Genetic variation in human telomerase is associated with telomere length in Ashkenazi centenarians

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Telomere length in humans is emerging as a biomarker of aging because its shortening is associated with aging-related diseases and early mortality. However, genetic mechanisms responsible for these associations are not known. Here, in a cohort of Ashkenazi Jewish centenarians, their offspring, and offspring-matched controls, we studied the inheritance and maintenance of telomere length and variations in two major genes associated with telomerase enzyme activity, *hTERT* and *hTERC*. We demonstrated that centenarians and their offspring maintain longer telomeres compared with controls with advancing age and that longer telomeres are associated with protection from age-related diseases, better cognitive function, and lipid profiles of healthy aging. Sequence analysis of *hTERT* and *hTERC* showed overrepresentation of synonymous and intronic mutations among centenarians relative to controls. Moreover, we identified a common *hTERT* haplotype that is associated with both exceptional longevity and longer telomere length. Thus, variations in human telomerase gene that are associated with better maintenance of telomere length may confer healthy aging and exceptional longevity in humans.

longevity | heritability | aging | biomarker

Telomeres consist of the TTAGGG tandem repeats at the ends of chromosomes and are known to protect these regions from degradation and DNA repair activities (1). Telomeres progressively shorten with each cell division in cultured primary human cells (2) until a critically shortened length is achieved, upon which the cells enter replicative senescence (3). Although the relevance of replicative senescence to in vivo aging remains poorly understood, numerous reports suggest that telomere shortening may be associated with organismal aging, with concomitant metabolic decline and increased risk for disease and death (4, 5). For example, several cross-sectional studies in humans have shown that telomere length in white blood cells is inversely related to the age of the cell donor (6–9). Likewise, shorter telomere length has been shown to be associated with age-related disease including coronary heart disease, hypertension, and dementia, as well as general risk factors for disease such as insulin resistance and obesity (10). Furthermore, oxidative stress and inflammation, two major postulated causal factors of aging, are known to accelerate telomere shortening, suggesting that telomere length may be an important biomarker of aging because it reflects the cumulative burden of oxidative stress and inflammation (4, 11). In addition, most (5, 12, 13) but not all studies (14, 15) have shown a positive association between telomere length and overall survival in humans. These results indicate that telomere shortening could be used as a biomarker of disease risk and progression as well as early mortality. However, biological mechanisms responsible for these associations are not known.

Telomere length varies among individuals and families and follows the polygenic mode of inheritance pattern typical of most quantitative traits (16). Heritability estimates for telomere length vary from 35 to 80% (9, 13, 17). Although several candidate genes have been identified as potential modulators of telomere length in humans (17, 18), none of these genes seem to play a direct role in maintenance of telomere length (19). Recently, one of the most obvious candidate genes of telomere maintenance, telomerase, has been shown to play a direct role in the maintenance of telomere length in humans (20). Telomerase is a specialized ribonucleoprotein enzyme complex that adds telomere repeats to the ends of chromosomes and has two essential components: a catalytic component encoded by the human telomerase reverse transcriptase (hTERT) and a human telomerase RNA component (hTERC). The latter component provides the template for nucleotide addition by hTERT. Heterozygote mutations in the *hTERT* and *hTERC* genes lead to short telomeres and are the major risk factors for rare hematopoietic disorders of bone marrow failure, including aplastic anemia and dyskeratosis congenital. These results indicate that the levels of functional telomerase are critical for telomere maintenance (21). Telomerase is expressed at high levels in specific germline cells, proliferating stem-like cells, and many cancers, whereas in normal adult cell types, it is either not expressed or is expressed at very low levels that are not sufficient to maintain telomere length. However, telomerase can be unregulated in these cells under certain conditions to maintain telomere length (22). This suggests that efficient regulation of telomerase gene expression in response to stresses that are known to reduce telomere length such as oxidative damage or inflammation would lead to better telomere maintenance.

We have previously shown that individuals in Ashkenazi families with exceptional longevity have generally been spared major age-related diseases such as cardiovascular disease and diabetes mellitus, which are largely responsible for mortality in the elderly, and that these features are heritable (23). Because studies on individuals with a normal life span suggest that

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common age-related diseases and a shorter life span are associated with shorter telomeres (24–28), we assessed the telomere length in blood leukocytes among subjects with exceptional longevity (centenarians) to investigate if centenarians survived with long telomeres, an indicator of better telomere length maintenance, or short telomeres reflecting chronologically old age. In addition, in view of a strong family history of exceptional longevity and genetic basis of telomere length maintenance, we tested if telomere length as a trait would be inherited by the offspring of centenarians and associated with the status of aging-related diseases. Finally, to study the genetic basis of telomere length maintenance, we took a candidate gene approach to discover all possible variations in two main genes associated with telomerase enzyme activity, *hTERT* and *hTERC*, in centenarians and younger controls for genetic association analysis with human exceptional longevity and telomere length. Our overall hypothesis is that maintenance of telomere length may indicate generalized genomic integrity, which, in turn, may have a profound influence on health and aging in humans.

