

# Pharmacogenetic Mechanism of ACE I/D Polymorphism Adversely Responding to ACE Inhibitors in Regulating the ACE Promoter Activity in Neurons

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**Abstract:** The ACE (angiotensin converting enzyme) inhibitors are not only drugs widely prescribed drugs in cardiovascular diseases, but also potentially therapeutic agents in dementia. Based on the findings that the ACE inhibitors could activate the c-Jun N-terminal kinase signal to increase the ACE gene expression and that the Alu element of the human ACE gene involved in regulating ACE promoter activity, we aimed to investigate whether there are different pharmacogenetic responses of ACE I/D polymorphism to the ACE inhibitors in neurons. The three reporter vectors, pACEpro(f)-SEAP, p-I-ACEpro-SEAP, and p-D-ACEpro-SEAP were used to examine the transcriptional activity of the vectors responding to the lisinopril treatment using a transient-transfection method in SH-SY5Y cells. Our results showed that lisinopril increased the promoter activity of an ACE gene by 16.7%. Additionally, we found the lisinopril enhanced the ACE promoter activity of the I-form vector by 17.2%, but adversely reduced that of the D-form vector by 16.8%, as compared with the respective control without the lisinopril treatment. Firstly, our findings had proved that the I/D polymorphism of ACE gene contrarily responds to the ACE inhibitors in regulating the ACE expression in neurons, which provide a novel insight suggesting genetic testing to tailor the treatment regimens in AD (Alzheimer's disease) patients.

**Key words:** ACE inhibitors, ACE I/D polymorphism, Alzheimer's disease, pharmacogenetics, promoter activity.

## 1. Introduction

The ACE (angiotensin converting enzyme) inhibitors are used extensively for the treatment of CVD (cardiovascular diseases) and kidney diseases to lower the risk of cardiovascular events and kidney failure [1]. Beyond their primary therapeutic goals,

ACE inhibitors are also considered as potentially therapeutic agents in dementia as per several clinical studies [2-5], especially for the centrally active ACE inhibitors [6]. Two large clinical trials indicated that the ACE inhibitors could secondarily reduce an incidence of cognitive impairment and the rate of cognitive decline, i.e., the PROGRESS (Perindopril Protection against Recurrent Stroke Study) [7] and the SYST-EUR (Systolic Hypertension in Europe)

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trial [8]. The similar pharmacologic effects of the ACE inhibitors on cognitive function were also found in recent clinical trials [9-11] and in animal models [12, 13]. It is noteworthy that some studies denoted that the direct effects of the ACE inhibitors to lower the risk of developing dementia could be independent from the controlling of cardiovascular disease and blood pressure control [2, 4, 14]. Nevertheless, these inconsistent results have also been reported in other studies [4, 15-18]. Whatever the positive or negative results, no clear mechanisms have been delineated.

Being the main target of the ACE inhibitors, ACE is a well-known zinc-containing metallopeptidase that plays a key role in the RAS (renin-angiotensin system) and performs diverse physiological functions in many tissues [19]. In fact, the local ACE expressed in brain tissue has also been identified in different neuron cells [20], and the increased ACE protein level and activity in the brain of AD (Alzheimer's disease) patients have been found in many studies [21-23]. In a recent multifaceted review [24], the principal enzymatic functions of ACE, i.e., converting decapeptide ANG I (Angiotensin I) into octapeptide ANG II (Angiotensin II) and hydrolyzing A $\beta$ 1-42 peptide to small amyloid peptide [25, 26], have been suggested to underpin the association with the risk of AD. However, the exact mechanism of how ACE is involved in the pathogenesis of AD is still a challenging fact to elucidate. Interestingly, and peculiarly noteworthy, the membrane-bound ACE has been found to function as a receptor that can be activated by the ACE inhibitors [27] or sheer stress [28] to induce the ACE signaling cascade, which in turn regulates the expression of the ACE protein itself. Therefore, it needs more experimental strategies to explore the unidentified role of ACE in the pathogenesis of AD, and the possible functions of the ACE inhibitors.

