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Biological aging analysis based on DNA methylation status for social anxiety disorder

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Abstract

Aim: Social anxiety disorder (SAD) is a common disorder characterized by excessive fear of scrutiny and embarrassment, leading to severe distress and avoidance behaviors or dysfunctions. SAD and other relevant diseases have been reported to be associated with a higher risk of aging-related diseases, such as Alzheimer's disease, Parkinson's disease, and diabetes mellitus. Recently, epigenetic clock analysis, which measures biological aging based on comprehensive DNA methylation (DNAm) status, has been widely conducted. We conducted epigenetic clock analyses in patients with SAD and controls, examining various epigenetic age acceleration and DNAm-based predictive values of aging-related proteins (GrimAge components and GrimAge2 components), including leptin level.

Methods: We used the publicly available DNAm dataset, GSE164056, which consists of 66 patients with SAD and 77 controls of Caucasian descent aged between 18 and 50 years. We conducted regression analyses investigating the association between SAD and various indices of epigenetic aging, using age and sex as covariates.

Results: None of the epigenetic clocks showed significant differences in age acceleration. Of the DNAm-based predictive values of aging-related proteins, leptin level in GrimAge components ($q = 0.0123$) and GrimAge2 components ($q = 0.0123$) were significantly lower in patients with SAD than in controls.

Conclusions: The results of this study suggested that leptin may be involved in SAD pathogenesis as an aging-related protein. Therefore, further studies with different designs are required.

KEYWORDS
aging, anxiety, hypothalamus, leptin, methylation

Nobuhiko Noguchi and Toshiyuki Shirai have equally contributed to this work.

1 | INTRODUCTION

Social anxiety disorder (SAD) is characterized by excessive fears of scrutiny, embarrassment, and humiliation in social situations, leading to severe distress and avoidance behaviors or dysfunctions. It is a common disorder that is estimated to occur in 3–7% of the adult population in the U.S. each year. SAD usually develops during childhood or adolescence and can subsequently lead to major depression, extensive dysfunction, and poor quality of life, especially if left untreated.[1] Both genetic and environmental factors have been reported to be implicated in the pathogenesis of SAD.[2] Functional neural circuits involving neurohormones and neurotransmitter systems, such as dopamine, glutamate, and oxytocin, as well as the amygdala, insular cortex, and prefrontal cortex, have been reported to be implicated in the pathogenesis of SAD.[3–6] Hypothalamic–pituitary axis (HPA) reactivity has also been suggested to be involved and is reported to influence avoidance behavior.[5] The average age at onset of SAD is younger, and the morbidity period is often longer.

Recently, aging research and the potential effects of epigenetic changes on aging through DNA methylation (DNAm) at cytosine-phosphate-guanine (CpG) sites have gained attention.[7,8] Several "epigenetic clocks" have been developed to predict biological aging based on DNAm patterns.[9–13] Estimates of epigenetic age acceleration have been associated with psychiatric disorders,[14–18] neurodegenerative disorders,[19,20] and all-cause mortality.[10]

The first generation of epigenetic clocks, including HorvathAge (based on 353 CpG sites),[21] HannumAge (based on 71 CpG sites),[9] and SkinBloodAge (based on 391 CpG sites),[22] were developed to predict chronological age itself. PhenoAge, developed by Levine et al.[11] based on 513 CpG sites, is associated with various aging outcomes, including all-cause mortality, cancer, physical functioning, coronary heart disease, and Alzheimer's disease.

GrimAge, developed by Lu et al.[12] based on 1030 CpG sites, incorporates several compositions, based on chronological age, sex, seven DNAm-based predictive values of aging-related plasma proteins' levels (adrenomedullin [ADM], beta-2-microglobulin [B2M], Cystatin C, growth differentiation factor-15 [GDF15], leptin, plasminogen activation inhibitor-1 [PAI1], and tissue inhibitor of metalloproteinases-1 [TIMP1]), and DNAm-based smoking pack-years (DNAmPACKYRS). More recently, Lu et al.[13] developed GrimAge2, an enhanced version of GrimAge, by incorporating hemoglobin A1c and C-reactive protein levels into its components. These components are called GrimAge components and GrimAge2 components. For instance, leptin, one of the proteins comprising these components, plays a protective role against aging-related diseases such as metabolic syndrome, diabetes, and cardiovascular disease.[23]

DNAm-based telomere length (DNAmTL) was developed by Lu et al.[24] based on 140 CpG sites and evaluates telomere lengths. DunedinPACE, developed based on 173 CpG sites, is a measure to quantify changes in the pace of biological aging according to the prediction of several physical function biomarkers.[25] Each algorithm differs in the type of epigenetic data on which it is trained. These estimators capture certain biological aspects of aging and have been successfully applied in the study of various psychiatric disorders.[26]

