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G82S RAGE polymorphism is associated with Alzheimer's disease

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TABLE OF CONTENTS

1. ABSTRACT

Receptor for advanced glycation end products (RAGE) has been implicated in the pathophysiology of Alzheimer's disease (AD) due to its ability to interact with amyloid beta and to elicit an inflammatory response. sRAGE, one of the splice variants of RAGE, has been reported to be a decoy receptor for amyloid beta peptides. The present study addresses the occurrence of G82S RAGE polymorphism in AD, and its association with the expression of sRAGE and amyloid beta load (Aβ peptide). The results indicated that the heterozygous genotype (GS) was distributed more than the wild genotype (GG) in patients with AD. Moreover, in patients with AD, there was decreased expression of sRAGE and increased expression of tRAGE and TNF-α. The data show that G82S RAGE polymorphism is highly associated with the development of AD, with decreased expression of sRAGE and increased expression of tRAGE and TNF-α.

2. INTRODUCTION

Alzheimer's disease (AD) is the sixth leading cause of death worldwide. In India, about 3.7 million people have AD and it is estimated that by 2030, the incidence will rise by two-fold (1). AD is a complex multistep and multifactorial disease involving genetic and environmental factors. Major pathological features of AD include the presence of senile plaques, neurofibrillary tangles (NFTs), and neuronal loss (2). In addition to the established pathology of senile plaques and NFT, there is a significant oxidative damage in the brains that are involved with AD (3). The pathology in the brain with AD is associated with the overproduction, deposition and failure in clearance of Aβ peptide (4).

Receptor for advanced glycation end products (RAGE) is a transmembrane receptor which binds to multiple ligands namely amyloid (Aβ), S100, amphoterin, and mac-1 and mediates inflammatory

response. It also acts as a cargo transporter for circulating plasma Aβ across blood brain barrier (BBB) into the brain (5). Activation of full-length RAGE (fRAGE) by Aβ binding, stimulates the expression of β site APP cleaving enzyme through T1 activation leading to enhanced Aβ production (6). RAGE also enhances the production of pro-inflammatory cytokines via the activation of the NF-κB pathway (7).

Within the brain, Aβ is cleared by phagocytosis, interstitial fluid drainage and also by crossing through the BBB to circulation. This is mediated by RAGE and the low-density lipoprotein receptor-related protein (LRP1) (8, 9). LRP1 and p-glycoprotein regulate the efflux of Aβ to circulation from the blood. Aβ is cleared out from systemic circulation by the liver/kidney. Soluble LPR (sLPR) in the blood prevents re-entry of Aβ back into the brain. However, the Aβ peptides also escape the sLRP-mediated mechanism and re-enter the brain through RAGE, further augmenting oxidative stress and neuronal damage (10).

There are about thirty single nucleotide polymorphisms (SNPs) reported to be present in the RAGE gene. The polymorphism in ligand-binding regions of the RAGE could affect the expression and function of RAGE (11). Several epidemiological studies have investigated the association between the G82S polymorphism in the RAGE gene and increased risk of AD development (12, 13, 14, and 15). A splice variant of RAGE termed soluble RAGE (sRAGE) acts as a decoy receptor for Aβ due to the absence of membrane - spanning domain. The present study focuses on analyzing the association of G82S RAGE with AD in south Indian population and to study the expression of RAGE variants and its correlation with Aβ in AD pathology.

3. MATERIALS AND METHODS

3.1. Study subjects

Individuals with AD (n=40) were recruited from PSG Institute of Medical Sciences and Research (PSG IMS&R), Coimbatore and Alzheimer's and Related Disorders Society of India (ARDSI), Thrissur, India. The present study was approved by the Institutional Ethics Committee of PSG IMS&R and all procedures involving human subjects were in accordance with the Declaration of Helsinki. Informed consents were obtained from control subjects and from the relatives/legal guardians of the AD patients for their inclusion in the study. The cognitive status of each subject was assessed using Mini-Mental State Examination (MMSE) (16) and the patients were categorized according to the criteria of NINCDS-ADRDA and DSM-IV for dementia (17). AD patients showing co-morbidity with cardiovascular disease and diabetes were excluded from the study. The control subjects (n=40) included in the study were in the age group ranging between 65-85 years with a negative history of cardiovascular disorders, diabetes and neurological disorders. Subjects with habits of smoking or alcohol consumption or having certain advanced malignancies such as AIDS and other major illnesses were excluded from the study. The blood samples (5 ml) from AD subjects and age matched healthy controls were collected individually in EDTA tubes and 4 ml was utilized for separation of plasma, PBMC and RNA. The remaining 1 ml was utilized for DNA isolation.