Results

Telomere Length, Heritability, and Longevity. Phenotypes of centenarians, their offspring (approximate age of 70 years), and offspring-matched controls without a family history of unusual longevity, all of Ashkenazi Jewish descent, are presented in Table 1. The control and offspring groups have similar characteristics of lipid profiles, except that controls have a slightly smaller percentage of large LDL particle size, but these differences are not significant (Table 1). The centenarian group displays a lower mean body mass index and higher HDL levels as compared with controls (Table 1).

When controls and centenarians are combined to represent cross-sectional age groups of unrelated individuals from middle age to oldest old (range: 43–105 years), telomere length declines with age until the age of 85 years (Fig. 1). However, individuals older than 86 years of age as well as the centenarians show longer telomere lengths compared with unrelated individuals younger than 85 years of age, with a mean difference of 0.17 ($P = 0.04$) in the linear regression model adjusting for age and gender. The telomere length of offspring exceeds that of controls in a hierarchical linear model, on average by 0.082 [95% confidence interval (CI): 0.017–0.148; $P = 0.014$]. Because centenarians and their offspring are appreciable singleton families, we used the regression approach for nonindependencies of the observed subjects. To account for parametric model bias as well as for unknown intrafamily correlation, we used a sandwich variance estimator equation and a generalized estimating equation (GEE), respectively. Both analyses repeated our first estimation supporting the observation of significant telomere length differences between offspring and controls, on average, by 0.081 (95% CI: 0.022–0.139; $P = 0.007$) for the sandwich estimation and by 0.082 (95% CI: 0.017–0.147; $P = 0.013$) for the GEE. Moreover, unlike controls, offspring of centenarians do not show an appreciable decline in telomere length with age as compared with controls (Fig. 1). However, the difference in age-related decline did not differ significantly between offspring and controls (adjusted mean difference = 0.002, 95% CI: −0.007 to +0.010; $P = 0.682$) by the same hierarchical linear model described earlier. High heritability (86%; $P = 0.005$) for telomere length between centenarians and their offspring was observed, suggesting that telomere length has a strong genetic component in families with exceptional longevity

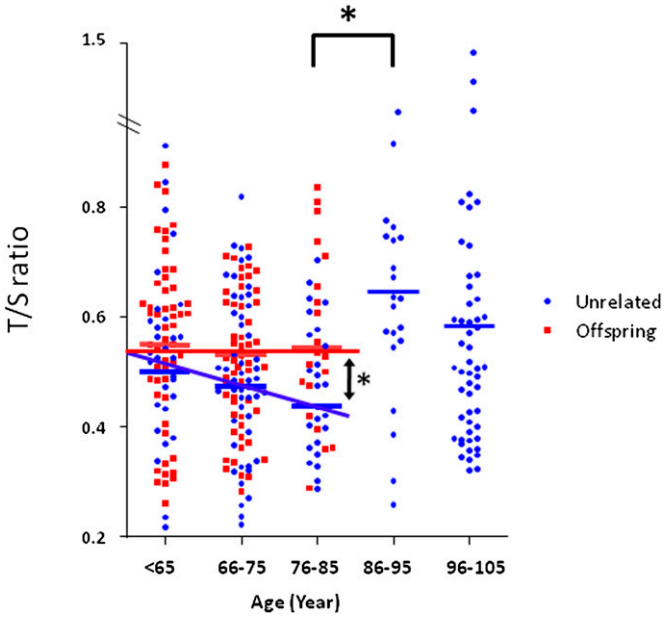

Fig. 1. Comparison of telomere length among Ashkenazi Jewish centenarians ($n = 86$), their offspring ($n = 175$), and controls ($n = 93$). Values are adjusted for age at recruitment and gender in the offspring and control groups and for gender alone in the centenarian group. *$P < 0.05$.

Table 1. Phenotypic characteristics of study subjects for telomere length measurements

				P values	
	Controls ($n = 93$)	Offspring ($n = 175$)	Centenarians ($n = 86$)	Offspring vs. controls	Centenarians vs. controls
Age range, years	43–94 (71.8)	44–85 (67.8)	95–105 (97.4)	0.0001	0.0001
Female, %	55	36	63	0.001	0.24
Body mass index, kg/m^2	25 (0.4)	26 (0.3)	22 (0.4)	0.28	0.0001
Waist circumference, inch	34 (0.4)	35 (0.3)	35 (0.5)	0.22	0.57
Cholesterol, mg/dL	197 (3.8)	202 (2.9)	191 (4)	0.28	0.26
Triglyceride, mg/dL	154 (8.9)	146 (6.7)	151 (9.6)	0.52	0.81
LDL, mg/dL	107 (3.5)	112 (2.6)	106 (3.6)	0.22	0.76
HDL, mg/dL	58 (1.7)	61 (1.3)	51 (1.8)	0.21	0.003
LDL particle size, nm	20.9 (0.12)	21.1 (0.08)	21.2 (0.1)	0.29	0.06
HDL particle size, nm	9.23 (0.07)	9.15 (0.05)	9.36 (0.06)	0.37	0.17
Large LDL particle size, % of total	52 (4.4)	62 (3.0)	62 (3.9)	0.07	0.1
Large HDL particle size, % of total	53 (2.3)	50 (1.6)	55 (2.1)	0.26	0.42

The phenotypic characteristics were adjusted for age and gender when controls were compared with offspring and for gender when controls were compared with centenarians. All values are mean (SE).

and that these families have better telomere length maintenance than controls.