To explore the plausible pathophysiological role of ACE in contributing to AD, the genetic marker of the

ACE I/D, an I (insertion) or D (deletion) of the Alu element located in Intron 16 has also been extensively examined in association with the risk for AD. The I allele of the ACE gene has been indicated as a potential susceptibility marker for AD in many previous studies [29-32]. However, some contradicting results keep the previous conclusions as pending [33]. In pharmacogenetic testing, a side study of the PROGRESS trail aimed to observe whether the ACE I/D influences the treatment response of macrovascular events and dementia to the ACE inhibitors for patients with a history of cerebrovascular disease, but this study provides no evidence that knowledge of ACE genotype is useful for predicting either the risk of disease or the benefits of perindopril-based blood pressure-lowering treatment [34]. This is a limited trail to test the cognitive function response to the ACE inhibitors according to the ACE I/D polymorphism of individuals. However, it is noteworthy that this study mainly focused on individuals with a history of a stroke, or a transient ischemic attack rather than specifying on AD or any characteristic types of dementia. Another similar pharmacogenetic cohort study indicated that the 6AG and M235T polymorphisms in the AGT gene were associated with a cognitive function response to the ACE inhibitors in a group of elderly and healthy participants (the Health, Aging and Body Composition Study) [35], but it was not applied for the ACE I/D genotype. Whereas AD is a complex polygenic disease, many candidate genes may contribute to each partial effect on the pathogenesis to the development of AD. Thus, it might be too early to reach a conclusion that the ACE I/D polymorphism is not associated with a cognitive improvement response to the ACE inhibitors by limited epidemiological studies.

Based on our recent report that the Alu element in the intron 16 of the ACE gene is involved in regulating the activity of the ACE promoter in neurons [36], the present study further aimed to examine whether the

ACE I/D polymorphism respectively responds to the ACE inhibitors in regulating the ACE promoter activity using reporter activity assay in SH-SY5Y cells. To unravel the feasible mechanisms of the pharmacogenetic response of ACE I/D to the ACE inhibitors in neurons will provide a helpful insight for further clinical trials.

## 2. Materials and Methods

### 2.1 Construction of the Luciferase Reporter Vectors

The detail of the three reporter vectors used in the present study was described in our previous work [36] and the modified schematic diagrams are illustrated in Fig. 1. Briefly, the backbone of our reporter vectors is the pSEAP-Basic2 vector (pSEAP-Bas) (BD Biosciences Clontech, Palo Alto, CA, USA). The promoter fragment of the human ACE (−760~+130) including many proved binding elements was amplified by a PCR (polymerase chain reaction) and first cloned into the multiple cloning site of the pSEAP-Bas vector using EcoRI restriction enzyme. The strain with forward direction of the ACE promoter,

pACEpro(f)-SEAP vector, were obtained. The I allele and the D allele fragments of human ACE Intron 16 were identified and amplified by the PCR, then 479 bp of I form fragment (with Alu element) and 192 bp of D form fragment were extracted and inserted between the Hind III and Xho I sites in front of the ACE promoter region in the pACEpro(f)-SEAP vector to obtain the p-I-ACEpro-SEAP vector and the p-D-ACEpro-SEAP vector, respectively. All these reporter constructs were verified by completely sequencing. The primers used for the PCR and DNA sequencing of the I and D fragments were as follows: forward primer, 5'-ACCGCTCGAGCTGGAGAGCCACTCCCATCC TTTCT-3'; reverse primer, 5'-CTAAAAGCTTGACG TGGCCATCACATTTCGTCAGAT-3'.

### 2.2 Cell Viability Assay

Lisinopril, a highly efficient inhibitor of ACE, and OA (okadaic acid) were purchased from Sigma-Aldrich, Inc (Saint Louis, USA). OA, a protein PP2A (Phosphatase-2A) inhibitor, could induce hyperphosphorylation of tau protein and cause neuronal

