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Estrogen-related gene *INPP5D* is a potential biomarker for Alzheimer's disease

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Abstract

Objective: This study aims to leverage bioinformatics methodologies to screen genes related to Alzheimer's disease (AD) and estrogen, unravel the interplay between estrogen and Alzheimer's pathophysiology, and furnish a foundation for the clinical treatment of AD. *Methods:* Through accessing AD transcriptomics data from the GEO database, we examined differentially expressed genes (DEGs) and elucidated their functional roles via GO and KEGG analyses. Also, we constructed a protein interaction network. Furthermore, we took the intersection between genes identified by the weighted gene co-expression network and estrogen-related DEGs, then obtained key genes through machine learning and verified them through internal data sets. Finally, immune infiltration analysis was performed on the data of patients with AD. *Results:* 326 DEGs were obtained from GSE118553, predominantly enriched in nucleotide diphosphorylation (biological process), nucleotide excision repair complexes (cellular composition), neurotrophic factor binding (molecular functions), and sugar metabolism (signaling pathway). Through weighted gene co-expression network analysis and the intersection of DEGs, three estrogen-related genes were screened out: *INPP5D*, *ENO1*, and *NOP16*. Next, *INPP5D* was selected as the pivotal gene by the random forest tree algorithm. The prediction model was built on *INPP5D*, and the AUC of the ROC curve equals 0.876. After single-sample gene enrichment analysis, the samples clustered into high- and low-immune groups. Intriguingly, there was a positive correlation between these two groups on activated NK cells and M1 macrophages, and a negative correlation between M1 macrophages and T cells CD4 memory resting.
Conclusion: The estrogen-related gene *INPP5D* is a potential biomarker for AD, which provides a new perspective and scientific basis for the development of AD treatment strategies based on hormone replacement therapy.

Keywords: Alzheimer's disease, hormone replacement therapy, bioinformatics, *INPP5D,* estrogen

INTRODUCTION

As the global population continues to grow and age, the number of people with dementia is expected to increase significantly. Surge from an estimated 57.4 million cases in 2019 to 152.8 million in 2050.[1] As of 2019, dementia is one of the leading causes of death globally, ranking seventh. It is also one of the main reasons why the elderly around the world lose the ability to live independently and need care from others. This disease costs the global economy $1.3 trillion, with roughly half of that cost borne by non-professional caregivers such as family members and friends. They spend an average of 5 hours a day caring for and monitoring patients.[2]

It is estimated that approximately 60%-70% of dementia cases can be attributed to Alzheimer's disease (AD). As a ubiquitous neurodegenerative disease, it is increasingly becoming a severe global public health challenge.[3] Over the past few decades, the scientific community has continued to explore various treatments. However, although some drugs with symptom-relieving effects have been developed, until now, no fundamental cure or significant improvement in the condition has

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been found.[4] Women account for two-thirds of all AD cases, reflecting that the risk of developing AD is 2-3 times greater than men, symptoms are often more severe and the disease progresses more quickly.[5] Although the mechanisms underlying these sex differences are not fully elucidated, there is evidence showing a link between menopause and a higher risk of developing AD, emphasizing the key role of reduced estrogen levels in the pathogenesis of AD.[5,6]

So recently hormone replacement therapy (HRT) has been increasingly used for neuroprotective treatment.[7] However, evidence regarding the therapeutic benefits of HRT is very scattered and conflicting.[8,9] A major challenge for future research will be to try to isolate the many factors that appear to contribute to the efficacy and vehicle of estrogen intervention to aid in attempts to establish reliable HRT treatment regimens to prevent cognitive decline. This article uses bioinformatics to conduct an integrated analysis of gene expression data related to AD, identify the key gene *INPP5D*, analyze its mechanism of action on AD, and provide evidence for HRT treatment of AD.

METHODS

Data download

Datasets GSE138260 and GSE118553 were downloaded by searching for 'Alzheimer's disease' and selecting the species 'Homo sapiens' in the Gene Expression Omnibus (GEO) public database (https://www.ncbi.nlm.nih.gov/geo/). GSE118553 was used as the validation dataset. Perform data processing on the downloaded gene chip. Search and download estrogen-related genes (ERGs) from the GeneCards public database (https://www.genecards.org/) using the keyword "Estrogen".

