Changes in Biothiol Levels are Closely Associated with Alzheimer's Disease

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Abstract

Background Alzheimer's disease (AD) is a progressive, neurodegenerative disease of the brain. Serum homocysteine (Hcy) level is considered to be an important biomarker for AD, however, the status of Hcy in brain tissue and neuronal cells, and the association between brain and serum levels of Hcy in clinical AD patients currently remain unclear.

Methods: The levels of Hcy and other biothiols such as cysteine (Cys) and glutathione (GSH) in clinical blood samples were evaluated using a non-cytotoxic and stable multi-signal fluorescent probe, ethyl (E)-3-(9-chloro-11-oxo-2,3,6,7-tetrahydro-1H,5H,11H-pyrano[2,3-f] pyrido[3,2,1 -ij]quinolin-10-yl)-2-cyanoacrylate (Probe 1), which were verified by ELISA.

Results: Measurement by Probe 1 revealed a significant increase in Hcy level, and a decrease in Cys and GSH levels in PC12 cells treated with in $A\beta_{1-42}$. The hippocampus and whole brain of APP/PS1 mice also showed a significant increase in Hcy level alongside the accumulation of age-related AD symptoms, while levels of Cys and GSH decreased simultaneously. This change was also observed in serum samples acquired from patients with AD. The upregulation of Hcy and the downregulation of Cys and GSH were reversed in the $A\beta_{1-42}$ -treated PC12 cells and the brain of APP/PS1 mice when supplemented with VB_6 , an important coenzyme in Hcy metabolism.

Conclusions: Changes in Hcy, Cys and GSH levels in the brain of APP/PS1 mice and $A\beta_{1-42}$ -treated PC12 cells were observed in situ with a new fluorescent probe, which were consistent with the abnormal changes in Hcy, Cys and GSH levels in the serum of AD patients. VB_6 supplementation was successful in ameliorating abnormal upregulation of Hcy in the brain of APP/PS1 mice and in $A\beta_{1-42}$ -treated PC12 cells.

Background

Sulphur amino acids, like homocysteine (Hcy), cysteine (Cys) and glutathione (GSH) are metabolically interrelated, low molecular weight thiols that are potential indicators of health status and disease risk [1]. Recent evidence has found that increased Hcy level is correlated with the onset of Alzheimer's disease (AD) [2]. In analysis of 40 studies covering 2619 AD patients and 2390 controls, Liang et al. showed that AD patients may have higher levels of Hcy in their plasma compared to controls [3]. Furthermore, Wan et al. found that Hcy levels were increased significantly in primary cultured astrocytes and HT22 cells after treatment with Amyloid beta peptide ($A\beta_{1-42}$). It was revealed that during the progression of AD, high levels of Hcy results in induction of a multitude of continuously generated reactive oxygen species (ROS) [4], thereby leading to consumption of Cys and GSH by reaction with superoxide anion radicals [5]. The process discussed is thought to markedly enhance the vulnerability of neurons, further resulting in hippocampal neuron injury, in turning cumulating in disruption to intelligence and memory in patients with AD [6, 7]. Meanwhile, lower levels of VB_6 and VB_{12} , the cofactors of enzymes for metabolizing Hcy,

were found to be more often associated with AD patients when compared to healthy individuals [8, 9]. Therefore, Hcy is considered to be a potential biomarker to predict early AD.

At present, high-performance liquid chromatography (HPLC), capillary electrophoresis and enzyme-linked immunosorbent assay (ELISA) have been used to detect biothiols in clinical trial [2, 10]. Through use of these methods, a positive correlation between Hcy in blood and the progression of AD was confirmed. An elevated plasma level of Hcy > 14.5 μ M was considered to be a strong risk factor for cognitive decline in AD patients [11].

Since AD is a neurodegenerative disease [12, 13], the changes in biothiols in the brain of AD patients may truly reflect the physiological and pathological changes that occur in the brain during the disease. However, until now, methods of sample collection used in clinical research to detect biothiol levels in the brain of AD patients have been limited to cerebrospinal fluid extraction [3]. Smach et al. have found that the concentration of Hcy in cerebrospinal fluid was increased in AD patients [14], however, the distribution and level of biothiols in the brain tissue of AD patients could not be directly observed and detected by collecting cerebrospinal fluid, which can be invasive and complicated.