3.2. Quantification of RAGE and TNF-α expression by qPCR

Peripheral blood mononuclear cells (PBMC) were isolated by HiSep LSM density gradient separation (Himedia, Mumbai, India). Total RNA from PBMC was isolated using RNA-XPress™ reagent (Himedia, Mumbai, India). Quality of RNA was analyzed using a nanospectrometer, (Imple, USA). 250 ng of total RNA was then reverse-transcribed using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA) and stored at -20º C until further use.

Expression analysis was performed for total RAGE (tRAGE), sRAGE and TNF-α using Quantitative real-time PCR (CFX96 Touch™ Real-Time PCR - Bio Rad) with 2X SYBR Green qPCR master mix (Applied Biosystems, USA). Information regarding the primer sequences used

Table 1. Primers sequence used for analysis of RAGE variants by qPCR

Gene	Forward (5'-3')	Primer (5'-3')	Product size (base pair)
Trage	GGGCAGTAGTAGGTGCTCAA	TCCGGCCTGTGTTCAGTTTC	120
Srage	AGCATCATCGAACCAGGCGA	TTTTCTGGGGCCTTCCATTC	134
TNF-α	CCCCAGGGACCTCTCTCTAATC	GGTTTGCTACAACATGGGCTACA	98
GAPDH	TGCACCACCAACTGCTTAG	GGATGCAGGGATGATGTTC	177

for real-time PCR analysis can be found in Table 1. The primers for tRAGE were designed with reference to the fRAGE gene (GenBank ID – NM001136) using Primer-Blast tool ensuring that it was comprised the exon-exon junction to avoid amplification of contaminating genomic DNA. On the other hand, the primers for soluble RAGE (containing intron 9) were designed to have an exon-intron junction and an exon-exon junction, ensuring it amplified all the prominent soluble isoforms (Figure 1). Though primers used for tRAGE can amplify other variants excluding sRAGE, the canonical variant fRAGE has been reported to be the major variant (70% to 80%) expressed in human tissues (18).

The PCR conditions were used as follows: 50ºC for 2 min, 95ºC for 10 min, followed by 40 cycles of 95ºC for 10s, and 60ºC for 1 min. Relative quantification of gene transcripts was calculated using $2^{-\Delta\Delta Ct}$ method and normalized to Glyceraldehyde-3-phosphate dehydrogenase (GADPH) transcripts.

3.3. Quantification of Aβ42 and sRAGE in plasma

Samples of blood collected in EDTA tubes were centrifuged at 400 g for 15 minutes to separate plasma, which was stored at -20 °C in aliquots till use. Aβ42 and sRAGE were quantified in plasma using a commercially available ELISA kit from CUSABIO (CSB-E10684h) and Quantikine R&D systems (DRG00, USA), respectively, according to the manufacturer's instructions.

3.4. G82S RAGE polymorphism

The Polymerase chain reaction – restriction fragment length polymorphism (PCR- RFLP) was designed to identify G82S RAGE polymorphism. The genomic DNA was isolated using Xpress DNA kit (Magenome, India), according to the manufacturer's instruction. The PCR was carried out in EmeraldAmp® PCR Master Mix (Takara, Japan) with genomic DNA (200 ng) and 10 µM of forward (GTCCCTCACCCACCCTGTC) and reverse primers (GCCACACACCCACACCCAC) for amplification of exon 3 region of RAGE gene containing G82S polymorphism. The amplified product (817 bp) was subjected to restriction digestion by *Alu I* (5 units) for 2h at 37 ºC followed by electrophoresis on 2% agarose gel.