Social anxiety disorder is closely associated with highly neurotic personality traits.[27,28] Neuroticism has been reported to increase the risk of future occurrence of aging-related diseases, such as Alzheimer's disease and Parkinson's disease.[29,30] Young cancer survivors are more likely to develop SAD.[31,32] Generalized anxiety disorder, which is categorized as the same type of neurosis as SAD, has been reported to be associated with a higher risk of aging-related diseases, such as diabetes mellitus and stroke.[33] Social anxiety is also frequently comorbid with major depressive disorder (MDD),[34] and we have previously found abnormalities in several epigenetic age acceleration and GrimAge components in patients with MDD.[17] Furthermore, social anxiety, especially among adolescents, increases the risk of suicide attempts, suicidal ideation, and suicide deaths.[35] Regarding suicide, we previously found significant shortening of telomere length, an indicator of aging, in suicide decedents.[36] Like these, SAD and various concomitant symptoms are reportedly associated with aging. To our knowledge, no study has performed an epigenetic clock analysis of SAD. We conducted biological age analyses based on the DNAm status of patients with SAD and healthy controls to investigate the association between this disease and aging from an epigenetic perspective. Additionally, we searched for specific findings in SAD by comparing our results of previous epigenetic clock study on MDD, which is often comorbid with SAD.

2 | METHODS

2.1 | Publicly available DNAm dataset (GSE164056)

We used publicly available DNAm data from the Gene Expression Omnibus database. The GSE164056 dataset was generated by Wiegand et al. and consists of 66 patients with SAD and 77 controls of Caucasian descent between the ages of 18 and 50 years (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE164056, last accessed on December 8, 2023). All participants were assessed with the Structured Clinical Interview for DSM 4th edition.[37] The severity of social anxiety was evaluated using the Liebowitz Social Anxiety Scale (LSAS).[38] All of the participants provided written informed consent, which was approved by the University of Tübingen local ethics committee in accordance with the Declaration of Helsinki. DNA was extracted using the QIAamp Blood Maxi Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Genomic DNA was bisulfite-converted using the EZ DNA Methylation™ Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. DNAm was determined using the Infinium MethylationEPIC Kit (Illumina, San Diego, CA, USA).

2.2 | Evaluation of epigenetic age acceleration and DNAm-based aging-predictive factors

As epigenetic aging evaluation, six epigenetic clocks (HorvathAge, HannumAge, SkinBloodAge, PhenoAge, GrimAge, and GrimAge2),

DNAmTL, GrimAge components, and GrimAge2 components were calculated using an online DNAm age calculator (https://horvath.genetics.ucla.edu/html/dnamage/ last accessed on July 23, 2024).[12,13,21] Furthermore, we calculated epigenetic age acceleration. AgeAccelHorvath, AgeAccelHannum, AgeAccelSkinBlood, AgeAccelPheno, AgeAccelGrim, and AgeAccelGrim2 were defined as the residual from regressing each DNAmAge on the chronological age. Positive and negative values indicated whether the epigenetic age is higher or lower than the expected age (based on chronological age), respectively. The age-adjusted estimate of DNAmTL (DNAmTLadjAge) was defined as the residual calculated by regressing DNAmTL on chronological age. Positive and negative values indicated whether DNAmTLadjAge was longer or shorter than the expected DNAmTL (based on chronological age), respectively. We calculated DunedinPACE with R package DunedinPACE. In addition, we could calculate predicted values for multiple cell compositions (CD8+ T cell, CD4+ T cell, Natural killer cell, B cell, Monocyte, Granulocyte, and PlasmaBlast) by using the Houseman method[21,39] in this calculator.

