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In Silico Analysis of Alzheimer's Disease Mechanism Through DNA Methylation and Gene Expression Data

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Alzheimer Hastalığı Mekanizmasının DNA Metilasyonu ve Gen Ekspresyon Verileri Üzerinden İn Silico Analizi

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Öz

Alzheimer Hastalığı (AD), şu anda tedavisi olmayan, hafızayı ve düşünce sürecini bozan, zayıflatıcı bir hastalıktır. Mevcut çalışmada, GEO veritabanında entegre bir araç olan GEO2R, AD ile ilişkili DNA metilasyonunu ve gen ekspresyonu veri kümelerini analiz etmek için kullanıldı. Diferansiyel olarak metillenmiş ve eksprese edilmiş AD genlerinden (DEMEG'ler) oluşan PPI ağı oluşturmak için BioGRID Veri Tabanından elde edilen veriler kullanıldı. PPI ağını topolojik olarak görüntülemek ve analiz etmek için Cytoscape kullanıldı. Hastalık ilişkilerini ve sinyal yolaklarını ortaya çıkarmak amacıyla zenginleştirme analizi yapmak için DAVID biyoinformatik programından yararlanıldı. Ayrıca, Connectivity Map 2 (Cmap 2) kullanılarak çalışmanın DEMEG'leri için farmakolojik hedefler olarak kullanılabilecek potansiyel terapötik ajanlar olarak küçük moleküller ortaya konuldu. Sonuç olarak, SMURF1 ve UBE2D2 gibi AD için yeni biyobelirteç adayları olarak daha fazla araştırılabilecek 502 ortak DEMEG ve çeşitli merkezi proteinler belirlendi. Alzheimer ile MAPK sinyal yolağının yanı sıra bağımlılık ve DEHB ve epilepsi gibi beyin hastalıkları arasındaki bağlantı da belirlendi. Ayrıca flukloksasilin, butamben, asetoheksamid gibi tedavi edici olarak kullanılabilecek aday küçük moleküller de önerilmiştir. Bu çalışma, AD hastalığı mekanizması hakkındaki bilgimizi ilerletmek için DNA metilasyonu ve gen ekspresyonu verilerini birleştirmiştir.

Anahtar Kelimeler: Alzheimer Hastalığı; Gen İfadesi; DNA Metilasyonu; Biyobelirteç; Küçük Moleküller

Abstract

Alzheimer's Disease (AD) is a debilitating disease impairing memory and thought process with currently no cure. In the current study, GEO2R, an integrated tool in the GEO database, was used to analyze DNA methylation and gene expression datasets associated with AD. Data from the BioGRID Database were used to create a PPI network of differentially methylation and expressed AD genes (DEMEGs). Cytoscape was used to image and analyze the PPI network topologically. The DAVID bioinformatics program was utilized to do enrichment analysis in order to uncover disease associations and signaling pathways. Furthermore, small molecules were predicted using Connectivity Map 2 (Cmap 2) as potential therapeutic agents that might be exploited as pharmacological targets for the study's DEMEGs. As a result, 502 mutual DEMEGs and several hubs that may be researched further as new biomarker candidates for AD such as SMURF1 and UBE2D2 were identified. The link between AD and the MAPK signaling pathway, as well as addiction and brain diseases such as ADHD and epilepsy has been established. Additionally, candidate small molecules that can be used as therapeutics such as flucloxacillin, butamben, and acetohexamide were proposed. This study integrated DNA methylation and gene expression data to further our knowledge of the AD disease mechanism.

Keywords: *Alzheimer's Disease; Gene Expression; DNA Methylation; Biomarker; Small Molecules*

1. Introduction

The process of transforming genes consisting of DNA sequences into proteins that affect the phenotype is called gene expression (Signor and Nuzhdin 2018). DNA methylation is required to regulate tissue-specific gene expression and genomic repression. DNA methylation at various genomic regions may utilize various impacts on gene activities considering underlying genetic sequence (Han *et al.* 2007). The details of the complex relationship between DNA methylation and gene expression are not fully resolved but are of great importance for disease development. DNA methylation plays a variety of roles in tissue-specific gene expression, including regulating the usage of alternative splicing sites during transcriptional splicing and selecting alternative promoters to increase or decrease gene expression. To fully understand disease mechanisms, it is necessary to understand the relationship between DNA methylation and gene expression (Ehrlich 2019, Sevimoglu 2023).

