Telomeres are complexes of tandem repeats of the sequence TTAGGG that cap chromosomes. They are essential for protecting chromosomal integrity (1), and shorten after every cell division. Short telomere length can cause genomic instability, which is associated with the initiation and progression of human cancers (2). At the same time, many incipient tumors can terminate their own growth by shortening their telomeres sufficiently to trigger replicative senescence or apoptosis (3). However, if sufficient numbers of mutations that promote growth and block cell senescence and apoptotic pathways accumulate in a cell before its telomeres shorten enough to trigger senescence or apoptosis and protect it from cancer, then unlimited proliferation may ensue. It follows that in some cell types, under some circumstances, long telomeres may actually increase the risk of cancer, by allowing more time and more cell divisions during which the cell can accumulate oncogenic mutations.

Most epidemiologic studies have reported that relatively shorter telomere length measured in peripheral WBC and in some instances buccal cells is associated with increased risk of cancer (4–10). In contrast, some recent reports have suggested that longer telomere length may be associated with increased risk of certain tumors, such as breast cancer and melanoma (11, 12). Most studies have used a case-control design (4–8), with several more recent reports using a prospective cohort design (9–11).

Given that peripheral WBC contain lymphocyte subsets that derive from lymphocytic stem cells and immunologically active tissue, we hypothesized that telomere length in peripheral WBC DNA might be particularly informative with regard to risk of developing non-Hodgkin lymphoma. To avoid potential disease bias, we carried out our study within a prospective cohort, the Alpha-Tocopherol, Beta-Carotene Cancer Prevention (ATBC) Study (13). In addition, we applied a modification of the original PCR-based assay developed by Cawthon (14), referred to as monochrome multiplex quantitative PCR (15), which has high assay precision and is highly correlated (Spearman $r = 0.91$, $P < 0.0001$) with the Southern blot method of measuring telomere length.

### Abstract

**Purpose:** Telomere length plays an important role in the maintenance of chromosomal stability and in tumorigenesis. We hypothesized that telomere length in peripheral WBC DNA obtained from healthy individuals would be a predictor of future risk of developing non-Hodgkin lymphoma.

**Experimental Design:** Using a new assay to measure relative telomere length, monochrome multiplex quantitative PCR, which strongly correlates with telomere length measured by Southern blot (Spearman $r = 0.91$, $P < 0.0001$) and has high precision (coefficient of variation = 7%), we compared telomere length in peripheral WBC DNA in 107 incident male non-Hodgkin lymphoma cases and 107 matched controls within the prospective Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study cohort.

**Results:** Median (10th, 90th percentile) telomere length was 1.10 (0.79, 1.43) in cases and 1.02 (0.78, 1.26) in controls ($P = 0.0017$, Wilcoxon sign test). There was a strong dose-response relationship between quartiles of telomere length and risk of non-Hodgkin lymphoma overall [odds ratios (95% confidence intervals) by quartile: 1.0; 1.1 (0.4-2.7); 1.8 (0.7-4.9); and 3.6 (1.4-8.9); $P$ trend = 0.003], and this association was similar across the most common non-Hodgkin lymphoma subtypes present in this study.

**Conclusion:** These results suggest that longer telomere length may be a potential predictor for future risk of non-Hodgkin lymphoma. (Clin Cancer Res 2009;15(23):7429–33)
Translational Relevance

We report that longer telomere length measured in peripheral WBC DNA is associated with future risk of developing non-Hodgkin lymphoma. This work contributes to a growing number of findings that biomarker assays that measure genomic damage and stability in peripheral WBC of healthy people (e.g., telomere length, global methylation, alteration in mitochondrial DNA copy number) can identify individuals who are at higher risk of developing certain types of cancer in the future. The translational implication of our findings, and this overall area of research, is that the general population will eventually be screened not only for inherited variation in genes, but for acquired alterations in DNA as well, which together will be powerfully predictive of future cancer risk. This will identify individuals who could benefit from various preventive strategies (e.g., focused carcinogen avoidance, chemoprevention) as well as targeted early disease detection.

Materials and Methods

Case and control enrollment. Details of the ATBC cohort study design have been described previously (16). Briefly, 29,133 male smokers, ages 50 to 69 y, were recruited from southwest Finland and randomized from 1985 to 1988. Subjects were provided α-tocopherol, β-carotene, both, or placebo. The study was approved by the Institutional Review Boards at the National Cancer Institute and the National Public Health Institute of Finland. Participants provided written informed consent.