Real-time, in-vivo monitoring of biothiol changes would be beneficial for early diagnosis, prevention and therapeutic development for personalized AD management. Recently, multi-signal fluorescent probes have been widely applied to observe and detect biothiols in-situ, due to their high selectivity, sensitivity, and operational simplicity [15, 16]. It is of great value to utilized multi-signal fluorescent probes to observe and quantify biothiols in AD patients and nerve cells, because this may help to confirm the associations between AD and Hcy levels by direct measurement in real-time. We previously developed a multi-signal fluorescent probe, ethyl (E)-3-(9-chloro-11-oxo-2,3,6,7-tetrahydro-1H,5H, 11H-pyrano[2,3-f]pyrido[3,2,1-ij]quinolin-10-yl)-2-cyanoacrylate (Probe 1), which can simultaneous detect Cys, GSH and Hcy via three different emission channels, and has been evaluated in Raw 264.7, L-02 and BEL-7402 cells [17]. In this study, using Probe 1, we measured the levels of Cys, GSH and Hcy in A β ₁₋₄₂-treated PC12 cells, the brain and hippocampus of an AD model (APP/PS1) mouse, and the serum of AD patients, which further enabled us to evaluate the effect of VB₆ supplementation on the level of expression of Hcy and related biothiols. Thus, we were able to verify the effectiveness of Hcy as a biomarker for early AD detection.

Materials And Methods

Participants

The serum of thirty-one consecutive patients (18 male and 13 female, ages range 26-70 years with average 52.57 $\pm$ 16.75) from the Ningbo Lihuili Hospital, was collected for measurement of Cys, GSH, and Hcy levels using an ELISA and Probe 1.

Patients (n = 31) sampled for Cys, GSH, and Hcy levels in serum were recruited from the memory clinic of Ningbo Kangning Hospital after meeting the diagnostic criteria for AD of the Diagnostic and Statistical Manual of Mental Disorders-V (DSM-V). Inclusion criteria required a diagnosis of mild to moderate dementia, with a disease duration of greater than three months prior the enrollment, a Behavioral Pathology in Alzheimer’s Disease Rating Scale score of equal to or greater than 8, and a Mini-Mental State Examination score of 10–26. Exclusion criteria included history of epileptic disorders and seizures, brain tumors or trauma, dementia caused by infection, depressive mood disorder and malnutrition. Age-, sex- and education-matched healthy subjects were recruited as a control cohort. The consort diagram of patient clinical data is presented in Table 1. All procedures were approved by the local Ethics Committee of Ningbo Kangning Hospital and Ningbo Lihuili Hospital, Zhejiang, China, and were in complete accordance with the ethical standards and regulations of human studies of the Helsinki Declaration. Informed consent was given by each individual as all experimental procedures were clearly explained to participants during the consent process.

Table 1
Clinical and electrophysiological data of patients with AD and the healthy control subjects

Subjects Variables	Participants		<i>P</i> value
	AD patients (n = 31)	Healthy control (n = 31)	
Age (years)	57.45±13.49	58.14±8.45	0.87
Gender (m/f)	16/15	14/17	0.76
Education (years)	8.35±2.48	7.63±2.15	0.81
MMSE (Mean± SD)	17.88±3.25	3.21±1.34	0.03
ADAS-Cog (Mean± SD)	24.55± 5.23	2.37± 2.72	0.02

Determination of Cys, GSH, and Hcy levels in human serum samples by fluorescence spectrophotometer and ELISA assay

All human blood was collected into anticoagulant tubes according to the standard procedures of Affiliated Hospital of Ningbo University. After a 4-fold dilution with acetonitrile, the serum was separated by spinning at 1, 4000 rpm in a centrifuge for 10 mins. The samples were incubated with Probe 1 (10 μM) at room temperature in the dark for 1 h. Then, the fluorescence values were recorded at λ_{ex} = 375 nm, λ_{em} = 467 nm for the Hcy; λ_{ex} = 500 nm, and λ_{em} = 568 nm for GSH; and λ_{ex} = 500 nm and λ_{em} = 568 nm for

Cys by F-4600 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). The levels in the serum were calculated based on standard curves.

The levels of Hcy, GSH and Cys were also detected by Hcy and Cys ELISA kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China), and a GSH ELISA kit (Beyotime Biotechnology, Shanghai, China). Methods were performed according to the manufacturer's instructions.