Alzheimer's disease is a type of dementia, which is a progressing neurodegenerative disease that generates brain cell destruction. The symptoms of AD include a decline in thought, memory and behavioral activities that develop progressively (Ulep *et al.* 2018). Various gene

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expression and DNA methylation studies have been carried out to understand the genetic mechanism of AD. For instance, a study regarding the AD disease epigenetic mechanisms has observed that the APOE gene plays an important role in methylation (Nourian *et al.* 2021). There are brain region-dependent differences in hypermethylation and hypomethylation in late-onset AD patients (Hanger and Wray 2010). Differences in DNA methylation and gene expression are related to disease processes such as neurodegeneration (Semick *et al.* 2019).

In this study DNA methylation and gene expression datasets of AD were analyzed and integrated to illuminate the mechanism associated with the disease. Construction of protein interaction networks and identification of hub proteins as well as enrichment analysis were achieved. In addition, small molecules that might be candidates in treatment of the disease were determined.

2. Material and Method

2.1. DNA Methylation and Gene Expression Data Acquisition

Raw data of high-throughput DNA methylation (GSE66351) and gene expression (GSE15222) datasets for AD were obtained from Gene Expression Omnibus (GEO) (Haertle et al 2019). Samples of these datasets are from frontal cortex tissue.

2.2. DNA Methylation and Transcriptome Data Analysis

DNA methylation and gene expression data analysis were performed with GEO2R tool embedded in GEO database. GEO2R is an online tool that compares groups of samples in a GEO Series to discover genes that differ in expression across experimental settings. This tool makes use of various R packages from the Bioconductor project. Bioconductor is an open-source software project built on the R programming language that offers tools for analyzing high-throughput genetic data. Statistical analysis of each dataset was performed individually. In the analysis of DNA methylation dataset, FDR was checked with Benjamini-Hochberg method (Zhang et al. 2019). Cut-off values for statistical significance were p-value <0.05 and | t |> 2. The genes obtained as a result of this analysis are named differentially methylated genes (DMGs). RMA normalization (Irizarry *et al.* 2003) and linear models' method for microarray data (LIMMA) (Wettenhall and Smyth 2004) was used to identify differentially expressed genes (DEGs). The DEGs with p value less than 0.05 and with fold change less than 0.5 were considered down-regulated and those with a fold change greater than 2 were considered up-regulated. Annotation conversions were performed using the HumanMethylation450 manifest file v1.2, bioDBnet (https://biodbnet-abcc.ncifcrf.gov/db/db2db.php), and gProfiler (Url-1). Mutual genes of the analyzed datasets are named differentially methylated and expressed genes (DEMEGs) of the study.

2.3. Protein-Protein Interaction (PPI) and Hub Protein Determination Related to the Diseases Under Study

The PPI network of the DEMEGs was constructed using protein interaction data from the BioGRID Database (Oughtred *et al.* 2018). Imaging and topological analysis of the network was performed using Cytoscape (Shannon *et al.* 2003). Hub proteins were identified by the dual metric approach utilizing the Cytohubba plug-in using the degree and betweenness centrality criteria (Chin et al. 2004). In a network, a node's degree is the number of links it has with other nodes. Betweenness centrality quantifies the degree to which the node is on the shortest path connecting additional sets of individuals in the network.

2.4. Enrichment Analysis for the Diseases Examined

Signaling pathways related to AD DEMEGs, and disease associations were carried out using DAVID bioinformatics tool, and results with p value <0.05 were accepted as significant (Huang *et al.* 2019). DAVID conducts analyses using a variety of databases. In this study, the KEGG (Kyoto Encyclopedia of Genes and Genomes) database was selected for signaling pathways (Url-2), and Genetic Association Database (GAD) (Becker et al. 2004) was selected for disease associations.