2.3 | Statistical analysis

Statistical analyses were performed using R version 4.3.1 (R Development Core Team, Vienna, Austria). In the demographic data, continuous variables were compared with Mann–Whitney U test and categorical variables with Fisher's exact test between patients and controls. The correlation between chronological age and DNAmAge was tested using the Spearman correlation analysis. For each epigenetic age acceleration, GrimAge component, and GrimAge2 component, the association with phenotype (SAD or control) was tested using multiple regression analyses with age and sex as confounders. In addition, as additional analyses, since cell compositions[40] and smoking status[41,42] could also affect DNA methylation, we also conducted multiple regression analyses, including DNAmPACKYRS, a predictor of cumulative smoking, and multiple cell compositions (CD8+ T cell, CD4+ T cell, Natural killer cell, B cell, Monocyte, Granulocyte, and Plasmablast) as additional confounding factors. Also, we compared the results of this analyses for SAD and the results of our previous epigenetic clock analyses for Caucasian patients with MDD, which has a high comorbidity rate with SAD.[34] We used the cohorts of GSE125105 dataset (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE125105, last accessed on July 23, 2024)[43,44] consisting of patients with MDD and controls and having been used in our previous analyses for MDD.[18] Furthermore, among patients with SAD, the association between the LSAS score and each epigenetic age acceleration, GrimAge component, and GrimAge2 component was tested using multiple regression analysis, adjusting for age and sex as confounding factors. For multiple testing, we calculated q values by adjusting the p values using the Benjamini Hochberg method. Statistical significance was defined as $q < 0.05$. Dummy variables for phenotype and sex were assigned as follows: sex: male = 0, female = 1; phenotype: control = 0, SAD = 1.

3 | RESULTS

There were no significant differences in age or sex between the patients with SAD and controls. The LSAS scores were significantly higher in patients with SAD than in controls ($p = 1.40 \times 10^{-22}$) (Table 1). We could obtain the predicted values of ADM, B2M, Cystatin C, GDF15, Leptin, PAI1, and TIMP1 levels, and DNAmPACKYRS as GrimAge components and the predicted values of ADM, Cystatin C, GDF15, Leptin, PAI1, and TIMP1 levels as GrimAge2 components. To be consistent with the notation in the online calculator, the components of GrimAge are denoted as DNAmADM, DNAmB2M, DNAmCystatinC, DNAmGDF15, DNAmLeptin, DNAmPAI1, DNAmTIMP1 and DNAmPACKYRS, and the components of GrimAge2 are denoted as DNAmadm, DNAmCystatin_C, DNAmGDF_15, DNAmleptin, DNAmpai_1, and DNAmTIMP_1. Demographic data, epigenetic clock, DNAmTL, epigenetic age acceleration, GrimAge components, and GrimAge2 components are listed in Table 1.

To confirm the suitability of the epigenetic clocks themselves for the data of this cohort, we found significant correlations between chronological age and each epigenetic clock and DNAmTL in the cohort (HorvathAge: $rho = 0.808$, $\mathbf{q = 3.99 \times 10^{-34}}$; HannumAge: rho = 0.855, $\mathbf{q = 7.51 \times 10^{-42}}$; SkinBloodAge: rho = 0.890, $\mathbf{q = 1.93 \times 10^{-49}}$; PhenoAge: rho = 0.789, $\mathbf{q = 1.66 \times 10^{-31}}$; GrimAge: rho = 0.907, $\mathbf{q = 3.16 \times 10^{-54}}$; GrimAge2: rho = 0.863, $\mathbf{q = 2.94 \times 10^{-43}}$; DNAmTL: rho = −0.668, $\mathbf{q = 7.98 \times 10^{-20}}$) (Figure S1).

Regarding epigenetic age acceleration, none of the clocks showed a significant association with SAD in multiple regression analysis using age and sex as confounders (AgeAccelHorvath: $q = 0.889$; AgeAccelHannum: $q = 0.649$; AgeAccelSkinBlood: $q = 0.770$; AgeAccelPheno: $q = 0.999$; AgeAccelGrim: $q = 0.770$; AgeAccelGrim2: $q = 0.585$; DNAmTLAdjAge: $q = 0.532$; DunedinPACE: $q = 0.999$) (Table 2 and Figure 1). Of the GrimAge components (DNAmADM: $q = 0.192$; DNAmB2M: $q = 0.404$; DNAmCystatinC: $q = 0.248$; DNAmGDF15: $q = 0.592$; DNAmLeptin: $\mathbf{q = 0.0123}$; DNAmPAI1: $q = 0.184$; DNAmTIMP1: $q = 0.192$; DNAmPACKYRS: $q = 0.192$) and GrimAge2 components (DNAmadm: $q = 0.248$; DNAmCystatin_C: $q = 0.413$; DNAmGDF_15: $q = 0.889$; DNAmleptin: $\mathbf{q = 0.0123}$; DNAmpai_1: $q = 0.192$; DNAmTIMP_1: $q = 0.446$), DNAmLeptin, one of the GrimAge components, and DNAmleptin, one of the GrimAge2 components showed significant associations with SAD (Table 2 and Figure 2).

