Epigenetics biomarkers of delirium: immune response, inflammatory response and cholinergic synaptic involvement evidenced by genome-wide DNA methylation analysis of delirious inpatients

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ABSTRACT

Background: The authors previously hypothesized the role of epigenetics in pathophysiology of delirium, and tested DNA methylation (DNAm) change among pro-inflammatory cytokines along with aging in blood, glia and neuron. The authors reported that DNAm level of the *TNF-alpha* decreases along with aging in blood and glia, but not in neuron; however, DNAm differences between delirium cases and non-delirium controls have not been investigated directly. Therefore, in the present study, DNAm differences in blood between delirium patients and controls without delirium were examined.

Methods: A case-control study with 92 subjects was conducted. Whole blood samples were collected and genome-wide DNAm was measured by the Infinium HumanMethylationEPIC BeadChip arrays. The correlation between DNAm levels in the *TNF-alpha* and age, network analysis, and the correlation between age and DNAm age were tested.

Results: Only delirium cases showed 3 CpGs sites in the *TNF-alpha* significantly correlated to age after multiple corrections. A genome-wide significant CpG site near the gene of *LDLRAD4* was identified. In addition, network analysis showed several significant pathways with false discovery rate adjusted p-value < 0.05. The top pathway with GO was immune response, and the second top pathway with KEGG was cholinergic synapse. Although there was no statistically significant difference, DNAm age among non-delirium controls showed “slower aging” compared to delirium cases.

Conclusions: DNAm differences were shown both at gene and network levels between delirium cases and non-delirium controls. This finding indicates that DNAm status in blood has a potential to be used as epigenetic biomarkers for delirium.

INTRODUCTION

Delirium among elderly patients is dangerous and common—it occurs in 15–53% of elderly patients after surgery, and in 70–87% of those in intensive care (1); however, it is underdiagnosed and undertreated (2). Delirium is notorious for its association with a long term cognitive decline (3) and high mortality (4, 5). Given that our society is aging, it is becoming increasingly important to predict which patients are at risk of experiencing delirium. Previous searches for biomarkers have revealed increased serum levels of inflammatory markers (6, 7) and cytokines (8, 9) among elderly patients with delirium. Similarly, studies in animal models have shown that cognitive disturbances (10) in response to exogenous insults increase with age and that cytokine release from microglia play a key role (11). Thus, it is possible that microglia and inflammatory cytokines play a role in the pathogenesis of delirium in humans. However, the mechanisms whereby cytokine release or neuro-inflammation is enhanced with aging remain unclear, and identifying the patients in whom this occurs and optimizing their care will require reliable biomarkers.

Given the fact that aging and inflammation are the key risk factors of delirium, we focused on the fact that DNA methylation (DNAm) changes dynamically over the human lifespan and that epigenetic mechanisms control the expression of genes including those of cytokines. Thus, we hypothesized that epigenetic modifications specific to aging and delirium susceptibility occur in microglia; that similar modifications occur in blood; and that these epigenetic changes enhance reactions to exogenous insult, resulting in increased cytokine expression and delirium susceptibility (12). In fact, no published study has assessed DNAm and its relationship to delirium in humans, especially with genome-wide DNAm investigation.

To support this hypothesis of DNAm change along with aging among pro-inflammatory cytokines, we previously reported that DNAm levels in blood decrease along with aging on the pro-inflammatory cytokine gene *TNF-alpha*, based on 265 participants from the Grady Trauma

Project (GTP) (12). We also showed that expression level of *TNF-alpha* among the same subjects increased with aging (12). This data supports our hypothesis that in pro-inflammatory cytokine genes, DNAm levels decrease along with aging, and expression level increase. In addition, using a unique dataset from our own study comparing DNAm status from neurosurgically resected live human brains followed by fluorescence-activated cell sorting (FACS), we showed that DNAm of the *TNF-alpha* universally decreases with age among the glial (neuronal negative) component, but no such patterns were found among neurons (neuronal positive component) (12). This data also supports our hypothesis that the DNAm in pro-inflammatory genes decrease in glia along with aging, making microglia potentially more prone to express those cytokine genes and to have highghtend inflammatory response when exposed to external stimuli such as surgery or infection leading to delirium.