Telomere Length, Age-Associated Diseases, and Lipid Profiles of Healthy Aging. Association analysis between telomere length and major age-related diseases among centenarians, their offspring, and controls indicated that significantly shorter telomere lengths (adjusted for age, gender, and group) are present in subjects with hypertension ($P = 0.006$), the metabolic syndrome ($P = 0.03$), or diabetes ($P = 0.03$) compared with subjects without these disorders (Fig. 2). Moreover, when we tested the relation between telomere length and cognitive function among centenarians, centenarians with impaired cognitive function [Mini-Mental State Examination (MMSE) score ≤25] have significantly shorter telomeres as compared with centenarians with normal cognitive function after adjustment for age and gender (Fig. 2; $P = 0.02$). We then tested whether telomere length correlated with the lipid profiles, which are known predictors of aging-related diseases such as coronary artery disease and metabolic syndromes (29). These analyses revealed that telomere length is associated with the lipid profiles of healthy aging, showing a positive association with increased particle sizes of LDL ($P = 0.0001$) and HDL ($P = 0.03$), percentage of large particle sizes of LDL ($P = 0.0002$) and HDL ($P = 0.038$), and levels of Apo-A1 ($P = 0.005$) and HDL ($P = 0.04$) (Table 2), whereas there is a negative association with very LDL levels ($P = 0.008$). Total cholesterol, triglycerides, LDL, and Apo-B levels show no significant correlation (Table 2). These results suggest that longer telomeres are positively correlated with the lipid profiles of healthy aging.

Variation in the Telomerase Genes, Longevity, and Telomere Length. We performed a comprehensive sequence analysis of *hTERT* and *hTERC* genes to detect all possible genetic variants, including the rare variants that may be enriched in centenarians, throughout the coding exons and exon–intron flanking regions in centenarians ($n = 100$) and controls ($n = 80$) using 2D gene scanning and DNA sequencing (Fig. 3). We found a total of 15 sequence variants in the *hTERT* gene, among which 5 were previously unknown unique variants that were not reported in various SNP databases (Table 3). The locations of these variants are shown in Fig. 4. Nine variants in the *hTERT* gene are in the coding region, including two nonsynonymous variants, 893 G > A (Ala-279 Thr) and 3242 G > A (Ala-1062 Thr), which are rare and found only in controls (Table 3). In silico analysis using SIFT (sorts intolerant from tolerant substitutions) (30) and PolyPhen (31), which predict the effects of amino acid changes on protein function, indicates that these changes are likely to be benign. Interestingly, rare synonymous or intronic variants in *hTERT* are enriched in centenarians ($n = 19$) compared with controls ($n = 3$) (Table 2; $P = 0.041$). In addition, three previously undescribed intronic variants in *hTERC* were also found, two of which were rare and found only in centenarians (Table 3).

We genotyped the four common *hTERT* variants, with a minor allele frequency (MAF) of >5% [IVS1–187 T > C, 973 G > A (Ala-305 Ala), 3097 C > T (His-1013 His), IVS16+99 C > T], in 73 centenarians and 49 controls. Information on telomere length was available for all these individuals. We did not find an association of the IVS1–187 T > C, 973 G > A (Ala-305 Ala), and IVS16+99 C > T with longevity. However, significant associations were detected with the 3097 C > T (His-1013 His) variant: the T allele is significantly enriched in centenarians (21.8%) compared with controls (8.2%; $P = 0.026$) (Table 3), and both CT and TT genotypes are significantly enriched in centenarians compared with controls (26.8% and 8.5% vs. 12.2% and 2%, respectively; $P = 0.035$) (Table 4). In contrast, no significant association between the *hTERC* common variant (IVS+63 T > C) and longevity was found (Table 4). Haplotype analysis [see SI Fig. S1 for a linkage disequilibrium (LD) plot derived from the four common variants] revealed that of the four most common haplotypes, Hap 1 is significantly depleted in centenarians compared with controls (34.9% vs. 53.1%; $P = 0.0056$), whereas Hap 3 is significantly enriched in centenarians compared with controls (13.7% vs. 8.2%; $P = 0.007$) when adjusted for age and gender (Table 5). Interestingly, a rare haplotype in controls, Hap 6, is found significantly more frequently in centenarians (1% vs. 6.8%; $P = 0.024$) (Table 5). Association analysis