2.5. Candidate Molecule Determination

Connectivity Map 2 (Cmap 2) was used to predict small molecules as candidate therapeutic agents that may serve as drug targets for the DEMEGs of the study (Lamb *et al.* 2006). This tool uses a Kolmogorov-Smirnov test statistic to rank molecular agents according to their similarity to the up/down-regulated DEMEGs provided. p value <0.05 was chosen for statistical significance, and top 20 molecules with negative mean values and negative enrichment values were selected.

3. Result

3.1. DNA Methylation and Transcriptome Data Analysis

In the present study GSE15222 and GSE66351 high-throughput DNA methylation and gene expression datasets were analyzed. Analysis revealed a total of 768 DEGs for GSE15222 dataset (580 up-regulated and 188 down-regulated) and 14230 DMGs for GSE66351 dataset. 502 mutual DEMEGs were uncovered (Figure 1). 367 of the mutual DEMEGs were upregulated and 135 were downregulated.

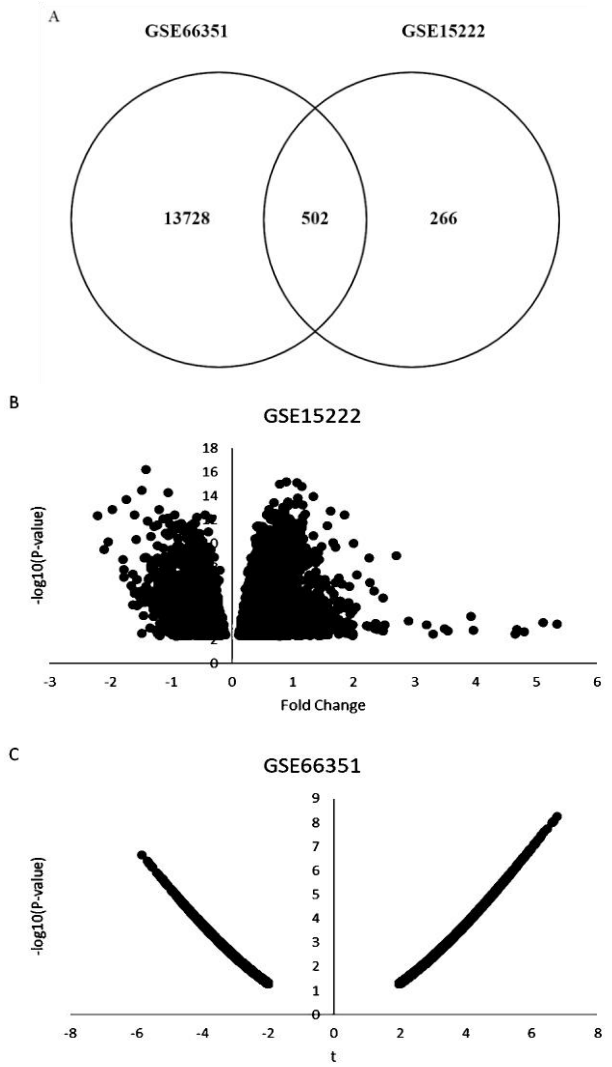


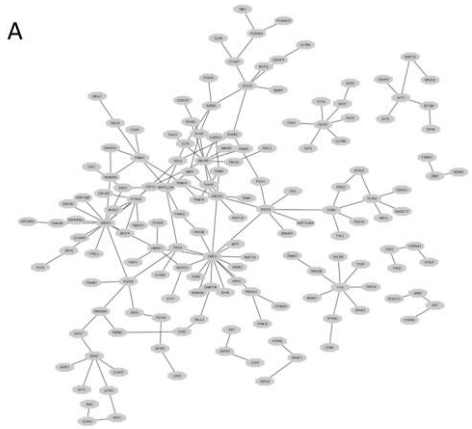
Figure 1. A) The mutual DEMEGs of AD B) volcano plot of DEGs in GSE15222 dataset and C) volcano plot of DMGs in GSE66351 dataset

3.2. PPI Network and Hub Proteins Related to the Disease Under Study

PPI network of the DEMEGs were constructed. There were 154 nodes and 165 edges of the network. Hub proteins were identified for the constructed DEMEG PPI network employing a dual metric approach with degree and betweenness centrality criteria (Figure 2). UBE2I, SRPK2, SMURF1 UBE2D2, RAD18 and CDC42 stood out as hubs of the network for both metrics.