However, what was lacking in our previous data was that we did not directly test DNAm differences between delirium cases and non-delirium controls. To fill this gap, we conducted the present study to compare DNAm status in blood from hospitalized patients with and without delirium to identify clinically useful epigenetic biomarkers for delirium from blood samples, which are routinely obtained from patients. We used blood for three reasons: 1) the function of monocytes in the blood is similar to that of microglia in that both release cytokines in response to exogenous stimulus, 2) our comparison of DNAm levels in live brain tissue (resected during neurosurgery) to those in blood from same individual collected at the same time point showed a high level of correlation genome-wide (13), and 3) our previous data showed a similar age-associated decrease in DNAm in the pro-inflammatory cytokine gene *TNF-alpha* among glia and blood (12). To accomplish these goals, we conducted the present study investigating DNAm differences in blood between delirium patients and controls without delirium.

To be comprehensive, we employed genome-wide approach using Illumina EPIC array. We first tested the pro-inflammatory cytokine gene, *TNF-alpha*, and its correlations with age between

groups with and without delirium. We also conducted network analysis by using the top differentially methylated CpGs between the two groups. Lastly, we compared DNAm age between the two groups.

METHODS AND MATERIALS

Subjects and Sample Collection

Study participants were co-enrolled when they were enrolled for a separate, ongoing study of delirium. A more detailed overview of study participants' recruitment process has been described previously (14). Briefly, 92 subjects were recruited for this epigenetics study between November 2017 and October 2018 at the University of Iowa Hospitals and Clinics. This study was approved by the University of Iowa's Human Subjects Research Institutional Review Board.

Delirium Status Definition

A more detailed overview of study participants' phenotyping has been described previously (14). Briefly, we screened potential study participants for the presence of delirium by reviewing hospital records and by administering the Confusion Assessment Method for Intensive Care Unit (CAM-ICU) (15), the Delirium Rating Scale - Revised-98 (DRS-R-98) (16), and the Delirium Observation Screening Scale (DOSS) (17). A final decision of delirium category was conducted by a trained psychiatrist (G.S.) with detailed chart review.

Sample Processing and Epigenetics Methods

Written informed consent was obtained, and whole blood samples were collected in EDTA tubes. All samples were stored at −80 °C. Methylome assays were performed as previously described (13). Briefly, genomic DNA was isolated from whole blood with the MasterPure™ DNA Purification kit (Epicentre, MCD85201). Genomic DNA was bisulfite-converted with the EZ DNA Methylation™ Kit (Zymo Research, D5002). DNAm of 93 samples (two samples were from one subject) was analyzed with the Infinium HumanMethylationEPIC BeadChip™ Kit (Illumina, WG-317-1002). Raw data was processed with the R packages ChAMP (18) and minfi (19, 20). One sample was filtered out because it had > 0.1 of CpG sites with detection *p*-values > 0.01,

and CpG sites were filtered out as described below with ChAMP. As a result, 92 samples and 701,196 probes remained. Then, quality control and exploratory analysis were conducted. The density and multidimensional scaling plots showed 5 outliers. Two of them were the duplicate samples from one subject. The remaining 87 samples were processed again using ChAMP. The probes were filtered out if they 1) had a detection *p*-value > 0.01 (12,903 probes), 2) had less than 3 beads in at least 5% of samples per probe (8,280 probes), 3) had non-CpG probes (2,911 probes), 4) had SNP-related probes (21) (94,425 probes), 5) had multi-hit probes (22) (11 probes), and 6) were located in chromosomes X or Y (16,356 probes). Eighty-seven samples and 731,032 probes remained by filtering. Beta mixture quantile dilation (23) was used to normalize samples. Batch effect was corrected with the Combat normalization method (24) as implanted in the package SVA (25).

Statistical Analysis

All statistical analyses were performed using R (26). Correlations between aging and DNAm levels of *TNF-alpha* at each CpGs were calculated with Pearson's correlation analysis. The categorical data was calculated with chi-square test. DNAm age was calculated using the online DNAm Age Calculator (https://dnamage.genetics.ucla.edu/) (27) after filtering and the quality control process. Cell type proportions of CD8 T cells, CD4 T cells, natural killer cells, B cells, monocytes, and granulocytes were estimated also using the online DNAm Age Calculator (https://dnamage.genetics.ucla.edu/) (27) by using the method described in the previous study (28). Differential DNA methylation at the level of individual CpGs was analyzed by RnBeads using the limma method (29, 30). Age, gender, and cell type proportions were included as covariates. Network analysis was conducted using the R package missMethyl (31) by correcting different numbers of probes per gene on the array for Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis.

