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Homocysteine-related hTERT DNA demethylation contributes to shortened leukocyte telomere length in atherosclerosis

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ABSTRACT

Aims: Leukocyte telomere length (LTL) is shortened in patients with clinical atherosclerosis (AS). Here we aimed to explore the contribution of elevated homocysteine (Hcy) level to LTL shortening in AS patients and the underlying mechanism.
Methods: Circulating leukocytes were collected from 197 patients with AS and 165 sex- and age-matched healthy subjects for LTL determination. mRNA expression or DNA methylation of human telomerase reverse transcriptase (hTERT) was determined by real-time PCR and methylation-specific PCR assay, respectively. We established a hyperhomocysteinemia (HHcy) mice model to confirm human results.
Results: Hcy was negatively correlated with LTL shortening in AS patients ($r = -0.179$, $p = 0.015$) and controls ($r = -0.146$, $p = 0.031$). Serum folate and high-sensitivity C-reactive protein levels significantly interacted with Hcy in LTL shortening. Hcy was related to hTERT mRNA downregulation and promoter demethylation, which combined was associated with LTL shortening in AS patients. Hcy-induced LTL shortening did not differ by sites of AS lesions or infarction. Similar to clinical observations, our HHcy mice model suggested that Hcy induced DNA demethylation and downregulation of mouse TERT and further contributed to LTL shortening.
Conclusions: Elevated Hcy level induced DNA demethylation of hTERT and was closely related with hTERT downregulation, which led to LTL shortening in AS. These findings provide novel insights into an epigenetic mechanism for Hcy-related AS.

1. Introduction

Cellular senescence derived from telomere attrition, dysfunction and shortening has a pivotal role in cardiovascular disease through mitotic and growth arrest and a chronic inflammatory state [1]. Because leukocyte are readily available and convenient to handle, leukocyte telomere length (LTL) has emerged as a potential biomarker of aging for the last decade. Indeed, most clinical studies have observed a consistent association of age- and/or sex-adjusted LTL shortening and atherosclerosis (AS) and its risk factors, such as homocysteine (Hcy) level [2,3], high body mass index [4], sedentary lifestyle [5], insulin resistance [6], smoking [4] and race/ethnicity [7]. However, an association does not prove causality, and the underlying mechanism remains undetermined.

Among the various components of human telomerase, the human telomerase reverse transcriptase (hTERT) gene, which encodes the catalytic subunit of telomerase holoenzyme has been defined as the rate-limiting factor in regulating telomerase activity and maintaining telomere length [8]. From characterization of the genomic sequence of hTERT, many studies suggested that hTERT epigenetic regulation by DNA methylation may be a new target of apoptosis in various cancer cells [9]. However, unlike most human gene promoters, in which CpG island demethylation leads to gene activation, hTERT control of regional demethylation by 5-aza-2-deoxycytodine or trichostatin A allows methylation-sensitive by repressor transcription factors such as CTCF to bind to the hTERT control region. This process is associated with reduced hTERT expression and LTL shortening [10–13].

Recently, our *in vivo* and *in vitro* experiments have documented that Hcy, as an independent risk factor of AS, could promote recruitment of methylation-sensitive transcription factors to the demethylated promoter of soluble epoxide hydrolase or platelet-derived growth factors via DNA methyltransferase 1. This process further affected endothelial cell dysfunction and vascular smooth muscle cell migration or proliferation [14,15]. Hcy accelerates the

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onset of human endothelial or progenitor cell senescence in an oxidative stress pathway dependent on telomerase inactivation leading to cellular dysfunction [16–19]. An epidemiological study also reported elevated serum Hcy level associated with human LTL shortening [3]. Therefore, DNA demethylation of hTERT may be a target of epigenetic regulation in Hcy-induced aging in AS.

Here in, we aimed to examine the contribution of elevated Hcy level to hTERT promoter methylation, mRNA expression and LTL shortening in clinical AS and confirm the process in an hyperhomocysteinemia (HHcy) mouse model.

2. Methods

2.1. Participants

We performed a hospital-based case–control study of 197 AS patients recruited from Peking Union Medical College Hospital, China. Patients had at least one atherosclerotic lesion or infarct in a cerebrovascular region ($n = 32$), carotid artery ($n = 56$), coronary artery ($n = 90$) or kidney artery ($n = 19$) confirmed by CT arteriography, aortic angiography, left ventriculography, MRI or symptoms (stroke, including transient ischemia). We excluded patients with cancer, uremia, aneurysm, genetic diabetes, Iatrogenic and traumatic aortic dissections. We used SPSS 16.0 to randomly select 165 sex- and age-matched healthy subjects who were visiting the hospital for a health examination as the normal control group.

Approximately 2 ml of venous blood was drawn from all subjects into EDTA vacutainers. After centrifugation at 3000 rpm for 10 min, serum was separated into a new tube for biochemical measurements. Leukocyte cell samples were separated into new tubes for DNA and RNA analysis, respectively. The serum and leukocytes were stored at −80 °C until used.

The study was designed in accordance with the principles of the Declaration of Helsinki and was approved by the ethics committee of Peking Union Medical College Hospital. All subjects provided informed written consent at entry or at specified intervals during the study.