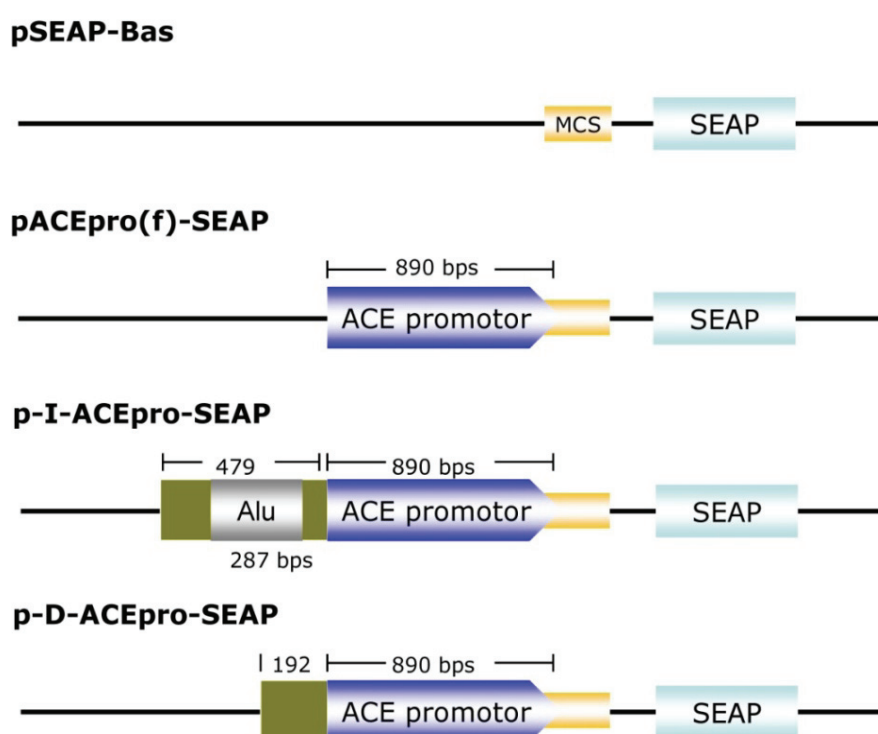


Fig. 1 Schematic diagrams of the reporter vectors in the present study modified from the figure in our previous work [36].

cell death in culture, thus it was selected as a positive control for the cell viability assay. Cell viability was detected using a PrestoBlue<sup>TM</sup> kit (Invitrogen, Carlsbad, USA). Prestoblue is quickly reduced by metabolically active cells, providing a quantitative measurement of viability and cytotoxicity. SH-SY5Y cells ( $1 \times 10^4$ ) were seeded in 96-well plates overnight before the experiment, and the cells were then treated with a serial working concentration of lisinopril (i.e., 10  $\mu$ M, 100  $\mu$ M, 1 mM and 10 mM) or 30 nM OA. After 24 h incubation, the cells were rinsed with PBS solution, and 10  $\mu$ L of prestoblue reagent was loaded. After 30 min of incubation at 37 °C, the cell viability was assessed by measuring the fluorescence of the PrestoBlue<sup>TM</sup> reaction (excitation at 515 nm, emission at 590 nm).

### 2.3 Cell Culture and Transient Transfection

The maintenance of SH-SY5Y cells, a neuroblastoma cell line, and transient transfection examination were also described in our previous work [36]. Briefly, the cells were maintained in MEM/F-12 (1:1) containing 10% fetal bovine serum in a humidified atmosphere of 95% air and 5% CO<sub>2</sub> at 37 °C. All experiments of the cell line were carried out within passage 12 to maintain uniform condition. The plasmid DNAs for transfection were prepared using QIAGEN columns (QIAGEN GmbH, Hilden, Germany). The cells were freshly subcultured at a density of  $2 \times 10^5$  cells on 6-well cassettes and were transfected using a transient liposome co-transfection protocol (TransFast<sup>TM</sup> Transfection Reagent, Promega, Charbonniere-les-bains, France) with 2.4  $\mu$ g of reporter plasmids and 0.6  $\mu$ g of pGL4 Luciferase Reporter Vectors for firefly luciferase gene expression as an internal control to normalize efficiency of transfection. All samples were run in quadruplicate in at least four different experiments.

### 2.4 Reporter Assay

The day after transfection, the medium was changed

to a completely fresh medium with the same volume per well. After another 48 h, the conditioned cell culture medium was collected for measurement of SEAP activity of each construct by a Great EscAPE<sup>TM</sup> SEAP Chemiluminescence Detection kit (BD Biosciences Clontech) and Luminometer (Labsystems Luminoskan). The luciferase assay of pGL4 firefly luciferase in cell lysate was performed according to the manufacturer's protocol from the commercial kit (Promega).

### 2.5 Statistics

Two-way ANOVA (analysis of variance) and Student's *t*-test were performed to compare the expression activity of reporter constructs in at least four independent transfection experiments as per the previous report [37]. Data are expressed as means  $\pm$  SE, and values of  $p < 0.05$  were considered statistically significant.

## 3. Results

### 3.1 The Effect of Lisinopril on the Cell Viability of SH-SY5Y Cells

Lisinopril, a centrally active ACE inhibitor with a high specificity to ACE, was selected to examine whether the ACE promoter responded to the ACE inhibitors in neuron. First, we analyzed the dose-dependent effects of lisinopril on the cell viability of SH-SY5Y cells using variant concentrations of lisinopril from 10  $\mu$ M to 10 mM (Fig. 2). Our results showed that even the high concentration of lisinopril (10 mM) did not affect the cell viability of SH-SY5Y cells, as compared to 40% of cell viability reduction in the treatment of 30 nM OA. In order to bind the membrane-bound ACE completely, 100  $\mu$ M of lisinopril was used to perform the following reporter assays of the ACE promoter activity.