RESULTS

Study Subjects Demographics

43 delirium case and 44 non-delirium control subjects were enrolled (age average 70.2 years, SD 10.2, range 42–101 years). Age and gender proportions were not statistically different between cases and controls. The total scores of the DRS-R-98 and DOSS were significantly higher in delirium cases than in non-delirium controls (Supplementary Table 1).

Comparison of Delirium Cases vs Non-delirium Controls in the *TNF-alpha* Gene

Whole blood samples from 87 samples (43 delirium and 44 non-delirium, age range 42–101 years) after filtering were analyzed using the EPIC array for genome-wide DNAm analysis. There were no significant differences in CD8 T cells, natural killer cells, and B cells between delirium cases and non-delirium controls (Supplementary Table 1). Among them, first, we specifically tested correlation between age and DNAm levels at 24 CpGs in the *TNF-alpha* gene tested on the Illumina EPIC array as shown in Table 1.Delirium cases showed 3 significant CpGs after correction for multiple testing level ($p < 0.05/24 = 0.00208$), whereas non-delirium controls showed no significant CpGs after correction for multiple testing level.

Network Analysis

Next, we directly tested genome-wide DNAm differences between delirium cases and non-delirium controls. Volcano plots for the distribution of individual CpG differences and their corresponding logarithmic transformed *p*-values are shown in Figure 1. The top 20 differentially methylated CpGs between delirium cases and non-delirium controls are shown in Supplementary Table 2. Genome-wide analysis showed a top hit at cg21295729 with genome-wide significance ($p = 5.07E-8$). This CpG is located near the gene *LDLRAD4*. Network analysis was conducted by using the CpGs with methylation level differences greater than 5.0% and *p*-

value < 0.0005 (n = 753). By using those genes, network analysis showed the following results of the top 20 pathways with GO (Table 2) and KEGG analysis (Table 3). The top pathways with GO were immune response and myeloid leukocyte activation, and with KEGG were aldosterone synthesis/secretion and cholinergic synapse. Supplementary Table 3 also shows the significant pathways of GO analysis with false discovery rate (FDR)-adjusted *p*-value < 0.05.

Comparison of Delirium Cases vs Non-delirium Controls: DNAm Age

We further tested differences of DNAm age between delirium cases and non-delirium controls. DNAm age showed signficant correlation with chronological age among delirium cases ($r = 0.78$, $p < 0.001$) and non-delirium controls ($r = 0.67$, $p < 0.001$). DNAm age among the non-delirium controls showed “slower aging” compared to the delirium cases, although there was no statistically significant difference (Figure 2).

DISCUSSION

This is the first study comparing epigenetics status, especially genome-wide DNAm, between patients with and without delirium. The data presented here is consistent with our hypothesis that decreased levels of DNAm on pro-inflammatory cytokines along with aging can lead to hightened inflammation associated with delirium, whereas in patients without delirium DNAm levels remain high, and thus inflammatory reaction could be suppressed and they are protected against delirium.

In the present study, only delirium cases showed significant CpGs with multiple comparison adjusted level between age and decreasing level of *TNF-alpha* DNAm. This result is consistent with our hypothesis that the DNAm level of pro-inflammatory genes decrease along with aging in delirium patients. We speculate that delirium patients may have persistent decline of DNAm levels in the *TNF-alpha* gene along with aging, and this may have led to onset of delirium with an additional medical condition, requiring them to be admitted to a hospital. On the other hand, patients without delirium had less decline of DNAm levels in the *TNF-alpha* gene with aging, thus it might have protected them from developing delirium. To confirm this speculation, we need to conduct a large, prospective study comparing patient population as similar as possible in terms of their medication conditions. Studying surgical patients where you can collect samples before their exposure to surgery would be idealy for such investigation.

From genome-wide DNAm analysis, one genome-wide significant signal in the gene of *LDLRAD4* was identified. *LDLRAD4*—low-density lipoprotein receptor class A domain containing 4—functions as a negative regulator of *TGF-beta* signaling that regulates the growth, differentiation, apoptosis, motility, and matrix protein production of a lot of cell types (32). Although it was not genome-wide significant, one of the top hit CpGs was near the gene *DAPK1*. *DAPK1*—death associated protein kinase 1—functions as regulating apotosis, autophagy and inflammation (33). It is reported that *DAPK1* levels are induced by *TNF-alpha* and *interferon-*

gamma (34). The other top hit CpG was near the gene *IRF8*. *IRF8*—interferon regulatory factor 8—functions as a modulating of the immune response, cell growth, and oncogenesis (35). It is reported that *IRF8* has an important role as a regulator of reactive microglia (36).

