Blood CDKN2A Gene Expression in Aging and Neurodegenerative Diseases

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Abstract

Background: Cyclin-dependent kinase inhibitor 2A (CDKN2A) is an important gene in cellular senescence and aging. Objective: This study assessed the utility of blood CDKN2A mRNA expression levels and methylation status as a potential biomarker for aging and the pathogenesis of Alzheimer’s disease (AD). Methods: The correlation between CDKN2A mRNA expression levels and age was examined in 45 healthy subjects, after which mRNA expression levels were compared among 46 AD patients, 20 mild cognitive impairment due to AD patients, 21 Parkinson’s disease patients, 21 dementia with Lewy bodies patients, and 55 older healthy controls. The methylation rates of the second exon of the CDKN2A gene, known to influence its expression levels, was also examined. Results: A significant correlation between CDKN2A mRNA expression levels and age was found (Spearman’s rank correlation coefficient: \( r = 0.407, p = 0.005 \)). CDKN2A mRNA expression levels in blood were significantly decreased in AD patients, although those of healthy controls were significantly increased with age. Further, only in AD patients were CDKN2A mRNA expression levels significantly and positively correlated with methylation rates. Conclusion: Although further research with a larger sample size is needed to elucidate the relationships between CDKN2A gene expression in blood and the development of other neurodegenerative diseases, CDKN2A mRNA expression in blood may be a biomarker for differentiating AD from normal aging and other neurodegenerative diseases.

Keywords: Aging, Alzheimer’s disease, blood, CDKN2A, gene expression

INTRODUCTION

Alzheimer’s disease (AD) is the most common cause of dementia; AD is a chronic neurodegenerative disease that results in progressive deterioration in cognitive function and behavior and is pathologically characterized by senile plaques and neurofibrillary tangles [1]. Senile plaques are extracellular accumulations of amyloid-β (Aβ), which is encoded by amyloid precursor protein (APP) on chromosome 21q21 [2]. Neurofibrillary tangles are areas of abnormal intracellular storage of hyperphosphorylated protein tau, which is encoded by microtubule-associated protein tau (MAPT) on chromosome 17q21 [3]. Genome-wide association studies (GWASs) have shown that the e4 allele of apolipoprotein E (APOE) is the strongest genetic risk for the development of late-onset AD, and dozens of other genes related to immunity, lipid metabolism, tau binding proteins, and the AβPP metabolism pathway are also implicated [4]. However, the molecular basis of AD pathogenesis has not yet been completely clarified.

A large body of literature has recently suggested the presence of an association between cellular...
senescence and age and several neurodegenerative diseases, including AD and Parkinson’s disease (PD) [5]. An emerging therapeutic target for age-related diseases is cellular senescence, a biological process that imposes perdurable proliferative arrest on damaged cells in response to various stressors [6]. The increased number of senescent cells in a variety of neurodegenerative diseases reflects the contribution of cellular senescence to the pathophysiology of such diseases [7]. Cyclin-dependent kinase inhibitor 2A (CDKN2A), a major tumor suppressor gene, is located at chromosome 9p21 in a region that has frequent loss of heterozygosity in several types of tumors [8] and encodes the CDKN2A protein. This protein typically blocks abnormal cell proliferation and growth by inhibiting the ability of cyclin-dependent kinases (CDKs) 4 and 6 to accelerate cell cycle G1 phase progression [9]. Because CDKN2A is considered to be an important gene in cellular senescence and aging [10], it is utilized as a molecular marker of cellular senescence [11]. The expression of CDKN2A protein has been shown to be elevated in cultured senescent cells [12, 13], as well as in the brains of patients with AD [14]. CDKN2A gene expression has also been reported to be elevated with age in the brain and lymphocytes of an AD mouse model [15].

Epigenetic changes are potentially heritable changes in gene expression that do not alter the DNA sequence. One epigenetic process that regulates gene expression is DNA methylation [16], which is associated with the pathogenesis of a number of brain diseases [17]. In previous studies, we reported that the DNA methylation and expression of several candidate genes were potential biological markers of neurodegenerative diseases [18–20]. Therefore, we hypothesized that CDKN2A mRNA expression levels in blood and CDKN2A methylation status could be associated with the pathogenesis of AD and may be potential biomarkers. However, notably, the blood methylation and expression levels of CDKN2A have not been examined in patients with AD and other neurodegenerative diseases.