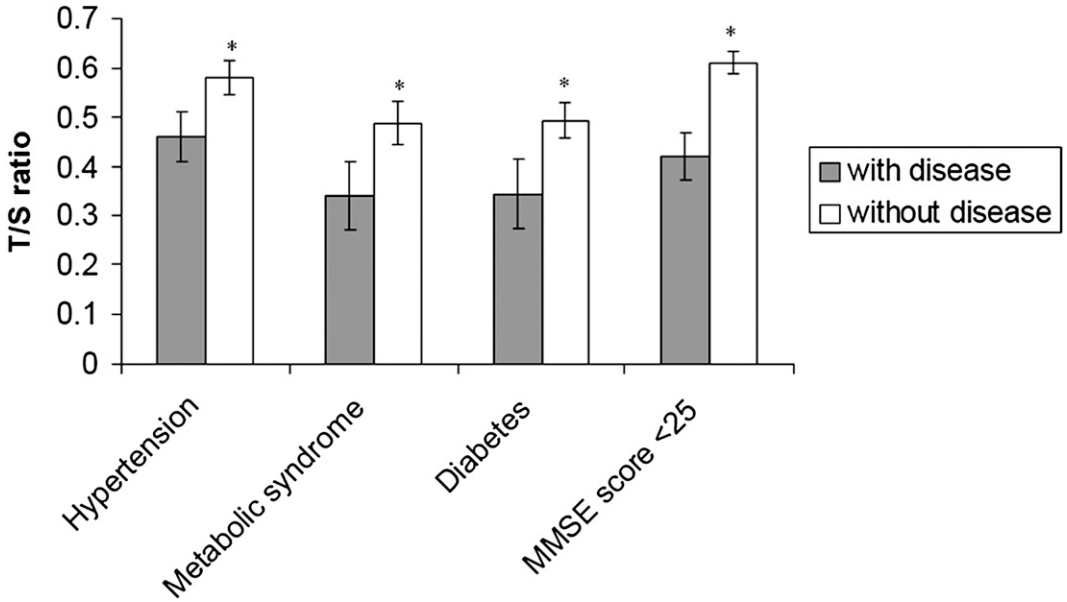

Fig. 2. Telomere length (adjusted for age, gender, and group) according to the absence (gray bars) or presence (white bars) of various age-related diseases in the study subjects. Number of individuals analyzed in each group: hypertension ($n = 125$), without hypertension ($n = 142$), with the metabolic syndrome ($n = 59$), without the metabolic syndrome ($n = 286$), diabetes ($n = 34$), without diabetes ($n = 276$). The MMSE was performed in centenarians only: MMSE score ≤25 (gray bars, $n = 46$) or >25 (white bars, $n = 32$). *$P < 0.05$.

Table 2. Telomere length and lipid profiles

	r	*P* value
Cholesterol, mg/dL	0.06	0.22
Triglyceride, mg/dL	−0.02	0.64
HDL, mg/dL	0.11	0.04
Large HDL, %	0.15	0.038
HDL size, nm	0.15	0.03
Apo-A, mg/dL	0.16	0.005
LDL, mg/dL	0.026	0.62
Large LDL, %	0.26	0.0002
LDL size, nm	0.29	0.0001
Apo-B, mg/dL	−0.003	0.95
Very LDL, mg/dL	−0.19	0.008

Telomere length was adjusted for group, gender, and age at recruitment.

of common *hTERT* or *hTERC* variants with telomere length indicates no significant association (Table S1). However, of the two haplotypes that are enriched in centenarians compared with controls, Hap 3 shows a positive association with telomere length after adjusting for age and gender ($P = 0.007$), whereas Hap 6 does not reach a significant threshold ($P = 0.59$) because of its low frequencies in the tested population (Table 6). In contrast, a centenarian-depleted haplotype, Hap 1, shows no association with telomere length (Table 5).

Discussion

Several studies have demonstrated a close relation between telomere length and life span in humans, including long-lived humans (4). However, interpretations of results from these studies are often confounded because of a lack of adequate controls. We employed a unique study design to overcome this shortcoming in a cohort of Ashkenazi Jewish individuals with exceptional longevity (centenarians), their offspring (approximate age of 70 years), and age- and gender-matched controls without a family history of unusual longevity. The use of offspring of individuals with exceptional longevity and their matched controls provides a powerful approach to identify genetically controlled longevity traits. This approach has previously led to the identification of longevity phenotypes such as lipoprotein sizes and the subsequent discovery of corresponding longevity genotypes (32–34). In the present investigation, we employed the same study design to delineate the relation between telomere lengths, longevity, and diseases of aging as well as to gain insights into the potential role(s) of genetic variations in the *hTERT* and *hTERC* genes on these phenotypes.

This study demonstrates that centenarians and their offspring have significantly longer telomeres than unrelated controls and that this trait is strongly heritable. Moreover, we have demonstrated that offspring of centenarians do not show an appreciable age-related decline in telomere length as is observed in our unrelated control population as well as in other cross-sectional studies (5). Because telomere length in younger persons (<75 years of age) is not significantly different between offspring of centenarians and unrelated controls, these results suggest that families with exceptional longevity have superior telomere length maintenance. Interestingly, unrelated controls older than 86 years of age exhibit longer telomeres than younger individuals (controls), suggesting that the rate of telomere attrition may be an important determinant of overall survival in the general population. The relatively older group may be affected by survival bias that selected out individuals with aging-related disease or those who would have died before reaching the age of 85 years, leaving the survivors with relatively longer telomeres.

Because shorter telomere length is associated with diseases of aging, including hypertension, the metabolic syndrome, and dementia (10), we studied the association between telomere length, major age-related diseases, and lipid profiles in centenarians, their offspring, and controls. Lipid profiles are known predictors of age-related diseases, and we have previously demonstrated that centenarians and their offspring have significantly larger HDL and LDL particle sizes and that these are heritable phenotypes of healthy aging associated with a lower prevalence of morbidity. We found that longer telomeres are indeed associated with lower prevalence of hypertension, the metabolic syndrome, type 2 diabetes, and better cognitive function as well as with healthier lipid profiles.