In our previous study, which conducted epigenetic clock analyses for MDD, prediction values of Cystatin C levels in GrimAge components and GrimAge2 components showed significant association with MDD in GSE125105 cohort. However, DNAmLeptin in GrimAge or DNAmleptin in GrimAge2 did not significantly associate with MDD in this cohort (Table S1).[18]

As additional regression analyses, we included DNAmPACKYRS and multiple cell compositions (CD8+ T cell, CD4+ T cell, Natural killer cell, B cell, Monocyte, Granulocyte, and Plasmablast) as additional confounding factors other than age and sex. As results, no epigenetic age acceleration showed significant association, but DNAmLeptin ($\mathbf{q = 0.0169}$) and DNAmPAI ($\mathbf{q = 0.048}$) in GrimAge components, and

TABLE 1 The demographic data for patients and controls.

	Controls	Patients	*p* Value
	n = 77	*n* = 66	
Age, years [IQR]	24 [22–28]	24 [20–28.75]	0.434*
Male, *n* (%)	29 (37.7)	20 (30.3)	0.328**
Male	*n* = 29	*n* = 20	
Age, years [IQR]	26 [24–28]	27.5 [21.75–31.25]	0.669*
Female	*n* = 48	*n* = 46	
Age, years [IQR]	24 [21–27]	23 [20–26.75]	0.468*
LSAS	10 [4–19]	72 [47.75–91.75]	1.40×10^{-22}*
Epigenetic Clock			
HorvathAge, median [IQR]	36.02 [32.23–38.68]	35.03 [31.40–39.89]	
HannumAge, median [IQR]	19.5 [16.10–22.06]	18.68 [16.19–21.91]	
SkinBloodAge, median [IQR]	26.04 [21.90–28.87]	24.35 [20.60–29.18]	
PhenoAge, median [IQR]	−51.46 [−54.98 – −47.79]	−51.62 [−55.37 – −47.34]	
GrimAge, median [IQR]	158.3 [155.3–161.6]	156.7 [155.1–160.9]	
GrimAge2, median [IQR]	123.7 [121.3–126.9]	122.9 [120.9–126.5]	
DNAmTL, median [IQR]	7.996 [7.896–8.111]	8.029 [7.910–8.118]	
Age acceleration			
AgeAccelHorvath, median [IQR]	0.1624 [−2.5117–2.5630]	−0.7956 [−2.8512–2.4867]	
AgeAccelHannum, median [IQR]	0.1228 [−1.5849–1.7260]	−0.2395 [−1.6476–1.4322]	
AgeAccelSkinBlood, median [IQR]	0.5905 [−1.8190–1.8554]	−1.0208 [−1.8787–1.7839]	
AgeAccelPheno, median [IQR]	−0.2437 [−2.1032–2.3366]	0.4141 [−2.92393–2.19582]	
AgeAccelGrim, median [IQR]	0.1317 [−1.1595–1.0166]	−0.2829 [−1.2980–1.0362]	
AgeAccelGrim2, median [IQR]	−0.3206 [−1.0402–1.0509]	0.02259 [−1.73342–1.09955]	
DNAmTLAdjAge, median [IQR]	−0.008025 [−0.0933831–0.0838027]	0.0073217 [−0.0733179–0.0666366]	
DunedinPACE, median [IQR]	0.8903 [0.8470–0.9544]	0.8955 [0.8365–0.9479]	
GrimAge component			
DNAmADM, median [IQR]	894.3 [889.3–900.9]	895.1 [887.8–900.3]	
DNAmB2M, median [IQR]	4 925 092 [4890497–4 974 689]	4 923 587 [4867137–4 973 240]	
DNAmCystatinC, median [IQR]	1 120 082 [1110581–1 131 725]	1 112 580 [1106972–1 130 566]	
DNAmGDF15, median [IQR]	2969 [2919–3007]	2961 [2921–3027]	
DNAmLeptin, median [IQR]	110 847 [110402–111 235]	110 483 [110106–110 921]	
DNAmPAI1, median [IQR]	120 673 [119865–121 451]	120 242 [119417–121 111]	
DNAmTIMP1, median [IQR]	33 270 [32891–33 873]	33 091 [32677–33 784]	
DNAmPACKYRS, median [IQR]	101.50 [99.26–103.56]	101.71 [99.47–105.56]	
GrimAge2 component			
DNAmadm, median [IQR]	863.4 [855.2–869.7]	862.5 [855.3–868.7]	
DNAmCystatin_C, median [IQR]	1 108 161 [1097476–1 124 090]	1 100 931 [1093208–1 120 628]	
DNAmGDF_15, median [IQR]	3030 [2961–3070]	3010 [2969–3071]	
DNAmleptin, median [IQR]	104 703 [104232–105 265]	104 503 [103972–104 944]	
DNAmpai_1, median [IQR]	112 086 [111327–113 062]	111 837 [110934–112 571]	
DNAmTIMP_1, median [IQR]	32 483 [31832–32 962]	32 188 [31731–32 920]	

Abbreviations: ADM, adrenomedullin; B2M, beta 2 microglobulin; DNAm, DNA methylation; DNAmPACKYRS, DNA methylation-based smoking pack-years; DNAmTLAdjAge, age-adjusted estimate of DNA methylation-based telomere length; GDF15, growth differentiation factor-15; LSAS, the Liebowitz Social Anxiety Scale; PAI1, plasminogen activation inhibitor-1; TIMP1, tissue inhibitor of metalloproteinases-1.

