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Original Article

Age-Dependent Decrease of DNA Hydroxymethylation in Human T Cells

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Hydroxymethylcytosine (hmC) is a natural nucleobase, which is converted from methylcytosine (mC) by tet methylcytosine dioxygenase (TET) family (TET1-3) enzymes. Decrease of genomic hmC is postulated to confer a risk for myeloid-lineage as well as T-cell neoplasms, based on the fact that loss-of-function mutations in the *TET2* gene were frequently identified in these diseases. The relationship between hmC and aging remains to be elucidated. Here, we demonstrated that hmC content decreased with age in the peripheral blood T cells of 53 human volunteers. We further identified that the mRNA expression levels of *TET1* and *TET3* decreased with age, while those of *TET2* were not influenced by age. The genomic hmC content was correlated with the mRNA expression level of *TET3*, but not those of *TET1* and *TET2*. Our study suggests the presence of new epigenetic regulatory mechanisms in aging T cells. 〔*J Clin Exp Hematop 55(1) : 1-6, 2015*〕

Keywords: hydroxymethylation, tet methylcytosine dioxygenase, aging

INTRODUCTION

Aging is accompanied by alteration of T-cell function, which leaves the elderly susceptible to infectious and autoimmune diseases. There have been several studies focused on age-dependent changes in T-cell function, including cytokine production and antigen-specific responses.[1-3] Furthermore, alteration in populations of functional T cells, such as an increase of regulatory T cells, has been described in the elderly.[4] Although these age-dependent changes in T cells have been attributed to alteration of transcriptional networks and other molecular pathways, the cues that may initiate such molecular events remain to be uncovered.

DNA methylation, one of the mechanisms for epigenetic regulation, plays important roles in diverse cellular processes, including cellular differentiation, senescence, and transformation.[5,6] Numerous regulators of T-cell function are known as targets of DNA methylation in T cells, including transcription factors such as FOXP3, cytokines such as interleukin-2, and micro-RNA such as miR-10b.[7-9]

Aging cells are also known to possess their own distinct methylation profiles when compared with young cells. Since the 1990s, age-dependent reduction of methylcytosine content in the genome or the mitochondria has been described in various tissues.[10-13] More recently, genome-wide methylation analysis using second-generation sequencing technologies dramatically changed our understanding of cytosine modification in aging cells.[14-20] Cytosine methylation is increased or decreased with age, depending on the tissue and the DNA region.[19,21,22]

Hydroxymethylcytosine (hmC) is an identified physiologic nucleotide contained in DNA, and an essential intermediate in the active and passive demethylation process.[23,24] Furthermore, hmC may serve as an epigenetic mark that regulates gene expression.[15,25] In mammalian cells, three tet methylcytosine dioxygenase (TET) proteins (TET1-3) function as converters of methylcytosine (mC) to hmC.

Alteration of the level of hmC has been analyzed in the brain and several organs/cells other than T cells. In the cerebellum, the hmC content in the genome was increased with age.[26] The link between hmC content and age has also been studied with mitochondrial DNA,[22,27,28] mitochondrial hmC was reported to be decreased in the frontal cortex but unchanged in the cerebellum during aging.[27] In the sperm, age-dependent increase in hmC content in the genome was

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reported.[28] These studies indicated the presence of distinct regulatory systems for the genomic hmC levels with age in each cell type.

MATERIALS AND METHODS

Samples

Twenty milliliters of peripheral blood was collected from 53 healthy volunteers (20-83 years, median 48.3 years) after written informed consent had been obtained. The experimental design was approved by the ethics committee of the University of Tsukuba Hospital.

Separation of human CD3$^+$ T cells

Mononuclear cells (MNCs) in the blood cells were separated by a gradient method using Ficoll-plus (GE Healthcare, Buckingham, UK). MNCs were then incubated with phycoerythrin (PE)-conjugated mouse anti-human CD3 antibody (BD Pharmingen, San Diego, CA, USA) for 30 min at 4℃, followed by incubation with anti-PE MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany). CD3$^+$ cells were manually separated using LS Columns (Miltenyi Biotec) according to the manufacturer's instructions. Purity of CD3$^+$ cells > 85% was confirmed using FACS Calibur (BD Biosciences, San Jose, CA, USA).