Stability and cytotoxic evaluation of Probe 1

Probe 1 dissolved in acetonitrile at 10 µg/mL was injected into an ACQUITY UPLC BEH C18 column (2.1 mm×100 mm, Waters Corp, Milford, MA, USA). A gradient elution was performed with MeCN (phase A) and deionized water (phase B) as follows: 0–0.4 min 25% A, 0.4–0.41 min 25–80% A, 0.41–2.0 min 80–95% A, 2.0–3.0 min 90% A, 3.0–4.0 min 25% A, 4.1–8.0 min 50% A, at flow rate of 0.3 mL/min at 25 °C. The mass spectrometer was operated in an ESI positive electrospray ionization mode. Mass spectrometry was carried out in scan mode from m/z 300 to m/z 500 with a capillary voltage of 4 kV and a gas (nitrogen) temperature of 500°C. Cone gas and desolvation gas flows were set at 50 and 600 L/h, respectively.

The effects of Probe 1 on cytotoxicity were determined using a CCK-8 assay according to instructions of the manufacturer of the CCK-8 kit (Beyotime, Shanghai, China). PC12 cells at 60%-70% confluence were treated with different concentrations of Probe 1 (1-10 µM) for 24 h. Then 20 µL of CCK-8 was added before incubation for 2 h. Absorbance was measured at 450 nm with the Varioskan Flash (Thermo Fisher Scientific, Waltham, MA, USA).

Imaging of endogenous and exogenous Cys, GSH and Hcy in PC12 cells

After being cultured in confocal plates until reaching 80 % confluence, PC-12 cells were pretreated with 100 µM Aβ₁₋₄₂ (A9810; Sigma-Aldrich, St. Louis, MO, USA) for 0-48 h. Nontreated cells were used as a control. All cells were then treated with 1 mM VB₆ for 2 h. Next, the cells were incubated with Probe 1 (10 µM) for 30 mins, and imaged after washing with PBS three times. To detect exogenous Cys, GSH and Hcy, PC-12 cells were pretreated with N-ethylmaleimide (NEM, 0.25 mM) for 30 min, and subsequently incubated with Cys, GSH and Hcy (100 µM, 30 min) and Probe 1 (10 µM, 30 min), before being washed with PBS three times. Cells were imaged by a fluorescence confocal microscope (Zeiss LSM 880, Oberkochen, Germany). (λ_{ex} = 405 nm, λ_{em} = 421-475 nm for the blue channel; λ_{ex} = 458 nm, λ_{em} = 500-550 nm for the green channel; and λ_{ex} = 543 nm, λ_{em} = 552-617 nm for the red channel). Using Zen Blue software, the mean fluorescence values were obtained by using the following formula: mean fluorescence values = Integrated Density / Area.

Experimental animals and experimental procedure

All mice were purchased from Hangzhou Ziyuan Experimental Animal Technology Co., Ltd. (SCXK 2019-0004), and housed in the animal center of Ningbo University Medicine College (Ningbo, China). All mice used were housed in a cage and maintained under a 12-h light/12-h dark cycle in temperature- and humidity-controlled environments (21 ± 2 °C and approximately 60%, respectively) with free access to food and water. All experimental procedures were performed in accordance with the health guide for the care and use of laboratory animals of national institutes, and animal protocols were approved by the ethical committee of animal use and protection.

Forty-Eight APP/PS1 (6, 9 and 12 month, $n = 12, 12, 24$, respectively) transgenic male mice and twelve C57BL/6 male mice (12-month-old) were sorted into five groups ($n = 12$ each group). APP/PS1 mice with different ages were given with or without 100 mg/kg of VB₆ (Shanghai Yuanye Bio-Technology, Shanghai, China) for 7 consecutive days (APP/PS1^{6M+}, APP/PS1^{9M+}, APP/PS1^{12M+} groups and APP/PS1^{12M+VB6+} group). Next, 6 mice in each group were injected intravenously with 2 mg/kg of Probe 1. After 30 min, the levels of Cys, GSH and Hcy in the brain were measured. All mice then underwent euthanasia and the hippocampus region was isolated by dissection and fixed in 4% paraformaldehyde (Merck, Darmstadt, Germany) solution for brain imaging.

