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Leucocyte telomere length in age-related macular degeneration

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ABSTRACT.

Purpose: **To evaluate the association between telomere length and age-related macular degeneration (AMD).**

Methods: **Circulating leucocyte telomere length and the proportion of telomeres < 5 kb were analysed in blood DNA samples taken from 121 patients with exudative AMD (83%), large drusen (14%) or central geographic atrophy (3%). Controls consisted of 77 age-matched subjects without AMD. The AMD status was assessed by a masked analysis of fundus photographs or angiographs. Telomere length was measured by Southern blotting.**

Results: **Mean (SD) telomere length was 7.76 kb (0.68) in AMD patients and 7.83 (0.69) in controls (p = 0.485). The corresponding proportions of telomeres < 5 kb were 10.60 (2.76) and 10.05 (2.64) (p = 0.197). In this material, there was no correlation between telomere length and age, gender or smoking status. There were no differences between the major AMD risk single-nucleotide polymorphisms (SNPs) of the CFH, HTRA1 or C3 genes, expect for somewhat longer telomeres in controls with the C3 risk SNP. There were no differences in telomere length between patients with drusen or exudative AMD.**

Conclusions: **Telomere length is not associated with exudative AMD or high-risk drusen.**

Key words: age-related macular degeneration – genetics – retina – telomere

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Introduction

Telomeres are structures capping the ends of the eukaryotic chromosomes. They function at preventing random attachment of DNA strands at chromosome ends and at helping to close the '5′ end gap' at the lagging DNA strand after DNA replication. The key structure in telomeres is the multiple repeat TTGGGG at the 3′ end of the replicated leading DNA strand. Telomere is formed by the enzyme of telomerase, modulated by an array of regulatory proteins (Blackburn 2001).

Without telomerase activity, the telomere shortens at each cell division. In somatic cells, telomerase activity is generally insufficient to fully compensate for the loss of telomere structure during cell division or occurring because of other factors, for example, by oxidative stress. Thus, length of the telomere has been shown to function as a 'biologic clock', correlating with ageing between and within species (Blasco 2005).

In humans, circulating leucocyte telomere attrition has been associated especially with age, smoking, insulin resistance and other risk factors for cardiovascular disease (Benetos et al. 2001; Shamani et al. 2001; Valdes et al. 2005; Fitzpatrick et al. 2007). Also, shortening of telomeres has been detected in conditions with oxidative stress and chronic inflammation (Schönland et al. 2003).

Age-related macular degeneration (AMD) is a disease leading to scarring and loss of function of the central retina. Oxidative stress attributed to light and the high metabolic rate in photoreception are thought to make the macular area especially susceptible to degenerative changes (Zarbin 2004). The risk of AMD is strongly determined by genetic factors and smoking. In addition, markers of cardiovascular disease and systemic inflammation, including leucocyte counts, modify the risk of AMD (Seddon et al. 2005; Boekhoorn et al. 2007; Shankar et al. 2007; Seitsonen et al. 2008).

We examined the possibility that telomere length might be associated with AMD risk. In this study, we report our results from telomere

length measurements in a group of patients with AMD and in age-matched control subjects.

Methods

Patients and controls

Venous blood was drawn from 121 AMD patients attending the Department of Ophthalmology, Helsinki University Hospital. AMD grade was determined by masked evaluation of fundus photographs of patients and control subjects. One hundred (82.6%) of the patients had neovascular AMD in the worse eye, 17 (14%) large drusen and 4 (3.3%) central geographic atrophy. Control subjects were attending the hospital for cataract surgery or were referred from private ophthalmology offices. Only hard drusen $< 63\ \mu$ or minimal pigmentary abnormalities were allowed in the controls. Most of the control subjects had neither drusen nor pigmentation in the macula. There were more women than men in both groups, and a higher proportion of the AMD cases were smokers (Table 1). All subjects gave their informed consent to participate in the study. The study protocol was approved by the local ethics committee, and the tenets of the Helsinki declaration were followed.