*The *p* values were calculated with Mann–Whitney *U* test.

**The *p* value was calculated with Fisher's exact test.

TABLE 2 The results of multiple regression analyses for the association with phenotype (patients with social anxiety disorder or controls), with age and sex as confounding factors.

	Coefficients	Standard error	*t* Value	*p* Value	*q* Value[a]
Epigenetic age acceleration					
AgeAccelHorvath	−0.149801	0.617335	−0.24266	0.808628	0.889491
AgeAccelHannum	−0.313635	0.434581	−0.72170	0.471693	0.648578
AgeAccelSkinBlood	−0.247913	0.513304	−0.48297	0.629874	0.769846
AgeAccelPheno	−0.000573	0.661913	−0.00087	0.999310	0.999310
AgeAccelGrim	−0.129518	0.261204	−0.49585	0.620785	0.769846
AgeAccelGrim2	−0.300824	0.336144	−0.89493	0.372374	0.585159
DNAmTLAdjAge	−0.012544	0.012419	−1.01009	0.314210	0.531740
DunedinPACE	−0.000073	0.019572	−0.00372	0.997036	0.999310
GrimAge component					
DNAmADM	−2.363555	1.171159	−2.01813	0.045501	0.191822
DNAmB2M	−10442.57	7812.820	−1.33659	0.183539	0.403785
DNAmCystatinC	−2327.380	1398.827	−1.66381	0.098404	0.247629
DNAmGDF15	4.794037	5.722406	0.83777	0.403600	0.591947
DNAmLeptin	−430.9424	124.1693	−3.47060	0.000692	**0.012320**
DNAmPAI1	−534.8867	236.1789	−2.26475	0.025075	0.183883
DNAmTIMP1	−88.76374	47.00069	−1.88856	0.061034	0.191822
DNAmPACKYRS	1.239601	0.633393	1.95708	0.052342	0.191822
GrimAge2 component					
DNAmadm	−2.265061	1.373178	−1.64950	0.101303	0.247629
DNAmCystatin_C	−2824.487	2224.994	−1.26944	0.206407	0.412814
DNAmGDF_15	1.987582	7.879241	0.25226	0.801216	0.889491
DNAmleptin	−369.8450	111.1765	−3.32665	0.001125	**0.012320**
DNAmpai_1	−448.4068	227.1757	−1.97383	0.050383	0.191822
DNAmTIMP_1	−110.2619	94.08117	−1.17199	0.243207	0.445880

Note: These values were calculated by multiple regression analyses examining the association between each item in the first column and the phenotype (patients or controls) using age and sex as confounding factors (Each item in the first column ~ phenotype + age + sex).

Abbreviations: ADM, adrenomedullin; B2M, beta 2 microglobulin; DNAm, DNA methylation; DNAmPACKYRS, DNA methylation-based smoking pack-years; DNAmTLAdjAge, age-adjusted estimate of DNA methylation-based telomere length; GDF15, growth differentiation factor-15; PAI1, plasminogen activation inhibitor-1; TIMP1, tissue inhibitor of metalloproteinases-1.

[a]The *q* values were calculated by adjusting the *p* values using the Benjamini Hochberg method. $q < 0.05$ is shown in bold.

DNAmleptin ($q = \mathbf{0.0281}$) in GrimAge2 components significantly associated with SAD (Table S2). In addition, we investigated correlations between DNAmLeptin or DNAmPAI1 in GrimAge components, and other confounding factors. Only sex was significantly correlated with DNAmLeptin ($q = \mathbf{1.17 \times 10^{-5}}$), and only DNAmPACKYRS ($q = \mathbf{0.0407}$) were significantly correlated with DNAmPAI1 (Table S3).

Furthermore, we investigated the association between LSAS score and each epigenetic age acceleration, GrimAge component, and GrimAge2 component. No age acceleration or components was significantly associated with LSAS score (Table S4 and Figures S2 and S3).