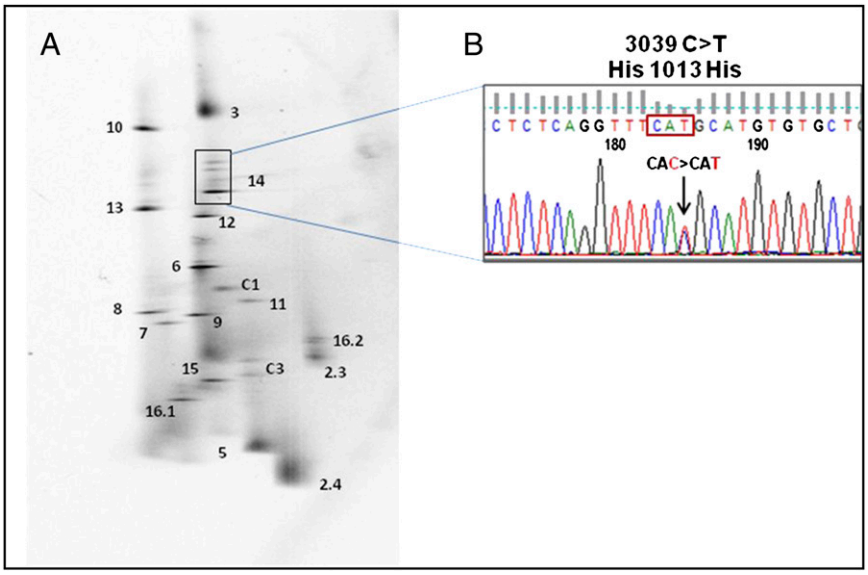

Fig. 3. 2D gene scanning of human telomerase gene (*hTERT* and *hTERC*). The coding regions and exon–intron junctions of the *hTERT* and *hTERC* were amplified by 2-step PCR. Eighteen PCR fragments were displayed in a 2D gel according to their size and melting temperature. (*A*) 2D gene scanning pattern from a centenarian subject with the fragment identification number and a heteroduplex band in exon 14 of the *hTERT*. (*B*) Common genetic variation in exon 14 was identified as 3097 C > T (His-1013 His) by nucleotide sequencing.

Table 3. Telomerase gene variants in centenarians and controls

	Nucleotide change	Protein change	No. of Heterozygotes: Centenarians (n = 100)	No. of Heterozygotes: Controls (n = 80)
hTERT				
Non-synonymous	835 G > A	Ala 279 Thr	0	1
	3148 G > A	Ala 1050 Thr	0	1
Synonymous	915 G > A	Ala 305 Ala	43	27
	1812 A > G	Ala 604 Ala	1	1
	1849 C > T	Leu 618 Leu	1	1
	2355 C > G	**Ser 785 Ser**	1	0
	2481 G > A	**Thr 827 Thr**	3	0
	2739 C > T	**His 913 His**	4	1
	3097 C > T	His 1013 His	28	11
Intronic	**IVS1-211G > A**		2	0
	IVS1-187 T > C		52	36
	IVS4+10 C > T		4	0
	IVS4+21 C > T		1	0
	IVS16+64 C > T		2	0
	IVS16+99 C > T		30	12
hTERC				
Intronic	**IVS-99 C > G**		1	0
	IVS+12 A > G		1	0
	IVS+63 T > C		30	27

Previously unknown unique variants are indicated in bold.

Telomere length, as a quantitative trait, is fairly well studied (19), but genetic factors that influence it are not well understood. Recent studies suggest that heterozygote mutation in *hTERT* and *hTERC* genes, which are the essential components of telomerase, show defective phenotypes in several diseases, indicating that half the usual dose of telomerase is inadequate for maintenance of telomeres with normal length (35, 36). Because living to 100 years of age is a rare phenotype in humans, with a prevalence of 1 in 10,000 individuals in the general population (37), we hypothesized that centenarians may harbor rare gain-of-function mutations in the telomerase genes that may also influence the length of telomeres. Sequence analysis of *hTERT* and *hTERC* genes revealed that rare synonymous or intronic variants in *hTERT* are enriched in centenarians (n = 19) compared with controls (n = 3) (Table 2; P = 0.041). In contrast, centenarians are completely devoid of nonsynonymous variants, whereas there are two control individuals who carry heterozygote nonsynonymous variants, 893 G > A (Ala-279 Thr) and 3148 G > A (Ala-1050 Thr). Although these nonsynonymous changes are predicted to be functionally benign, they are also found in idiopathic pulmonary fibrosis (IPF) patients with a MAF >5% (38), raising the possibility that these variants may have negative