Measurement of telomere length

DNA was extracted from venous blood using PureGene, Gentra method (Gentra systems, Minneapolis, MN, USA). Telomere length was measured using TeloTAGGG Telomere length assay kits (Roche Molecular Biochemicals, Basel, Switzerland), as described (Fyhrquist et al. 2010). Briefly, an aliquot (1 μg) of DNA (sample or control DNA) was digested with HinfI and RsaI (20 U/μg DNA each) at 37°C for 2 hr. Separation of digested DNA (1 μg) was carried out by 0.8% agarose gel electrophoresis at 5 V/cm in 0.04 M Tris–acetate and 0.001 M EDTA, pH 8.0 buffer for 2–3 hr.

After electrophoresis, the DNA fragments were transferred by Southern blotting (by capillary transfer) to a positively charged nylon membrane (Hybond N+; Amersham, Little Chalfont, UK) at room temperature using 3 M NaCl and 0.3 M sodium citrate, pH 7.0. The transferred DNA was then fixed on the blotting membrane by UV-cross-linking (UV Stratalinker 1800; Stratagene, La Jolla, CA, USA). The blotted DNA fragments were hybridized to a digoxigenin-labelled probe specific for telomeric repeats in hybridization oven (Techne, Burlington, NJ, USA) at 42°C for 3 hr.

The membrane was incubated with a DIG-specific antibody covalently coupled to alkaline phosphatase and then visualized by virtue of alkaline phosphatase metabolizing CDP-*Star* (Sigma-Aldrich, St Louis, MO, USA), a highly sensitive chemiluminescence substrate. The membrane was exposed to a hyperfilm ECL (Amersham), and the films were analysed using Adobe PhotoShop and Science Lab 99 Image gauge software (Fuji Photo Film Ltd, Tokyo, Japan).

The mean size of the telomere restriction fragment (TRF) was estimated using the formula {sum}(ODi – background)/{sum}(ODi – background/Li), where ODi is the chemiluminescent signal and Li is the length of the TRF fragment at position i.

There is increasing evidence suggesting that regardless of mean telomere length, one critically short telomere may cause a cell to enter senescence (Hemann et al. 2001; Zou et al. 2004). Therefore, using the same films as in the mean TRF analysis, we also calculated the percentage of short telomeres, shorter than 5 kb, in each telomeric sample.

Briefly, the total chemiluminescence intensity of each sample was measured and the signal intensity below molecular size marker 5 kb. Percentage of short telomeres = (intensity of chemiluminescence signal below 5 kb – background) × 100/(total signal intensity – background).

Genotyping for AMD risk single-nucleotide polymorphisms

The AMD patients and controls were genotyped for the major AMD risk SNPs (CFH, rs1061170, HTRA–I, rs11200638 and C3, rs2230199), as described previously (Seitsonen et al. 2008).

Statistical analysis

Normality of variables was tested with Kolmogorov–Smirnov test. For continuous variables, differences between two groups were analysed with Student's *t*-test and between multiple groups with ANOVA. Appropriate non-parametric tests were used when necessary. Chi-square test or Fisher's exact test was used for categorical data. Statistical analyses were performed using SPSS versions 14.0, 15.0 and 17.0 (SPSS, Chicago, IL, USA).

Power analysis, made with the PS Power and Sample analyzer, obtained from http://biostat.mc.vanderbilt.edu/twiki/bin/view/Main/PowerSampleSize, indicated that with an SD of about 10% of the mean and the numbers of cases and controls used in this study, a difference of about 4% between the groups could be detected with a power of 0.8 and a p-value of 0.05.

Table 1. Distribution of potential nongenetic risk factors for age-related macular degeneration in the study subjects.

