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# Reduced telomere length in subjects with dementia and diabetes mellitus type 2 is independent of apolipoprotein E4 genotype

Lakshmi Narayanan Kota, Srikala Bharath *, Meera Purushottam, Pradip Paul, Palanimuthu Thangaraju Sivakumar, Mathew Varghese, Sanjeev Jain

*Department of Psychiatry, National Institute of Mental Health and Neurosciences, Bangalore, India*

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ABSTRACT

Apolipoprotein E4 gene is associated with increased risk of dementia with comorbid diabetes mellitus. Both dementia and diabetes mellitus type 2 are independently associated with telomere shortening. We assessed relative telomere length and apolipoprotein E genotype in subjects with dementia ($n = 70$) and cognitively normal control groups ($n = 55$) with and without comorbid diabetes mellitus type 2. Relative telomere length was highest in the control group ($Q2 = 0.91$) followed by dementia ($Q2 = 0.48$) and dementia with comorbid diabetes mellitus type 2 ($Q2 = 0.39$). Apolipoprotein E4 allele frequency was highest in dementia with comorbid diabetes mellitus type 2 (0.26). Apolipoprotein E4 allele was not significantly associated with telomere attrition in both dementia and cognitively normal group irrespective of comorbid diabetes mellitus type 2 ($P > 0.05$). The findings suggest that relative telomere length is unrelated to apolipoprotein E4 genotype in dementia and cognitive normal subjects with or without comorbid diabetes mellitus type 2.



## 1. Introduction

Telomeres are repetitive DNA sequences at the ends of eukaryotic chromosomes (Moyzis et al., 1988). Without telomeres, the ends of chromosomes would be recognized as double stranded DNA breaks by DNA repair machinery. During S phase of every cell replication, the telomeres replicate incompletely and become shortened by a few base pairs (Olovnikov, 1971, 1973; Watson, 1972). Under normal circumstances, this shortening is corrected by an RNA enzyme complex called telomerase (Greider and Blackburn, 1985, 1987). The telomerase and telomere associated shelterin proteins are suggested to have an important role in telomere homeostasis influenced by specific haplotypes representing these proteins (Codd et al., 2010). Short telomeres are associated with aging and degenerative phenotypes like dementia, diabetes and atherosclerosis (Von Zglinicki et al., 2000; Thomas et al., 2008; Löf-Ohlin et al., 2008; Kume et al., 2012; Honig et al., 2012; Balasubramanyam et al., 2007).

Apolipoprotein E4 allele is an established risk factor for developing dementia (Bharath et al., 2010). Apolipoprotein E4 isoform is involved in the build-up of insoluble Aβ amyloid protein in neurons (Jiang et al., 2008a) and also increased plasma cholesterol levels (Lane and Farlow, 2005). These events end in disruption of cellular homeostasis as the effects of increased amyloid or plasma cholesterol contribute to degenerative phenotype leading to neuronal loss or atherosclerosis.

As dementia is associated with apolipoprotein E4 genotype and low telomere length, it is important to explore influence of apolipoprotein E4 genotype on telomere length. Earlier studies had suggested significant association to no association of telomere attrition with apolipoprotein E4 status in dementia and cognitively normal group (Takata et al., 2012; Honig et al., 2006; Starr et al., 2008; Jacobs et al., 2013). One study had

*Abbreviations:* AD, Alzheimer's disease; $C_t$, cycle threshold; DM, diabetes mellitus type 2; ICD-10, international classification of diseases-10; PBL, peripheral blood leukocyte; PGC1α and PGC1β, PPARγ co-activators; PPARγ, peroxisome proliferator activated receptors; Relative T/S, relative telomere/single copy gene; RTPCR, real time polymerase chain reaction.

* Corresponding author at: National Institute of Mental Health and Neurosciences (Deemed University), Bangalore 560 029, India. Tel.: +91 80 2699 5271; fax: +91 80 2656 2121/4830.
*E-mail addresses:* lakshmineuroscience@gmail.com (L.N. Kota), srikala.bharath@gmail.com (S. Bharath), meera.purushottam@gmail.com (M. Purushottam), paul.pradipp@gmail.com (P. Paul), sivakumar.nimhans@gmail.com (P.T. Sivakumar), mat.varg@yahoo.com (M. Varghese), sjain.nimhans@gmail.com (S. Jain).

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suggested longer relative telomere length with apolipoprotein E4 genotype in cognitively normal (Wikgren et al., 2012). These findings suggest that the relationship between telomere length and apolipoprotein E genotypes may not be linear and warrants further scrutiny. Further studies could unravel intermediate cellular pathways and other phenotypes linking apolipoprotein E gene with telomere length explain whether longer or shorter telomere length observed is a consequence of low replicative potential (as replication events tend to shorten telomere) or merely reflect higher replicative capacity (longer telomere mean higher replicative potential).

Diabetes mellitus type 2 (DM) is associated with dementia (Peila et al., 2002) and telomere attrition (Balasubramanyam et al., 2007; Adaikalakoteswari et al., 2005). The Indian population has higher rates of DM (Diabetes, 2006) but lower frequencies of apolipoprotein E4 compared to western populations (Bharath et al., 2010). Our earlier study had shown higher rates of DM in dementia subjects with apolipoprotein E4 allele (Kota et al., 2012). With increasing rates of dementia (Shaji et al., 2010) and DM (Diabetes, 2006) in India, it is necessary to understand the molecular basis of these diseases.