Although a potential role of these specific genes in pathophysiology of delirium requires further investigation, network analysis identified several top pathways relevant to neurofunction and inflammatory/immune processes, including immune response, leukocyte activation, neutrophil activation, and myeloid cell activation involved in immune response from GO analysis. Many pathways relevant to delirium and neural function were also identified from KEGG analysis, including cholinergic synapse, serotonergic synapse, and leukocyte transendothelial migration. The cholinergic synapse was second from the top KEGG pathways. The cholinergic system is one of the most important neurotransmitter systems in the brain, and deficiency of acetylcholine is well known to be associated with delirium (37, 38). The results of the present study are further supporting the relevance of cholinergic function in potential pathophysiology of delirium. Furthermore, the pathways of positive regulation and regulation of interleukin-10 production, and positive regulation of interleukin-17 production, were significant (FDR-adjusted p-value < 0.05) from GO analysis. This result is also suggesting the role of pro-inflammatory cytokines and neuro-inflammation in pathophysiology of delirium, consistent with our hypothesis. These findings may support the validity of this epigenetic investigation of delirium pathophysiology.

We showed that DNAm age was significantly correlated with chronological age in both delirium cases and non-delirium controls. However, non-delirium cases showed relatively slower progression of DNAm aging along with chronological aging than delirium cases. DNAm age measures the accumulative effect of an epigenetic maintenance system (27) and predicts mortality (39, 40). We speculate that non-delirium controls may have a protective mechanism against DNAm aging, which can also prevent developing delirium. With a larger sample size study we can test if DNAm aging is in fact different between delirium cases versus controls.

There are several limitations in our study. First, the sample size is relatively small. To overcome this limitation, we need to increase the sample size in the future. Second, medical conditions required them to be hospitalized were diverse among study subjects including in the present study. As mentioned previously, comparing those with and without delirium after same type of surgery (post-operative delirium) would help minimize confounding factors. However, even with these limitation, the presented data showed supporting evidence of epigenetic differences in the pro-inflammatory cytokine gene *TNF-alpha* and in the immune/inflammatory response network and cholinergic system. Lastly, we used only blood samples and did not investigate brain tissues directly. However, there is a significant correlation between brain tissue and blood in DNAm levels, as shown in our previous study (13). Also, as the goal of our study is to identify potentially clinically useful biomarkers, we believe that investigating DNAm differences in blood associated with delirium is important to improve our future clinical practice.

In conclusion, to the best of our knowledge, this is the first epigenetics study of delirium. The DNAm was investigated genome-wide. The results were consistent with our previous work and hypothesis (12). Despite these limitations mentioned above, we showed evidence of epigenetic differences both at gene levels and network levels between delirium cases and non-delirium controls. This finding indicates that DNAm status in blood may become a useful epigenetic biomarker for delirium.

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DISCLOSURES

Dr. Shinozaki G is co-founder of Predelix Medical LLC, and reports U.S. Provisional Patent Application No. 62/731599, titled “Epigenetic biomarker of delirium risk.” The other authors report no biomedical financial interests or potential conflicts of interest.

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Figure legends

Figure 1 Volcano plots for the distribution of individual CpG differences and their corresponding logarithmic transformed *p*-values (n = 87)

Figure 2 Correlation between chronological age and DNAm age in delirium (n = 43) vs controls (n = 44)

Abbreviation: DNAm; DNA methylation.