Dot blot analysis

Genomic DNA was extracted from CD3$^+$ cells using the phenol-chloroform method. Dot blot analysis was performed as previously described with minor modifications.[25] Briefly, after DNA denaturation, 2-fold serial dilutions were spotted onto a Z probe membrane (Bio-Rad, Munich, Germany). Membranes were incubated with rabbit anti-hmC antibody (Active Motif, Carlsbad, CA, USA) overnight at 4℃, followed by incubation with horseradish peroxidase-conjugated anti-rabbit immunoglobulin G secondary antibody (DAKO, Glostrup, Denmark). The membrane was incubated with an enhanced chemiluminescence reagent (Immobilon Western Millipore, Billerica, MA, USA) and then visualized using Image Quant LAS 4000 (GE Healthcare).

mRNA expression analysis

Total RNA was extracted from CD3$^+$ T cells using an RNAeasy mini kit (Qiagen, Venlo, The Netherlands). cDNA was synthesized with random primers using SuperScript III (Life Technologies, Carlsbad, CA, USA). mRNA expression was analyzed using SyberGreen reagent (Roche, Mannheim, Germany) detected with ABI7500 (Life Technologies, Carlsbad, CA, USA). Data were normalized using glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Primer sequences for human TET1, TET2, TET3, and GAPDH are listed in Table 1.

Table 1. Primer sequences

Primers	Forward Sequence (5'-3')	Reverse Sequence (5'-3')
TET1	GGGCAGTGGAAAAGAAACCT	GGGGTTCGGTTTCACTTTTT
TET2	CCATCTTGCAGATGTGTAGAGC	CCCTGAGAACTTTTGCCTTC
TET3	CCACAAGGACCAGCATAACCTC	CTCGCTACCAAACTCATCCGTG
GAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC

Statistical analysis

Data were analyzed by Pearson's chi-squared test using Predictive Analytics Software (PASW) Statistics 18 (Japan International Business Machines, Tokyo). All p values were from 2-tailed tests. P values < 0.05 were considered significant.

RESULTS

Age-dependent decrease of hmC in human T cells

In each series of dilution, we chose 3 points that gave the most linear correlation between the dilution and the concentration measurement. This procedure provided reproducible values for the hmC content, which were significantly decreased with age ($R^2 = 0.395$, $p < 0.01$) (Fig. 1a). The hmC content was linearly decreased when the volunteers were analyzed in 10-year intervals of age (Fig. 1b). In contrast, the hmC content was not influenced by gender ($p = 0.36$) (Fig. 1a).

Age-dependent decrease of TET1 and TET3 mRNA expression levels in human T cells

TET family proteins (TET1-3) are the only enzymes identified to convert mC to hmC. Therefore, we examined the relationship between the expression levels of *TET* family genes and age. We found that the mRNA expression levels of *TET1* and *TET3* were significantly decreased with age ($R^2 = 0.127$, $p < 0.01$; $R^2 = 0.260$, $p < 0.01$, respectively), while those of *TET2* were unchanged ($R^2 = 0.022$, $p = 0.28$) (Fig. 2).

Correlation between the TET3 mRNA expression levels and the genomic hmC content in human T cells

Then, we compared the genomic hmC content with the mRNA expression levels of *TET1*, *TET2*, and *TET3*. This indicated that the mRNA expression level of *TET3* was significantly correlated with the hmC content ($R^2 = 0.097$, $p =$

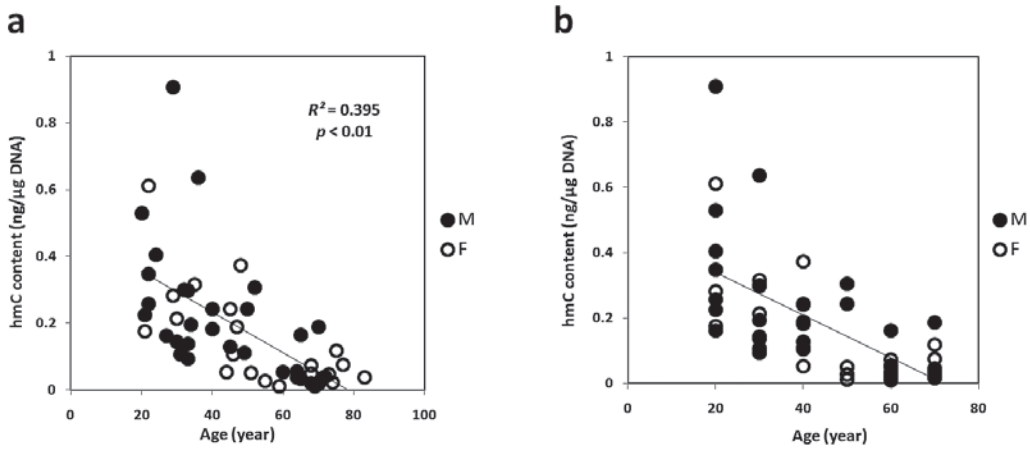

Fig. 1. Age-dependent decrease of hmC in human T cells. hmC content in T cells was measured by dot blot analysis. (***1a***) hmC content *vs*. age. (***1b***) hmC content *vs*. 10-year intervals of age. M, male; F, female.