Incident non-Hodgkin lymphoma cases were identified using the Finnish Cancer Registry, which provides nearly 100% of case ascertainment in Finland (17). The hospital records of the identified non-Hodgkin lymphoma cases were reviewed by an expert study oncologist for confirmation of lymphoma diagnosis and histology. Through April 30, 2002, incident cases of non-Hodgkin lymphoma (n = 107) diagnosed after providing a whole blood sample in 1992 or 1993 were identified based on the histology information coded in the International Classification of Disease-Oncology second edition (ICD-O-2: 9590-9820, 9940-9941; ref. 18). Cases were further grouped into non-Hodgkin lymphoma subtypes according to WHO guidelines (18). Controls were selected from the ATBC study participants who were alive and free of cancer at the time of the case diagnosis, and were individually matched to cases on date of birth (±5 y). DNA was extracted from whole blood samples using the phenol-chloroform method, and a monochrome multiplex quantitative PCR assay was used to determine the telomere measurements (15).

Telomere assay. In brief, the reagents in the 25 μL PCR were 10 mM Tris-HCl (pH 8.3), 50 mM sodium KCl, 3 mM MgCl₂, 0.2 mM each dNTP, 1 mM dNTP, 1 mM betaine, 0.25× SYBR Green I, and AmpliTaq Gold DNA polymerase, 0.625 U. The four primer sets were (5′ to 3′): telg (at 900 nmol/L), ACACAAAGGTTGGTTGGTTGGTTGGTTGGT; telc (at 900 nmol/L), TGGTACCTATCTCCTATTCTATTCTATTCTATAACA; hbg (at 500 nmol/L), CGGGGCCGGGCCGGGCCGGGCCGGGCTCGTATCCAGCTTACCTGTGG; and hbgd (at 500 nmol/L), GCCGGCGGCCGGGCCGGGCCGGGCCGGGCTCGTATCCAGCTTACCTGTGG. From 5 to 70 ng of human genomic DNA were added per reaction well. Three-fold serial dilutions of a reference genomic DNA sample were used to generate two standard curves for each PCR plate (five concentrations with a high of 150 ng/reaction and a low of 1.85 ng/reaction). Thermal cycling: 1 cycle of 15 min at 95°C; 2 cycles of 15 s at 94°C, 15 s at 49°C, 32 cycles of 15 s at 94°C, 10 s at 62°C, 15 s at 74°C with signal acquisition, 10 s at 84°C, and 15 s at 88°C with signal acquisition. The 74°C reads provided the crossing thresholds (Cts) for telomeres; the 88°C reads provided the Cts for the single copy gene (β-globin). After the run was complete, the MyiQ software (Bio-Rad IQ5 2.0 Standard Edition Optical System Software) was used to determine the T (telomere) and S (single copy gene) values for each experimental sample by the Standard Curve method. Figure 1 presents the principle of telomere length measurement by quantitative PCR. The ratio of the telomere PCR signal to the single copy gene (in our case, β-globin) PCR signal (i.e. the T/S ratio) is proportional to the average telomere length per cell. All T/S ratios of experimental DNA samples are expressed relative to the T/S ratio of the same reference DNA sample, which, by definition, is assigned a T/S ratio of 1.0. For a given experimental sample, the T value is the number of nanograms of the reference DNA that matches the experimental sample for copy number of the telomere template, and the S value is the number of nanograms of the reference DNA that matches that experimental sample for copy number of the single copy gene template. T/S, therefore, is a relative and dimensionless value. Samples with a T/S >1.0 have an average telomere length greater than that of the standard DNA; samples with a T/S <1.0 have an average telomere length shorter than that of the standard DNA. Multiplex quantitative PCR eliminates a major source of variation present in monoplex quantitative PCR. In monoplex quantitative PCR, variation in the amount of DNA pipetted into the T and S reaction wells results in variation in T/S, but in multiplex quantitative PCR both T and S are measured in each reaction well, so the pipetting variation between wells does not affect T/S.

Statistical analysis. Cases and their matched controls were assayed consecutively within each batch. Blinded quality control samples were interspersed across batches to evaluate assay reproducibility, and samples were analyzed one to two times. The assay intraclass correlation coefficient was 80% and the coefficient of variation was 7%. Odds ratios and 95% confidence intervals were estimated using conditional logistic regression models. Telomere length was categorized into quartiles based on the distribution among controls. Tests for trend were calculated using the median value for each telomere length quartile. Variables that resulted in a ≥10% change in the β-coefficient of telomere length in the base model that adjusted for matched age were considered confounders and included in final, multivariable models.

Age at randomization, body mass index, smoking, physical activity, dietary intake of alcohol, blood pressure, and mitochondrial DNA copy number were not confounders in our sample. All P values are two-sided.

Results

Cases were comparable to control subjects (Table 1). Telomere length was very weakly and inversely correlated with age (Spearman correlation r = –0.07, P = 0.46), and weakly and positively correlated with pack-years of smoking (Spearman correlation, r = 0.12, P = 0.20) among controls, which would be expected given the relatively narrow age range of this older cohort of all tobacco smokers.