3.5. Statistical analysis

Statistical analysis was performed using the statistical package SPSS Version.16, Chicago, USA. An independent sample t-test was used to compare cases between AD and control. Receiver operating characteristic (ROC) curve analysis was performed to study the reliability of sRAGE in plasma as marker for AD diagnosis. Pearson's correlation was done wherever applicable. For genotype and allelic frequency, the level of significance was analyzed using chi-squared test. The disease relationship with genotype and allelic frequencies is presented as an odds ratio with 95% confidence interval.

4. RESULTS

The baseline clinical characteristics of the study groups (AD and control) are shown in Table 2. The MMSE score in AD group indicated severe cognitive dysfunction. The plasma Aβ 42 level was found to be significantly higher in the AD group compared to controls ($p < 0.001$).

4.1. Analysis of expression of RAGE variants and TNF-α in PBMC

Expression of tRAGE, sRAGE and TNF-α were quantified using qPCR with gene specific primers. To analyze mRNA expression of RAGE

Table 2. Clinical characteristics of study groups

Parameters	Alzheimer's patients	Control subjects
Number of subjects	40	40
Sex (W/M)	23/17	15/25
Age (Years)	74.45 ±0.95[b]	70.4 ±1.4[b]
MMSE[a]	2.0 ± 0.28[b]	25.9 ± 0.32[b]
Aβ42 (pg/ml)	191.57 ± 9.5 [b]	88.48 ± 6.7 [b]
Abbreviations: W = Women, M = Men; [a] Mini Mental State Examination (MMSE) score = Profound 0-4, Severe 4-13, Moderate 13-16, Mild 17-23, Normal 24-30; [b] Mean ± SEM.		

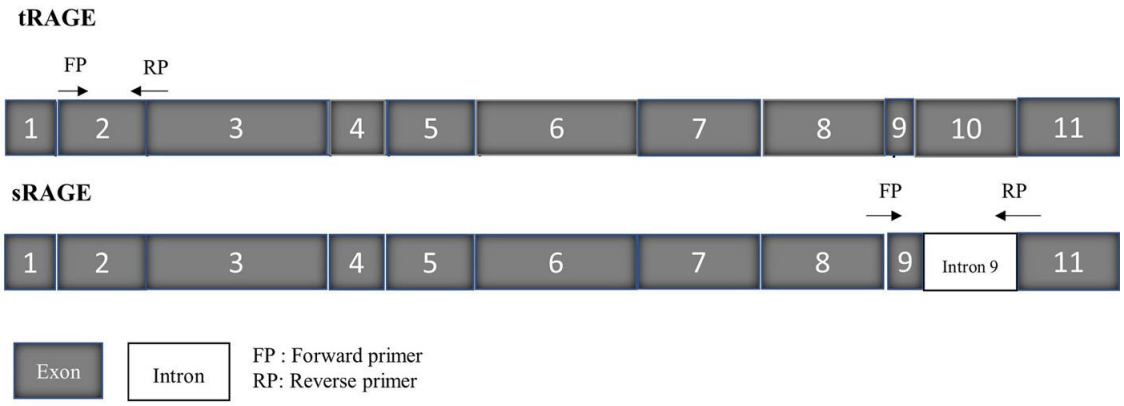

Figure 1. Schematic representation of qPCR primer designed to amplify tRAGE and sRAGE variants. RAGE gene exons, intron; Forward primer: FP, Reverse primer: RP.

variants, the comparative threshold cycle (Ct) method was applied with GAPDH as the endogenous reference gene. The first, synthesized strand cDNA was verified by the amplification of GAPDH. Melt curve analysis of sRAGE, tRAGE and TNF-α revealed a single peak which represents a single amplicon and specific amplification (Figure 2A). The results revealed that while the expression sRAGE was down regulated by 3.4-fold, tRAGE expression was upregulated by 1.9-fold in AD samples compared to controls. There was also an upregulation in the expression of TNF-α (2.2 fold) in the AD group associated with lower expression of sRAGE in AD (Figure 2B, Table 3).