Thus, in this study, the correlation between CDKN2A mRNA expression levels and age was examined in healthy subjects. Then, mRNA expression levels were compared among patients with AD, patients with mild cognitive impairment (MCI) due to AD, patients with PD, patients with dementia with Lewy bodies (DLB), and older healthy controls (HCs). The methylation status of the second exon of the CDKN2A gene, known to influence its expression levels, was also examined.

MATERIALS AND METHODS

Participants

First, to examine the correlation between CDKN2A mRNA expression levels and age, 45 healthy subjects aged 20–69 years old (15 males and 30 females, mean age ± standard deviation (SD) = 44.5 ± 14.5 years) who had no cognitive impairment, psychiatric signs, or a past history of mental diseases and who were diagnosed as mentally and cognitively healthy by at least two certified psychiatrists based on clinical interviews were enrolled. The demographic data of the healthy subjects are shown in Table 1.

Next, to examine the blood levels of CDKN2A mRNA in neurodegenerative diseases, 46 AD patients (15 males and 31 females, mean age ± SD = 77.4 ± 4.40 years), 20 MCI due to AD patients (7 males and 13 females, 81.9 ± 4.93 years), 21 PD patients (7 males and 14 females, 76.0 ± 4.28 years), and 21 DLB patients (7 males and 14 females, 77.8 ± 7.50 years) who were outpatients at Ehime University Hospital and Zaidan Niihama Hospital, Ehime, Japan, were enrolled. All patients met the diagnostic criteria for AD, PD, and DLB according to the National Institute on Aging/Alzheimer’s Association, UK Parkinson’s Disease Society Brain Bank, and 2017 DLB clinical diagnostic criteria, respectively. A diagnosis of amnestic MCI was made for patients who satisfied the following criteria: 1) normal general cognitive function with a Mini-Mental State Examination (MMSE) score ≥ 23; 2) on the delayed recall test of the Wechsler Memory Scale-Revised, the cutoff scores for any cognitive impairment were selected according to the education status as follows: ≤ 8 points for 16 years of education, ≤ 4 points for 8–15 years, and ≤ 2 points for 0–7 years; 3) neuropsychiatric examination: an absence of dementia or depression as determined by geriatric neuropsychiatrists according to the Diagnostic and Statistical Manual of Mental

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Healthy volunteers</th>
</tr>
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<tbody>
<tr>
<td>Age (y)</td>
<td>44.53 ± 14.5</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>15/30</td>
</tr>
<tr>
<td>Number of 20- to 29-year-old subjects</td>
<td>9</td>
</tr>
<tr>
<td>Number of 30- to 39-year-old subjects</td>
<td>9</td>
</tr>
<tr>
<td>Number of 40- to 49-year-old subjects</td>
<td>9</td>
</tr>
<tr>
<td>Number of 50- to 59-year-old subjects</td>
<td>9</td>
</tr>
<tr>
<td>Number of 60- to 69-year-old subjects</td>
<td>9</td>
</tr>
</tbody>
</table>

Values are the means ± the standard deviations.
Disorders, 3rd edition, revised (DSM-III R) [22] criteria; 4) no ADL impairment; and 5) hippocampal and parietal lobe atrophy without apparent cerebrovascular disease assessed by brain MRI. AD patients were evaluated with the MMSE and the Alzheimer’s Disease Assessment Scale (ADAS) to assess their cognitive function [21, 23], the Montgomery-Asberg Depression Rating Scale (MADRS) to assess their depression symptoms [24], the Clinical Dementia Rating (CDR) as measured by family caregivers [25], and the Neuropsychiatric Inventory (NPI) to assess their psychological symptoms [26]. The HCs were 55 elderly participants (18 males and 37 females, 77.1 ± 5.61 years) without cognitive impairment, psychiatric signs, or a history of mental disorders who were diagnosed as mentally and cognitively healthy by at least two certified psychiatrists based on clinical interviews.

All participants were unrelated and of Japanese origin and provided written, informed consent using forms approved by the institutional ethics committees of Ehime University Hospital (Approval Number: 31–K8 and R2-5). This study was conducted in accordance with the Declaration of Helsinki. The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Blood sample collection and the processing of complementary DNA (cDNA) and genomic DNA (gDNA)

Total RNA was obtained from whole peripheral blood samples using PaxGene Blood RNA Systems tubes (BD, Tokyo, Japan). The RNA concentration and purity were measured with a NanoDrop-1000 (Thermo Fisher Scientific, Yokohama, Japan), and acceptable 260/280 ratios were within 1.8–2.0. For each 40-µl reaction, 1.0 µg RNA was applied as a template to synthesize cDNAs with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). The gDNA samples were obtained from peripheral blood samples using EDTA tubes and extracted using the QIAamp DNA Blood Mini Kit (Qiagen, Tokyo, Japan).