### 3.2 Lisinopril Enhances the ACE Promoter Activity

The promoter activity of the pACEpro(f)-SEAP

vector was assayed after the transfected cells were exposed to plus or minus 100  $\mu\text{M}$  of lisinopril for 24 h. We found that lisinopril significantly up-regulated the promoter activity of the pACEpro(f)-SEAP vector by approximately 17% ( $p < 0.01$ ) (Fig. 3 and Table 1), which is consistent with the previous finding that the ACE gene was increased by the ACE inhibitors activation [38].

### 3.3 Contrary Response of I and D Form Fragment to Lisinopril Treatment

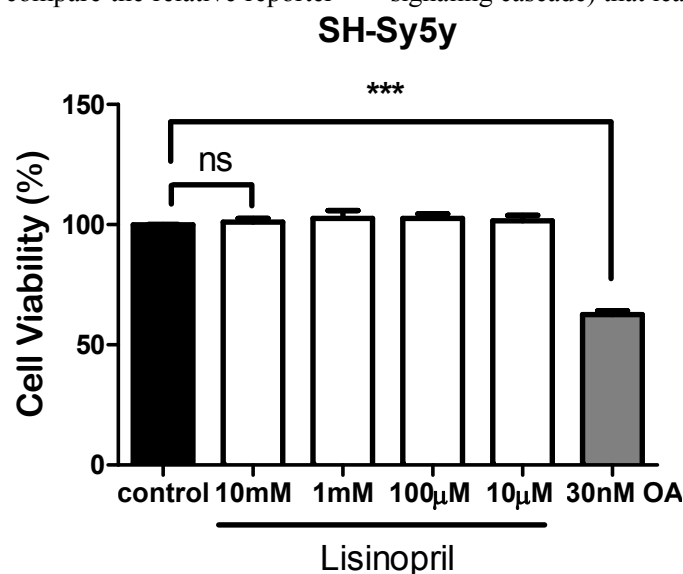
Furthermore, SH-SY5Y cells were transiently transfected with p-I-ACEpro-SEAP or p-D-ACEpro-SEAP respectively, and then the cells were parallelly treated with or without 100  $\mu\text{M}$  of lisinopril for 24 h in each set of the experiment. The reporter assay showed that the promoter activity of p-I-ACEpro-SEAP was increased to 117% responding to lisinopril treating in comparison to the control group (Fig. 4 and Table 2). In contrast, we found that lisinopril suppressed approximately 18% of the ACE promoter activity in the cells transfected with p-D-ACEpro-SEAP (Fig. 5 and Table 3).

Further, we wanted to compare the relative reporter

activity of I form and D form vectors responding to lisinopril. There, we designated the promoter activity of the pACEpro(f)-SEAP group without treating lisinopril as 100%, and then merged the present results above and our previous results (Fig. 6a) [36] into one figure (Fig. 6b). After recalculating the relative percentage of the promoter activity of the three reporter vectors in SH-SY5Y cells incubated with or without lisinopril, we found that the I form reporter vector (p-I-ACEpro-SEAP) showed 2-fold higher promoter activity in responding to lisinopril as compared with the D form vector (p-D-ACEpro-SEAP) in SH-SY5Y cells.

