HHS Public Access

Author manuscript

Am J Hematol. Author manuscript; available in PMC 2014 June 01.

Published in final edited form as:

Am J Hematol. 2013 June ; 88(6): 492–496. doi:10.1002/ajh.23438.

Telomere length and elevated iron: The influence of phenotype and *HFE* genotype

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Abstract

Elevated body iron stores are associated with morbidity and mortality due to oxidative stress. Hereditary hemochromatosis, a common condition caused by *HFE* gene mutations, can lead to excess iron storage and disease but clinical penetrance of *HFE* gene mutations is low and many people with elevated iron stores lack *HFE* mutations. We analyzed data from the Hemochromatosis and Iron Overload Screening Study to assess the relationship among *HFE* genotype (individuals with either homozygous or compound heterozygous status for C282Y and/or H63D *HFE* mutations were defined as genotype positive, or G+), elevated iron phenotype (individuals exceeding gender-specific transferrin saturation and serum ferritin threshold levels were considered phenotype positive, or P+), and leukocyte telomere length, a marker of biological aging and cumulative oxidative stress. In unadjusted analyses in comparison to individuals who were G−P−, G+P− were not significantly different (OR 0.74; 95% CI 0.26–2.04), while the G+P+ (OR 2.03; 95% CI 1.15–3.56), and G−P+ (OR 2.24; 95% CI 1.5–3.29) had increased risk of short telomeres (<=25th percentile) rather than long telomeres (>=75th percentile). In analyses adjusting for age, gender, and race/ethnicity, the effect of individuals with elevated iron phenotypes having short telomeres persisted with G+P+ individuals (OR 1.94; 95% CI 1.02–3.72), and G−P+ individuals (OR 2.17; 95% CI 1.39–3.39) being significantly different from the G−P− group. In conclusion, elevated iron phenotype, but not *HFE* genotype, was associated with shortened

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Conflict of interest: Nothing to report.

CDC disclaimer: The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

telomeres. Further studies will be needed to determine whether telomere length provides a marker for morbidities specifically associated with iron overload.

Introduction

Certain inherited genetic mutations are associated with hereditary hemochromatosis (HH), a condition of excess body iron [1,2]. Type 1 HH, or *HFE* hemochromatosis, is one of the most common autosomal recessive disorders in the US [3,4]. Mutations on the *HFE* gene, especially the C282Y homozygous variant found most often in populations of northern European descent, can lead to excessive dietary iron absorption and progressive accumulation of iron in the body, potentially reaching toxic levels by middle life [4,5]. Other *HFE* mutations, like H63D, and non-*HFE* HH mutations (Types 2,3, and 4 HH) are likely more common in non-northern European populations than the C282Y mutation [1,6–15]. The excess iron is deposited in multiple organs, causing oxidative tissue damage which can lead to health conditions, including cirrhosis of the liver, cancer, and cardiomyopathy [16–21]. However, once the diagnosis is made, liver and heart function can be improved with periodic phlebotomy or erythrocytapheresis to remove excess iron [22–24].

Despite the common usage of *HFE* mutation identification to assess risk of clinical iron overload in at-risk individuals with potential iron overload, not all persons with elevated body iron stores have mutations in the *HFE* gene and elevated body iron stores are still associated with an increased risk of morbidity (cancer, cardiovascular disease, inflammation, and dementia) and mortality [1,2,25–30].

Excess iron deposition is associated with biomolecular oxidative damage and mimics physiologic changes that occur with aging and leads to age-related conditions [31–35]. Telomere length has emerged as a marker for cumulative oxidative stress and biological aging, which is the key to age-related morbidity [36–38]. Shortened telomere length has been associated with shorter life span as well as a wide variety of age-related diseases and conditions, including cardiovascular disease, diabetes, insulin resistance, and hypertension [39–41].

Because not everyone with *HFE* gene mutations develops elevated iron stores and not everyone with elevated body iron stores has *HFE* gene mutations, the relationship among *HFE* gene mutations, elevated body iron, and cumulative oxidative stress represented by telomere length is uncertain. The purpose of this study was to examine the relationship among *HFE* genotypes, elevated iron phenotypes, and telomere length.

Methods

This study used existing data included in the Hemochromatosis and Iron Overload Screening (HEIRS) Study and computed additional assays of the linked, stored specimens. The HEIRS Study evaluated a multicenter, multiracial-ethnic sample of 101,168 primary care adults 25 years of age or older in the United States and Canada. Interview data were obtained from initial screening of all participants and a subsequent Comprehensive Clinical Exam (CCE) for C282Y homozygotes, non-C282Y homozygote participants with elevations of serum

biochemical tests of iron status, and control subjects. DNA specimens collected from each participant during the CCE were obtained from the Biologic Specimen and Data Repository Information Coordinating Center (BioLINCC) at the National Heart Lung and Blood Institute and used for the telomere assays. The data obtained in the present study were then merged with the other variables contained in the HEIRS data sets.