Table 1: Correlation of age and blood DNAm at 24 CpGs in the *TNF-alpha* gene compared between delirium cases vs non-delirium controls

Gene	Delirium Cases (N=43)			Non Delirium Controls (N=44)		
	CpG	*r*	*p*-value	CpG	*r*	*p*-value
TNF-alpha	cg26729380*	-0.53	2.33E-04	cg12681001	-0.36	0.017
	cg10650821*	-0.53	2.58E-04	cg10650821	-0.32	0.036
	cg04425624*	-0.51	4.34E-04	cg21467614	-0.31	0.040
	cg08553327	-0.46	0.002	cg19648923	-0.30	0.047
	cg21467614	-0.40	0.007	cg04425624	-0.28	0.064
	cg21222743	-0.38	0.011	cg10717214	-0.28	0.067
	cg12681001	-0.38	0.013	cg26729380	-0.27	0.072
	cg10717214	-0.38	0.013	cg01569083	-0.23	0.130
	cg01569083	-0.25	0.102	cg19124225	-0.17	0.264
	cg03037030	-0.25	0.109	cg21222743	-0.17	0.269
	cg19648923	-0.19	0.233	cg08553327	-0.16	0.285
	cg21370522	-0.13	0.424	cg21370522	-0.14	0.374
	cg06825478	-0.11	0.464	cg26736341	-0.13	0.405
	cg02137984	-0.09	0.582	cg23384708	-0.10	0.504
	cg04472685	-0.09	0.587	cg19978379	-0.08	0.584
	cg15989608	-0.06	0.708	cg15989608	-0.08	0.623
	cg23384708	-0.04	0.801	cg03037030	-0.08	0.628
	cg20477259	-0.03	0.830	cg01360627	-0.07	0.645
	cg01360627	-0.03	0.857	cg06825478	-0.05	0.724
	cg26736341	-0.02	0.910	cg20477259	-0.02	0.920

	cg19978379	-0.01	0.945	cg24452282	0.00	0.998
	cg24452282	0.03	0.835	cg02137984	0.02	0.899
	cg08639424	0.04	0.812	cg04472685	0.02	0.891
	cg19124225	0.08	0.617	cg08639424	0.08	0.627

Notes: Pink highlights a negative correlation, and blue highlights a positive correlation.

*Significant after correction for multiple testing level ($p < 0.05/24$)

Table 2: Result of the top 20 pathways of GO analysis with differentially methylated CpGs between delirium cases and non-delirium controls

Term	Ont	N	DE	*p* value	FDR
immune response	BP	1925	82	3.99E-10	7.11E-06
myeloid leukocyte activation	BP	635	41	6.24E-10	7.11E-06
cell activation involved in immune response	BP	698	42	1.60E-09	1.21E-05
leukocyte activation involved in immune response	BP	694	41	3.60E-09	2.05E-05
cell activation	BP	1331	65	8.84E-09	4.03E-05
neutrophil activation	BP	496	33	1.13E-08	4.28E-05
granulocyte activation	BP	503	33	1.40E-08	4.56E-05
leukocyte activation	BP	1181	58	2.17E-08	6.18E-05
neutrophil degranulation	BP	483	32	2.57E-08	6.31E-05
neutrophil activation involved in immune response	BP	486	32	2.77E-08	6.31E-05
myeloid cell activation involved in immune response	BP	538	34	3.11E-08	6.44E-05
cytoplasmic vesicle	CC	2261	96	4.05E-08	7.13E-05
intracellular vesicle	CC	2264	96	4.21E-08	7.13E-05