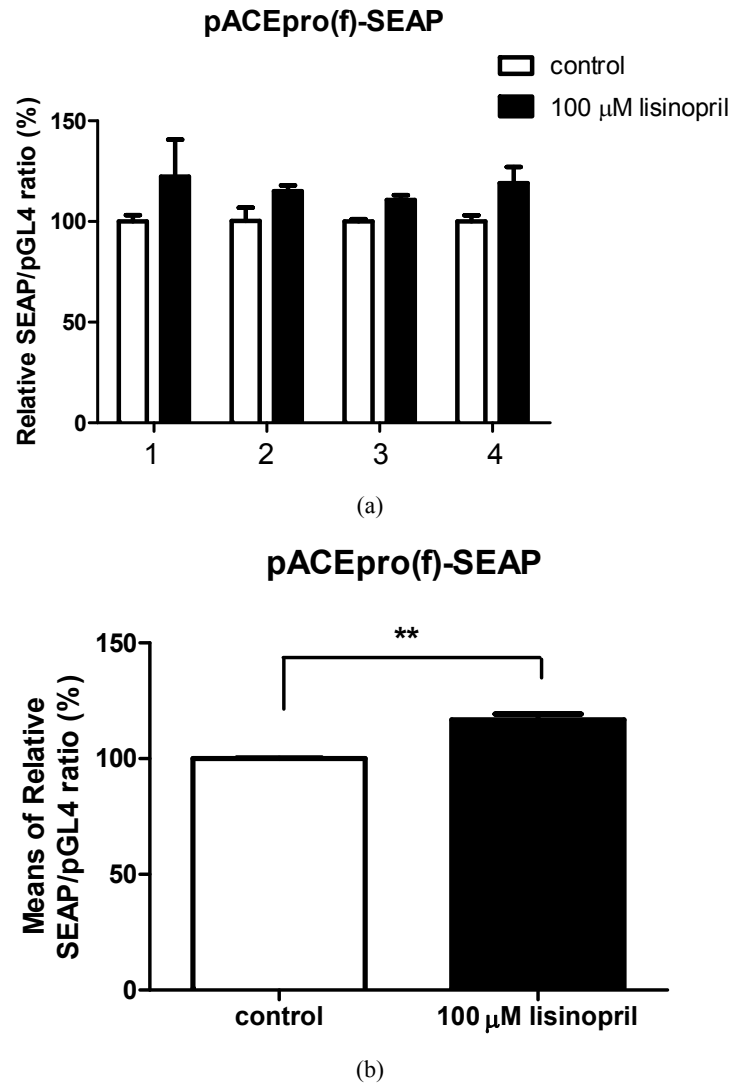
## 4. Discussion

In the present study, we first demonstrate the mechanisms that the ACE inhibitors could increase the promoter activity of the ACE gene in neurons, and that I and D form alleles exert an adverse response to lisinopril in regulating the ACE promoter activity. We propose that the binding of lisinopril to membrane-bound ACE might trigger phosphorylation of c-Jun and the activation of AP-1 (intracellular signaling cascade) that leads to an increase of the ACE



**Fig. 2** Effects of lisinopril on the cell viability of SH-SY5Y cells. SH-SY5Y cells were stimulated with lisinopril (10  $\mu\text{M}$ , 100  $\mu\text{M}$ , 1mM or 10 mM) or 30 nM OA, respectively. After 24 h, the cell viability was detected by prestobule reagent. The control group without lisinopril or OA treatment is designated as 100%. Data represent the mean  $\pm$  SEM of six independent experiments.

“ns”: no significant; “\*\*\*”:  $p < 0.001$  relative to control group.

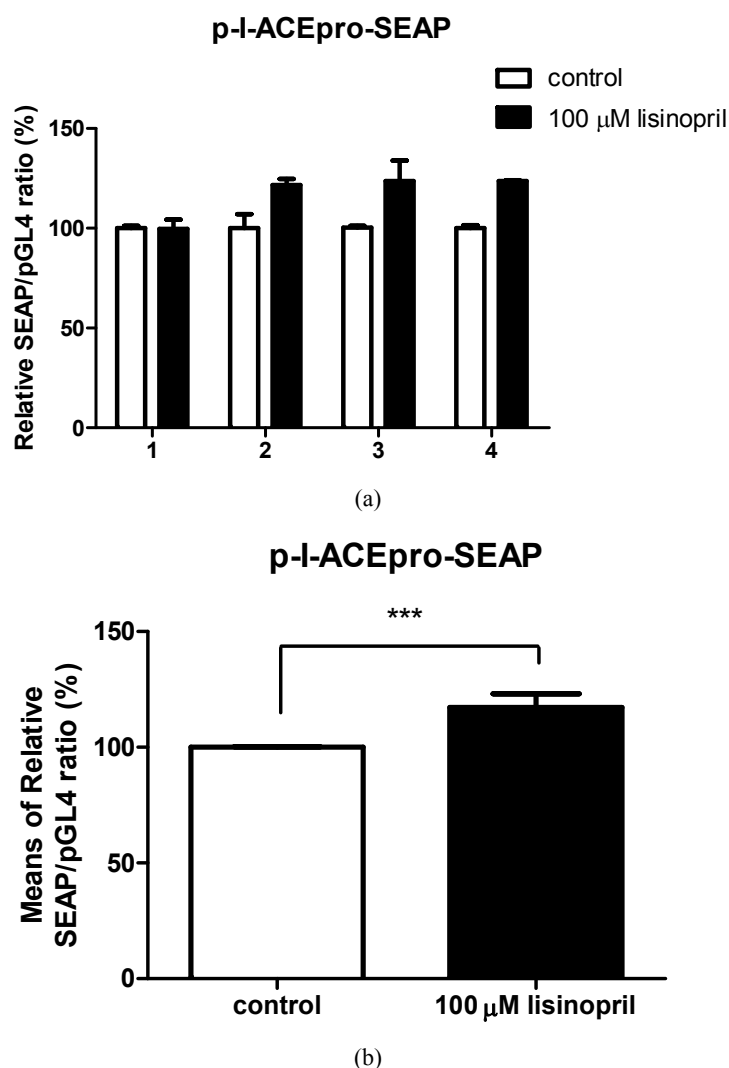


**Fig. 3** Effects of lisinopril on regulating the transcriptional activity of human ACE promoter in SH-SY5Y cells: (a) each relative SEAP/pGL-4 ratio of four independent experiments when the cells were transfected with pACEpro(f)-SEAP vector and treated with or without 100 μM lisinopril. For each construct, the experiments were performed in quadruplicate; (b) mean relative ratios of all four independent experiments from Fig. 3a. The control group without treating lisinopril is designated as 100%, and the values are expressed with standard error.