Screen for differentially expressed genes (DEGs)

The R language limma package was used to screen the DEGs of GSE138260. The screening threshold was set to adjusted P value (P adj.) < 0. 05 and fold change (FC).) Logarithmic absolute value | logFC | >1. DEGs were visualized using the ggplot2 package.

Enrichment analysis

Based on GO and KEGG databases, the clusterProfiler package was used to perform functional enrichment analysis on DEGs and visualize the enrichment results.

PPI network analysis

In order to explore whether there is an interaction relationship between characteristic genes, the STRING database (https://string-db.org) was used to construct a PPI network for the characteristic gene (AD-Estrogen), and Cytoscape software was used to analyze protein interactions. Make a network diagram and process it to get the PPI network diagram.

Weighted gene co-expression network analysis (WGCNA)

The R package WGCNA was used to analyze the gene co-expression of AD in the data set. After data processing, based on the approximate scale-free topological network and the obtained soft threshold, a hierarchical clustering tree diagram is generated, the key modules are identified, and the online Venn diagram tool (http//bioinformatics. psb.ugent.be/ webtools/Venn/) to obtain key modules and characteristic genes of estrogen genes.

Screening of key genes

Compare two machine learning methods, random forest (RF) and support vector machine (SVM), through cross-validation to screen key genes. Use the R language meta package, rms package, and rmda package to evaluate the importance, predictive performance, and clinical utility of key genes (Hub-gene) through nomogram, calibration curve, and decision curve (DCA) analysis, and through statistical significance (*P<0.05) to evaluate the difference.

Verification of key genes

Verify the key genes through the data set GSE118553, draw the ROC curve, and calculate the area under it (AUC) to evaluate the consistency and stability of the key genes on different data sets. The closer the AUC value is to 1, the model The better the performance.

Immune infiltration analysis

Single sample set enrichment analysis (ssGSEA) was performed to evaluate the correlation of 28 types of immune cells, and clusters AD group samples through cluster analysis to discover relevant immune cell subpopulations, and at the same time screen The key genes identified were analyzed for immune infiltration.

Cell culture and model establishment

PC12 cells were purchased from the Chinese Academy of Sciences. Cells in logarithmic growth phase were seeded into 6-well plates at a density of 2×104. A mixture of Aβ oligomers was added to the culture medium at a concentration of 100 μmol/L. The experiment was conducted after incubation at 37°C for 24 hours.

Western blot

Total proteins from the samples were extracted using RIPA lysis buffer and quantified using the BCA method. Subsequently, protein denaturation was performed, and the samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis before being transferred to a polyvinylidene fluoride membrane. Blocking was carried out with 5% skim milk at room temperature for 1 hour. The primary antibody was added and incubated overnight, followed by the addition of the secondary antibody and incubation at room temperature for 1 hour. Imaging was then performed using electrochemiluminescence. Quantitative analysis of fluorescence intensity was conducted using the image analysis software ImageJ.

Statistical analysis

R software (https://www.r-projec t.org/, version 4.1.2) was used for analysis. Continuous values between the two groups were analyzed using the t test, and non-parametric comparisons between the two groups were performed using the Wilcoxon test. *P<0.05 was considered to be statistically significant.

RESULTS

Screening and functional analysis of DEGs

The R language limma package was used to screen the DEGs of the data set GSE138260. There were a total of 326 genes, including 256 up-regulated genes and 70 down-regulated genes. The results were displayed through volcano plots and heat maps (Figure 1A-B). Perform GO and KEGG functional enrichment analysis on the screened DEGs. The results of GO enrichment analysis (Figure 1C) showed that biological processes (BP) were mainly enriched in nucleotide diphosphorylation. The cellular component (CC) is mainly enriched in nucleotide excision repair complexes. Molecular functions (MF) are mainly enriched in neurotrophic factor binding. The results of KEGG enrichment analysis (Figure 1D) showed that two important parts of the sugar metabolism pathway, Glycolysis and Gluconeogenesis, were the main enriched pathways. The STRING database was used to construct a PPI network for DEGs (Figure 1E), and Cytoscape software was used to process the protein interaction network diagram to obtain a PPI network diagram.

