Published in final edited form as:

Int J Biol Markers. ; 27(4): . doi:10.5301/IBJBM.2012.9347.

Shorter peripheral blood telomeres are a potential biomarker for patients with advanced colorectal adenomas

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Abstract

BACKGROUND—Colorectal cancer (CRC) can be prevented by the early detection and removal of advanced adenomas (AAs) by colonoscopy. Our aim was to evaluate peripheral blood leukocyte (PBL) telomere length as a potential biomarker for the presence of AAs.

METHODS—PBL telomere length was measured in patients with AAs (n = 35), in a control group of similar-aged patients who had a normal colonoscopy (n = 145) and in a separate population group with no history of cancer, again similarly aged (n = 495). Telomere measurements were performed using a quantitative PCR assay and reported a ratio of telomere and single copy gene measurements.

RESULTS—Telomere lengths tended to be lower in the patients with AAs than in patients in the normal colonoscopy group (p < 0.001) as well as those in the population group (p = 0.011). A telomere/single copy gene ratio of 0.5 was found to have an estimated 94% sensitivity and 56% specificity for AAs; a combination of sensitivity and specificity for which a value of >0.5 would reduce the odds of a patient having AAs by a factor of 0.11 (the negative likelihood ratio). Thirty three percent of individuals in the population group tested above this cut off and could be considered at low risk for AAs.

CONCLUSIONS—PBL telomeres are shortened in patients with colorectal neoplasia suggesting that PBL telomere length could be a promising non-invasive blood biomarker to pre-screen for risk of AAs prior to colonoscopy.

Keywords

Telomeres; Colorectal polyps; Colorectal adenomas

INTRODUCTION

About 10% of the cancers diagnosed in the world are colorectal cancers (CRCs) ¹. CRC can be prevented by removal of precancerous, adenomatous, colorectal polyps using colonoscopy. Patients with colorectal polyps that have advanced neoplastic features, termed advanced adenomas (AAs), benefit the most from colonoscopy. Those found to have an AA

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Ethical Statement: Approved by the Institutional Review Board.

Conflicts of interest: None.

may have avoided a diagnosis of CRC and are identified as needing closer follow up for recurrent colorectal neoplasia ².

Population screening for colorectal neoplasia is recommended and is usually done either by noninvasive tests for stool blood or by more invasive colonoscopy. Colonoscopy is the most commonly used screening test in the United States ^{3,4} and about 5–10% of individuals undergoing screening colonoscopy have AAs identified ^{5,6}. The remaining majority of patients who have colonoscopy and do not have AAs, endure the inconvenience of colon cleansing and are put at risk for colon perforation.

Therefore, biomarkers for colorectal neoplasia are of intense interest as they would allow for targeting of colonoscopy to those at higher risk and deferring or limiting its use in those at lower risk. The obvious benefits would be decreased costs and an increase in the number of patients opting for CRC screening (40% or more of patients recommended to have colonoscopy do not have the test) ⁴.

Shorter peripheral blood leukocyte (PBL) telomere length has been reported as a biomarker for precancerous lesions, cancer diagnosis and cancer mortality. Telomeres are repetitive DNA sequences that cap the ends of chromosomes, protecting them from damage and regulating cellular senescence. Telomere shortening predisposes chromosomes to instability, the molecular mechanism for the development of most cancers ⁷. Shorter PBL telomere length is associated with the progression of Barrett's esophagus, a precancerous lesion analogous to the AAs of the colon and rectum, to esophageal adenocarcinoma ⁸. A large prospective study showed individuals with shorter telomeres had increased overall cancer morbidity and mortality ⁹. Case control studies have associated shortened PBL telomere length with specific cancer diagnoses including gastric, head and neck, renal cell, lung, and bladder cancer ^{10,11}.

We hypothesize that shorter PBL telomeres may be a useful biomarker to risk stratify the general population for AAs. To test our hypothesis, we measured PBL telomere lengths in patients with AAs and compared them with PBL telomere lengths previously obtained in patients with normal colonoscopies. We also had previously measured PBL lengths from a population reference group.

The goal of this study was to examine the feasibility of PBL telomere length measurements to identify a subset of those tested who may be at particularly low risk for AA. If such a subset could be identified of sufficient size and of sufficiently lowered risk for AAs then there would be the potential to consider initiating colonoscopy at a later age than those at higher risk or having a longer interval between colonoscopies. From a more technical standpoint, the goal was to assess whether there may exist a threshold telomere length measure that may have a low negative likelihood ratio ((100%-sensitivity)/specificity) for AAs.