3.3. Enrichment Analysis

Signaling pathways and associated diseases were explored through enrichment analysis of the mutual DEMEG list. Signaling pathways were detected employing KEGG database and accepting p<0.05 (Table 1). There are 12 signaling pathways associated with the data. Prominent signaling pathways such as MAPK signaling pathway and PI3K-AKt emerged as a result of the analysis. Addictions such as nicotine and morphine also came into view. The disease associations (GAD-Genetic Association Database) of the mutual DEMEGs were obtained using DAVID Bioinformatics tool with a p<0.05 cut-off value (Table 2).



B

Hub	Degree	Hub	Betweenness
UBE2I	15	UBE2I	4720.33
SRPK2	14	SRPK2	4156.13
SMURF1	10	RAD18	3476.33
UBE2D2	9	ALDOA	3252.17
FYN	7	UBE2D2	2366.07
RAD18	7	SMURF1	2145.67
MAP1LC3A	6	PIAS4	2115.07
PSMA1	6	RMND5A	2106.17
CDC42	6	ITGB1	2104
FGFR1	6	CDC42	2002.67

Figure 2. A) The constructed Protein-Protein Interaction Network for AD DEMEGs, B) hubs according to degree and betweenness metrics.

Table 1. Signaling pathway associations of the mutual DEMEGs

Signaling Pathway	P-Value
Neuroactive ligand-receptor interaction	2.02E-06
Nicotine addiction	0.0055
MAPK signaling pathway	0.0060
PI3K-Akt signaling pathway	0.0075
GABAergic synapse	0.0139
Morphine addiction	0.0156
Axon guidance	0.0158
Rap1 signaling pathway	0.0179
Arrhythmogenic right ventricular cardiomyopathy	0.0335
Taste transduction	0.0370
Butanoate metabolism	0.0413
ECM-receptor interaction	0.0427

There are 42 disease associations. Brain diseases such as ADHD, bipolar disorder, schizophrenia, autism, and epilepsy stood out. In line with signaling pathway associations, addictions such as alcoholism, heroin and marijuana abuse and tobacco use came into prominence in our enrichment analysis.

Table 2. Top 20 Disease Associations of the mutual DEMEGs

Disease	P-Value
several psychiatric disorders	2.80E-08
alcoholism	1.33E-06
Tobacco Use Disorder	2.85E-06
Cell Adhesion Molecules	1.51E-05
alcohol consumption	1.86E-05
ADHD	3.32E-05
cirrhosis, alcoholic; alcoholism	4.05E-05
schizophrenia	5.93E-05
Cleft Lip|Cleft Palate	1.49E-04
Autism	1.98E-04
Bulimia	2.22E-04
Bipolar Disorder	9.97E-04
heroin abuse	0.0011
personality disorder	0.0014
epilepsy	0.0014
Marijuana Abuse|Psychoses, Substance-Induced	0.0016
alcohol dependence	0.0019
Body Weight	0.0066
Alzheimer's disease	0.0075
Adiponectin	0.0086

Table 3. Candidate Therapeutic Agents for AD

Candidate Agent	*Average Value*	*P-Value*
Methotrexate	-0.421	0.0125
Acetohexamide	-0.399	0.0133
Proscillaridin	-0.550	0.0147
Ricinine	-0.195	0.0157
Telenzepine	-0.390	0.0161
Isoniazid	-0.305	0.0163
Rifampicin	-0.501	0.0168
Flutamide	-0.325	0.0174
Butamben	-0.412	0.0186
Flucloxacillin	-0.355	0.0239
Strophanthidin	-0.386	0.0245
Lomustine	-0.314	0.0269
Dacarbazine	-0.257	0.0329
Timolol	-0.284	0.0485

3.4. Candidate Small Molecule Determination

Using Cmap, small molecules were unearthed that could be researched further as candidate therapeutic agents with $p<0.05$ and a negative mean value. 14 candidate agents and their p-values are shown in Table 3.