Imaging of endogenous and exogenous Cys, GSH and Hcy in the hippocampus of mice

The mouse hippocampal tissue was frozen, embedded and sliced (8- μ m-thick). Probe 1 solution (10 μ M) was added and the sections were incubated for 60 min at 4 °C. For imaging of endogenous Cys, GSH and Hcy, the hippocampal slices were incubated with Probe 1 (10 μ M) for 60 min, and then washed with PBS three times. For monitoring exogenous Cys, Hcy and GSH, the hippocampal slices were pre-incubated with NEM (500 μ M) for 30 min, and then washed with PBS three times. Next, Cys, GSH and Hcy (500 μ M) were added and the slices were incubated for 30 min, separately, before each section was washed with PBS three times. Finally, sections were imaged by a fluorescence confocal microscope (Zeiss LSM 880, Oberkochen, Germany). Using Zen Blue software, the mean fluorescence values were obtained using the following formula: mean fluorescence values = Integrated Density / Area.

In vivo whole brain fluorescence imaging

An injection solution of Probe 1 (2 mg/kg) was freshly prepared in saline with 15% DMSO, and the solution was stabilized for 20 min before injection. The APP/PS1 and C57BL/6 mice were anesthetized with ketamine (40 mg/kg) before a 1.0 cm cranial imaging window was surgically prepared. Each mouse was injected intravenously with 100 μ L of Probe 1. At 0, 15, 30, 60 and 120 min, the same regions of brain were imaged using an IVIS®Lumina[®] animal imaging system (Caliper LifeSciences, Perkin Elmer,

Hopkinton, MA) with the same parameters. Data analysis was performed using LivingImage® 4.2.1 software.

Determination of Cys, GSH and Hcy levels in PC12 cells and the hippocampus of mice by Probe 1

Cultured PC12 cells (1×10^4 cells/ μL , 10 μL) or murine hippocampal cells (10 mg) were collected and lysed/homogenized. After centrifugation at 1,4000 g for 10 min at 4 °C, the supernatant was collected for the quantitative analysis of Cys, GSH and Hcy by Probe 1 as previously described.

Statistical analysis

All cellular -based assays were performed in triplicate. Data were analyzed using SPSS software, version 16.0 (SPSS Inc., Chicago, IL, USA). The results were expressed as mean $\pm$ SD. Values among different groups were analyzed using one-way ANOVA, with correction for multiple comparisons using the Sidak method. $P < 0.05$ was considered statistically significant.

Results

Probe 1 is feasible for evaluation of Cys, GSH and Hcy levels in PC12 cells and in vivo

In order to evaluate the chemical stability of Probe 1, UPLC-MS was utilized to analyze the changes in the retention time (RT) and mass spectrum before and after deposition at room temperature for 48 h (Fig. S1a and b). There were no changes in retention time observed pre and post -deposition, with an RT of 2.13 min, and the difference between the two peak areas was less than 5%. The mass spectrum of the compound after deposition was also not changed, with a $[\text{M} + \text{H}]^+$ m/z of 399, indicating that Probe 1 has excellent stability in practical application.

After PC12 cells were treated with 1–10 μM of Probe 1 for 48 h the cell survival rate was greater than 90% (Fig. S1c), indicating that, at these concentrations, Probe 1 is not cytotoxic to PC12 cells.

The accuracy and validity of Probe 1 for use in measuring clinical serum samples was also verified. We firstly used the commercial ELISA method to measure the serum Cys, GSH and Hcy concentrations in the clinical patient samples, and compared the results when Probe 1 was used for measurement. There was a similar trend in the data obtained using both methods and the data were closely matched (Table S1), indicating Probe 1 is suitable for serum Cys, GSH and Hcy detection in clinical applications.

Probe 1 can additionally be used to detect thiols in PC12 cells. NEM was added to remove the endogenous thiol, and when exogenous Hcy, Cys or GSH was added, Probe 1 could induce the production

of corresponding blue, green and red fluorescence. Furthermore, after storage at room temperature for 48 h, the exogenous thiols in PC12 cells could still be detected by Probe 1 with no attenuation in fluorescence intensity (Fig. S2a and b).

Moreover, in this study we elucidate that Probe 1 can be used to detect thiol groups in the hippocampus of mice. Firstly, NEM was used to suppress signal from endogenous biothiols, before Hcy, Cys or GSH were added to stimulate the bright blue, green and red fluorescence through activity of Probe 1, which confirmed the excellent response of Probe 1 to Cys, Hcy and GSH in hippocampal tissue (Fig. S2c). Similarly, the intensity of fluorescence in hippocampal tissue was not attenuated after being deposited for 48 h at room temperature (Fig. S2d). However, in-vivo, we observed that the intensity of fluorescence of produced by Probe 1 activity in the brain peaked at 30 min before being attenuated, while, the fluorescent produced by Probe 1 activity did continue for 120 min before fading completely (Fig. S3). This data ensures that adequate testing time will be available when using Probe 1 to detect thiol groups in brain tissue.