4 | DISCUSSION

To our knowledge, this is the first study to conduct epigenetic age analyses of SAD including GrimAge and GrimAge2 components. Epigenetic age acceleration was not significantly different between patients with SAD and controls. Among the GrimAge components and GrimAge2 components, the DNAm-based prediction values of leptin levels were significantly lower in patients with SAD than in controls, even after adjusting with the Benjamini Hochberg method. Although the DNAm-based prediction values of leptin level are less correlated with age than the other components, leptin is believed to suppress hunger and is negatively correlated with disease or mortality risk.[12,13] Although SAD is often associated with MDD,[34] the results of this study were different from those of our previous epigenetic clock analyses for MDD.[18] Our findings on leptin may be specific to SAD. The fact that SAD has a younger onset, and a longer duration of disease may be partial factors in the specify.[1] Furthermore, the significance of association between predicted values of leptin levels and SAD remained even after including confounding factors such as smoking status and cell composition, and

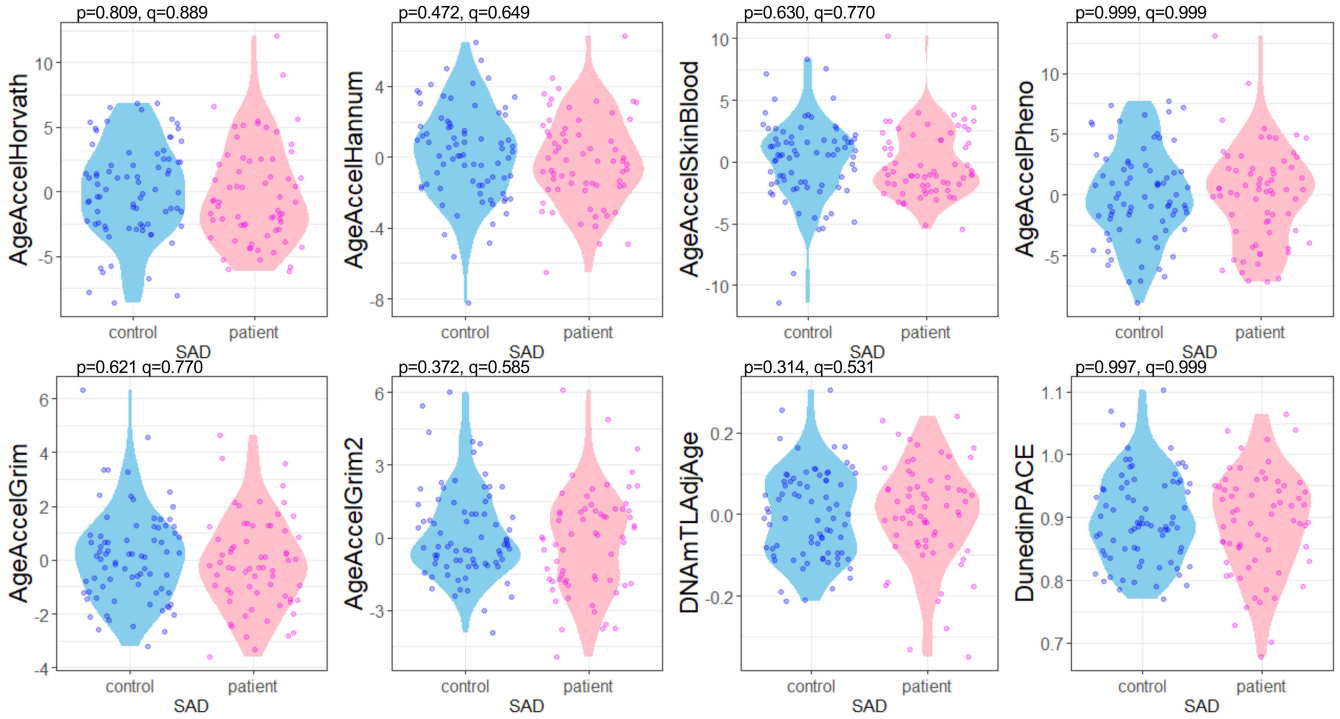

FIGURE 1 Violin plot showing age acceleration for each group. The *p* values were calculated by multiple regression analyses to test the association between each epigenetic age acceleration and phenotype (SAD or controls), and the *q* values were calculated with Benjamini Hochberg adjustment. Blue dots indicate data from the control group, and magenta dots indicate data from the patient group. This diagram was created using the R package ggplot2. DNAmTLAdjAge, age-adjusted estimate of DNA methylation-based telomere length; SAD, social anxiety disorder.

we found PAI1 also had significant association with SAD in additional regression analyses.