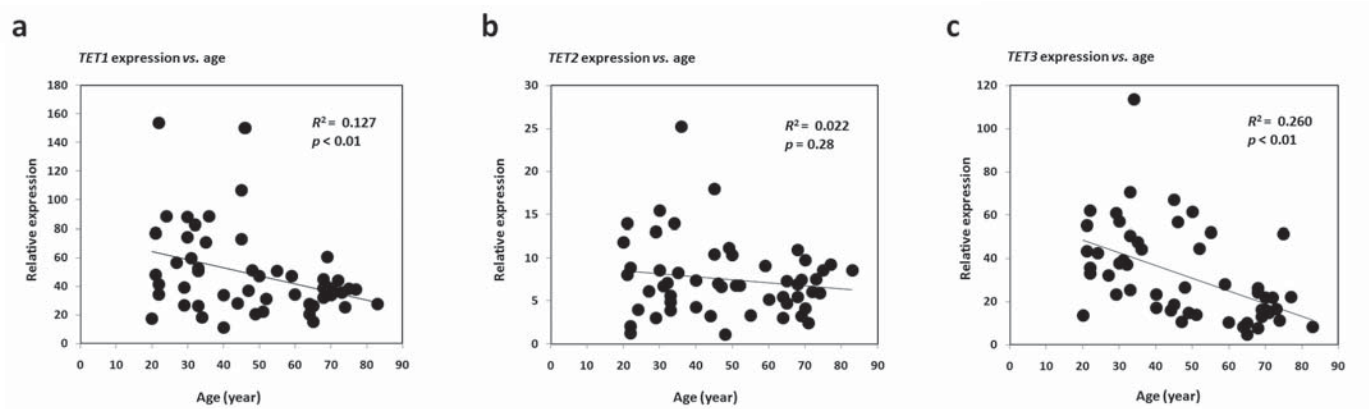

Fig. 2. Age-dependent decreases of *TET*1 and *TET*3 mRNA levels. mRNA expression levels of *TET*1, *TET*2, and *TET*3 in T cells were analyzed by qRT-PCR. *GAPDH* was used for normalization. (***2a***) *TET*1 relative expression *vs*. age. (***2b***) *TET*2 relative expression *vs*. age. (***2c***) *TET*3 relative expression *vs*. age.

0.02), while those of *TET1* and *TET2* were not (R^2 = 0.020, p = 0.30; and R^2 = 0.024, p = 0.26, respectively) (Fig. 3).

DISCUSSION

We demonstrated a dramatic age-dependent decrease in the genomic hmC content in human peripheral blood T cells. This could be caused by the gradual decrease in *TET3* expression level during aging (Fig. 4). The decrease in genomic hmC content might play a role in alteration of the T-cell function in elderly people, although the possibility has not been ruled out that the changes in both *TET3* mRNA level and hmC content represent a secondary observation reflecting a population change within the T-cell compartment. Nevertheless, we have identified an interesting link between epigenetic change in aged T cells and the increase in T-cell malignancies in the elderly. Extremely frequent *TET2* mutations have been reported in angioimmunoblastic T-cell lymphoma (AITL) and peripheral T-cell lymphoma, not otherwise specified (PTCL-NOS), having AITL-like features by us (83%)[29] and others (30-76%).[30-32] Given the fact that aging is an overwhelming risk factor for both AITL and PTCL-NOS, it is speculated that the combinatorial hmC decreases caused by age-dependent impairment of TET3 function due to its reduced expression and by impairment of TET2 function due to mutations might provide a certain T cell with transforming ability. Because *TET2* mutations are also known to be common in myeloid malignancies,[29-31] similar combinatorial hmC decreases in hematopoietic stem/myeloid progenitor cells might cause these cancers. Mutations in *TET* family genes are rare in malignancies other than those of the hematopoietic lineages,[33-36] but a reduction in genomic hmC content has