The results and discussion may be presented separately, or in one combined section, and may optionally be divided into headed subsections.

A β ₁₋₄₂ induced changes in Hcy, Cys and GSH expression in PC12 cells

Probe 1 can detect endogenous Hcy, Cys, and GSH in PC12 cell (Fig. 1a). A β ₁₋₄₂ can exacerbate oxidative stress by decreasing endogenous antioxidant levels and resulting in induction of apoptosis in PC 12 cells [2, 18–20]. When PC12 cells were treated with A β ₁₋₄₂ for 24–48 h, the fluorescence intensity from the blue channel (Hcy) was enhanced with increasing treatment time, and correspondingly, fluorescence from the green and red channels (Cys and GSH) broadly decreased (Fig. 1a). Next, we quantified intracellular levels of the three biothiols using Probe 1 and observed that treatment of PC 12 cells with A β ₁₋₄₂ for 48 h resulted in significantly increased levels of Hcy ($\sim 2.44 \pm 0.37$ fold, $P < 0.01$), and decreased Cys and GSH levels ($\sim 32.70\% \pm 0.03$ and $61.57\% \pm 0.05$) compared to levels identified in nontreated control cells (Fig. 1b).

Biothiol level changes in the brains of AD mice

The three different fluorescent signals resulting from detection of Cys, GSH and Hcy were shown in the different layers of the hippocampal tissue slices from the C57BL/6 mice imaged by the Z-stack mode of a confocal microscope (Fig. 2a). In these C57BL/6 hippocampal sections, there was greener and red fluorescence representing endogenous Cys and GSH, while there was less blue fluorescence representing Hcy, indicating that Hcy was not abundant in the hippocampus of normal mice (Fig. 2b). This was converse to the data indicating the Cys, GSH and Hcy levels in the hippocampal slices of APP/PS1 mice

at different ages. The green (Cys) and red fluorescent (GSH) signals were gradually attenuated with the age in APP/PS1 mice, and this attenuation was more obvious in the APP/PS1^{12M+} group compared to in the hippocampus of the C57BL/6^{12M+} group. Meanwhile, the blue fluorescence (representing Hcy) was enhanced with increasing age, indicating that the level of Hcy in the hippocampus rises with the progression of AD pathology in this mouse model (Fig. 2b). Meanwhile, we quantified Cys, GSH and Hcy concentrations in the hippocampus of APP/PS1 and C57BL/6 mice using Probe 1, and found that Cys and GSH levels were reduced by 39.54%±0.05 and 15.24%±0.02, but Hcy level were increased by 1.46 ± 0.31-fold, in the APP/PS1^{12M+} group when compared to the C57BL/6^{12M+} group (Fig. 2c). These results are consistent with the trend of fluorescence staining observations we described using Probe 1 for detection.

In order to further monitor the changes in Cys, GSH and Hcy levels in the brain of AD model mice at different ages, we used the in-vivo imaging technique and found differences in the fluorescence intensities of the 3 biothiols when comparing the brains of normal C57BL/6 mice and APP/PS1 mice with AD symptoms. When the APP/PS1 mice were 6 months old, there was little to no difference in biothiol level expression compared to the C57BL/6^{12M+} group. However, the brains of 9 month APP/PS1 mice showed significant enhancement of Hcy fluorescence, while Cys and GSH signal was attenuated. In the brains of 12 month old APP/PS1 mice, the fluorescent signals indicating Cys and GSH presence were decreased by 62.50%±0.07 and 44.83%±0.05, while Hcy signal was increased by 1.60 ± 0.19-fold, when compared with those of C57BL/612M + group (Fig. 2d).

Cys, GSH, and Hcy levels are abnormal in the serum of AD patients

The levels of Cys, GSH, and Hcy in the serum samples acquired from healthy individuals and AD patients were measured using Probe 1. Table 2 shows that the average level of Hcy in the serum of healthy individuals was just below 8 μM, whereas Cys and GSH concentrations were just over 39 μM and 700 μM, respectively. Meanwhile, in AD patients, average Hcy was over 22 μM, which is an increase of 2.78 ± 0.83 fold, and Cys and GSH were just below 24 μM and 662 μM, which is a decrease 36.2%±0.04 and 43.3% ±0.06, compared to the control cohort, respectively (Table 2, S2 and S3).