Leptin regulates food intake and energy expenditure,[45] mediated by its binding to leptin receptors expressed throughout the central and peripheral nervous systems. In the central nervous system, the leptin receptor gene is highly expressed in the hypothalamic nuclei, including the paraventricular nucleus (PVN), a region that plays important roles in the maintenance of homeostasis, such as driver of the HPA response.[46,47] Hyperactivity of the HPA axis results in excessive secretion of adrenocorticotropic hormone (ACTH) and cortisol. Acute elevations in cortisol levels have great importance as a protective response to stress, but long-term exposure to cortisol can lead to not only physical problems such as obesity, cancer, and cardiovascular disease, but also anxiety and depression through hippocampal and other neurological injuries.[48–51] Cortisol and ACTH levels were inversely correlated with leptin level.[52] Given these findings, we would expect that while the HPA system in patients with SAD is hyperactive due to various anxiety or stress conditions, leptin is inversely related; however, the results on leptin in anxiety are inconsistent among studies.[53–59] Due to complex feedback mechanisms, low leptin levels may be the secondary result of abnormal activation of the HPA system, and/or high leptin levels may be the result of increased leptin resistance. This study showed a significant negative association between SAD and leptin based on DNAm status; however, the severity of social anxiety was not significantly associated. The results from the various study designs should be confirmed in future studies.

PAI1 is an important inhibitor of tissue plasminogen activator and is increased in a variety of clinical situations related to aging.[60,61] PAI1 has also been reported to effect on brain neurons adversely.[62] In our previous study, DNAm-based predictive value of PAI1 level was significantly elevated in patients with autism spectrum disorders than controls.[63]

This study had several limitations. The sample in this study was obtained only from single cohort of Caucasians, and it is unknown whether the results can be adapted to other cohorts or racial groups. Among epigenetic clocks, there are several negative values that are often far from the actual age. However, in relative terms, all clocks showed a significant correlation with the actual age (Figure S1). The GrimAge and GrimAge2 components are protein predictors based on DNAm status and require actual test values. GrimAge components and GrimAge2 components reflect levels of proteins that were selected as reliable aging predictors by machine learning when GrimAge and GrimAge2 were developed, respectively.[12,13] However, in this study, the prediction values of leptin and PAI1 levels were not significantly correlated with actual age in the cohort. We thought the reason is that GrimAge indicates susceptibility to mortality diseases such as cardiovascular disease and life expectancy rather than actual chronological age. We were unable to obtain background information that might have affected the DNAm status, such as actual smoking history.[64] For smoking, we substituted DNAmPACKYRS. as a confounding factor for additional regression analyses.