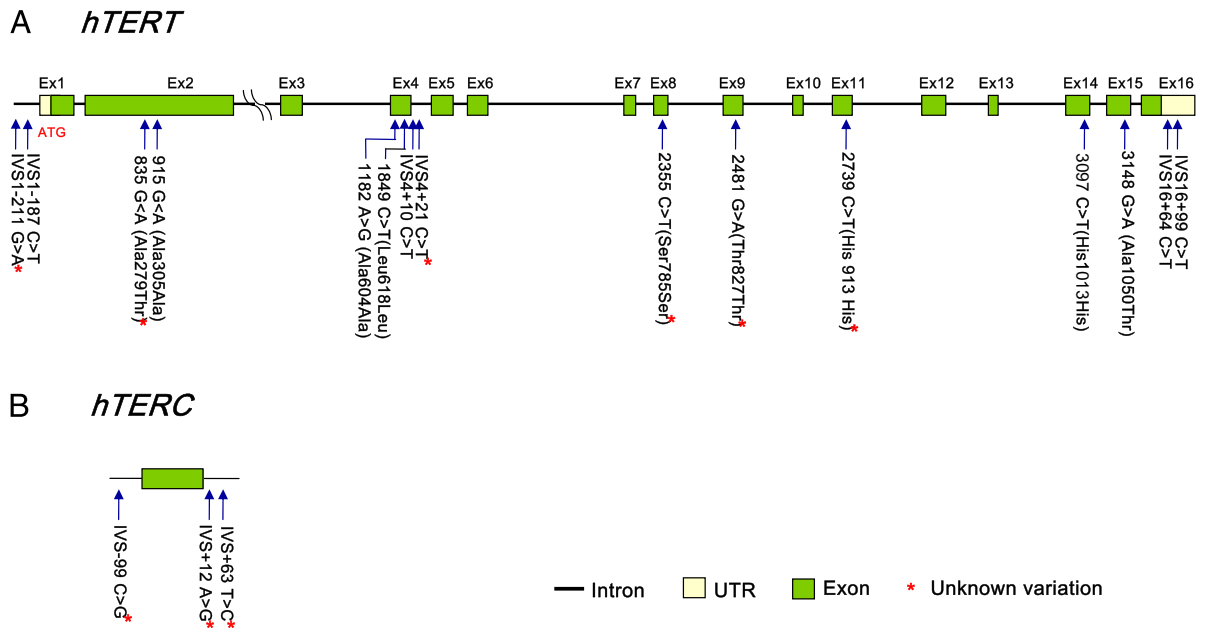

Fig. 4. Schematic representation of exons, introns, UTRs, and variants discovered. (*A*) *hTERT*. (*B*) *hTERC*. Green blocks, coding exons; white blocks, 5′ and 3′ UTRs; *previously unknown variants.

Table 4. Association of hTERT common alleles/genotypes with exceptional longevity

Nature of variation				Controls (n = 49)	Centenarians (n = 73)	P value[†]
				Allele, %		
TERT	Exon 1	IVS1–187 T > C	C	36.7	42.5	0.3717
	Exon 2	973 G > A (A 305 A)	A	30.6	34.9	0.4834
	Exon 14	3097 C > T (H 1013 H)	T	8.1	21.9	0.0049
	Exon 16	IVS16+99 C > T	T	11.2	16.4	0.2556
TERC		IVS+63 T > C	C	18.5	18.8	0.9402
				Genotype, %		
TERT	Exon 1	IVS1–187 T > C	TT	40.8	31.5	0.4494
			CT	44.9	52.1	
			CC	14.3	16.4	
	Exon 2	973 G > A (A 305 A)	GG	53.1	43.8	0.5313
			GA	32.7	42.5	
			AA	14.3	13.7	
	Exon 14	3097 C > T (H 1013 H)	CC	85.7	63.9	0.035
			CT	12.2	26.8	
			TT	2	8.5	
	Exon 16	IVS16+99 C > T	CC	81.6	68.5	0.0972
			CT	14.3	30.1	
			TT	4.1	1.4	
TERC		IVS+63 T > C	TT	64.4	66.2	0.5653
			CT	34.2	29.9	
			CC	1.4	3.9	

[†]Adjusted for age and gender.

functional effects. In addition, although we did not find a single coding variation in the *hTERC* gene, we found two rare intronic variants only in centenarians. The enrichment of both synonymous and intronic variants of *hTERT* and *hTERC* genes in centenarians is intriguing because these variants are known to play a functional role in the regulation of gene expression through modulation of mRNA stability, mRNA secondary structure, alternative splicing, or translational efficiency (39, 40).

The expression level of hTERT is a major determinant of telomerase activity (41). Recent studies indicate that telomerase activation can be regulated by environmental interventions such as lifestyle changes or stress management (42). There are functional hTERT promoter variants known to influence telomerase expression and telomere length (43). However, because there is very little LD in the *hTERT* gene, it is not likely that the centenarian-enriched rare variants are proxies of functional variants in the *hTERT* promoter. It is tempting to speculate that the rare variants might influence expression of *hTERT* in response to environmental stresses such as inflammation or oxidative stress, which then promotes better maintenance of telomere length. Indeed, mean telomere length is much greater among the carriers of the rare variant compared with the noncarriers after adjusting for age and gender [telomere repeat copy number to single-copy gene copy number (T/S) ratio of 0.62:0.41, crude difference = 0.21; $P = 0.02$ by Student t test, $P = 0.037$ by Mann-Whitney U test], raising the possibility that these rare variants may have a positive functional impact on telomere maintenance. Our study also revealed that common genetic variations in hTERT may influence the telomere length maintenance associated with longer telomeres in families with exceptional longevity.