Table 2
The Hcy, Cys and GSH levels in the serum from AD patients and healthy controls (μM, mean ± SD).

Group	n	Hcy	Cys	GSH
Control	31	7.99 ± 3.24	39.40 ± 5.51	749.53 ± 20.14
AD	31	22.17 ± 4.02**	24.94 ± 4.28**	662.23 ± 25.51**
Note: * <i>P</i> < 0.005; ** <i>P</i> < 0.001, compared with that of the Control group.				

VB₆ reverses the changes in Hcy, Cys and GSH levels in Aβ₁₋₄₂-treated PC12 cells and brains of AD model mice

VB₆ is an important coenzyme that can promote cystathionine beta-synthase-mediated catalysis of serine and Hcy into Cys [8, 9]. PC12 cells were pretreated with Aβ₁₋₄₂ before being supplemented with VB₆ for 2 h. This supplementation with VB₆ resulted in significantly increased levels of Cys and GSH, by 1.73 ± 0.25 and 1.25 ± 0.18 fold, and a significantly decreased Hcy level, by $30.44\% \pm 0.06$ fold, when compared to those Aβ₁₋₄₂ treated cells with no VB₆ supplementation (Fig. 3a). Furthermore, when detected by Probe 1, we found that VB₆ treatment led to significantly increased amounts of fluorescence signal indicating Cys and GSH, and decreased levels of Hcy fluorescence, compared to the Aβ₁₋₄₂ treated cells with no VB₆ (Fig. 3b).

12-month-old APP/PS1 mice were treated with VB₆. After 7 days of VB₆ (100 mg/kg) intervention, compared with the APP/PS1^{12M+} group with no treatment, the brain levels of Cys and GSH had increased by 1.84 ± 0.27 -fold and 1.21 ± 0.14 -fold, and the level of Hcy had decreased by $9.80\% \pm 0.02$, respectively, in the APP/PS1^{12M+VB6+} group (Fig. 3c). Similarly, the fluorescent signals indicating Cys and GSH were significantly increased, while Hcy signal was decreased in the APP/PS1^{12M+VB6+} group compared to the APP/PS1^{12M+} group (Fig. 3d).

Brain imaging showed that pre-treatment with VB₆ (100 mg/kg, 7 d) effectively changed the Epi-fluorescence levels of Cys, GSH, and Hcy in the brain compared of the APP/PS1^{12M+} mice. Cys and GSH levels were increased by 1.80 ± 0.20 -fold and 1.25 ± 0.16 -fold, respectively, while the level of Hcy was found to be decreased by $27.08\% \pm 0.03$, in the APP/PS1^{12M+VB6+} group compared to the APP/PS1^{12M+} group (Fig. 3e).

Discussion

The most abundant intracellular biothiols, Cys, GSH, and Hcy, are widely associated with physiological and pathological processes in complex biological environments [8, 21]. Hcy and serine are converted to cystathionine by transsulfuration in a reaction catalyzed by cystathionine β-synthase, which is further converted to Cys by cystathionine γ-lyase[22]. Cys then acts as a substrate for glutamate cysteine ligase and glutathione synthetase and will eventually be converted to GSH [2, 23]. Therefore, Hcy levels are tightly associated with the levels of Cys and GSH. Evidence has indicated that aberrant levels of Cys, Hcy, and GSH contribute substantially to AD pathology [24]. During the progression of AD, the accumulation of Aβ may lead to an increase in ROS generation, and a decrease in the levels of endogenous antioxidants, such as GSH [25–27], resulting in induction of additional oxidative stress [25]. For the three biothiols, Hcy is most strongly correlated with oxidative stress, since auto-oxidation of Hcy can produce ROS, which

causes an increase in the level of free radicals locally in the brain, aggravating nerve cell damage and causing neuronal cell death in severe cases [28]. Elly et al. demonstrated that the combined effects of Hcy and local oxidative stress contribute to the damage of presynaptic terminals in neurodegenerative motor neuron diseases [29]. Moreover, Liang et al. analyzed data from 239 AD patients and 226 controls, and found that AD patients have higher cerebrospinal fluid levels of Hcy (0.11–0.19 $\mu\text{mol/L}$) compared to healthy controls (0.07–0.08 $\mu\text{mol/L}$) [3], and the change was consistent with measurements in serum. Therefore, it is generally considered that the level of Hcy is an important diagnostic indicator for AD [30].