WGCNA and screening of characteristic genes

Through WGCNA analysis (Figure 2A-E), the samples are clustered, outliers are removed and the sample clustering tree is drawn. A soft threshold $\beta = 2$ (scale-free $R^2 = 0.9$) was selected to construct the network, and the relationship between sample features and modules was analyzed. A total of 5 expression modules were identified, based on module membership (MM) and gene significance (GS). Through calculation, the cyan (turquoise) module with the highest matrix score correlation can be obtained (cor = 0.25, P = 0.0032). The 137 genes and 326 DEGs of the cyan module are combined with 9335 ERGs. The intersection was taken and a Venn diagram was drawn (Figure 1H). As a result, 108 AD and nutrition characteristic genes (AD-Estrogen) were screened out. The three genes obtained from the intersection of the three were *INPP5D*, *ENO1*, and *NOP16*.

Screening of key genes

The results based on the comparison between the SVM-RFE algorithm and the random forest tree (RF) algorithm (Figure 3A) show that the error of the random forest tree algorithm is small. At the same time, according to the reverse cumulative distribution diagram of the residuals (Figure 3B), the RF algorithm (Figure 3C-D) was selected to screen key genes. Based on 108 characteristic genes, the top 5 key genes were selected: *INPP5D*, *XKR8*, *NOP16*, *ENO1*, and *RHBG*. A nomogram of key genes (Hub-genes) was constructed through the "rms" package (Figure 4E). The results showed that as the score increased, the expression levels of genes *INPP5D*, *NOP16*, and RHBG showed an upward trend, and the expression levels of *XKR8* and *ENO1* showed a downward trend, *INPP5D* is the most important Hub-gene affecting the score. The calibration curve calibration result graph (Figure 4F) shows that the actual and predicted probabilities are in good agreement. The decision curve analysis (DCA) results showed good clinical practicality (Figure 4G). The data set GSE118553 was used to verify *INPP5D* (Figure 4H). The

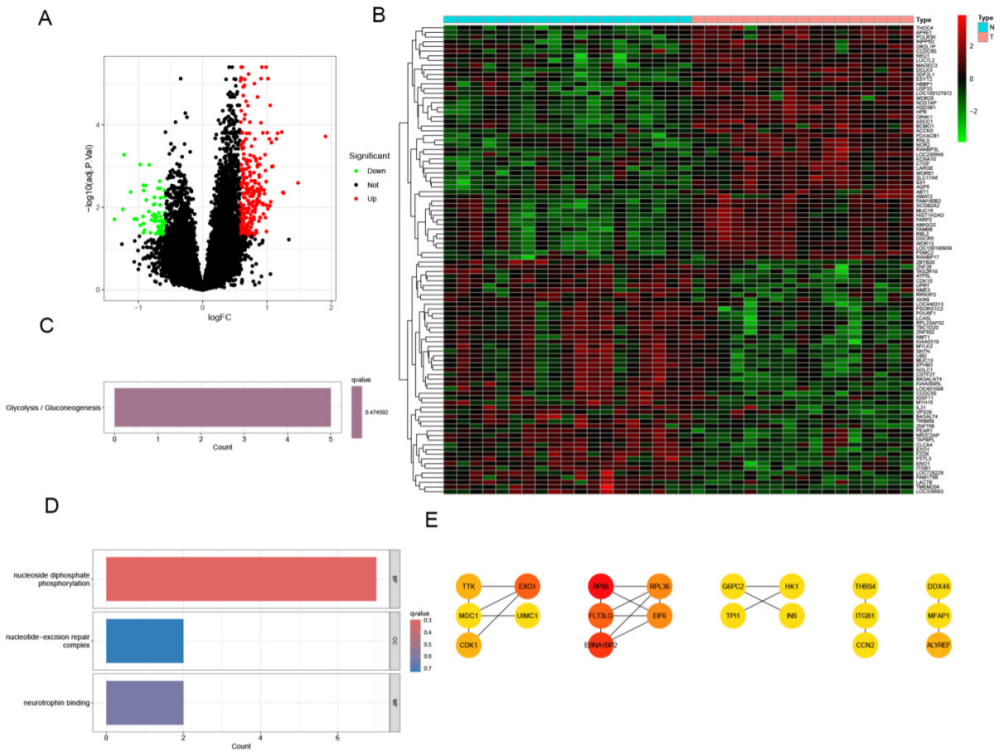

Figure 1. Screening and functional analysis of differentially expressed genes (DEGs). (A) Volcano plot of DEGs. (B) DEGs heat map. (C) GO enrichment analysis. (D) KEGG enrichment analysis. (E) PPI network interaction diagram.

