBX5
	NKX2-8	0.021	DGKQ;SLC6A15
	MEF2A	0.030	GRIA2;CMTM4;SLC6A15;GPR22;ANK2;RORA
	BCL6	0.033	GRIA2;TMEM67;SLC6A15;GPR22
	HNF4A	0.035	NTNG1;TMEM67;SLC6A15;ANK2;RORA
	GATA6	0.036	NTNG1;CA12;ANK2;RORA
	RELB	0.038	KL;DGKQ;SLC6A15;COPS7B
Hypo-HGs	TFAP2C	0.004	BMP2;RASSF1;CYHR1;GATA4
	HINFP	0.010	CREB3;HDAC11;CYHR1;COPS7B;CYBA
	SP3	0.024	HDAC11;CYHR1;CYBA
	NR5A1	0.027	CREB3;RASSF1;POLR3E
	SP1	0.028	HDAC11;CYHR1;CYBA

With regard to the Hyper-LGs that we detected, one study detected identified loci in *EIF4EBP1* as associated with late onset AD (Nalls et al., 2009). A study has suggested that XBP1 was a risk factor for developing AD (Duran-Aniotz et al., 2017). In addition, XBP1 dysregulation has a profound impact on immune systems, inflammatory response and implicated in complexes diseases including AD (Cisse et al., 2017). A polymorphism in *MEF2A* could be involved in AD pathogenesis (González et al., 2007). Variation in *RELB* impacts upon hippocampal function in late onset AD (Xiao et al., 2017). With regard to Hypo-HGs, increased expression of *SP3* observed in brains of AD patients (Boutillier et al., 2007). The overexpression of *SP1* in AD subjects was reported and suggested as a therapeutic target to help prevent AD (Citron et al., 2008). However, it should be noted that our study has some limitations in that it does not include data on gene expression or the methylation profiles of the genes in the same brain region. This is due to the lack of any available datasets to investigate these aspects. Thus, although we have uncovered a number of potentially important hub genes and pathways, they require further experimental verifications to establish them as having a definite role AD pathobiology.

5. Conclusions

In the present study, we have analyzed gene expression and DNA methylation profiling in AD. We identified 28 MDEGs and pathway analysis revealed significant enrichment of pathways related to AD pathogenesis. The PPI analysis revealed hub Hyper-LGs of AD included *TOMM22, TBX5, ANK2, GRIA2, COPS7B, RORA*; such genes for Hypo-HGs included *BMP2, GATA4, HDAC11, GGA2, CREB3, RASSF1*. Regulatory TFs (EIF4EBP1, XBP1, NKX2-8, MEF2A, BCL6, HNF4A, GATA6, RELB) were identified among the hyper-LGs; similarly we identified TFs (TFAP2C, HINFP, SP1, SP3, NR5A1) influencing Hypo-HGs. Since these are robust candidate genes based on dysregulated methylation, it is possible that these or some significant downstream gene transcription targets of these TFs may be useful for diagnostics (in the case of secreted TFs detectable in the blood) and possibly as treatment targets for AD. The present study improves our understanding of the epigenetic in the pathobiology of AD and identified a number of potential AD biomarkers for further investigation in experimental studies.

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Author contributions. MRR conceived and designed the study; MRR and TI analyzed data; MRR wrote the draft manuscript; EG, JMWQ, and MAM reviewed and edited the manuscript; MAM supervised the project.

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Conflict of interest. The authors declare that there is no conflict of interest.

Data availability. Gene expression profiling data with accession GSE4757 and DNA methylation profiling data with accession GSE45775 are publicly available at the Gene Expression Omnibus database (https://www.ncbi.nlm.nih.gov/geo/).

Supplementary Materials. To view supplementary material for this article, please visit http://dx.doi.org/10.1017/exp.2020.65.