4.2. Quantification of sRAGE and Aβ42 in plasma samples

The levels of sRAGE in plasma was significantly lower in patients AD (151.91±15.9 pg/mL, $p<.001$) as compared to the levels found in cognitively healthy age matched control (753.79±58.12 pg/mL) (Figure 3A). The plasma Aβ42 level was found to be higher in AD (191.57 ± 9.5 pg/ml, $p<.0001$) compared to controls (88.48 ± 6.7 pg/ml) Table 4. The correlation analysis between plasma sRAGE and Aβ levels has shown no significant relation (AD R = 0.122; age matched healthy control subjects R = -0.080) (Figure 3B). ROC curve analysis was conducted to assess the cutoff points of sRAGE to differentiate the AD group from the controls. The area under the curve was found to be 0.981, indicating good diagnostic accuracy for sRAGE with a cutoff value of ≤ 278.09 pg/ml (Sensitivity 100 %, Specificity 85%) suggesting that plasma sRAGE could be used to distinguish AD from controls ($p = 0.001$; 95% CI, 0.960 – 1.00, Figure 3C).

4.3. Analysis of RAGE G82S polymorphism

In the present study PCR-RFLP was adopted to analyze the G82S polymorphism.

Table 3. The expression levels of tRAGE, sRAGE and TNF-α mRNA

Gene	Values in AD compared to age matched healthy controls
Trage	1.9-fold
Srage	3.4-fold
TNF-α	2.2-fold

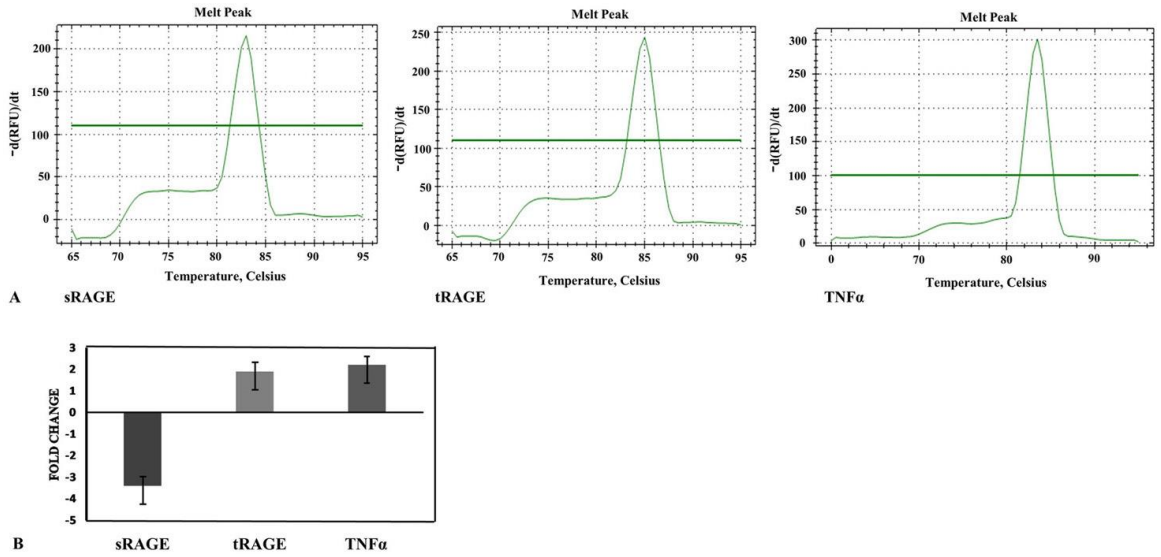

Figure 2. Expression profile of RAGE variants and TNF-α. (A) Melt curves from qPCR for sRAGE, tRAGE and TNF-α. (B) Analysis of RAGE variants and TNF-α mRNA expression in PBMC. Bar graph depicts mean gene expression levels and error bars represents the standard error mean obtained from the average of 40 nos. Expression values are obtained by $2^{-\Delta\Delta CT}$.