MATERIALS AND METHODS

This study was approved by the Mayo Clinic Institutional Review Board.

Patient Samples

All subjects were enrolled retrospectively from biobanks. The AA and normal colonoscopy control samples were obtained from the Mayo Clinic Biobank for Gastrointestinal Health Research. Population reference group samples were obtained from the Mayo Clinic Specialized Programs of Research Excellence (SPORE) Pancreas biobank. AA subjects had AAs as defined by the accepted criteria of having any of the following: size equal to or

larger than 10 mm, a significant villous component (> 20%), or high grade dysplasia (US Multi-Society Task Force on Colorectal Cancer and the American Cancer Society ¹²). Normal colonoscopy controls had had a colonoscopy with no adenomatous colon polyps found. Population reference group subjects had no history of cancer, but no information on history of colonoscopy or colon polyps was available for this group. Both the normal colonoscopy control group and the population group were selected so as to have similar age distribution to the group with AAs.

Measurement of PBL telomere length

DNA was extracted by standard methods from all PBLs (AutoGen FlexStar, Holliston MA with Flexigene chemistry from Qiagen Chemistries, Valencia, CA). Measurement of PBL telomere length was by the quantitative PCR telomere assay technique originally developed by Cawthorn¹³. A standard curve was used to allow transformation of cycle threshold (Ct) into nanograms of DNA for telomere (T) and standard (S) gene reactions. A minor modification from Cawthorn's method was that the T/S ratios reported are the ratio of the median of three T measurements and the median of three S measurements.

Two master mixes of PCR reagents were prepared, one with the T primer pair, the other with the S primer pair. Fifteen microliters (μl) of the T master mix were added to each sample well, control well and standard curve well of the first plate and 15 μl of the S master mix were added to each sample well, control well and standard curve well of the second plate. For each sample assayed, three identical 5 μl aliquots of the DNA sample (15ng/aliquot) were added to plate 1 and another three aliquots were added to the same well position in plate 2. For each standard curve, one reference DNA sample was serially diluted in TE by 1:2 fold per dilution to produce six concentrations of DNA ranging from 0.78 to 25ng/ μl . Five microliters of each concentration was distributed to the standard curve wells on each plate. The plates were then sealed with a transparent adhesive cover, centrifuged briefly at 800 g and transported on ice to the ABI 7900HT instrument for analysis (Applied Biosystems, Carlsbad, CA).

The T and S PCRs were prepared identically with the exception of the oligonucleotide primers. The final concentrations of the reagents in the PCR were 20mM Tris-HCl, 0.2mM each dNTP's, 2.0mM MgCl₂, 1% DMSO, 150nM ROX dye, 0.2X SYBR Green I (Invitrogen, Carlsbad, CA), 5mM DTT, 1.25 U AmpliTaq Gold DNA polymerase (Applied Biosystems). The final T primer concentrations were tel 1b, 600 nM; tel 2b 900 nM. The final S primer concentrations were *HBB1* 300 nM; *HBB2* 700 nM. The primer sequences was tel 1b 5'-CGGTTTGTGGGTTGGGTTGGGTTGGGTTGGGTT-3'; tel 2b 5'-GGCTTGCCCTACCCCTACCCTACCCTACCCTACCCT-3'; *HBB1* 5'-GCTTCTGACAAGTGTGTTCACTAGC-3'; *HBB2* 5'-CACCAACTTCATCCACGTTACC-3'. The thermal cycle conditions for both primers pairs begin with a 95° C incubation for 10 min to activate the AmpliTaq Gold DNA polymerase. For T PCR, this was followed by 40 cycles of 95° C for 15 s, 54° C for 2 minutes. For the S PCR, this was followed by 40 cycles of 95 ° C for 15 s, 58 ° C for 60 s, and 72° C for 30 s. The data were then analyzed with the ABI SDS software.

Assay inter- and intra-assay variability were 3.0% and 3.5%, respectively, and similar to that reported in other studies. Intra- and inter-assay variability were determined by using stock DNA from one individual. For intra-assay variability, 25 telomere length measurements were compared from the one pair of T and S PCR plates. To determine inter-assay variability, 25 telomere length measurements were determined from one pair of T and S PCR plates and compared to a separate set of 25 measurements from another pair of T and S PCR plates.