	Cases (n = 121)	Controls (n = 77)	p (χ^2 or t-test)
Age (Mean, SD)	75.4 (6.3)	76.7 (4.1)	0.099
Female	98/121 (81%)	65/77 (84%)	0.538
Never smoked (data missing in 12 subjects)	64/112 (57%)	63/74 (85%)	0.000
Diabetes type II	16/121 (13%)	8/77 (10%)	0.551
Treatment for hypertension	47/121 (39%)	38/77 (49%)	0.145
Statin users	30/121 (25%)	30/77 (39%)	0.034
History of coronary artery disease	13/121 (11%)	14/77 (18%)	0.137

Results

In our material, 100/121 (83%) of the AMD patients had exudative AMD, 17 (14%) had high-risk drusen, and 4 (3%) had geographic atrophy.

The AMD patients tended to be slightly younger than control subjects (mean ages 75.4 and 76.7 years, respectively, p = 0.099, *I*-test). There were more smokers and less statin users in the AMD patients than in controls (Table 1.). There was no dif-

Table 2. Telomere lengths and potential non-genetic risk factors for age-related macular degeneration in the whole study material.

Telomere length in subgroups	All subjects	p (*t*-test)
Gender		
Female	7.80 (0.70)	0.423
Male	7.78 (0.69)	
Ever smoked		
No	7.81 (0.72)	0.246
Yes	7.70 (0.59)	
Diabetes II		
Yes	7.71 (0.58)	0.573
No	7.79 (0.70)	
Treatment for hypertension		
Yes	7.84 (0.64)	0.358
No	7.74 (0.72)	
Statin use		
Yes	7.81 (0.77)	0.742
No	7.77 (0.65)	
History of coronary artery disease		
Yes	7.76 (0.63)	0.854
No	7.79 (0.70)	

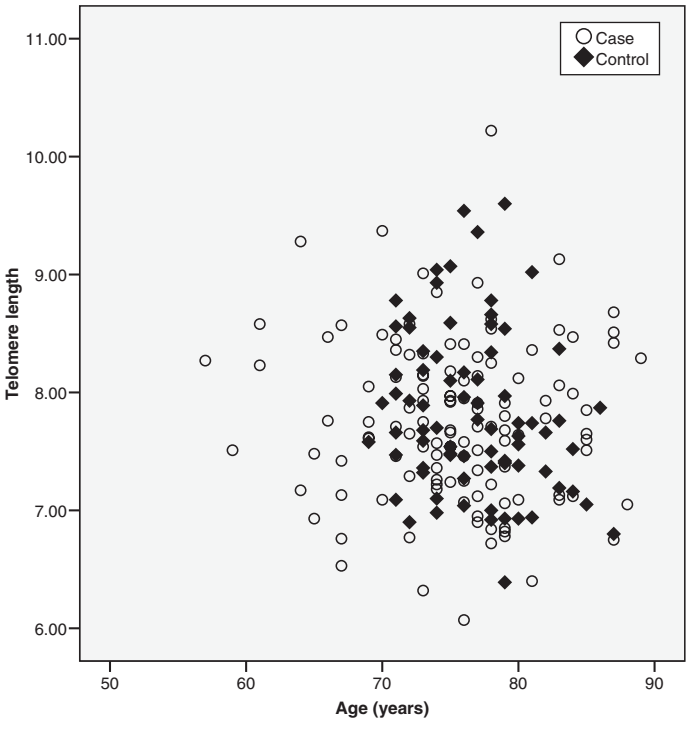

Fig. 1. Telomere length in relation to age in patients with AMD (open circles) and control subjects (black diamonds). $r = 0.08$, p = 0.24 (all subjects); $r = 0.041$, p = 0.65 (AMD patients); and $r = 0.211$, p = 0.07 (control subjects) (Pearson correlation).

ference in the prevalence of type II diabetes, hypertension or coronary artery disease between the groups. In our material, with a quite narrow age range, there was no correlation between age and telomere length (Fig. 1). Also, the telomere length was unaffected by smoking, type II diabetes or treatment for arterial hypertension (Table 2.).