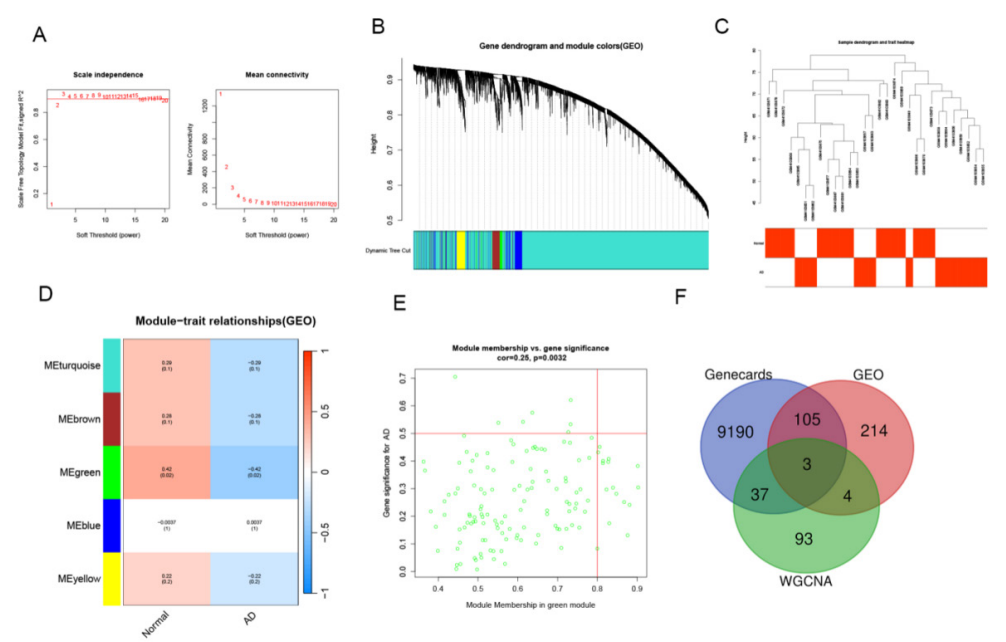

Figure 2. WGCNA construction and screening of characteristic genes (AD-Estrogen). (A) Soft threshold network topology analysis with soft threshold β = 2 (scale-free R2 = 0.9). (B) WGCNA gene dendrogram and module colors. (C) Clustering tree diagram. (D) Heat map of important module feature relationships. (E) Scatter plot of module eigengenes in WGCNA cyan module. (F) Venn diagram of WGCNA cyan module and DEGs versus estrogen genes.