Information on the primers used, nucleotide sequence of PCR products and *AluI* restriction site in the PCR product are all represented in Figure 4A. The representative gel image of six samples after digestion of the PCR-amplified products (Figure 4B) and schematic pattern of RFLP after digestion with *AluI* enzyme can be found in Figure 4C. The distribution of genotype was in accordance with the Hardy–Weinberg equilibrium. The genotypic and allelic frequency of G82S RAGE polymorphism in AD and control group along with the resulting odds ratio and significance levels is represented in Table 5**.**

The S82S genotype was not observed in both groups. Allelic frequency of S allele in the AD and control group was 0.225 and 0.037 respectively, whereas for G allele, it was 0.775 and 0.963 respectively. The observed difference in genotype distribution and allelic frequency between controls and the AD group was statistically significant (χ^2 test, p <0.05) (Table 5). Reduced sRAGE in subjects with GS genotype was also observed compared to GG genotype and the results were statistically significant (Figure 5).

5. DISCUSSION

In the present study, the differential expression of tRAGE and sRAGE was analyzed in PBMC of AD and age matched control. The upregulation of fRAGE and down regulation of sRAGE in AD patient highlights the role of RAGE variants in AD pathogenesis. sRAGE being the circulatory form, was analyzed in plasma, also indicated down regulation of its expression and this was correlated with increased Aβ load in AD. The graphical representation of these results represented in Figure 6.

The risk association of G82S carrier with

Table 4. Plasma levels of Aβ42 and sRAGE

Marker	Plasma levels	
	Age matched healthy controls (pg/mL)	Individuals with AD (pg/mL)
Aβ42	88.48 ± 6.7	191.57 ± 9.5
Srage	753.79±58.12	151.91±15.9