Details of study design and sampling methods have been published and can be found in the HEIRS Protocol [42,43].

Subjects

Of the 1,157 subject DNA specimens sent to the investigators by BioLINCC, 1,146 samples had verified telomere values, of which, 137 (12% of the original sample) were excluded due to potential risk of misclassification due to phlebotomy treatment. Individuals from the CCE who could be classified into one of four groups representing both *HFE* genotype and phenotypic expression of elevated body iron (elevated transferrin saturation (TS), and serum ferritin (SF) levels) were identified. Our final sample for analysis consisted of 1,009 subjects.

Elevated iron phenotype

Subjects' phenotype status was determined by gender-specific threshold values for serum biochemical tests for iron status. Males were considered "phenotype positive" (P+) for the elevated iron phenotype if their TS level was 50% or above and SF level was above 300 ng/mL. Females were considered "phenotype positive" (P+) for the elevated iron phenotype if their TS level was 45% or above and SF level was above 200 ng/mL. Individuals who had SF and TS levels below these gender-specific thresholds were considered "phenotype negative" (P−) for the elevated iron phenotype.

HFE genotype

Subjects were considered to be "genotype positive" (G+) for *HFE* genotype if they were homozygous or compound heterozygous for *HFE* gene mutations, expressed as C282Y/C282Y, H63D/H63D, and C282Y/H63D alleles, respectively. H63D homozygotes were considered G+ as the genotype has been associated with elevated body iron indicators in certain race-ethnicities [7–9]. Compound heterozygotes were considered G+ as the genotype has been associated with elevated body iron indicators relative to wild-type [44]. All other subjects were defined as "genotype negative" (G−).

Analysis groups

The first of four groups consisted of individuals who were G+P+. The second group consisted of individuals that were G−P+. These were individuals with either one copy of either the C282Y or H63D allele (heterozygous carriers of *HFE* mutation) or neither allele affected by the two common *HFE* gene mutations and were included in the CCE because they had both elevated TS and elevated SF.

The third group consisted of individuals who were G+P−. This group was composed of persons homozygous or compound heterozygous for the C282Y or H63D alleles with

normal TS (<50% in men and <45% in women) and SF (<300 ng/mL in men, and <200 ng/mL in women).

The fourth group consisted of individuals who were G−P−. These were individuals who had no allele mutations and normal TS and SF. They participated in the CCE as control subjects. We randomly selected individuals from all of the groups except for the G+P− group which had a small sample size. For the G+P− group, we used the specimens from all available subjects.

Telomere length via real time PCR analysis

Leukocyte telomere length was measured with a quantitative PCR-based technique (qPCR) that compares telomere repeat sequence copy number to single-copy gene (36b4) copy number in a given sample [45]. Triplicate DNA samples were amplified in parallel in 20 μL reaction using SsoFast EvaGreen real-time PCR supermix (Bio-Rad, Hercules, USA) containing 20 ng of sample DNA. The telomere-specific reaction included 500 nM of telomere-specific primers (forward: 5′GGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGT T3′; reverse: 5′GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACC CT3′). The 36b4 reaction included 300 nM of the forward (5′CAGCAAG TGGGAAGGTGTAATCC3′) and reverse (5′CCCATTCTATCATCAACGG GTACAA3′) primers. The qPCR/primer supermix (19 uL) was aliquoted into PCR multiwell plates using an EpMotion 5070 robotic liquid handling unit (Eppendorf, Germany), and then 1 uL of sample DNA (20 ng) was added to each well. All qPCR reactions were run using a CFX96 real-time thermal cycler (Bio-Rad). The thermal cycling profile for both amplicons began with 95°C incubation for 3 min and then 30 cycles of 10 sec at 95°C and 1 min at 58°C. The specificity of all amplifications was determined by melting curve analysis. A total of 14 study samples and 2 calibrator samples (all in triplicate) were processed per plate.

Analysis of qPCR data—Analysis of sample telomere length and 36b4 expression levels was done using the PCR Miner algorithm developed by Zhao and Fernald [46]. Values derived for telomere (T) were normalized for each sample with the corresponding expression of 36b4 gene (S) as T/S ratio.

Telomere length status was defined according to intra-sample telomere length percentiles as the bottom, or “short,” quartile (<=25th percentile), middle quartiles (26–74th percentile), and top, or “long,” quartile (>=75th percentile). The “short” quartile was considered to have more cumulative oxidative stress than the “long” quartile [37,38].