Haplotypes of the *hTERT* gene derived from four common variants showed associations with longevity and/or telomere length. These are two intronic variants, IVS1–187 T > C and IVS16+99 C > T, and two synonymous variants, 973 G > A (Ala-305 Ala) and 3097 C > T (His-1013 His). These common variants may contribute to the regulation of *hTERT* gene expression. A common intronic *hTERT* variant shown to be associated with susceptibility to IPF may also affect the expression levels of hTERT (44). Taken together, our study demonstrates that centenarians may harbor individually rare but collectively more common genetic variations in genes involved in the telomere maintenance pathway, implicating a role of regulatory variants in

Table 5. hTERT haplotypes and association with exceptional longevity

Hap	IVS1–187 T > C	973 G > A (A305A)	3097 C > T (H1013H)	IVS16+99 C > T	Haplotype frequency, %		
					Centenarians (n = 74)	Controls (n = 49)	P value*
1	T	G	C	C	34.9	53.1	0.008
2	C	A	C	C	21.9	23.5	0.322
3	T	G	C	T	13.7	9.2	0.007
4	C	A	T	C	10.3	5.1	0.392
5	C	G	C	C	6.2	5.1	0.749
6	T	G	T	C	6.8	1.0	0.024
7–9			Others		6.2	3.1	0.203

*Adjusted for age and gender.

Table 6. hTERT haplotypes and association with telomere length

Haplotype	Hap	Increase (+) or decrease (–) in telomere length (T/S ratio)	*P* value
T G C C	1	–0.0307	0.742
C A C C	2	–0.0759	0.447
T G C T	3	0.4040	0.007
C A T C	4	–0.0222	0.899
C G C C	5	–0.1280	0.648
T G T C	6	0.1235	0.591
Rare	7–9	–0.0837	0.340

the telomerase gene in human longevity and maintenance of telomere length.

In summary, we provide strong correlative evidence that individuals in Ashkenazi Jewish families with exceptional longevity have better maintenance of telomere length and that the telomerase genes may function as important genetic determinants of both human longevity and telomere length. Additionally, our data suggest that both telomere length and variants of telomerase genes may have a cumulative influence on lower disease prevalence and a favorable lipid profile in centenarians and their offspring. Additional comprehensive studies on genetic and genomic variation of centenarians and their offspring comprising candidate genes, especially those known to play a role in telomere maintenance in model organisms, may reveal previously undescribed genomic regions and molecules that are operative in human health and longevity.

Materials and Methods

Study Design and Subjects. A cohort of Ashkenazi Jews with exceptional longevity was recruited as previously described (34). Probands were required to be living independently at 95 years of age as a reflection of good health, although at the time of recruitment, they could be at any level of dependency. In addition, probands had to have a first-degree offspring who was willing to participate in the study. Birth certificates or dates of birth as stated on passports were used to verify the participants' ages. The Ashkenazi population is descended from a founder population (estimated to be several thousands) originating in the 15th century. This "founder effect" resulted in a population both culturally and genetically homogeneous, from which several disease-related genes have been successfully identified (45). A majority of these individuals were born in the United States or moved there before World War II. Telomere measurements (see below) were made on 86 Ashkenazi probands (aged 97.4 ± 0.3 years) defined as having exceptional longevity, 175 offspring of parents with exceptional longevity (aged 67.8 ± 0.6 years), and 93 controls who were offspring of parents with usual survival (aged 71.8 ± 1 years). By definition, parents of controls survived to the age of 85 years or less. The control group consisted largely of spouses of the offspring group. Informed written consent was obtained in accordance with the policy of the Committee on Clinical Investigations of the Albert Einstein College of Medicine.

For each subject, a detailed medical history, physical examination, and blood sample collection were performed as previously described (23, 32, 34). The presence of hypertension, the metabolic syndrome, and diabetes was determined using National Cholesterol Education Program (NCEP), Adult Treatment Panel III (ATP III) criteria (46). Scores on the MMSE (47) ≤25 were considered to indicate impaired cognitive function. Triglyceride, LDL-cholesterol (LDL), and HDL-cholesterol (HDL) levels and average particle sizes were determined by NMR spectroscopy at LipoScience, Inc., as previously described (34). Large lipoprotein particle sizes were defined as >8.9 nm for HDL and >21.3 nm for LDL.