4. Discussions and Conclusions

The course of Alzheimer's disease and its genetic underpinnings remain unclear. The illumination of AD disease mechanism may help identify therapeutics that might be a cure for the disease. With this aim, DNA methylation and gene expression datasets of AD were analyzed. A protein interaction network of the mutual DEMEGs was constructed and hub proteins were identified. Signaling pathways and diseases associated with AD DEMEGs were put forward and several small molecules that may be regarded as candidate therapeutics have been identified. Several DEMEGs stood out as hubs (UBE2I, SRPK2, SMURF1 UBE2D2, RAD18 and CDC42) of the DEMEG network. For instance, an upregulated DEMEG, UBE2I (ubiquitin conjugating enzyme E2 I), encodes a member of the E2 ubiquitin-conjugating enzyme family. In a study done by Ahn and colleagues, UBE2I (also known as Ubc9) polymorphism is associated with late onset AD (Ahn *et al.* 2009). Another upregulated DEMEG, SRPK2 (Serine/Arginine-Rich Protein-Specific Kinase 2), is a cell cycle regulated kinase that phosphorylates Serine/arginine domain-containing proteins and mediates pre-mRNA splicing with unclear function in neurons. Hong and coworkers propose that SRPK2 may play a role in Alzheimer's disease etiology (Hong *et al.* 2012). CDC42 (Cell Division Cycle 42), another hub, modulates signaling pathways that affect several biological processes, including cell morphology, migration, endocytosis, and cell cycle progression (Zhang and Niu 2022). In a recent study CDC42, which is a downregulated hub, is linked with the progression of AD (Zhu *et al.* 2023). The upregulated hub SMURF1 (SMAD Specific E3 Ubiquitin Protein Ligase 1), encodes a ubiquitin ligase that is specific for SMAD proteins, and this hub has a critical function in the control of cell motility, signaling, and cell polarity, as well as being an essential factor in mitophagy (Shao *et al.* 2017). Recent studies suggest that mitophagy, which is a mechanism for replacing damaged mitochondria and protecting cells from abnormal cell death signaling might be a player in AD disease progression (Sharma *et al.* 2022). UBE2D2 is an upregulated hub which is a E2 ubiquitin-conjugating enzyme (Critchley *et al.* 2022). RAD18 (RAD18 E3 ubiquitin protein ligase) is also an upregulated DEMEG which promotes glioma growth and lowers glioma cells' sensitivity to radiation. Parenti and coworkers identified RAD18 as a marker in AD disease progression (Parenti *et al.* 2007).

SMURF1 and UBE2D2 may be examined further for their roles in AD disease progression and biomarker capabilities. Enrichment analysis associated with the mutual DEMEGs identified various signaling pathways. These associated pathways give us a glimpse of the disease mechanism. For instance, an association of MAPK signaling pathway was identified. In neurons, MAPK activation are important for long term potentiation (Tsutsui and Hays 2018). Mitogen-activated protein kinases (MAPKs) regulate a variety of cellular activities such as proliferation, differentiation, apoptosis, survival, inflammation, and innate immunity. Oxidative stress which is a crucial risk factor in the pathogenesis of AD is induced by Aβ resulting in the activation of p38 MAPK as well as subsequent hyperphosphorylation of tau (Kim and Choi 2015). The PI3K/Akt signaling pathway regulates cell

survival, proliferation, growth, differentiation, motility, intracellular trafficking, and dendritic and axonal extension. It plays a distinct role in the preservation of synaptic plasticity and has a significant impact on memory processes. The involvement of the PI3K/Akt signaling axis in the advancement or repression of AD disease is very context-dependent. In general, PI3K/Akt activation in neurons and neural stem cells is beneficial, whereas activation in microglia cells may be detrimental (Razani *et al.* 2021). Also we have to mention the pathways that are related to morphine and nicotine addiction. While there are not many studies linking AD and opioid use research suggest patients with a history for opioid use should be targeted for early dementia detection (Oh and Song 2024). Even though a higher prevalence of Alzheimer's disease among individuals who smoke has been revealed (Wallin *et al.* 2017), there is also research suggesting nicotine itself (not the cigarette) may be helpful in protecting neuronal stem cells when exposed to cytotoxic or inflammatory agents (Brooks and Henderson 2021).