PCR procedure

mRNA expression was analyzed by real-time quantitative PCR using a StepOnePlus Real-Time PCR System (Applied Biosystems). The specific TaqMan probes were Hs00923894 m1 for CDKN2A, Hs00153451_m1 for E2F1, Hs01078066_m1 for RB1 and Hs99999905 m1 for GAPDH (Applied Biosystems). GAPDH was used for the normalization of quantitative RT-PCR and was used as the reference gene in the study [27]. The final volume of each reaction was 20 µl and contained TaqMan Universal Master Mix (Applied Biosystems). Expression levels were examined in duplicate. The ΔΔCt method and StepOne software (Applied Biosystems) were used to determine relative expression levels.

Bisulfite conversion and pyrosequencing

The primers were consistent with previous studies showing a correlation between methylation status and CDKN2A expression [28]. The sequence was localized in the second exon in the CDKN2A gene and contained seven CpG sites (Fig. 1). The EpiTect Plus DNA Bisulfite Kit (Qiagen, Valencia, CA, USA) was used to convert the gDNA (1000 ng/sample) with bisulfate. Converted gDNA was then used as the template for PCR amplification with a forward primer (5-AGGGGTTGGTTGGTTATTAG-3) and a reverse primer (5-[Biotin]-CTACCTACTCTCCCCCTCTC-3). The final volume of each PCR was 25.0 µL, and each reaction contained 2.0 µL gDNAs, 0.5 µM forward and reverse primers, 0.2 U AmpliTaq gold...
Statistical analysis

Statistical analyses were performed with SPSS 22.0 software (IBM Japan, Tokyo, Japan), except for the post hoc Steel’s test, which was conducted with EZR version 1.26 [29]. Normality was tested by the Shapiro-Wilk test. Sex differences were analyzed using Pearson’s chi-squared test. Comparisons of CDKN2A mRNA expression between patients with AD, MCI, PD, and DLB and HCs were performed by the Kruskal-Wallis test with the post hoc Steel’s test. Comparisons of CDKN2A mRNA expression by sex were conducted with the Mann-Whitney U test. To validate the utility of the CDKN2A mRNA expression level for the diagnosis of AD, receiver operating characteristic curve analysis was used. The optimal cutoff value was set using Youden’s index. Comparisons of the methylation rate at each CpG site between AD patients and control subjects were conducted with the Mann-Whitney U test. Correlations of CDKN2A mRNA expression with age, the age of onset, the duration of illness, MMSE score, NPI score, ADAS score, MADRS score, methylation rates at each CpG site, and average total methylation of all sites were conducted using Spearman’s rank correlation coefficient. Correlations between the methylation rate at each of the 7 CpG sites were also examined using Spearman’s rank correlation coefficient. Significance was defined at the 95% level ($p=0.05$), and it was set at $p=0.007$ after Bonferroni corrections for comparisons of each methylation rate and correlations between each methylation rate at 7 CpG sites.

RESULTS

Correlation between CDKN2A mRNA expression levels and age in healthy volunteers

A significant correlation between CDKN2A mRNA expression levels and age was found (Spearman’s rank correlation coefficient: $r=0.407$, $p=0.005$, Fig. 2). CDKN2A mRNA expression levels did not differ by sex ($p=0.268$).

Comparison of CDKN2A mRNA expression levels among neurodegenerative diseases

CDKN2A mRNA expression was examined in the blood of patients with AD, MCI, PD, and DLB and in HCs. The demographic data and clinical characteristics of the participants are shown in Table 2. There was no difference by sex (Pearson’s chi-squared test: $p=1.00$). The MCI group was significantly older than the other groups (MCI versus HCs: 81.9 ± 4.93 versus 77.1 ± 5.61 years, Kruskal-Wallis $p=0.005$, Steel’s test: $p=0.005$), whereas the other groups showed no significant differences in age (AD versus HCs: 77.4 ± 4.40 versus 77.1 ± 5.61 years, Steel’s test: $p=0.99$, DLB versus HCs: 77.8 ± 7.50 versus 77.1 ± 5.61 years, Steel’s test: $p=0.92$, PD versus HCs: 76.0 ± 4.30 versus 177.1 ± 5.61 years, Steel’s test: $p=0.92$).