The risk SNPs studied were associated with AMD in this material (CFH: p -, HTRA1: p = 0.001 and C3 p = 0.01 (χ^2-test).

The average mean telomere length in the samples was 7.76 kb (SD = 0.64) in AMD patients and 7.83 (0.69) in controls (p = 0.485). The corresponding proportions of short telomeres < 5 kb were 10.60% (2.77) and 10.05% (2.64) (p = 0.197, *t*-test). Of the shortest quartile of telomeres in the whole material, 27% was in the AMD group and 21% in the control subjects (p = 0.306, χ^2 -test). For the longest quartile of telomeres, the corresponding percentages were 24% and 26% (p = 0.750). The telomere parameters did not correlate with the CFH, HTRA1 or C3 AMD risk SNPs, except that in the control subjects the C3 AMD risk allele G was associated with longer telomeres: the telomere lengths (mean [SD, N]) were 7.69 (0.56, 57), 8.05 (0.85, 16) and 9.20 (0.69, 3) in the controls with the C3 genotypes CC, CG and GG, respectively. The mean telomere length was 8.23 (0.89, 19) in controls with the G allele, compared to 7.69 (0.57, 57) in those without (p = 0.0003, Student's *t*-test). Similar effect was not present for the mp 5 proportion in control subjects.

Discussion

Telomere shortening has been found in conditions associated with AMD, such as hypertension, cardio- and cerebrovascular disease and smoking (Benetos et al. 2001; Shamani et al. 2001; Fitzpatrick et al. 2007). Thus, we hypothesized that a connection with AMD might also be present.

In our material, we could not find any association between the telomere parameters studied and AMD. The study was sufficiently powered for detecting even relatively small differences in telomere length between the groups.

This is a case–control study with both cases and controls being obtained from several sources. Thus, it is difficult to evaluate how well the study sample presents AMD and non-AMD subjects in the general population. We could, however, reproduce the main genetic risk factors as well as smoking in this material. Thus, we feel that it is unlikely that a significant contribution of telomere length would be present in the general population, but not be detected in our sample.

The association of longer telomeres with the C3 risk allele in the control group could be interpreted as the result of a protective effect, with the longer telomeres preventing subjects with the risk allele from developing AMD. In view of the multiple subgroup testing, this result may also be due to chance. Anyhow, it did not affect the overall results.

In their normal state, the cells of the retina and especially the RPE cells do not proliferate in significant amounts. Thus, local telomere attrition at the level of the RPE would occur first during early phases of AMD, when there are proliferative changes in the RPE (Matsunaga et al. 1999), although oxidative pressure and light exposure may affect telomeres also in the absence of cell proliferation.

We did not measure local telomeres, but analysed white cell telomeres from peripheral blood samples. The correlations between telomere length and, for example, cardiovascular disease are present in analyses made from periph-

eral white blood cells (Shamani et al. 2001; Fitzpatrick et al. 2007). It has been thought that the blood telomere length may reflect systemic telomerase capacity of an individual. Alternatively, shortened white blood cell telomeres may be associated with chronic activation of the immune system, beyond the reparative capacity of telomerases (Blasco 2005). Such a chronic systemic inflammation has been reported in the pathogenesis of cardiovascular disease and also AMD (Zarbin 2004). The AMD patients in our material had mostly neovascular AMD. Telomere shortening inhibits neovascularization in experimental set-ups (Pallini et al. 2006). It is thus possible that telomere shortening might have a role in the pathogenesis of geographic atrophy. The number of patients with geographic atrophy in our study was so small that this hypothesis cannot be tested here. The fact that no correlation between telomere length, gender and age emerged in our material may be due to the gender imbalance and narrow age scale in our subjects. It is also known that telomere length does not correlate with mortality and morbidity in the very old (Martin-Ruiz et al. 2005).

In any case, our data do not suggest a specific connection between AMD and telomere length.

However, normal age-related shortening of telomeres may still be one of the ageing changes that make conditions favourable to the action of specific pathogenetic factors for the development of AMD.

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