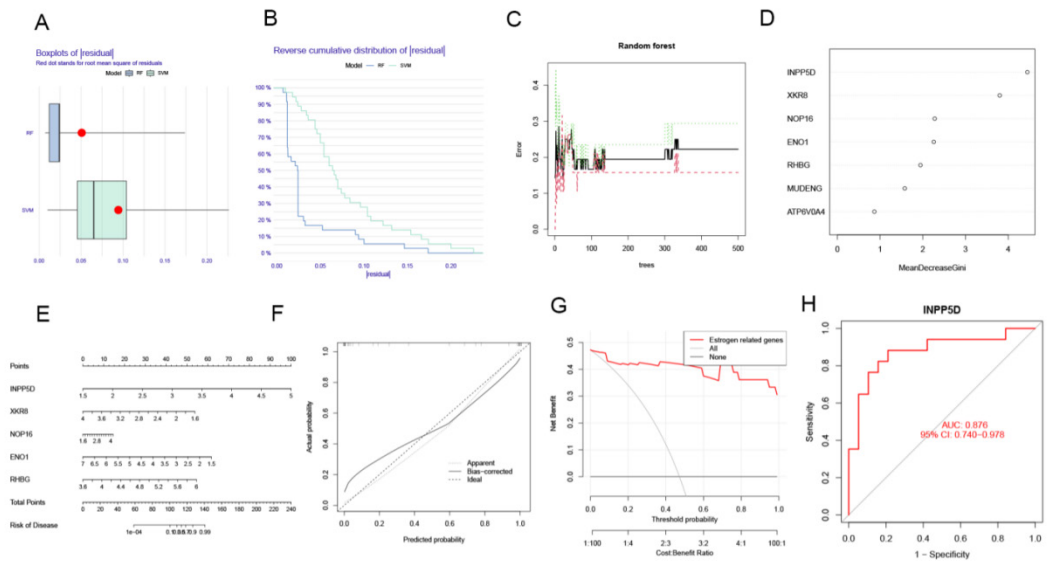

Figure 3. Screening and evaluation of key genes. (A) Boxplots of two machine learning methods showing the distribution of residuals. (B) Inverse cumulative distribution plot of residuals for two machine learning methods. (C) RF algorithm error rate versus the number of classification trees. (D) Feature importance plot of 7 genes in the RF algorithm. (E) Nomogram scores for five key genes. (F) Calibration curve plot assessing accuracy. (G) Evaluation using DCA curve. (H) Data set validation ROC curve of Hub-gene.

results showed that the AUC of the ROC curve of *INPP5D* = 0.876, indicating that the *INPP5D* gene has a good predictive ability for AD.

Immune infiltration analysis

Immune signature analysis was performed through single-sample enrichment analysis, and the results showed (Figure 4A) that there was no significant difference in immune scores between Alzheimer's disease patients and the normal group. Immune cell correlation analysis showed (Figure 4B) that NK cells activated and Macrophages M1 were positively correlated, and Macrophages M1 and T cells CD4 memory resting were negatively correlated. High levels of infiltration of activated plasma cells and activated mast cells (dark red blocks). Neutrophils and memory T cells CD4 positive (T cells CD4 memory resting) are also expressed at high levels in some samples. Activated plasma cells had a value of 0.3 in the first sample and values close to 1 in the other samples. This suggests that the level of infiltration of activated plasma cells is significantly increased under these specific conditions. The results of immune cell infiltration analysis of the key gene *INPP5D* (Figure 4C) showed that it was related to the activation status of neutrophils, dendritic cells and natural killer cells, *P < 0.05.

Verification of INPP5D expression

We verified the expression of *INPP5D* using an external dataset, GSE118553, and the results showed an increase in *INPP5D* expression (Figure 5A). To further validate the reliability, we established an in vitro model of Alzheimer's disease using PC12 cells and detected the expression of *INPP5D* by Western blot. The results indicated elevated expression of *INPP5D* protein (Figure 5B, 5C).

DISCUSSION

Significant differences exist between men and women regarding endocrine function and fluctuations in adult hormone levels.[10] Estrogen is the main sex hormone in women, one of its main functions is to promote the maturation of primary follicles during their development and to control the ovulation process.[11] Unlike androgens, whose expression remains relatively stable throughout adulthood, the natural depletion of the follicular pool in primordial women during menopause results in the cessation of estrogen production and a rapid decline in endogenous hormone levels.[12] Both androgens and estrogens are neuroactive and are known to have significant neuroprotective effects. However, as those neuroprotective factors decrease during menopause, drastic changes