The CDKN2A mRNA expression level was significantly lower in patients with AD than in HCs (AD versus HCs: 1.38 ± 0.55 versus 1.73 ± 0.67, Kruskal-Wallis test: $p=0.014$, Steel’s test: $p=0.034$, Fig. 3). No significant differences in CDKN2A mRNA expression level were observed between MCI, PD, or DLB patients and HCs (MCI versus HCs: 1.62 ± 1.25 versus 1.73 ± 0.67, Steel’s test: $p=0.31$, DLB versus HCs: 1.81 ± 1.17 versus 1.73 ± 0.67, Steel’s test: $p=0.97$, PD versus HCs: 1.94 ± 0.75 versus 1.73 ± 0.67, Steel’s test: $p=0.69$). CDKN2A mRNA expression levels were not correlated with age in any patients or control subjects (Spearman’s rank correlation coefficient: $r=-0.163, p=0.833$).

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Table 2
Demographic and clinical data of Alzheimer’s disease patients

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>HCs</th>
<th>AD</th>
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<td>N</td>
<td>55</td>
<td>46</td>
<td>20</td>
<td>21</td>
<td>21</td>
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</tr>
<tr>
<td>Age (y)</td>
<td>77.0±5.6</td>
<td>77.4±4.40</td>
<td>81.9±4.93*</td>
<td>76.0±4.3</td>
<td>77.8±7.5</td>
<td>p=0.005*</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>18/37</td>
<td>15/31</td>
<td>7/13</td>
<td>7/14</td>
<td>7/14</td>
<td>p=1.0b</td>
</tr>
<tr>
<td>MMSE total score</td>
<td>18.3±5.45</td>
<td>25.4±1.88</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duration (y) (n=39)</td>
<td>4.2±4.07</td>
<td></td>
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<tr>
<td>NPI-10 total score (n=44)</td>
<td>13.8±16.30</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>MADRS total score (n=33)</td>
<td>5.9±4.86</td>
<td></td>
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<tr>
<td>ADAS total score (n=34)</td>
<td>19.5±9.0</td>
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<tr>
<td>CDR score (0:0.5:1:2:3) (n=44)</td>
<td>1.0±23:18:2</td>
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</table>

Values are the means ± the standard deviations. HCs, healthy control subjects; AD, Alzheimer’s disease; MCI, mild cognitive impairment due to AD; PD, Parkinson’s disease; DLB, dementia with Lewy bodies; MMSE, Mini-Mental State Examination; ADAS, Alzheimer’s Disease Assessment Scale; NPI, Neuropsychiatric Inventory; MADRS, Montgomery-Asberg Depression Rating Scale; CDR, Clinical Dementia Rating. *Steel’s test, p=0.005 (HCs versus MCI). a Kruskal-Wallis p=0.005. b Pearson’s chi-squared test for independence.

Comparison of the methylation status of CDKN2A between patients with AD and HCs

The sequence and position of the 7 CpG sites in CDKN2A exon 2 are shown in Fig. 1. CDKN2A methylation rates at each CpG site in patients with AD and HCs are shown in Fig. 4. Methylation rates at almost all CpG sites were correlated with each other in the AD group and HCs (Fig. 1). The methylation rates of the AD group were not different from those of the control group at CpG1 (AD versus HCs: mean ± SD = 3.08 ± 1.35 versus 2.91 ± 1.12, p = 0.77), CpG2 (4.94 ± 2.76 versus 4.93 ± 2.45, p = 0.85), CpG3 (2.92 ± 1.46 versus 2.72 ± 1.23, p = 0.73), CpG4 (3.19 ± 1.67 versus 3.03 ± 1.35, p = 0.87), CpG5 (4.67 ± 2.35 versus 4.59 ± 2.18, p = 0.87), CpG6 (2.70 ± 1.39 versus 2.50 ± 1.17, p = 0.63), or CpG7 (2.98 ± 1.47 versus 2.82 ± 1.18, p = 0.84), nor was the average CpG methylation rate
Comparisons of E2F1 and RB1 mRNA expression levels between patients with AD and HCs

Since CDKN2A gene is capable of inducing cell cycle arrest in G1 and G2 phases, and its functions have been connected with E2F1 and RB1 genes, we conducted their gene expression analyses between patients with AD and HCs. No significant differences in either E2F1 or RB1 mRNA expression levels were observed between patients with AD and HCs (E2F1: AD versus HCs: 0.82 ± 0.33 versus 0.85 ± 0.39, Mann-Whitney test: p = 0.98, RB1: AD versus HCs: 0.88 ± 0.15 versus 0.89 ± 0.17, Mann-Whitney test: p = 0.86, Fig. 3). Interestingly, E2F1 mRNA expression level showed significantly positive associations between both CDKN2A mRNA expression level (Spearman’s rank correlation coefficient: r = 0.27, p = 0.006) and RB1 mRNA expression level (Spearman’s rank correlation coefficient: r = 0.24, p = 0.018).