Measuring/Estimating Length by Quantitative Real-Time PCR. Genomic DNA was extracted from blood samples and stored at −80°C. The average (of triplicate) telomere length in leukocytes was estimated using a quantitative PCR (qPCR) method (48). This approach provides relative average telomere lengths in genomic DNA by determining the T/S ratio in experimental samples relative to a reference sample. Briefly, standard curves were generated for telomere lengths and for the single-gene copy amplification reactions from a reference DNA sample that was serially diluted with double-distilled water by 1.68-fold per dilution to produce 5 concentrations of DNA ranging from 7.7 to 61 ng/μL. For analysis of subjects' DNA, triplicate PCR reactions using 2 μL of each DNA dilution were carried out in a 20-μL volume using the LightCycler FastStart DNA Master SYBR Green kit (Roche Diagnostics), with $MgCl_2$ added to a final concentration of 3 mM. Primers for telomeres and the single-copy gene HGB (β-globin gene) were added to final concentrations of 0.1 μM per 0.9 μM and 0.3 μM per 0.7 μM, respectively. The primer sequences are TEL 1b, 5′-CGGTTTGTTTGGGTTTGGGTTTGGGTTT-GGGTT-3′; TEL 2b, 5′-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTT-ACCCT-3′; HGB1, 5′-GCTTCTGACACAACTGTGTTCACTAGC-3′; and HGB2, 5′-CACCAACTT-CATCCACGTTC-ACC-3′ (48). The enzyme was activated at 95°C for 10 min, followed by 30 cycles at 95°C for 5 s, 58°C for 10 s, and 72°C for 40 s for the HGB reaction or for 20 cycles at 95°C for 5 s, 56°C for 10 s, and 72°C for 60 s for the telomere reaction. All transition rates were set to 20°C/s with the exception of the annealing transition rate in the telomere reaction, which was set to 4°C/s. Forty samples (mixed samples from probands, offspring, and controls) were analyzed by qPCR, both in our laboratory and by one of the authors (R.M.C.) for validation purposes, yielding a correlation of 0.89 ($P < 0.001$). In addition, to ensure reproducibility, when measuring previously unanalyzed samples, and to account for assay variability between batches, we used an anchor sample that was previously measured. The reproducibility between the two measurements was high ($r = 0.94$, $P < 0.001$). Results obtained using this qPCR were highly correlated with those obtained by the traditional terminal restriction fragment length approaches (48). One T/S ratio unit is equivalent to a mean telomere length of 4,270 bp in the leukocytes. The qPCR telomere length determination does not include subtelomeric lengths.

Mutation Screening and Genotyping of hTERT and hTERC. We sequenced genomic DNA isolated from blood of centenarians and controls to discover all possible genetic variations in the exonic regions and intron–exon boundaries of the telomerase genes (*hTERT* and *hTERC*) by a 2D gene scanning (TDGS) method (49). To increase specificity, PCR amplification was designed in two steps. To begin with, triple-multiplex long-distance PCR coamplifies eight large fragments encompassing the coding and splicing junction regions of *hTERT* and *hTERC*. These products serve as a template for quadruple-multiplex short PCRs to amplify target regions in 18 fragments; 16 fragments for hTERT and two fragments for hTERC. Primers are listed in Table S2. The mixture of amplicons after heteroduplexing was separated on the basis of size and melting temperature in a 2D gel. Then, DNA fragments containing heterozygous sequence variation were reamplified from the genomic DNA and sequenced to identify the position and nature of sequence variation. For *hTERT*, exon 1 was analyzed by nucleotide sequencing and exons 2, 4, and 5 were separately analyzed by denaturing gradient gel electrophoresis (DGGE) because of ambiguous melting patterns on a TDGS gel. Genotypes of the common variants in the telomerase gene were determined by DGGE using the same primers designed for the TDGS assay.

Statistical Analyses. Two comparisons have been established: (*i*) between probands and controls for whom crude data were adjusted for gender and (*ii*) between offspring and controls for whom crude data were adjusted for age and gender. Individual phenotypes that were not normally distributed were log natural-transformed for analysis and back-transformed for the current presentation. Associations of telomere length with other variables were estimated using regression on an indicator variable for group, age, and gender. In defining strata for age, cutoff points of 65, 75, 85, 95, and 105 years were used (Fig. 1). Telomere length distribution in probands appears to be nonnormally distributed; thus, to test for statistical significance, we applied nonparametric statistics analysis, and pairwise crude comparisons of telomeres between the study groups were carried out using the Wilcoxon signed-rank test. Parameters were estimated by maximum likelihood methods. The significance of the parameter (association) was evaluated using the likelihood ratio test by comparing the model in which the

parameter is set to 0 with the model in which the parameter is estimated. In addition, we compared telomere length between offspring and controls using a hierarchical linear regression model to account for clustering within families. The regression equation adjusted for age and gender included an indicator variable distinguishing offspring from controls and an offspring vs. controls × age interaction term. We tested the hypotheses that offspring have higher mean telomere length than controls and that the age-related decline in telomere length is less in offspring than in controls. To determine the effect of lipid profile on telomere length, a general linear model adjusted for group, gender, and age at recruitment was used. Correlations of telomere length and lipid profile adjusted for group, gender, and age at recruitment were evaluated using Spearman rank correlation coefficients. Heritability of telomere length was assessed in two ways: First, it was computed in the ASSOC module of SAGE (v6.0.1) (http://darwin.cwru.edu/sage/). Second, narrow sense heritability was estimated from the slope of the linear regression of the traits of each parent on the mean value of the offspring group (16). Data are expressed as mean (SE) as appropriate. Statistical analyses were performed using SAS 9.1 (SAS Institute).

Comparison of the observed numbers of each genotype with those expected under Hardy-Weinberg equilibrium was tested using χ^2 tests for each of the three groups: centenarians, their offspring, and controls. Haplotypes were constructed using the PHASE algorithm (50). The haplotype trend regression analysis for logistical (case-control), linear (telomere length), and proportional hazard with telomere length was performed using JMP genomics 4 (SAS Inc., Cary, NC).

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