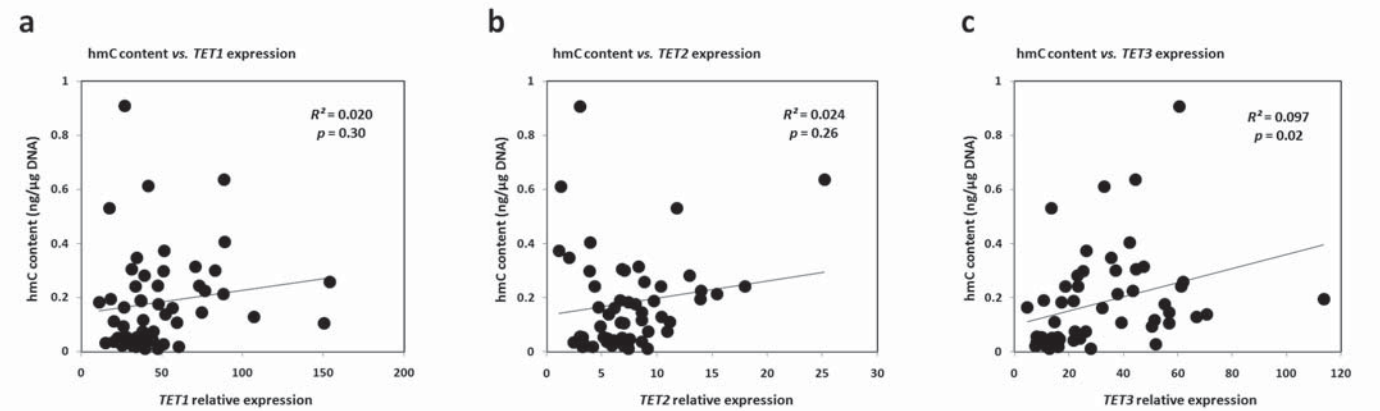

Fig. 3. Correlation between the *TET1*, *TET2*, and *TET3* mRNA expression levels and the genomic hmC content in human T cells. mRNA expression levels of *TET1*, *TET2*, and *TET3* were compared with hmC content in human T cells. (***3a***) *TET1* relative expression *vs*. hmC content. (***3b***) *TET2* relative expression *vs*. hmC content. (***3c***) *TET3* relative expression *vs*. hmC content.

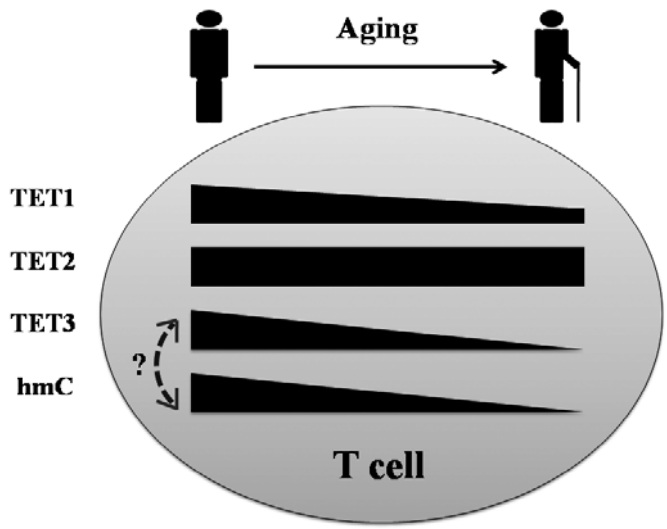

Fig. 4. Schematic illustration of correlation between DNA hydroxymethylation and TET3 expression in the aging process in human T cells. mRNA expression levels of *TET1* and *TET3* decreased with age, while those of *TET2* were not influenced by age. The genomic hmC content was correlated with the *TET3* mRNA expression level, but not with *TET1* and *TET2* mRNA expression levels.

been described in colorectal,[35,37] gastric,[35,38] prostate, breast,[39] hepatocellular,[40] and lung[34] carcinomas, as well as brain tumor[34] and melanoma.[33] Taking these findings together, appropriate regulation of mC to hmC conversion may be necessary to prevent cells from undergoing neoplastic transformation beyond the cell types, and dysregulation of mC to hmC conversion might, at least in part, explain why aging is an ultimate risk factor in various types of cancer.

Our study implies that *TET3* expression is important for genomic hmC decrease during aging in T cells. Decrease in the hmC content of mitochondrial DNA in the frontal cortex, however, was not accompanied by alteration of *TET1-3* mRNA levels.[27] Thus, the hmC content could be changed in an age-dependent manner in multiple cell systems, but the changes in regulation of hmC are variable according to the cellular context. The mechanisms of the age-dependent decrease of hmC content and the impact of such alteration in age-related T-cell dysfunction should be clarified in future study.

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