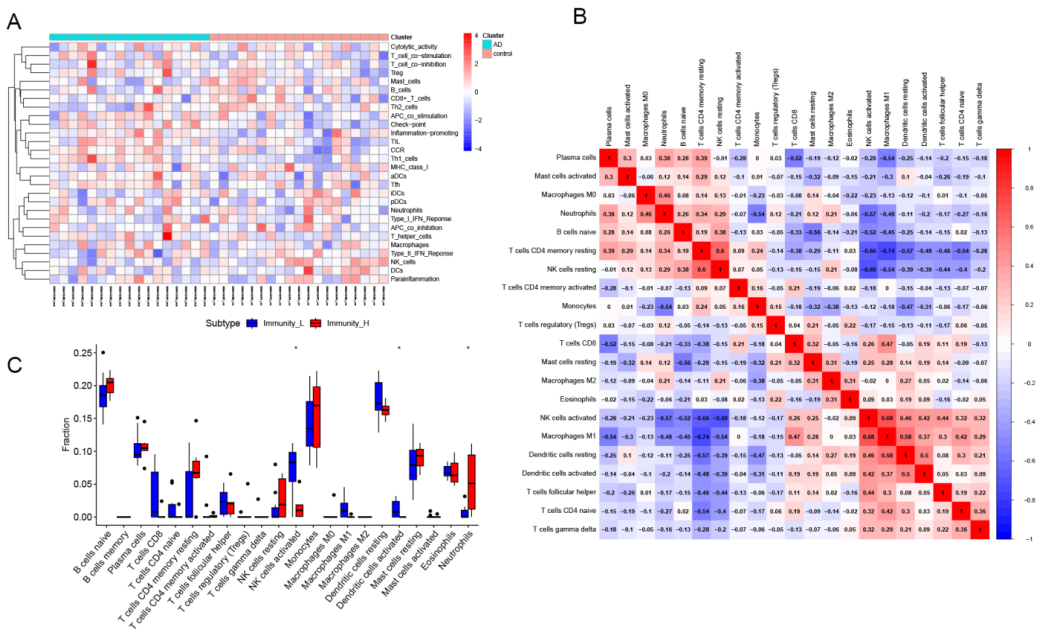

Figure 4. Immune infiltration analysis. (A) Heat map of immune cell infiltration by ssGSEA in AD healthy and diseased groups. (B) Heat map of immune cell correlation matrix in the AD disease group, with red indicating positive correlation and blue indicating negative correlation. (C) Box plot of differential expression of immune cell infiltration of *INPP5D*.

in the estrogen milieu may increase women's susceptibility to AD.[13-15]

In the 1990s, HRT attracted widespread attention as a neuroprotective treatment. This interest has prompted researchers to further explore the mechanism of action of estrogen at the laboratory level and has led to the development of many clinical trials to confirm relevant theoretical hypotheses.[16] A meta-analysis of studies from that period revealed that HRT can improve cognitive function in female patients with AD.[17] This result provides strong evidence support for the positive impact of HRT in maintaining cognitive function.[5]

The Women's Health Initiative Memory Study (WHIMS) is a randomized controlled trial enrolling women over 65 who are not showing

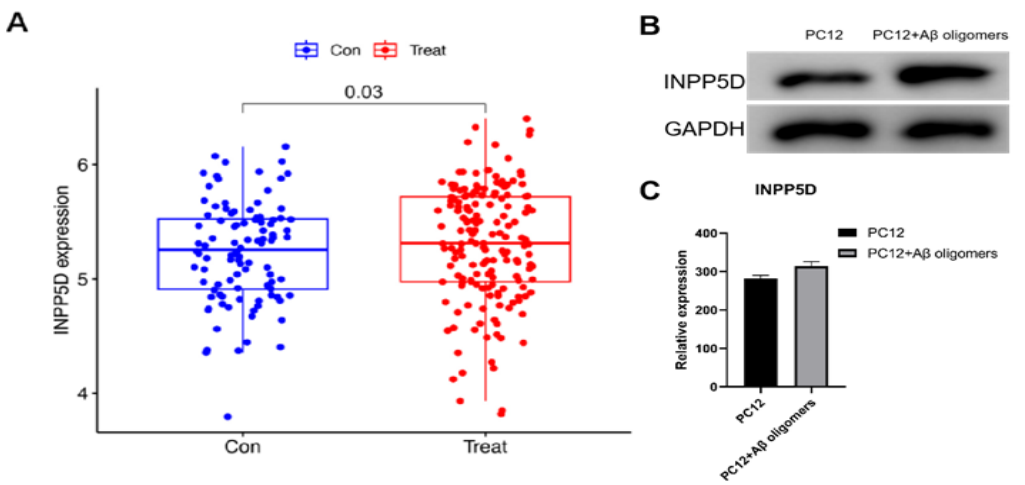

Figure 5. Verification of INPP5D expression. (A) Expression of INPP5D in dataset GSE118553. (B) Western blot detection of INPP5D protein expression in the AD cell model. (C) Bar chart of INPP5D protein expression in the AD cell model.