“\*\*\*”:  $p < 0.01$  relative to control group.

**Table 1** The statistic results from two-way ANOVA analysis in Fig. 3.

Effect		Mean		Difference	
Control		100.1	-	-	
100 μM lisinopril		116.8	-	+16.67	
Source of variation	DF	MS	F	P	
Residual	16	179.8	-	-	
Experiment	3	37.61	0.2092	0.8885	
Control vs. 100 μM lisinopril		1667	9.272	0.0077	
Interaction		38.78	0.2157	0.884	

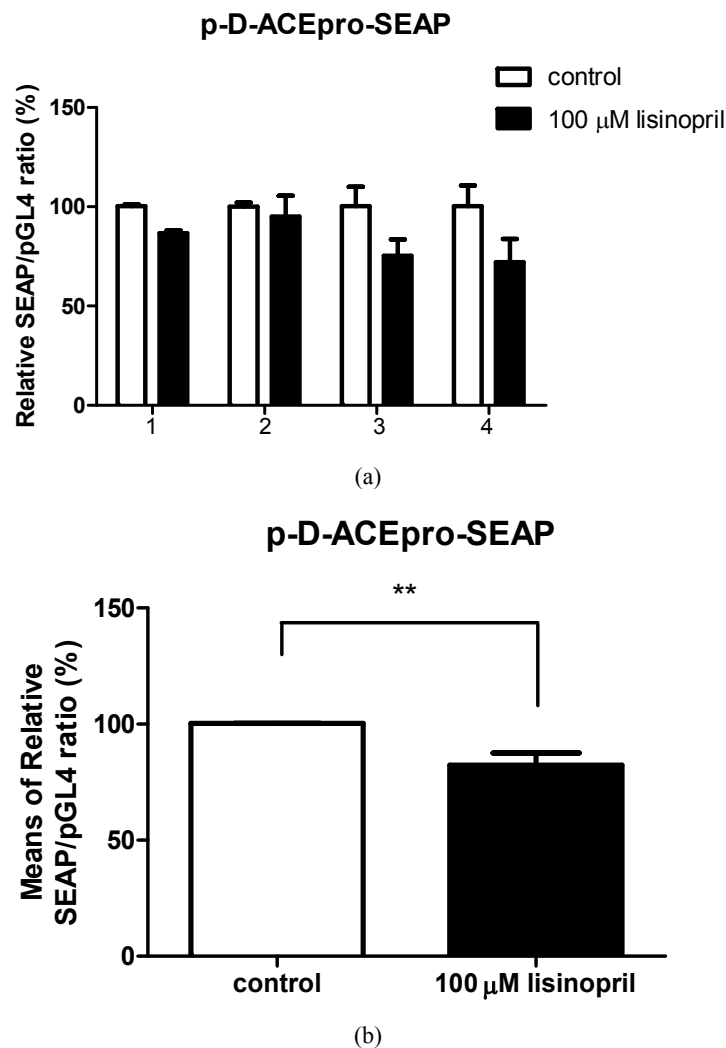


**Fig. 4** Effects of lisinopril on regulating the transcriptional activity of p-I-ACEpro-SEAP in SH-SY5Y cells: (a) each relative SEAP/pGL-4 ratio of four independent experiments when the cells were transfected with pACEpro(f)-SEAP vector and treated with or without 100  $\mu$ M lisinopril. For each construct, the experiments were performed in quadruplicate; (b) mean relative ratio of all four independent experiments from Fig. 4a. The control group without treating lisinopril is designated as 100%, and the values are expressed with standard error.

“\*\*\*”:  $p < 0.001$  relative to control group.

**Table 2** The statistic results from two-way ANOVA analysis in Fig. 4.

Effect		Mean		Difference
Control		100.1	-	-
100 $\mu$ M lisinopril		117.2	-	+17.2
Source of variation	DF	MS	F	P
Residual	16	71.21	-	-
Experiment	3	207.7	2.917	0.0662
Control vs. 100 $\mu$ M lisinopril	1	1751	24.59	0.0001
Interaction	3	203.4	2.856	0.0699