Covariates

Demographics—Age is associated with both increasing body iron stores and shorter telomeres [47,48]. For analysis, age was analyzed as a continuous variable in the logistic regression model. Subjects’ race-ethnicity was categorized as either Non-Hispanic White or Other, as hemochromatosis-related genetic mutations differ by race-ethnicity [6–8,11,14,15]. Subjects’ gender was categorized as either male or female, as iron stores and telomere length may be associated with gender [49,50]. Subjects’ health insurance status was categorized as

either “insured” or “uninsured” and education attainment as “less than high school,” “high school,” or “more than high school.”

Statistical analysis

Analyses were conducted using SAS 9.2 (SAS Institute, Cary NC). Bivariate analyses with Chi-square and Fisher’s exact test for rare outcomes were conducted to compare genotype-phenotype groups. An adjusted multivariate logistic regression model (N = 511) for the odds of having “Short telomere” versus “Long telomere” was generated to compare genotype-phenotype status after controlling for significant differences in covariates among groups (age, race-ethnicity, and gender). Intra-sample telomere quartiles (< =25th percentile and > =75th percentile) were compared to examine extremes in telomere length to detect an association between genotype-phenotype group and telomere quartile if one were present. Age was included in the model because it is linked to biological aging and telomere length [47,48]. Although education and insurance were examined to describe the individuals, they were not entered into the multivariate model because of their lack of previously identified relationship with telomere length.

An additional subgroup analysis was conducted of only the C282Y homozygotes. In this subgroup analysis, we examined elevated iron phenotype with telomere length through several strategies. First, *t*-tests were conducted comparing mean telomere length between individuals who had the elevated iron phenotype and those who did not have elevated iron. Second, we computed *t*-tests comparing individuals with SF > 1000 ng/mL with those with non-elevated SF (<300 ng/mL in men, and <200 ng/mL in women).

Results

Demographic characteristics of the groups under investigation are featured in Table I. Genotype-phenotype groups were significantly different by gender, race-ethnicity, and health insurance. Groups carrying the *HFE* mutations were much more likely to be Non-Hispanic White than groups without *HFE* mutations. No differences were observed between genotype-phenotype groups in regards to education status.

In a sub-analysis of the C282Y/C282Y subgroup (n = 82) (data not shown), although the mean telomere length was shorter in the elevated iron group (272.2) than the phenotype negative group (290.1), the relationship in this small subgroup was not statistically significant (P = 0.44). Similarly, among individuals with SF levels >1,000 ng/mL (n = 11) telomere length was shorter (255.3) than those with non-elevated SF (<300 ng/mL in men, and <200 ng/mL in women) (n = 29) whose mean telomere length was 292.5 but once again this did not reach statistical significance (P = 0.29).

Elevated iron phenotype, but not *HFE* genotype, was associated with shorter telomeres in bivariate analyses (Table II). Elevated iron phenotype, but not hemochromatosis genotype, was associated with shorter telomeres in both crude and adjusted logistic regression models (Table III). Adjusting for age, gender, and race-ethnicity attenuated the relationship slightly but both groups with elevated iron phenotype (G+P+ and G−P+) remained significantly different from the control group (G−P−) in terms of the likelihood of having short telomeres.

Discussion

The results of this study showed that elevated serum biochemical tests of iron status were associated with shorter telomere length. To the best of our knowledge, this is the first study to examine telomere length in relation to *HFE* genotypes or elevated serum tests of iron status. The result was independent of *HFE* gene mutations and existed even after controlling for age, gender, and race-ethnicity. *HFE* mutations were not significantly associated with telomere length in this sample of adults.

Regarding *HFE* genotype, the results further support the idea of gene–environment interactions in that the *HFE* genotype appears to primarily impact health through the pathway of elevated body iron and subsequently increased oxidative stress [2]. It is unclear whether lifestyle variables may increase the propensity for phenotypic expression among these individuals. Finding interventions to minimize the expression of the genes and control iron levels is a reasonable approach to health promotion in individuals with *HFE* genotypes.

The results also indicate that individuals who did not have the *HFE* gene mutation but had elevated iron had shorter telomeres. This suggests that there are other precursors to elevated iron in the general population that need to be monitored beyond the *HFE* gene mutations. Previous research indicated that although women who used multivitamins had longer telomere length than those who didn't, women who used iron supplements had a shorter telomere length than nonusers [51]. There is some evidence that certain non-*HFE* gene mutations associated with elevated iron stores are more common in different ethnic groups [1,6–12]. It is possible that other unidentified genotypes associated with elevated iron were present in the genotype negative group and that these groups attenuated the relationship between genotype and telomere length.