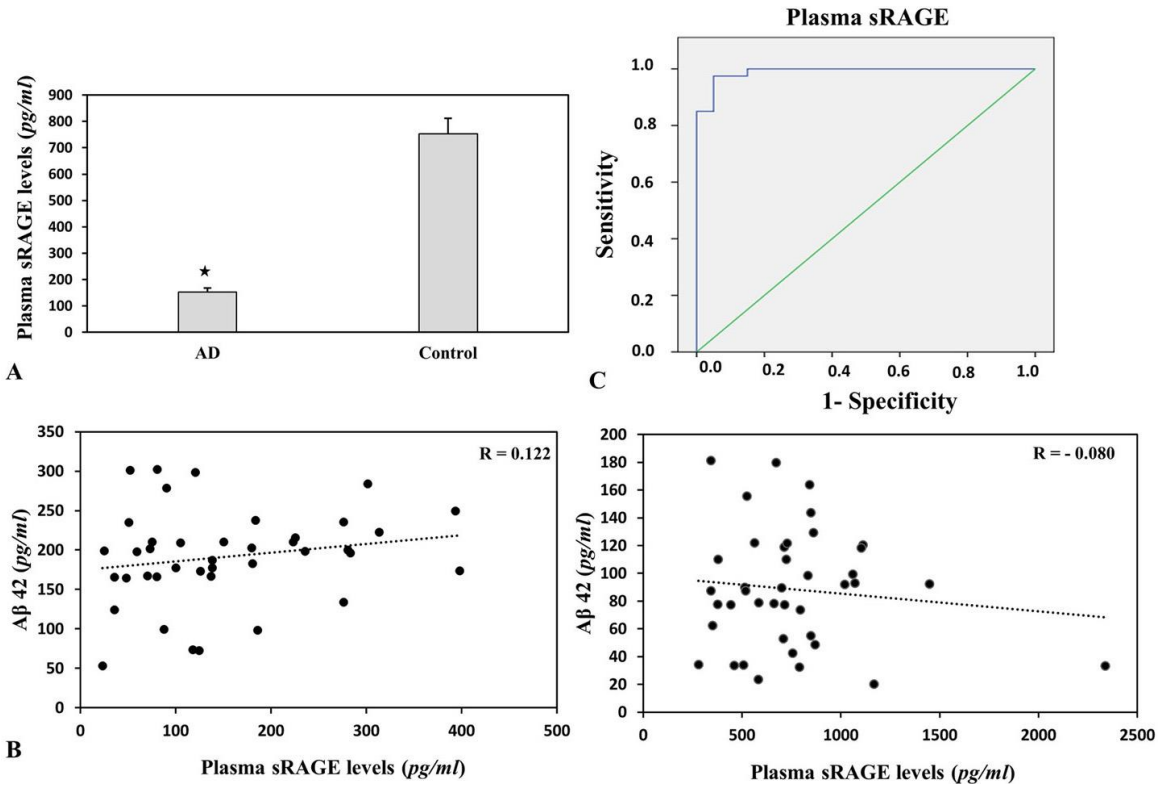

Figure 3. Plasma sRAGE levels in AD. (A) Plasma sRAGE in AD and control. The values are shown as mean ± SEM and were found to be statistically significant compared to control, (p<0.001). (B) The correlation between concentration of sRAGE and Aβ42 (i) AD (R=0.122) ii) controls (R= -0.080). (C) Receiver operating characteristic curve analysis of plasma sRAGE: AUC: 0.981 (p < 0.001), cutoff value of ≤ 278.09 (Sensitivity 100 %, Specificity 85%). The quality of diagnosis test is determined by AUC: 0.9-1 excellent, 0.8-0.9 good, 0.7-0.8 fair, 0.6-0.7 poor, 0.5-0.6 fail.

AD was analyzed in South Indian population. The frequency of occurrence of 82S risk allele (heterozygous form) is higher in AD compared to age matched control indicating that 82S RAGE allele is probably the risk factor for AD. Association of G82S polymorphism with AD is also reported in Chinese (12, 13), Korean (14) and Turkish population (15). sRAGE by binding to circulating Aβ in the plasma regulates Aβ equilibrium between brain and peripheral circulation thus enhanced Aβ clearance (19). The ELISA and qPCR analysis of RAGE variants showed significant decrease in sRAGE in plasma along with an increase in tRAGE in PBMC in AD compared to controls. Both sRAGE and sLRP was found to be decreased in Chinese population (20,21). Reduced sRAGE might interfere with the clearance of Aβ. The decreased level of sRAGE could be responsible for increased load of Aβ observed in plasma of AD patients and ROC analysis supports the use of plasma sRAGE as a diagnostic marker for identifying the cases of AD with sensitivity and specificity of 100 % and 85% respectively. The