In this study, we observed significant changes in Cys, GSH, and Hcy levels in $\text{A}\beta_{1-42}$ -treated PC12 cells, in the brains of APP/PS1 AD model mice and the serum of clinical AD patients. The Hcy level was significantly increased, and Cys and GSH levels were significantly reduced in $\text{A}\beta_{1-42}$ -treated PC12 cells, which would be repeated in APP/PS1 mice and exacerbated by age in these mice. These results further verified the relationship between neuronal cell and brain levels of Hcy, Cys and GSH and AD pathology. However, as a limitation to the study, these evidences are circumstantial unless changes in biothiols can be observed directly in situ.

Probe 1 can simultaneously detect Cys, GSH, and Hcy via three emission channels with properties of high quantum yield and minimal fluorescence background signal [17]. In our study, we found that Probe 1 had good stability and low cytotoxicity and we firstly tested the effectiveness of Probe 1 in clinical serum samples. The levels of Cys, GSH, and Hcy detected by Probe 1 showed a similar trend when compared to levels detected by the ELISA, and the data were consistent in terms of the concentration given for each test sample. Perhaps most importantly, we found that Probe 1 could reach the brain in a short time by passing through the blood brain barrier after intravenous injection and could then be imaged clearly. Furthermore, we used probe one and ELISA to successfully observe changes in levels of three biothiols in PC12 cells and the hippocampal regions and brains of mice after various treatments. These results indicate that Probe 1 is an effective tool for observation of Cys, GSH, and Hcy in vivo and in vitro.

Using confocal imaging, we found that the 3 biothiols of interest were not present at noticeably different levels in the brains of 6-month-old APP/PS1 mice compared to control mice. However, significant differences were observed in 9-month-old APP/PS1 mice compared to controls, and the fluorescence intensity of Hcy in the brain of 12 month-old APP/PS1 mice was significantly enhanced, while Cys and GSH levels were reduced compared to controls, which is consistent with the age of onset of behavioral changes observed in APP/PS1 mice during AD disease progression [31]. It has been reported that there are no behavioral differences between APP/PS1 mice and WT mice before 9 months of age, while apparent abnormalities, including changes in memory function, could be observed after 9 months in APP/PS1 mice [22]. Notably, Hcy content in the serum of APP/PS1 mice increased rapidly from 9 to 12 months of age [32].

VB_6 is a coenzyme required for proper function of cystathionine β -synthase. Studies have indicated that lowering levels of plasma Hcy by VB_6 treatment could markedly slow cognitive decline in humans [9]. Encouragingly, it's been observed that VB_6 (at 100 mg/kg) can significantly reverse the alterations in Hcy

to Cys and GSH levels in the hippocampus of AD model mice and A β_{1-42} -treated PC12 cells. Our observation provided strong support for VB₆, as an anti-AD candidate drug, which may reduce the risk of AD and improve AD prognosis in humans.

Conclusions

In summary, we firstly confirmed that Hcy is an effective biomarker for AD disease. Changes in Cys, GSH and Hcy were observed in situ in the brains of APP/PS1 mice and A β_{1-42} -treated PC12 cells by a novel multi-signal fluorescent Probe 1. With the progression of AD, it was observed that Hcy levels were upregulated while Cys and GDH levels fell in the hippocampus and brain of APP/PS1 mice. These changes were consistent with abnormal alterations in Hcy levels in the serum of AD patients. Meanwhile, intervention using VB₆ treatment could effectively reverse the observed abnormal Cys, GSH and Hcy levels in A β_{1-42} -treated PC12 cells and in the brains of APP/PS1 mice.

Abbreviations

AD	Alzheimer’s disease
Hcy	homocysteine
Cys	cysteine
GSH	glutathione
Probe 1	ethyl(E)-3-(9-chloro-11-oxo-2,3,6,7-tetrahydro-1H,5H,11H-pyrano[2,3-f]pyrido[3,2,1-ij]quinolin-10-yl)-2-cyanoacrylate
VB ₆	Vitamin B6
A β_{1-42}	Amyloid beta peptide
ROS	reactive oxygen species
HPLC	high-performance liquid chromatography
ELISA	enzyme-linked immunosorbent assay
NEM	N-ethylmaleimide

Declarations

Acknowledgments

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Authors' contributions

Han Hui: methodology, investigation, writing of the original draft. Feng Wang: methodology, investigation. Juanjuan Chen: methodology, formal analysis. Xingxing Li: investigation. Gaoqing Fu: data curation. Denghui Gu: methodology. Jiawei Zhou: methodology, data curation, formal analysis. Dongsheng Zhou: investigation, conceptualization. Haimin Chen: supervision, text review & editing, funding acquisition. Wei Wu: methodology, project administration.