Though C282Y homozygosity is associated with more severely elevated body iron stores and greater incidence of clinical iron overload than other *HFE* mutations, many populations with low or no occurrence of this mutation, especially non-northern Europeans, experience elevated body iron stores [6–15]. It is possible that other *HFE* mutations, such as H63D homozygosity, and non-*HFE* mutations, such as Types 2,3, and 4 HH (related to gene mutations on the *hepcidin*, *TfR2*, and *Fpn* genes, respectively), are contributing to elevated iron storage in persons of non-northern European descent [1,2]. However, given that elevated body iron, even below clinical iron overload, is associated with greater morbidity, mortality, and shorter telomeres, it is important to discover environmental factors contributing to elevated iron storage in multiracial-ethnic groups [25–32].

These results in humans focusing on leukocyte telomere length are somewhat different from evidence from a rat model examining liver cell telomere length. In a rat model of iron overload examining liver cell telomere length, iron overloaded rats had significantly increased telomerase activity but no difference in telomere length [52]. As a response to iron overload, enhanced telomerase activity may be a response to iron-induced damage in specific organs.

There are several limitations to this study. First, although the H63D *HFE* gene mutation is commonly used as an indicator of hemochromatosis, its relationship to iron overload is less

clear than that of the C282Y *HFE* gene mutation [7,44]. In this study, both genotypes were evaluated together as well as separately, in relationship to elevated iron with the results suggesting that combining the two did not lead to a bias in the results. Second, this study is cross-sectional and thus allows us to only impute associations. Telomere length evaluates cumulative exposure to oxidative stress and in this study is significantly associated with currently elevated iron. Future studies will increase our knowledge regarding changes in iron levels and changes in the slope of telomere shortening. Third, we did not control for lifestyle variables and some outcome conditions like diabetes, which may be associated with both elevated iron and shorter telomere length. These variables may be in the causal pathway between elevated iron and telomere length [37,53,54].

In conclusion, this study suggests that the presence of *HFE* genotypes does not have an inherent physiologic impact on telomere length, whereas elevated body iron test results are associated with shorter telomere length. Given the consistent association observed between elevated body iron and morbidity and mortality, it is important to discover the physiological and environmental factors contributing to iron loading to prevent future disease [25–30].

Acknowledgments

Samples were provided by Biologic Specimen and Data Repository Information Coordinating Center (BioLNCC).

Contract grant sponsor: Centers for Disease Control and Prevention (CDC); Contract grant number: 1U01DD000754.

Contract grant sponsor: National Heart Lung and Blood Institute (NHLBI).

Contract grant sponsor: NIH/NCRR South Carolina COBRE for Cardiovascular Disease; Contract grant number: NIH/NCRR P20 RR016434.

Contract grant sponsor: South Carolina IDeA Networks of Biomedical Research Excellence (INBRE); Contract grant number: NIH/NCRR P20 RR16461.

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TABLE I

Covariates by Genotype–Phenotype Status (%)

	Genotype+ Phenotype+	Genotype+ Phenotype−	Genotype− Phenotype+	Genotype− Phenotype−	*P*-value
Sample size = 1,009	110	45	397	457	–
Total (%)	10.9	4.5	39.3	45.3	–
Gender (%)					<0.001
Men	46.4	28.9	58.2	50.1	
Women	53.6	71.1	41.8	49.9	
Race-ethnicity (%)					<0.0001
White, non-Hispanic	88.2	91.1	26.7	53.6	
Other	11.8	8.9	73.3	46.4	
Education (%)					0.13
Less than HS	9.8	17.9	16.3	10.1	
High school	18.3	25.0	26.2	23.5	
More than HS	71.9	57.1	57.5	66.3	
Health insurance					<0.001
Insured	92.7	78.6	72.8	84.4	
Uninsured	7.3	21.4	27.2	15.6	

TABLE II

Genotype or Phenotype Status by Telomere Length (%)

	Short telomere (< =25th pctl)	Long telomere (> =75th pctl)	*P*-value
Sample size = 511	257	254	–
Total	50.3	49.7	–
HFE genotype			0.67
Positive (*n* = 82)	52.4	47.6	
Negative (*n* = 429)	49.9	50.1	
Elevated iron phenotype			<0.0001
Positive (*n* = 270)	59.6	40.4	
Negative (*n* = 241)	39.8	60.2	

TABLE III

Logistic Regression Predicting Short Telomere Length by Genotype Phenotype Group (n = 511)

Crude model	**Odds ratio**	**95% Confidence interval**
Genotype–henotype		
G+ P+ (n = 64)	2.03	1.15–3.56
G+ P− (n = 18)	0.74	0.26–2.04
G− P+ (n = 206)	2.24	1.5–3.29
G− P− (n = 223)	–	Reference
Adjusted model[a]	Odds ratio	95% Confidence interval
Genotype–henotype		
G+ P+ (n = 64)	1.94	1.02–3.72
G+ P− (n = 18)	1.27	0.42–3.89
G− P+ (n = 206)	2.17	1.39–3.39
G− P− (n = 223)	–	Reference

[a]Adjusted for age group, gender, and race-ethnicity.