Telomere length was statistically significantly longer among cases than controls [median (10th, 90th percentile), 1.10 (0.79, 1.43) in cases and 1.02 (0.78, 1.26) in controls, Wilcoxon sign test; P = 0.0017; Table 1]. The risk of non-Hodgkin lymphoma was significantly increased with longer telomere length, compared with the lowest quartile of telomere length (P trend = 0.003), and the association was consistent across the predominant non-Hodgkin lymphoma subtypes in this series of cases (Table 2). Adjustment for demographic factors shown in Table 1 had a negligible impact on the results (data not shown).
To determine if the association might be driven in part by longer telomere length among cases undiagnosed at the time of blood sample collection, we excluded cases diagnosed within the first year of follow-up (n = 17) after blood sample collection, and found that results were very similar, with odds ratio (95% confidence interval) of 0.8 (0.3-3.1), 1.5 (0.5-4.1), and 3.1 (1.2-8.2) for the second, third, and fourth quartiles of telomere length, compared with the lowest quartile of telomere length (P trend = 0.01).

To evaluate the potential effects of the trial vitamin supplementation on the relationship between telomere length and risk of non-Hodgkin lymphoma, we carried out further analyses stratified by α-tocopherol versus no α-tocopherol supplementation, and β-carotene versus no β-carotene supplementation. Risks were similar in each group, and tests for interactions were not statistically significant (data not shown).

**Discussion**

To the best of our knowledge, this is the first prospective study addressing the relationship between telomere length and the risk of non-Hodgkin lymphoma. Although the sample size is modest, we detected relatively strong effects, which were consistent across major non-Hodgkin lymphoma subtypes. One previous report of telomere length and non-Hodgkin lymphoma by Widmann et al. of 40 cases and 40 controls using a case-control design found that shorter telomere length was associated with increased risk of aggressive non-Hodgkin lymphoma (7). The reasons for this discrepant result with our study are not immediately apparent. Given that relatively shorter telomere length was present in DNA extracted from B cells, T cells, and granulocytes in cases versus controls in the initial report (7), it would be expected that measuring telomere length in an aggregated “buffy coat” containing all peripheral leukocytes, as we did in our study, would give similar results. Widmann et al. measured telomere length by the Flow-Fish method whereas we used a new PCR-based method, which correlates almost perfectly with the Southern blot assay of telomere length. The correlation between the assay used in our report and the Flow-Fish method is not known. The prospective nature of our study is another potential difference between the two reports.

It is possible that tumor cells were present in the peripheral blood of undiagnosed patients in our study, particularly those with the more indolent non-Hodgkin lymphoma histologies. There is much evidence, however, that telomere length is shorter in lymphoma cells (19–21). As a consequence, if tumor cells were circulating in the blood of undiagnosed patients, the average telomere length of their peripheral WBC DNA would tend to be shorter, not longer. This would have biased our results towards the null, rather than create the relatively strong association we report with longer telomere length. At the same time, given that we observed similar associations between longer telomere length and risk of non-Hodgkin lymphoma for a...
Table 2. Odds ratio and 95% confidence interval for telomere length and non-Hodgkin lymphoma

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Telomere length quartile*</th>
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<tbody>
<tr>
<td></td>
<td>Q1 (≤0.88)</td>
</tr>
<tr>
<td>NHL</td>
<td></td>
</tr>
<tr>
<td>Cases/controls</td>
<td>16/27</td>
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<tr>
<td>OR (95% CI)</td>
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<tr>
<td>DLBCL</td>
<td></td>
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<tr>
<td>Cases/controls</td>
<td>1/5</td>
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<tr>
<td>OR (95% CI)</td>
<td>1.0</td>
</tr>
<tr>
<td>CLL/SLL</td>
<td></td>
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<tr>
<td>Cases/controls</td>
<td>6/11</td>
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<tr>
<td>OR (95% CI)</td>
<td>1.0</td>
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<tr>
<td>NHL excluding DLBCL and CLL/SLL</td>
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<tr>
<td>Cases/controls</td>
<td>9/11</td>
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<tr>
<td>OR (95% CI)</td>
<td>1.0</td>
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NOTE: The model was adjusted for age at randomization. Abbreviations: NHL, non-Hodgkin lymphoma; DLBCL, diffuse large B-cell lymphoma; CLL/SLL, chronic lymphocytic leukemia/small lymphocytic lymphoma; OR, odds ratio; 95% CI, 95% confidence interval.

*See Table 1 footnote for definition of telomere length.

References


Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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A Prospective Study of Telomere Length Measured by Monochrome Multiplex Quantitative PCR and Risk of Non-Hodgkin Lymphoma

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