DISCUSSION

There were three major findings in this study. First, this is the first study showing significantly increased blood CDKN2A mRNA expression levels with age in healthy persons. Because CDKN2A is a well-known senescence marker and senescent cells accumulate in tissues with age, CDKN2A mRNA expression levels in blood may be a new biomarker for human biological aging. Consistently, in single-cell gene expression datasets from blood cells, the fraction of cells expressing CDKN2A was reported to increase with age [30]. It has also been reported that elderly people showed a significantly higher level of CDKN2A gene expression in endothelial progenitor cells than younger persons. Because our preceding study showed that the expression in blood was correlated with that in the brain in 3xTg AD mice [31], CDKN2A mRNA expression levels in blood may be correlated with those in the brain in humans.

Second, blood CDKN2A mRNA expression levels were significantly decreased in patients with AD and significantly increased with age in healthy controls. Because the risk of cancer among patients with AD and the risk of AD among patients with cancer have been reported to be lower [32–34], the inverse relationship between AD and cancer is speculated [35]. Although there were significant correlations between E2F1 mRNA expression and both CDKN2A and RB1 mRNA expressions, significant differences between the two groups (3.50 ± 1.72 versus 3.36 ± 1.47, p = 0.83). In patients with AD, CDKN2A mRNA expression was significantly correlated with methylation rates at CpG1 (Spearman’s rank correlation coefficient: r = 0.41, p = 0.005), CpG2 (r = 0.40, p = 0.005), CpG3 (r = 0.40, p = 0.005), CpG4 (r = 0.41, p = 0.005), CpG6 (r = 0.42, p = 0.004), and CpG7 (r = 0.41, p = 0.005) and with the average total methylation of all sites (r = 0.42, p = 0.003; Fig. 5). On the other hand, methylation rates at all CpG sites and the average total methylation of all sites were not correlated with CDKN2A mRNA expression in HCs (Spearman’s rank correlation coefficient: r = 0.02, p = 0.87; Fig. 6).
changes were observed only in CDKN2A but not in either E2F1 or RB1 mRNA expressions. CDKN2A is well known to be a major tumor suppressor gene; therefore, the decreased CDKN2A gene expression seen in patients with AD could reflect aberrant cellular senescence and oncogenesis in patients with AD. A similar inverse association with cancer was previously reported for PD [36] and other neurodegenerative diseases, including multiple sclerosis and Huntington’s disease [37], but such an association was not found for MCI, PD, or DLB. Claiming that CDKN2A expression changes may be a “biomarker for AD” is difficult in consideration of the subtle changes (approximately 20%) in CDKN2A expression with numerous control subjects and cases overlapping; however, this could be an element of the panel of biomarkers for AD [27].

Third, only in AD patients were CDKN2A mRNA expression levels significantly and positively correlated with methylation rates. The CpG sites were selected according to preceding studies showing correlations between methylation status and CDKN2A expression [28]. Two studies showed positive correlations between the methylation of these sites and immunoexpression or mRNA levels in cancer cells [28, 38], whereas one study showed a positive correlation between CDKN2A expression and methylation in cancer cells [39]. Although hypermethylation in the promoter region is usually related to transcriptional silencing, CDKN2A mRNA expression was significantly and positively correlated with methylation rates only in AD patients. This result may be associated with aberrant epigenetic changes in the CDKN2A gene in AD.

This study had some limitations. Since few studies reported relationships between CDKN2A and neurodegenerative diseases, patients in various stages should be recruited, and the effects of medication should be considered in future studies. Due to the small sample size and limited power, the negative findings should not be interpreted as showing no associations. Our results should be reproduced in a larger cohort.

CONCLUSIONS

The results of the present study demonstrated that CDKN2A mRNA expression in blood was lower in patients with AD, compared with healthy controls, but it was unchanged in patients with other neurodegenerative diseases. Although CDKN2A mRNA levels in blood may be a biomarker for differentiating AD from other neurodegenerative diseases, further research with a larger sample size is needed to elucidate the relationships between CDKN2A gene expression in blood and the development of other neurodegenerative diseases.

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