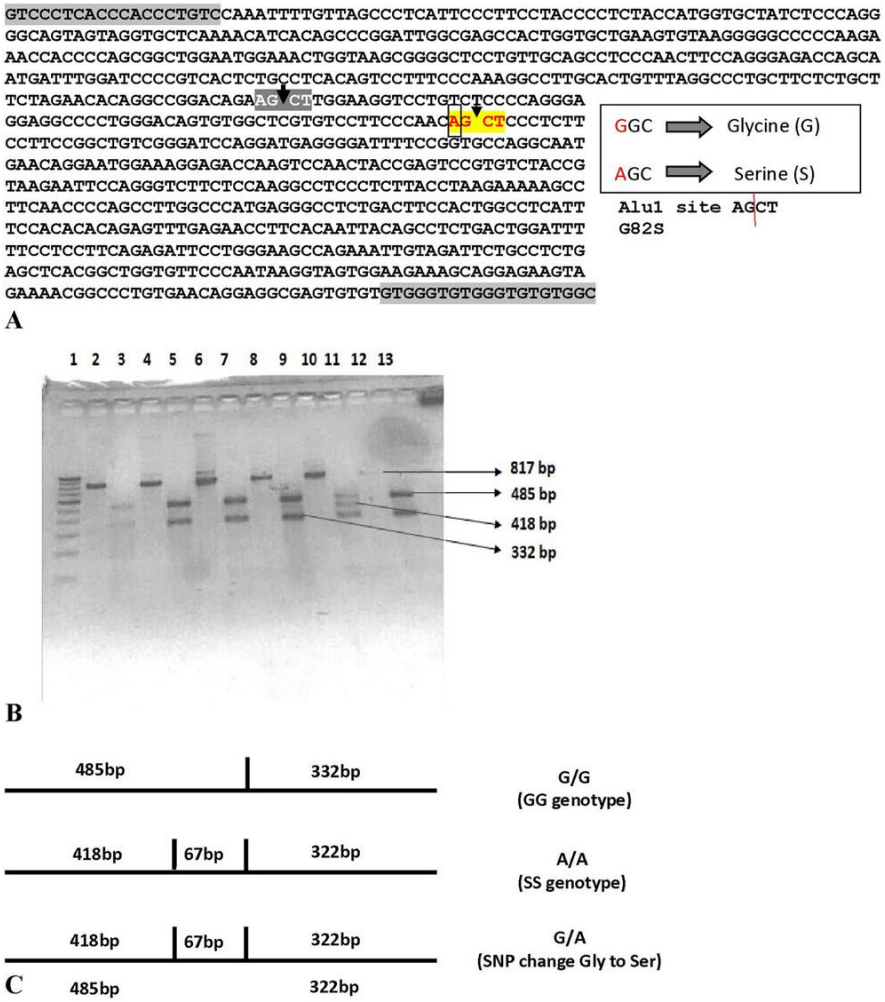

Figure 4. G82S RAGE Polymorphism**.** (A) Nucleotide sequence of the PCR-amplified RAGE gene showing the restriction site of *AluI* that are highlighted in grey and yellow color and indicated by arrow. The yellow color represents the restriction site that occurred due to single nucleotide change from G to A that shifted Gly 82 to Ser. Primer sequences are highlighted in gray. (B) PCR RFLP results of G82S RAGE gene polymorphism. PCR product: Lane - 2,4,6,8,10,12; Restricted samples (*AluI* digest): Lane 3,5,7,9,13- Genotype GG, Lane 11 - Genotype GS, Lane 1- 100 bp Ladder. (C) Restriction pattern of RAGE for homozygous GG and SS and heterozygous GS. After *AluI* digestion, the G82G genotype of RAGE gives two fragments and S82S genotype gives three fragments. Due to single nucleotide polymorphism, the heterozygous GS allele gives four fragments.

observed increase in fRAGE expression may be due to the consequences of Aβ overload, since it has been reported that Aβ toxicity induces fRAGE expression leading to further oxidative damage. While plasma TNF-α was shown to be enhanced during aging (22) the observed increase in TNF-α (2.2-fold) levels in AD compared to age matched healthy controls indicate the association of TNF-α in AD probably due to its effect as an early mediator of acute phase response (2,12, 23, 24).

sRAGE was decreased in G82S RAGE genotype in both AD and control subjects compared to the G82G genotype. The decreased sRAGE observed in plasma could be due to alteration in N81 glycosylation of RAGE caused by G82S polymorphism which makes it more susceptible for metalloprotinases (25) and also due to alteration in transcription and expression of RAGE gene. Park *et al.* had confirmed that the Asn^{81} site is fully glycosylated in G82S RAGE with significant amounts