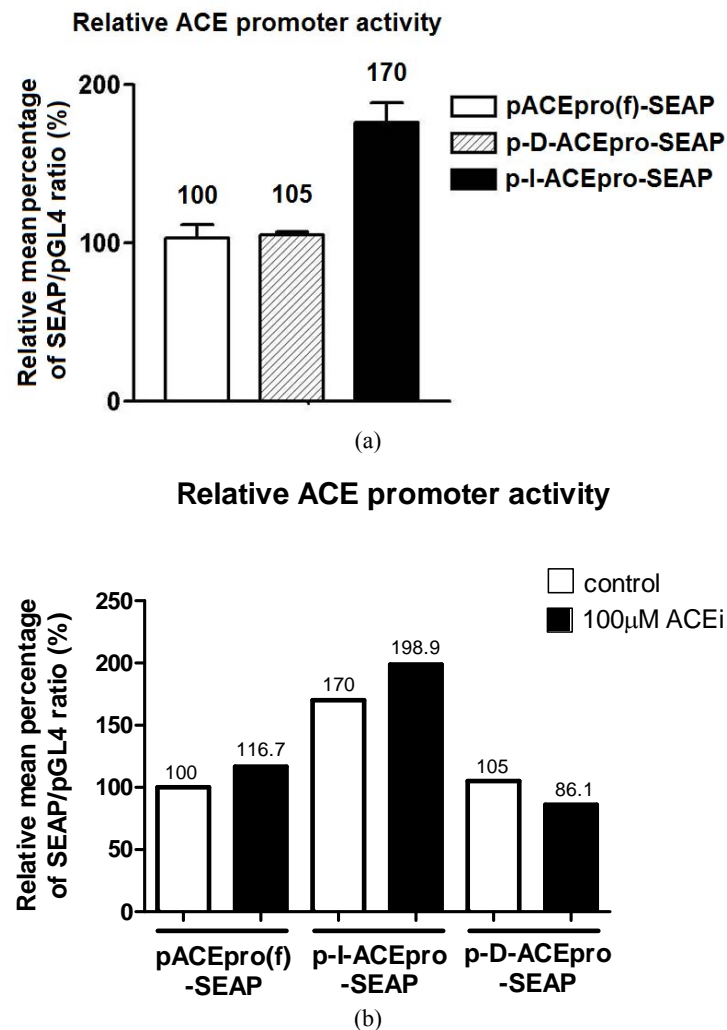
**Fig. 5** Effects of lisinopril on regulating the transcriptional activity of p-D-ACEpro-SEAP in SH-SY5Y cells: (a) each relative SEAP/pGL-4 ratio of four independent experiments when the cells were transfected with pACEpro(f)-SEAP vector and treated with or without 100 μM lisinopril. For each construct, the experiments were performed in quadruplicate; (b) mean relative ratio of all four independent experiments from Fig. 5a. The control group without treating lisinopril is designated as 100%, and the values are expressed with standard error.

“\*\*\*”:  $p < 0.01$  relative to control group.

**Table 3** The statistic results from two-way ANOVA analysis in Fig. 5.

Effect		Mean		Difference	
Control		100.3	-	-	
100 μM lisinopril		82.25	-	-18.00	
Source of variation	DF	MS	F	P	
Residual	16	197.2	-	-	
Experiment	3	163.3	0.8279	0.4977	
Control vs. 100 μM lisinopril		1	1944	9.858	0.0063
Interaction		3	171.8	0.871	0.4765





**Fig. 6** The relative response of I and D form to ACE inhibitor in ACE promoter activation: (a) to represent the relative percentage of three relative ACE promoter activities when the transfected cells were not treated with lisinopril, which was modified from Fig. 2 of our previous report [36]; (b) to compare the relative promoter activities of the three vectors in responding to lisinopril, we recalculated the relative percentage of their promoter activities from the results in Figs. 3-5 and 6a. The basal promoter activity of pACEpro(f)-SEAP without lisinopril treatment is designed as 100% control.

expression as previously reported [39]. Pharmacogenetic testing brings the potential for personalized therapeutic strategies not only to improve health outcomes and efficacy of prescribed medications but also reduce the cost of care. However, there is substantial debate about the clinical utility of pharmacogenetic testing, due in part to the lack of direct evidence or defined mechanisms. Presently, no clinical pharmacogenetic practice is currently applied for AD treatment and even for cardiovascular medicines [40]. Herein, our study first provides the direct molecular evidence that I/D polymorphism in

Intron 16 of an ACE gene may play a vital role in adversely responding to the ACE inhibitors in regulating the ACE expression in neurons.