symptoms of dementia to explore the potential effects of HRT on dementia and cognitive function.[18] In this study, 4532 participants were randomly assigned to receive continuous combined HRT or placebo, while 2974 women who had undergone hysterectomy were assigned to continuous unopposed HRT or placebo. After 4 years of observation, the results showed that in the group receiving HRT, the risk of dementia diagnosis increased twice, and cognitive decline was more severe than in the control group.[19,20] The findings of WHIMS sparked great controversy, and until now, there is still great uncertainty about whether estrogen has any beneficial effects on cognitive health.

In order to gain a deeper understanding of the link between estrogen and AD, this study selected AD-related data sets that met specific criteria from the GEO public database and carefully analyzed the DEGs in the selected data sets. Through this process, we identified a key gene related to estrogen, inositol polyphosphate-5-phosphatase (*INPP5D*). *INPP5D* may play an important role in the pathophysiological process of AD. It is worth noting that *INPP5D* serves as a direct target of p53, and considering the direct interaction between p53 and the estrogen receptor, the two positively regulate the expression levels of target genes.[21] Therefore, estrogen may regulate *INPP5D* expression through p53.

In the brain, *INPP5D* is mainly expressed in microglia.[22] Recent genetic and proteomic research on AD has pushed microglia to the forefront of research because genetic loci associated with AD risk are heavily concentrated in genes expressed by microglia.[23-25] These genes are valued because of their central role in microglial function. Microglia are resident macrophages in the central nervous system and have become a major participant in the pathogenesis of AD.[26] Microglia play a key role in the pathological process of AD, involving the production of cytokines and the response to Aβ and other neurotoxic substances.[27] A hallmark feature of AD is sustained glial activation and a state of neuroinflammation.[26] This long-term immune activation is caused in part by ineffective clearance of pathological aggregates like Aβ. Microglia will migrate to Aβ-rich plaques, surround and engulf the dynamically released Aβ in the plaques. Consistent with this, an increase in the number of microglia can be observed near Aβ plaques in AD patients.[28,29]

More and more studies have found that the protein SHIP1 encoded by the AD-related gene *INPP5D* plays a key regulatory role in the phagocytosis function and immune response of microglia.[29] This suggests that SHIP1 may play an important role in pathological processes related to Alzheimer's disease. Therefore, current research focuses on the development of small molecule inhibitors targeting SHIP1 protein as potential immunotherapeutic strategies based on AD.[30,31] In addition, SHIP1 has the ability to promote the differentiation of macrophage progenitor cells into M1 macrophages in cancer.[32] In our study, through immune infiltration analysis, we found a positive correlation between NK cells and M1 macrophages. Based on this, it is inferred that the protein SHIP1 encoded by the *INPP5D* gene may promote the increase of M1 macrophages, thereby leading to an increase in the expression of NK cells. This change may further promote the progression of AD by exacerbating the inflammatory response. This has been confirmed in previous studies. Increased expression of *INPP5D* may be a risk factor for AD.[33] In mice with β-amyloidosis, *INPP5D* is up-regulated during Aβ deposition and negatively regulates microglia aggregation to amyloid plaques block.[34] Deletion of SHIP-1 encoded by *INPP5D* leads to significantly enhanced recruitment of microglia to Aβ plaques, changes in microglial gene expression, and significantly improves neuronal health.[27] It has also been shown that *INPP5D* deficiency alleviates amyloid pathology in a mouse model of Alzheimer's disease.[35] This is consistent with our finding that increased expression levels of *INPP5D* were observed in the AD patient population, which may indicate its role as a potential risk factor for the development of AD.

In conclusion, the estrogen-related gene *INPP5D* is a potential biomarker for Alzheimer's disease, providing a new perspective and scientific basis for the development of AD treatment strategies based on hormone replacement therapy.

DISCLOSURE

Data Availability: Additional data to that included in the manuscript can be provided upon request.

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Conflicts of interest: None

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