In the current study, the disease associations can be divided into two distinct groups: brain diseases (ADHD, bipolar disorder, schizophrenia, autism and epilepsy) and addictions (alcoholism, tobacco use, marijuana abuse and heroin abuse). Although no common gene has previously been identified between ADHD (Attention-Deficit/Hyperactivity Disorder) and AD, it is thought that SORC2 and SORC3 genes may play a role in amyloid precursor protein. Increasing degree of consanguinity is linked to both ADHD and AD (Zhang *et al.* 2022). Diniz and coworkers suggest that having a history of bipolar disorder, which is another brain disease linked to this study, dramatically raised the likelihood of being diagnosed with AD (*Diniz et al.* 2021). Recent research suggests epilepsy is linked to late onset AD (Kamondi *et al.* 2024). It is safe to state that those with the aforementioned brain conditions are more prone to develop AD. So, early detection and intervention may be beneficial in slowing disease progression. Addictions were the second group of diseases linked to Alzheimer's disease DEMEGS. Excessive alcohol use causes microglial activation, neuroinflammation and neuronal cell death (Venkataraman *et al.* 2017). Previous studies have shown a link between alcoholism and AD (Hoffman *et al.* 2019).

Small molecules are defined as compounds that can modulate biochemical processes to diagnose, treat or prevent diseases. Some target therapeutics were identified such as telenzepine, which is a thienobenzodiazepine that acts as a selective antimuscarinic. Telenzepine may contribute to improved memory performance in mild AD patients (Savelkoul *et al.* 2012). While testosterone has a protective effect on AD, flutamide, another candidate agent, inhibits this positive effect (Yan *et al.* 2019). In addition to its anti-infectious properties, rifampicin, also in our list, displayed neuroprotective effects in various models of neurodegeneration as well. Pilot studies show that rifampicin treatment may be beneficial for AD patients (Yulug *et al.* 2018). Agents that affect more than one AD-related target may have a potential to increase efficacy and/or improve safety in AD treatment. For this, various isoniazid-derived acylhydrazones have been shown to have positive effects as potential acetylcholinesterase and myeloperoxidase inhibitors with antioxidant activity (Santos *et al.* 2020). The potential of methotrexate to protect against dementia has been demonstrated, but its mechanism has not yet been elucidated. In this study Proscillaridine, flucloxacillin, butamben, acetohexamide, strophanthidine, lomustine, dacarbazine, ricinine and timolol have been determined as candidate molecules which were not previously associated with AD, which indicates that these molecules can be researched further as candidate therapeutics for this disease.

In this in silico study, analysis of DNA Methylation and gene expression datasets of AD was achieved. The results indicated various hub proteins with biomarker potential. SMURF1 and UBE2D2 in particular might be investigated further for their roles in Alzheimer's disease development and biomarker capabilities. The relationship between psychiatric disorders and AD as well as various addictions and AD also attracted our attention. Another outcome of the study is that the MAPK signaling pathway was dysregulated in AD patients. There is a need for future experimental studies on the relationship of AD and MAPK signaling pathways. Enrichment analysis has also revealed two separate categories of disorders linked to AD DEMEGs: brain diseases and addictions. Individuals with a previous diagnosis of the brain diseases mentioned in the study are more likely to develop AD in the future. In the light of the results obtained; proscillaridin, flucloxacillin, butamben, acetohexamide, strophanthidine, lomustine, dacarbazine, ricinine and timolol may be further researched as candidate molecules for the treatment of AD.

Declaration of Ethical Standards

This study is derived from master thesis (thesis number: 678313) under the supervision of Assist. Prof. Dr. Tuba Sevimoğlu by Fatih Özen on date of 2021, Titled: "Alzheimer ve MS hastalıklarında DNA metilasyon ve gen ekspresyon verileri incelenerek ortak mekanizma belirlenmesi".

Credit Authorship Contribution Statement

Author-1: Investigation, methodology and analysis, visualization and writing – original draft.

Author-2: Conceptualization, methodology and analysis, supervision – review and editing.

Declaration of Competing Interest

The authors have no conflicts of interest to declare regarding the content of this article.

Data Availability Statement

The raw data supporting the conclusions of this article are available by the authors upon reasonable request.

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