Peripheral blood leukocyte telomere length (PBL) is suggested to be a surrogate for global cellular replicative capacity (Sahin and DePinho, 2012) and altered PBL cellular viability was established in subjects with dementia (Yoon et al., 2010). We studied apolipoprotein E4 allele frequencies and PBL relative telomere length in cognitively normal subjects & people with dementia with and without comorbid DM from a predominantly southern Indian population.

## 2. Subjects and methods

### 2.1. Subjects

Subjects ($N = 125$) were recruited from the geriatric clinical services of the Department of Psychiatry, National Institute of Mental Health and Neurosciences, Bangalore. Dementia cases ($n = 70$) were diagnosed clinically by psychiatrists using international classification of diseases (ICD-10) criteria. Hindi Mental Status Examination (Ganguli et al., 1995) was used to assess cognitive ability of the subjects. Cognitively normal subjects ($n = 55$) with no lifetime history of neuropsychiatric illness or family history of neuropsychiatric illness were recruited after age group matching with dementia subjects. Comorbid DM, hypertension and ischemic heart disease were ascertained by clinical history.

### 2.2. Methods

The study was approved by institutional ethics committee. After informed written consent, ten milliliters of venous blood was collected, and DNA was isolated using salting out method (Miller et al., 1988). The real time quantitative polymerase chain reaction (RTPCR) was setup for relative telomere length estimation using Applied Biosystems 7500 real time PCR system (Cawthon, 2002). The telomere sequence and a single copy gene in a known quantity of DNA were amplified simultaneously and the results were recorded as number of cycles of RTPCR required to reach threshold fluorescence ($C_t$ or cycle threshold). The $C_t$ value is inversely related to the initial copy number of telomere or single copy gene. The T/S ratio (or $\Delta C_t = C_t$ of telomere amplification $- C_t$ of single copy gene amplification) reflects number of copies of telomere in each cell in an assay. The relative T/S ratio which was calculated as difference between the $\Delta C_t$ of unknown sample and normalized for $\Delta C_t$ of reference DNA enables comparison of relative T/S ratio across assays. The following formula was used to calculate the relative T/S ratios. $\Delta C_t$ (unknown sample) $- \Delta C_t$ (reference sample) $= \Delta\Delta C_t$; rT/S ratio $= 2^{(-\Delta\Delta C_t)}$.

The reference sample was an arbitrary sample that was included in all experimental assays. Each sample was assayed in duplicate and $C_t$ values which showed s.d. $> 1$ between the technical replicates were not considered for analysis. Experiments were repeated after recoding the DNA samples to eliminate sample bias.

To detect the apolipoprotein E genotype, polymerase chain reaction (PCR) was done using sequence-specific primer PCR methodology (Pantelidis et al., 2003). The presence of 173-bp band was indicative of the specific apoliopoprotein E haplotype. The results were corroborated with RTPCR results for 10% of the samples in every experiment (Kota et al., 2012).

### 2.3. Reproducibility of the measured relative T/S ratios

Replicate assays of sample and reference were setup at different times to calculate the inter assay variation. The coefficient of variation (square root of variance/mean) as calculated by measuring relative T/S ratios of a sample repeated over four different assays was 6.7%. Samples differing in average telomere length by as little as 13% ($1.96 \times$ s.d.) should be distinguishable by this method at the 95% confidence interval (Cawthon, 2002).

### 2.4. Statistical analysis

Statistical tests were done using R software (Fox, 2005) and QTI-plot (Vasilief, 2011). Chi square test ($\chi^2$) was used for comparing categorical variables. Mann Whitney $U$ test ($U$, two tailed $p$) and Kruskal Wallis $H$ test were used for comparison between groups of continuous variables as the relative T/S ratios and log transformed relative T/S ratios calculated were not normally distributed and non-parametric tests are most useful for small samples (Fagerland, 2012; Hart, 2001). Spearman's rank correlation ($r_s$, two tailed $p$) and partial correlation ($r$, two tailed $p$) coefficients were used for correlation analysis.

## 3. Results

### 3.1. Comparison of relative T/S ratios: age, gender and phenotype

The compared groups (Table 1) were age group and gender matched. There was a trend of age related decline of relative T/S ratios in the cognitively normal group ($r_s = -0.218$; $p = 0.11$). There was a trend of lower relative T/S ratios in those who develop dementia earlier in their lives ($r_s = 0.104$, $p = 0.39$). Dementia group showed significantly lower relative T/S ratio compared to the cognitively normal group after adjusting for age, gender, hypertension, diabetes and ischemic heart disease ($p = 0.001$; Fig. 1A). The relative T/S ratios compared between subtypes of dementia or severity of dementia showed no significant association within the sub-groups ($p > 0.05$). Relative T/S ratios in DM group did not differ significantly from non-diabetic group (Fig. 1A). Relative T/S ratios were significantly lower in dementia with co-morbid DM (Fig. 1B).

### 3.2. Relative T/S ratios comparison: apolipoprotein E4 and phenotypes

The apolipoprotein E4 allele frequency was significantly higher in dementia group (0.21) compared to the cognitively normal group (0.055). Dementia subjects with comorbid DM had higher apolipoprotein E4 allele frequency (0.26) compared to cognitively normal with DM (0.07) though dementia group had significantly low comorbid DM compared to cognitively normal group. There