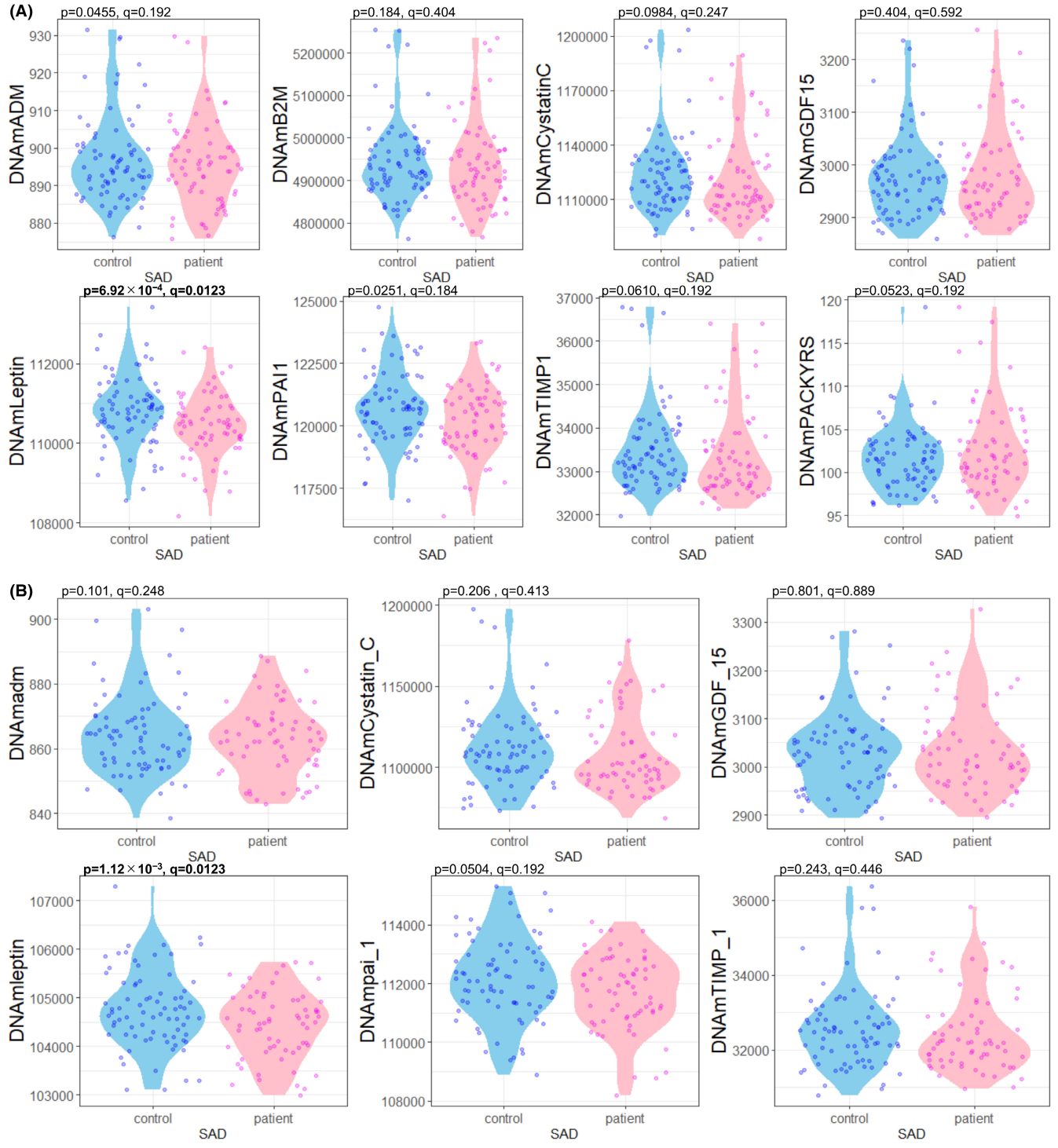

FIGURE 2 Violin plot showing (A) each GrimAge component and (B) each GrimAge2 component for each group. The *p* values were calculated by multiple regression analyses to test the association between each component and phenotype (SAD or controls), and the *q* values were calculated with Benjamini Hochberg adjustment. Blue dots indicate data from the control group, and magenta dots indicate data from the patient group. This diagram was created using the R package ggplot2. ADM, adrenomedullin; B2M, beta 2 microglobulin; DNAm, DNA methylation; DNAmPACKYRS, DNA methylation-based smoking pack-years; DNAmTLAdjAge, age-adjusted estimate of DNA methylation-based telomere length; GDF15, growth differentiation factor-15; PAI1, plasminogen activation inhibitor-1; SAD, social anxiety disorder; TIMP1, tissue inhibitor of metalloproteinases-1.

5 | CONCLUSION

Despite these limitations, we believe this study provides findings on SAD from a biological perspective based on DNAm status. Although we did not observe a significant acceleration of DNA methylation age in patients with SAD, we observed significant differences in leptin levels. We hypothesize that leptin is centrally involved in the pathogenesis of SAD as one of the aging-related proteins. We believe that

this study provides a basis for future studies on the pathogenesis of SAD. Further studies are required from various perspectives, including biological aging.

AUTHOR CONTRIBUTIONS

Nobuhiko Noguchi: Data curation, Formal analysis, Investigation, Visualization, Writing – original draft. Toshiyuki Shirai: Data curation, Formal analysis, Investigation, Visualization, Writing – original draft. Akira Suda: Data curation, Formal analysis. Saki Hattori: Data curation, Formal analysis. Masatoshi Miyauchi: Data curation, Formal analysis. Satoshi Okazaki: Data curation, Formal analysis. Junichi Fujita: Data curation, Formal analysis. Takeshi Asami: Data curation, Formal analysis. Ikuo Otsuka: Conceptualization, Methodology, Data curation, Formal analysis, Writing – review & editing. Akitoyo Hishimoto: Conceptualization, Supervision, Writing – review & editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

We used the publicly available GSE164056 dataset, which was generated in a previous study[37] and can be found in the Gene Expression Omnibus database (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE164056).

ETHICS STATEMENT

All the participants whose data were recorded in GSE164056 provided written informed consent which was approved by the University of Tübingen local ethics committee in accordance with the Declaration of Helsinki.

Approval of the Research Protocol by an Institutional Reviewer Board: All the participants whose data were recorded in GSE164056 participated in the research approved by the University of Tübingen local ethics committee.

Informed Consent: All the participants provided written informed consent.

Registry and the Registration No. of the Study/Trial: N/A.

Animal Studies: N/A.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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