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Peer Reviews

Reviewing editor: Dr. Sourav Kolay
UT Southwestern, 5323 Harry Hines Blvd, Dallas, Texas, United States, 75390-9096

This article has been accepted because it is deemed to be scientifically sound, has the correct controls, has appropriate methodology and is statistically valid, and has been sent for additional statistical evaluation and met required revisions.

doi:10.1017/exp.2020.65.pr1

Review 1: Identifying the Function of Methylated Genes in Alzheimer's Disease to Determine Epigenetic Signatures: A Comprehensive Bioinformatics Analysis

Reviewer: Dr. Md. Ataur Rahman
Korea Institute of Science and Technology, Neuroscience Centre

Date of review: 08 November 2020

Conflict of interest statement. Reviewer declares none

Comments to the Author: Title: Identifying the Function of Methylated Genes in Alzheimer's Disease to Determine Epigenetic Signatures: A Comprehensive Bioinformatics Analysis

In the present manuscript the authors apply a variety of bioinformatics approaches to identify differentially methylated genes and differentially expressed genes from available datasets on Alzheimer's disease to understand their biological pathways and interconnections. This manuscript is very interesting and advance epigenetics filed of Alzheimer's disease. The manuscript is clearly written and the results are well presented, but I suggest some minor revisions:

1) The manuscript should be improved in the level of detail and description of both materials and method and results, including figure captions.

2) The significance of the hub genes should be stressed in the discussion section.

3) The discussion section should be concise

4) Please check for grammar and typos. (for example, interaction is misspelt in discussion).

Score Card

Presentation

Is the article written in clear and proper English? (30%)	5/5
Is the data presented in the most useful manner? (40%)	5/5
Does the paper cite relevant and related articles appropriately? (30%)	4/5

Context

Does the title suitably represent the article? (25%)	5/5
Does the abstract correctly embody the content of the article? (25%)	5/5
Does the introduction give appropriate context? (25%)	5/5
Is the objective of the experiment clearly defined? (25%)	5/5

Analysis

Does the discussion adequately interpret the results presented? (40%)	5/5
Is the conclusion consistent with the results and discussion? (40%)	3/5
Are the limitations of the experiment as well as the contributions of the experiment clearly outlined? (20%)	2/5

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Review 2: Identifying the Function of Methylated Genes in Alzheimer's Disease to Determine Epigenetic Signatures: A Comprehensive Bioinformatics Analysis

Reviewer: Dr. Kichang Kwak

Date of review: 14 November 2020

Conflict of interest statement. Reviewer declares none

Comments to the Author: The paper describes the application of gene expression data to identify differentially expressed genes and DNA methylation data to identify differentially methylated genes to identify overlapping methylated differentially expressed genes to provide novel insights in AD pathology. Overall, the paper is fairly organized. But before publication, the authors should address the following points:

1. Add the reference for first sentence "Alzheimer's disease (AD) is a neurodegenerative ~" in introduction (page 3).
2. Rephrase the sentence "In this study, we analyzed gene expression ~" in objective (page 4).
3. Why did you use a threshold value for degree as 20? Clarify a threshold value for degree (page 6).
4. What is the regulatory TFs? Define "regulatory TFs" (page 6).
5. How did you get the adjusted p value? Clarify the statistical methods, for example multiple comparison correction or covariates.
6. Make sure to discuss the limitations of this study.
7. Proof-read the entire text for minor grammatical errors, especially abbreviations.

Score Card

Presentation

4.0 /5

Is the article written in clear and proper English? (30%)	4/5
Is the data presented in the most useful manner? (40%)	4/5
Does the paper cite relevant and related articles appropriately? (30%)	4/5

Context

3.8 /5

Does the title suitably represent the article? (25%)	4/5
Does the abstract correctly embody the content of the article? (25%)	4/5
Does the introduction give appropriate context? (25%)	4/5
Is the objective of the experiment clearly defined? (25%)	3/5

Analysis

Does the discussion adequately interpret the results presented? (40%)	4/5
Is the conclusion consistent with the results and discussion? (40%)	4/5
Are the limitations of the experiment as well as the contributions of the experiment clearly outlined? (20%)	3/5

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