2.2. Biochemical measurements

Biochemical variables, including serum total Hcy, blood glucose, total cholesterol, triglycerides (TG), high-density lipoprotein (HDL) cholesterol, and blood urea nitrogen (BUN) were assayed by use of an automatic analyzer (Hitachi 7060, Tokyo) in the clinical laboratory of Peking Union Medical College Hospital. Briefly, serum total Hcy was determined by use of an enzymatic commercial kit (Carolina Liquid Chemistries, USA). Levels of TG, HDL, low-density lipoprotein (LDL), alanine aminotransferase, γ-glutamyl transpeptida, creatinine, blood urea nitrogen, glucose, high-sensitivity C-reactive protein (hs-CRP), folate and vitamin B12 were determined by enzymatic commercial tests, and all reagents were from Olympus Diagnostics. Coefficients of variation were <10% for homocysteine, hs-CRP and folate, and <5% for all other variables.

2.3. LTL determination

Blood global DNA was extracted from leukocytes by use of the TIANamp Genomic DNA kit (Beijing, China). Telomere length ratio (*T*/*S* value) was measured by a quantitative PCR (qPCR) method as described [20], which compares the ratio of the telomere repeat copy number (*T*) to single-copy gene copy number (*S*) in a given sample. The specificity of all amplifications was determined by melting curve analysis. A reference DNA sample (transformed kidney epithelial cells: HEK293T cells) was included with each measurement to control interassay variability. Water was the negative control in 3 tubes. Seven concentrations from 2 to 200 ng/μl of a reference DNA sample were serially diluted and analyzed in triplicate in every 96-well plate to obtain good linearity ($R^2 = 0.99$). For quality control, all reference DNA samples were checked for concordance between triplicate values. The final coefficient of variation for the *T* or *S* amplicon and *T*/*S* ratio was 1.73% and 1.08% and 2.96%, respectively. All experimental DNA samples were assayed in triplicate. qPCR amplification involved use of LightCycler 480 (Roche, Switzerland). *T*/*S* values were converted to kilobases (Kb) by the following formula: telomere base pairs (Kb) = 1.585*T/S ratio +3.582 [21].

2.4. Quantitative real-time RT-PCR (qRT-PCR)

Total RNA of leukocytes was extracted by the Trizol reagent method (Invitrogen, USA) and converted to cDNA with 0.5 μg RNA samples. Quantification of hTERT-encoding mRNA involved use of the PrimeScript Real-Time RT-PCR reagent kit (Takara Biotechnology [DALIAN] Co.). hTERT and mouse TERT (mTERT) primer sequences were as described [22]. The PCR conditions were 40 cycles at 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s, and performed with LightCycler 480 (Roche, Switzerland). Relative mRNA expression was normalized to that of β-actin. All experimental DNA samples were assayed in triplicate. Water was a negative control.

2.5. Methylation-specific PCR (MSP)

Blood genome DNA was extracted as above described and modified with use of the BisulFlash DNA Modification kit (Epigentek Group Inc., USA). The modified DNA was used as a template for MSP amplification. As we previously described [23], we designed methylated and unmethylated primers based on binding sites of CTCF (a suppressor of hTERT expression) on the promoter of hTERT by using the bioinformatics program (http://www.urogene.org/methprimer/index1.html) (supplement Fig. 1). Bisulfite-treated DNA was amplified by using Premix Ex Ta, Hot Start Version (Takara Biotechnology [DALIAN] Co., China) and analyzed by agarose gels. All positive PCR products were ligated into the pGEM-T vector and confirmed by sequencing. MSP replications were repeated twice for each sample. PCR products were separated on agarose gels, and bands were visualized by staining with ethidium bromide and quantified by densitometry with use of NIH Image J. The methylation pattern represented the ratio of DNA methylation to total methylation and unmethylation and was calculated as methylation/(methylation and unmethylation) ×100% [23]. Water was a negative control.

2.6. Animal model

The hyperhomocysteinemia (HHcy) mouse model was described in our previous study [14,15]. Briefly, male 6- to 8-week-old C57BL/6J mice were fed standard mouse chow diet with or without 2% (wt/wt) L-methionine for 4 or 8 weeks ($n = 8$ in each group). Mouse serum was harvested for measuring concentrations of total Hcy before mice were anesthetized (intraperitoneal 100 mg/kg ketamine and 10 mg/kg xylazine) until loss of forepaw righting reflex. Total RNA and genomic DNA were extracted from circulating leukocytes for qRT-PCR or MSP assay. For telomere length detection, primer sequences were for mouse TEL, forward, 5′-GGTTTTTGAGGGTGAGGCTGA GGGTGAGGGTGAGGGT-3′ and reverse, 5′-TCCCGACTATCCCTATCCCTATCCCTA TCCC TATCCCTA-3′; and mouse 36b4t forward, 5′-ACTGGTCTAGGACCCGAG AAG-3′ and reverse, 5′-TCAATGGTGCCTCTGGAGATT-3′.

Our treatment and procedures for the *in vivo* mouse study followed the Guide for the Care and Use of the Laboratory Animals