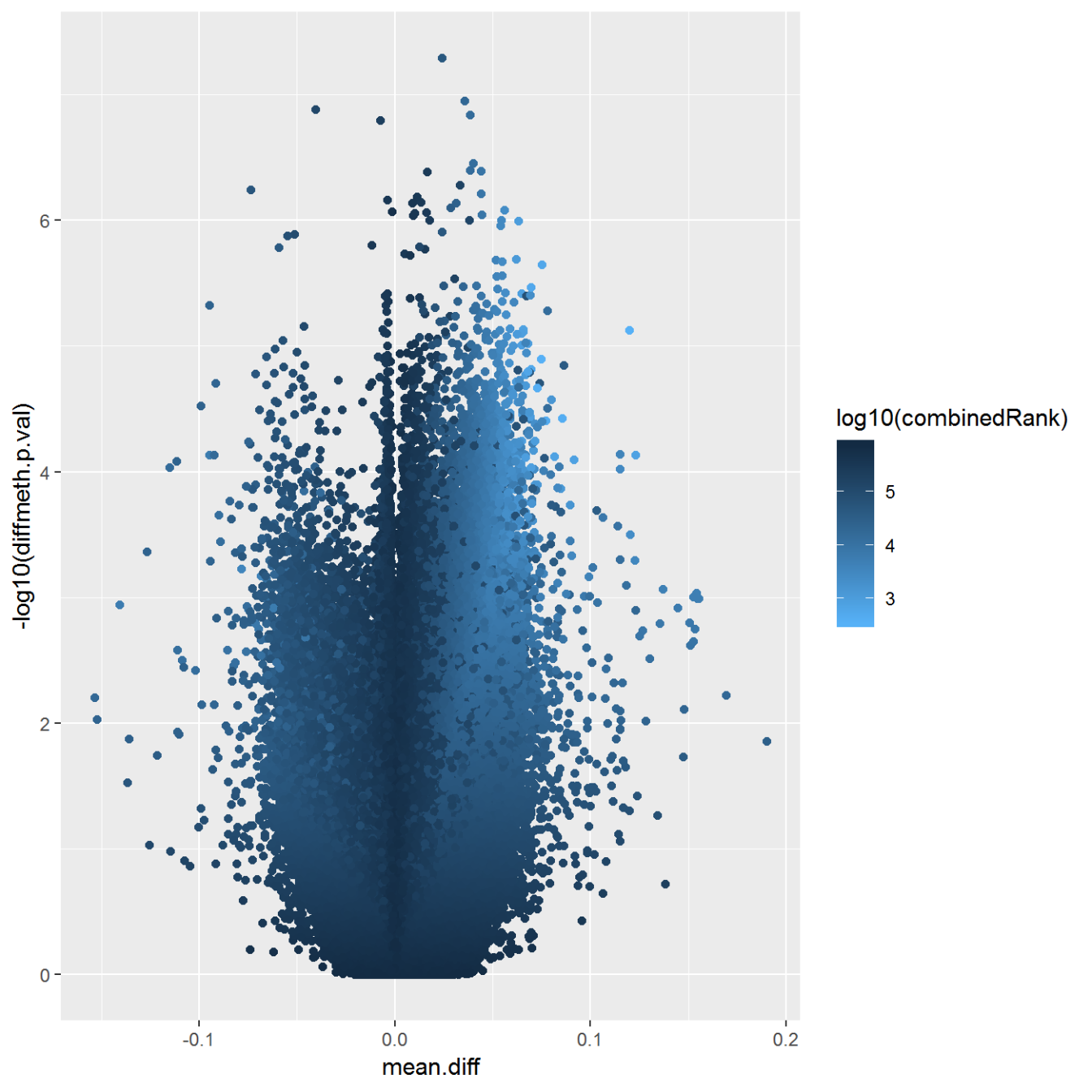
neutrophil mediated immunity	BP	497	32	4.39E-08	7.13E-05
leukocyte degranulation	BP	529	32	2.13E-07	2.93E-04
immune system process	BP	2807	103	2.17E-07	2.93E-04
tertiary granule	CC	164	16	2.29E-07	2.93E-04
immune effector process	BP	1139	52	2.45E-07	2.93E-04
cytoplasmic vesicle part	CC	1462	67	2.49E-07	2.93E-04
leukocyte mediated immunity	BP	762	39	2.58E-07	2.93E-04

Abbreviations: GO; Gene Ontology, FDR; false discovery rate.

Table 3: Result of the top 20 pathways of KEGG analysis with differentially methylated CpGs between delirium cases and non-delirium controls

Pathway	N	DE	*p* value	FDR
Aldosterone synthesis and secretion	98	12	1.58E-04	0.038
Cholinergic synapse	112	13	2.28E-04	0.038
Long-term depression	60	8	0.001	0.086
Apelin signaling pathway	137	12	0.001	0.086
Parathyroid hormone synthesis, secretion and action	106	11	0.001	0.086
Pantothenate and CoA biosynthesis	19	4	0.002	0.088
Circadian entrainment	97	10	0.003	0.123
Fc gamma R-mediated phagocytosis	90	9	0.003	0.123
Gastric acid secretion	75	8	0.004	0.123
Melanogenesis	101	9	0.004	0.123
Sphingolipid signaling pathway	118	10	0.004	0.123
Serotonergic synapse	113	9	0.005	0.130
Adrenergic signaling in cardiomyocytes	144	11	0.007	0.166
Retrograde endocannabinoid signaling	141	10	0.007	0.166
Amoebiasis	94	8	0.008	0.166
Gap junction	88	8	0.008	0.167
GnRH signaling pathway	93	8	0.010	0.179
Pathogenic Escherichia coli infection	55	5	0.010	0.179
Leukocyte transendothelial migration	111	8	0.011	0.179
Cushing syndrome	155	11	0.011	0.179