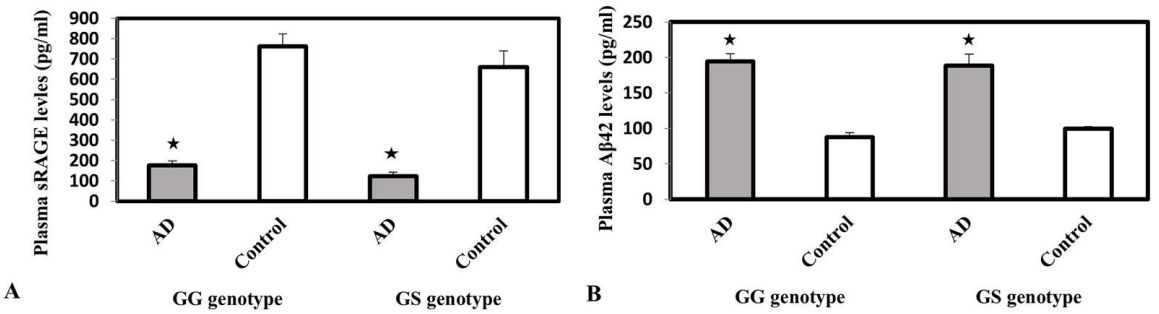

Figure 5. Association of G82S RAGE genotype on sRAGE and Aβ42 expression in AD. (A) sRAGE in different RAGE genotypes. It is found that the expression of sRAGE was reduced in AD, which is further reduced in G82S RAGE genotype compared to G82G RAGE in AD and control. (B) Aβ42 levels in different RAGE genotypes. The expression of Aβ42 was observed to be high in AD in both genotypes compared to control. The values are shown as mean ± SEM. The effects of G82S polymorphism on the levels of sRAGE and Aβ42 expression in AD were found to be statistically significant with the p value, $p<0.0001$

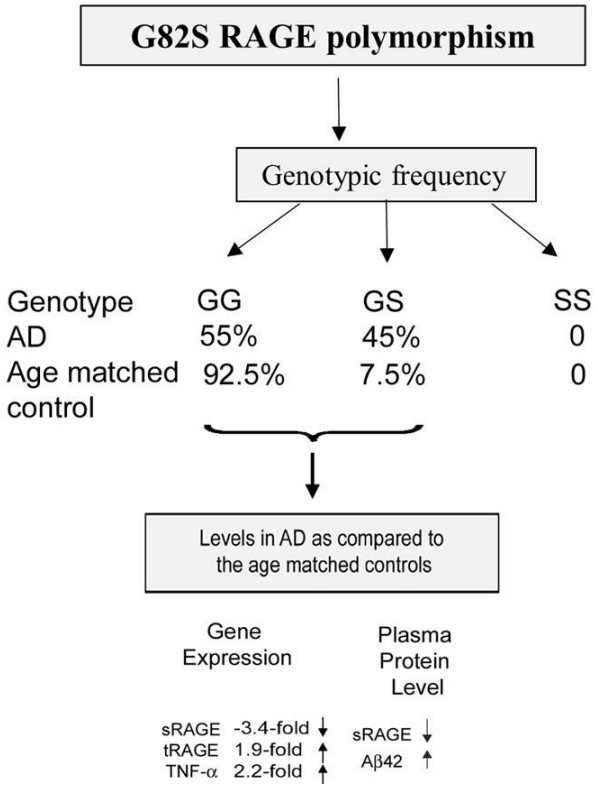

Figure 6. The G82S RAGE polymorphism showing genotypic frequency, and levels of sRAGE, tRAGE, TNF-α. Arrows point to up or downregulation.

of *N*-linked glycan, proved through the combination of MS analysis and PNGase-F digestion (26). Asn^{81} is located in close proximity to a hydrophobic cavity in the RAGE V-domain. Hydrophobic interactions involving residues within this cavity are critical for RAGE- S100B protein ligand binding (27) and are those most likely to be influenced by Asn^{81} glycosylation. The effect of *N*-linked glycosylation on the outcome of the RAGE-S100B ligand interaction was confirmed through measures of NF-κB

activation. Based on these studies, the change in the structure and conformation of RAGE due to alteration in glycosylation in G82S RAGE polymorphism has been hypothesized for RAGE- Aβ interaction as well. Higher level of 82S risk allele with lower levels of sRAGE would further favor the formation of Aβ fibrils significantly based on the concept that Chaney *et al.* (2005) (28) established the abolishment of Aβ filamentous aggregation, when Aβ peptide is incubated with sRAGE for longer time. A longitudinal study with the incorporation of MCI to AD is needed to use the circulating sRAGE as a preliminary marker for AD diagnosis.

6. ACKNOWLEDGMENTS

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Abbreviations: AD: Alzheimer's disease; RAGE: Receptor for advanced glycation end products; Aβ 42: Amyloid peptide 1-42; CSF: Cerebral spinal fluid; BBB: Blood brain barrier; LRP: low-density lipoprotein receptor-related protein, sRAGE: soluble receptor for advanced glycation end products, tRAGE: total receptor for advanced glycation end product, TNF-α: tumor necrosis factor alpha, Aβ42: amyloid peptide

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