Statistical analysis and evaluation of PBL telomere cutoffs for AAs

Comparisons in PBL telomere length between pairs of groups were conducted using a Wilcoxon rank sum test. Analyses assessing the effect of age on telomere length used the logarithm (base 2) of T/S ratios as the response variable in linear regression analyses due to the highly skewed nature of the distributions. Estimates of sensitivity, specificity, and the proportion of the population reference group with telomere lengths below a given cut-off were estimated for potential cut-off values along with exact binomial 95% confidence intervals (CI). A receiver-operating-characteristic (ROC) curve was created to explore the sensitivity and specificity for various thresholds as well as identifying whether a threshold may exist with low negative likelihood ratio ((100% - sensitivity)/specificity), whilst not overly compromising specificity. Statistical analyses were performed using SAS version 9.2 (Cary, NC) and R statistical software version 2.11.0 (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Demographic summaries were similar in the three groups (Table 1). As expected, telomere lengths were lower at higher ages in the population group, with an estimated reduction in the log (base 2) T/S ratio of 0.21 (95% CI: 0.08 to 0.34) per 10 years, which is a small effect with age relative to the variability in telomere lengths overall (Figure 1). No decrease in telomere length with age was apparent in the normal colonoscopy control group and the advanced adenoma group, however power was limited due to the smaller sample sizes and the high variability.

All three groups had a skewed distribution of T/S ratios. Skewing was most pronounced in the population control group and inspection of Figure 1 shows three extreme outliers in this group. These outliers had S values below the 5th percentile for samples in that group and T values above the 75th percentile. The quantitative PCR amplification plots for these outliers appeared appropriate. The likely explanation for the low S values would be a technical issue such as partial failure of the S PCR reaction. Since this data was obtained and analyzed our protocol has changed and retesting is considered for samples where the T/S ratio falls within the upper or lower 10th percentile for our laboratory. Also, since these experiments were conducted with singleplex PCR, a multiplex method has been reported with improved reliability and correlation with telomere length measurements by Southern Blot¹⁴.

Patients with AAs had a clear tendency toward having shorter telomere lengths compared to those with normal colonoscopies ($p < 0.001$, Table 1, Figure 1). Additionally those with AAs tended to have lower telomere lengths than the population controls ($p = 0.011$), and those with normal colonoscopies tended to have higher telomere lengths than the population controls ($p < 0.001$). This would be consistent with the hypothesis of an association between telomere length and the presence of adenomas because the population group will consist of individuals who would have the full range of colonoscopy results: some normal, some with AAs.

The area under the receiver-operator characteristic (ROC) curve (Figure 2) was 0.77 (95% CI: 0.69 to 0.85), again indicating a fair degree of separation of the distributions of telomere lengths in the AA group compared to the normal colonoscopy group. The graph also shows lines corresponding to combinations of sensitivity and specificity that yield negative likelihood ratios of 0.1, 0.2, and 0.3. Careful examination of Figures 2 and 3 indicate that there may exist a threshold for the T/S ratio of 0.5 that has low negative likelihood ratio and that may be of utility for screening.

At this cut-off value, the estimated sensitivity is 94% (95% CI: 81% to 99%) and the specificity is 56% (95% CI: 47% to 64%), a combination that yields an estimated negative likelihood ratio of 0.11. Of the general population reference group, 33% (95% CI: 29% to 37%) had T/S ratio greater than 0.5 and who could be considered to be at substantially lowered risk for AAs.

DISCUSSION

Shortening of PBL telomeres was associated with the presence of advanced colorectal adenomas. Further, the results suggest that one third of patients may have PBL telomere lengths above a threshold for which the negative likelihood ratio, or ratio of post- to pre-test odds of AAs is 0.11. In this scenario, if say 6% of the tested population have AAs, the one third of the population with a negative test result would have a reduced post-test probability of having AAs of 0.7%. This low risk group could consider deferring colonoscopy to a later date or using less invasive colorectal neoplasia screening.

The putative pathway for telomere associated neoplasia is that shortening of telomeres in the colorectal mucosa increases chromosomal susceptibility to instability. However, experimental evidence is mixed on if PBL telomere length is truly a surrogate for colorectal mucosal telomere length^{15,16}. Chromosomal instability is the molecular mechanism for 80% of colorectal cancers with microsatellite instability the mechanism for the remaining 20%¹⁷. There is a recent report that telomeres are also shortened in microsatellite unstable CRCs and thus telomere shortening may be a common element shared by both the chromosomal and microsatellite instability pathways⁷.