It is noteworthy that the pharmacological role and plausible mechanism of ACE inhibitors in AD remain unsolved paradoxes, although Kehoe and Wilcock thoughtfully discussed this issue in their review article based upon the clinical trials and laboratory-based findings [41]. They indicated that the introduction of an ACE inhibitor in order to block the ACE activity might not only reduce the benefits to A $\beta$  degradation [42, 43], but also decrease the potential

harm of Angiotensin II action on either acetylcholine release or hypertension. In addition, the inhibition of an ACE activity in the brain has also been reported to associate with cognitive improvement via increasing substance P, diminishing inflammatory cytokines, suppressing reactive oxygen species generation, or improving cerebral blood flow [2, 4, 6, 12]. In addition to the well-known functions of an ACE enzyme as mentioned above, the physiological implications of ACE continue to expand, in which ACE has been found to exert many other functions than is usual and is localized in different brain tissues. For instance, Paul et al. [44] indicated that “intracellular” ACE converting ANG I to intracellular ANG II in brain neurons may facilitate learning and memory. A functional RAS in mitochondria was demonstrated to have a role in nitric oxide production in many tissues including neurons [45]. Moreover, Bernstein et al. recently presented that overexpression of ACE in myelomonocytes could prevent Alzheimer’s-like cognitive decline through degrading A $\beta$  and enhancing immunity functions [46]. Taken together, it keeps pending to conclude if the induction of an ACE expression by the ACE inhibitors in neurons is beneficial to cognitive decline or otherwise. Thus, to further clarify the pharmacological role of the ACE inhibitors in increasing its target, an ACE protein could be challengeable but very helpful to understand the clinical application of the ACE inhibitors.

According to our present findings, lisinopril positively enhances I allele-increased promoter activity of the ACE gene because of the Alu element. In contrast, D allele seems to restrict the promoter activity responding to lisinopril in neurons. Due to the paradoxical hypothesis of the ACE function relevant with AD pathogenesis as discussed above, there would be two novel contrary perspectives to interpret our results: (1) if the increased ANG II is the main pathogenesis consideration for some subgroups of AD patients, then the individuals with D allele genotype in

the subgroups would be suggested to have the introduction of the ACE inhibitors to restrain their ACE expression; (2) in contrast, if the clearance of A $\beta$  aggregation is the priority for some subpopulations of AD patients, then it might be beneficial for the individuals with I allele to take the ACE inhibitors in order to produce more ACE molecules. Therefore, before an associated pharmacogenetic trial of an ACE I/D with better cognitive response to the ACE inhibitors in AD, we suggest in advance an effective strategy to subgroup the pathological type of AD individuals.

However, the inconsistently associated results of the ACE I/D polymorphism with a specific disease (e.g., CVD or AD) have led to some arguable issues existing for more than a decade. First, the main question is that none of the available direct evidence has been provided to delineate the definite molecular mechanism by which D allele was pointed out to be associated with a higher ACE activity or concentration. The second vital question is whether the secreted ACE in serum could represent the amount of all ACE protein expressed from all tissues of the individual. Recently, a study provided an important evidence that the secreted ACE in human serum is very minor part of expressed ACE in comparison to membrane-bound ACE [47]. Besides, Fagyas and his colleagues [48, 49] showed that most of the secreted ACE in body fluid are blocked by natural ACE inhibitors in serum (e.g., serum albumins). Moreover, according to Chattopadhyay’s report [50], the secreted form of ACE can not replace the tissue-bound ACE for maintaining normal blood pressure. According to the above, it might not be the best investigative strategy to use the concentration of serum ACE to represent the function of systemic ACE, and it might be even unwarrantable to use serum ACE to interpret the pathophysiological role of a specific local ACE expression. Therefore, those previous studies that examined the association between the serum ACE concentration and chronic diseases might need to be

reevaluated. Conceptually, it would be more questionable to use serum ACE concentration, but not CSF ACE, to explore the association of an ACE function with neurodegenerative diseases (e.g., AD) without taking into consideration of the blood-brain-barrier function and the independent role of local RAS in the brain tissue.

## 5. Conclusions

Our findings first proved that lisinopril increased the promoter activity of an ACE gene in neurons, and that the I/D polymorphism of the ACE gene contrarily responds to the ACE inhibitors in regulating the ACE expression in neurons. Hitherto, our study moves an important step forward to provide a deep insight for the future pharmacogenetic testing of the ACE I/D polymorphism responding to the ACE inhibitors in AD patients.

Herein, we also propose that first it may be necessary to subgroup the same pathological type of AD before the researchers tackle a pharmacogenetic trial to clarify the response of an ACE I/D to ACE the inhibitors. For instance, the subgroup could be the individuals of AD with hypertension, AD with amyloid burden, or AD without macrovascular events. As doing such, it may provide new experimental evidence to guide the clinical doctors to do personalized therapy in the future.

## Disclosure Statement

The authors have no actual or potential conflicts of interest to disclose. No writing assistance was utilized in the production of this manuscript.

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