Abbreviations: KEGG; Kyoto Encyclopedia of Genes and Genomes, FDR; false discovery rate.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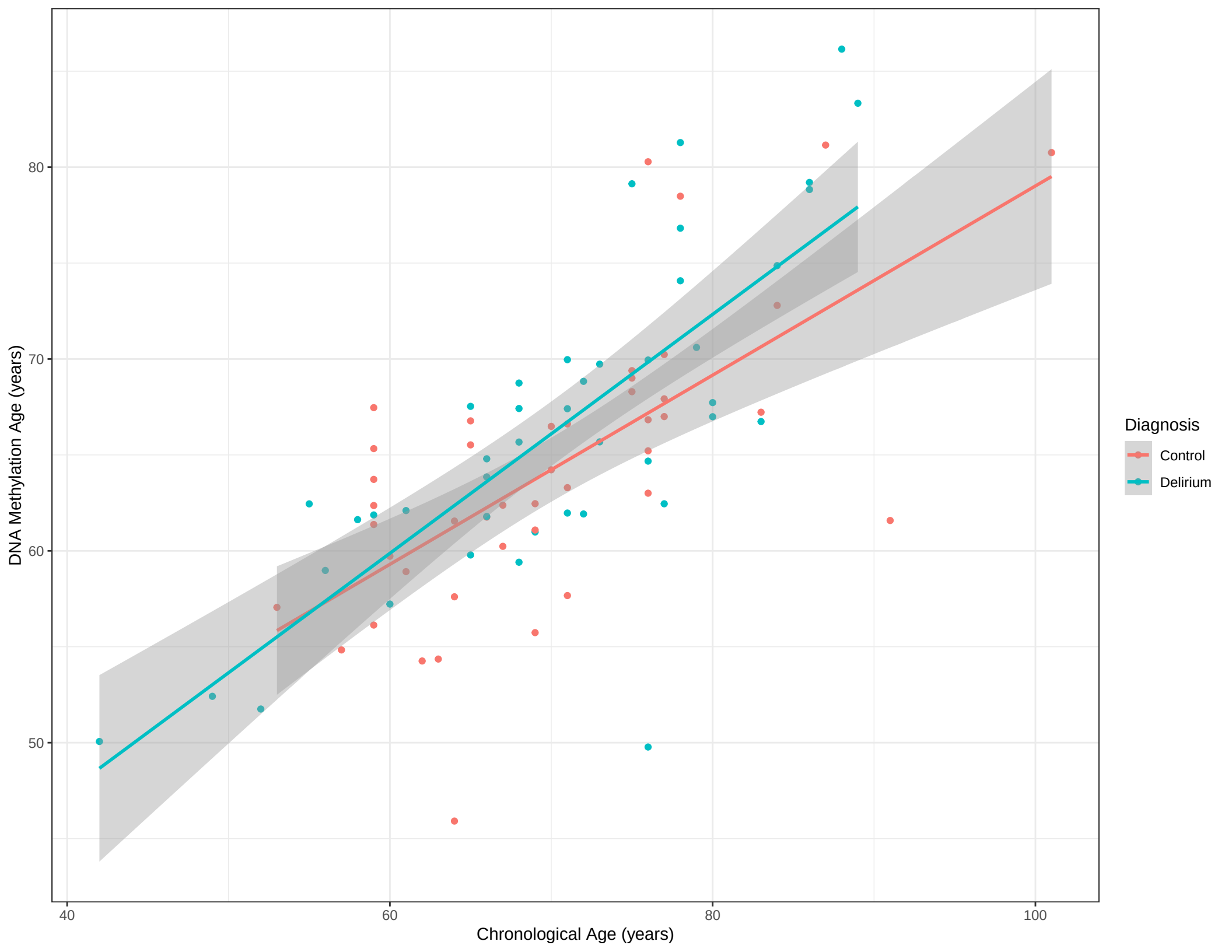
6
4
2
0
-0.1
0.0
0.1
0.2
mean.diff
log10(combinedRank)
5
4
3


DNA Methylation Age (years)
80
70
60
50
40
60
80
100
Chronological Age (years)
Diagnosis
Control
Delirium