If shortened PBL telomere length is associated with AAs it would be expected that it would also be associated with CRC. However, studies have shown CRC is associated with shortened PBL telomere length, longer PBL telomere length or no association at all. One has demonstrated an association¹⁸, two did not^{19,20} and one showed an association with retrospectively but not prospectively obtained samples²¹ and finally one has shown association with PBL telomere lengthening²². An explanation for the negative studies could be “colonoscopy bias”. Neither of the two negative studies controlled for a history of colonoscopy which has strong protective effect for CRC, decreasing the risk of death by CRC by at least 60%²³. Those patients who underwent colonoscopy, regardless of how short their telomeres were, would be unlikely to be diagnosed with colon cancer. Another possibility would be that the AAs seen in those with shortened telomeres have a lower potential to transition to cancer with telomere shortening in some cases leading to a “dead end” where adenomas but not cancer is the endpoint.

Most preliminary studies of CRC biomarkers test for CRC detection rather than AA detection, as AAs close similarity to normal mucosa makes their identification much more problematic. The recommended stool blood test for colorectal neoplasia at this time, the fecal immunohistochemistry test (FIT), is reported as 87% sensitive for CRC but only 35% sensitive for AAs²⁴. Our results are too preliminary to make a meaningful comparison to FIT but are promising for the ability of PBL telomere length to stratify patients for risk of AAs and assess who may most benefit from colonoscopy.

Limitations of the study include that the groups were measured separately for telomere length and the small number of AA patients with the lack of ability to control for factors known to affect telomere length such as smoking. Larger scaled more controlled studies in clinical settings would be expected to show lower sensitivity and specificity. From other reports, it appears that telomere length is an independent, additional marker of neoplasia. A large prospective study has showed that the association between a cancer diagnosis and

shortened PBL telomeres remains even after controlling for factors (e.g. age) associated with PBL telomere shortening ⁹.

We report positive exploratory results on the potential for use of PBL telomere length to risk stratify for AAs. A prospective validation of the association of AAs and PBL telomere length is ongoing (clinical trials registration NCT01062035).

Acknowledgments

GRANT SUPPORT

This project was supported by a Mayo Clinic clinical research grant and the National Cancer Institute R01 CA132718.

Financial support: Mayo Clinic clinical research grant and the National Cancer Institute R01 CA132718

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Figure 1.

Distributions of peripheral blood leukocyte (PBL) telomere lengths for the three study groups. The horizontal line corresponds to a potential T/S cut-off value of 0.5 that may be useful to screen for individuals at higher vs. lower likelihood of having advanced adenomas. There are statistically significant differences between the advanced adenoma group compared to both the normal colonoscopy group ($p < 0.001$) and the population control group ($p = 0.011$). There is also evidence of a difference between the normal colonoscopy group and population group ($p < 0.001$).

Figure 2.

Receiver operating characteristic curve showing sensitivity and specificity for different thresholds for the T/S ratio. The straight lines show combinations that correspond to a negative likelihood ratio of 0.1, 0.2 and 0.3; the points highlight certain cut-points for the T/S ratio. A threshold of 0.5 provides a low negative likelihood ratio while not overly compromising specificity.

Figure 3.

Estimated sensitivity and specificity along with 95% confidence intervals for potential peripheral blood telomere length cut-offs. The vertical line is identified as a telomere length cutoff that may provide a suitable balance of sensitivity (94%; 95% CI: 81% to 99%) and specificity (56%; 95% CI: 47% to 64%) that could be of potential utility in identifying individuals at higher vs. lower likelihood of having advanced adenomas.

Table 1

Group demographics and peripheral blood leukocyte telomere lengths.

Variable	Summary	Advanced adenoma (N=35)	Normal colonoscopy (N=145)	Population group (N=495)
Gender (female)	No. (%)	15 (43%)	70 (48%)	233 (47%)
Age (yrs)	Median (range)	69 (44, 82)	68 (44, 82)	67 (38, 91)
Telomere length (T/S ratio) ¹	Median (range) (IQR) ²	0.28 (0.18, 1.06) (0.22, 0.37)	0.54 (0.12, 2.90) (0.35, 0.69)	0.38 (0.02, 12.3) (0.24, 0.62)

¹ Mean and standard deviation not shown for telomere length on this scale since distribution is highly skewed.

² IQR = 'Interquartile range'.