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Availability of data and materials

All data generated or analysed during this study are included in this published article.

Ethics approval and consent to participate

The Chinese Alzheimer's Biomarker and Life style study was approved by the Institutional Ethics Committee of Ningbo Kangning Hospital.

Consent for publication

All authors have given consent for publication.

Conflicts of Interest

The authors declare no conflict of interests, financial or otherwise.

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Figures

Figure 1

Changes in Cys, GSH and Hcy levels in A β_{1-42} -treated PC12 cells. a Confocal fluorescence images of endogenous Cys, GSH and Hcy in PC12 cells. PC12 cells were incubated with A β_{1-42} (100 μ M) for 12-48 h, then incubated with Probe 1 (10 μ M, 30 min), and imaged. (λ_{ex} = 405 nm, λ_{em} = 421-475 nm for the Hcy; λ_{ex} = 458 nm, λ_{em} = 500-550 nm for the Cys; and λ_{ex} = 543 nm, λ_{em} = 552-617 nm for the GSH). Scale bar: 20 μ m. b Cys, GSH and Hcy levels in PC12 cells as detected by the ELISA method.

Figure 2

Changes in Cys, GSH and Hcy levels in the hippocampus and brain of APP/PS1 mice. a The imaging of mice hippocampal tissue. Confocal z-scan imaging of sections between a depth of 0 to 8 μm . b Confocal fluorescence images of endogenous Cys, GSH and Hcy in the hippocampus of APP/PS1 and C57BL/6 mice. The hippocampal region of mice was isolated, sliced, and the slices were incubated with Probe 1 (10 μM , 30 min), then imaged. (λ_{ex} = 405 nm, λ_{em} = 421-475 nm for the Hcy; λ_{ex} = 458 nm, λ_{em} = 500-

550 nm for the Cys; and λ_{ex} = 543 nm, λ_{em} = 552-617 nm for the GSH). Scale bar: 200 μ m. c Cys, GSH and Hcy levels in hippocampal homogenate of APP/PS1 mice and C57BL/6 mice was tested by Probe 1. d The in situ fluorescence image of brains of C57BL/6 mice and APP/PS1 mice after 30 min of Probe 1 intravenous injection. The Epi-fluorescence value (radiant efficiency) was calculated through the formula $\frac{\text{Epi-fluorescence value}}{\text{radiant efficiency}}$

Figure 3

The effect of VB6 treatment on the levels of Cys, GSH and Hcy. a Effect of VB6 on the Cys, GSH and Hcy levels in A β 1-42–treated PC12 cells. b Effect of VB6 treatment on the fluorescence images of endogenous Cys, GSH and Hcy in A β 1-42–treated PC12 cells. PC12 cells were incubated with VB6 (1 mM) and A β 1-42 (100 μ M) for 48 h, then imaged. (λ_{ex} = 405 nm, λ_{em} = 421-475 nm for the Hcy; λ_{ex} = 458 nm, λ_{em} = 500-550 nm for the Cys; and λ_{ex} = 543 nm, λ_{em} = 552-617 nm for the GSH). Scale bar: 20 μ m. c Effect of VB6 on the Cys, GSH and Hcy levels in the hippocampus of APP/PS1 mice. Mice were treated with or without VB6 for 7 days. The levels of Cys, GSH and Hcy were tested by Probe 1. d Effect of VB6 treatment on the fluorescence images of endogenous Cys, GSH and Hcy in hippocampus of APP/PS1 mice. APP/PS1 mice were treated with or without VB6 for 7 days, then hippocampus region was isolated, sliced, hippocampus incubated with Probe 1 (10 μ M, 30 min), imaged. Scale bar: 200 μ m. e After 30 min of Probe 1 intravenous injection, the effect of VB6 on the in situ fluorescence image of brains of 12-month age APP/PS1 mice. The Epi-fluorescence value reflects the levels of Cys, GSH and Hcy.

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