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Research Article

Telomere Length Varies By DNA Extraction Method: Implications for Epidemiologic Research

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Abstract

Background: Both shorter and longer telomeres in peripheral blood leukocyte (PBL) DNA have been associated with cancer risk. However, associations remain inconsistent across studies of the same cancer type. This study compares DNA preparation methods to determine telomere length from patients with colorectal cancer.

Methods: We examined PBL relative telomere length (RTL) measured by quantitative PCR (qPCR) in 1,033 patients with colorectal cancer and 2,952 healthy controls. DNA was extracted with phenol/chloroform, PureGene, or QIAamp.

Results: We observed differences in RTL depending on DNA extraction method ($P < 0.001$). Phenol/chloroform-extracted DNA had a mean RTL (T/S ratio) of 0.78 (range 0.01–6.54) compared with PureGene-extracted DNA (mean RTL of 0.75; range 0.00–12.33). DNA extracted by QIAamp yielded a mean RTL of 0.38 (range 0.02–3.69). We subsequently compared RTL measured by qPCR from an independent set of 20 colorectal cancer cases and 24 normal controls in PBL DNA extracted by each of the three extraction methods. The range of RTL measured by qPCR from QIAamp-extracted DNA (0.17–0.58) was less than from either PureGene or phenol/chloroform (ranges, 0.04–2.67 and 0.32–2.81, respectively).

Conclusions: RTL measured by qPCR from QIAamp-extracted DNA was less than from either PureGene or phenol/chloroform ($P < 0.001$).

Impact: Differences in DNA extraction method may contribute to the discrepancies between studies seeking to find an association between the risk of cancer or other diseases and RTL. *Cancer Epidemiol Biomarkers Prev; 22(11); 2047–54. ©2013 AACR.*

Introduction

Constitutional telomere length and its association with cancer risk has been the focus of multiple studies seeking to identify predictive and prognostic biomarkers for malignancy, targets for prevention and treatment, and a better understanding of the mechanisms underlying carcinogenesis. Telomeres are tandem repeated DNA sequences (TTAGGG) that cap linear chromosomes and protect them from unraveling; telomeres shorten with each cell division and with oxidative damage (1). In healthy cells, telomere-shortening results in regulated cell senescence and apoptosis. Conversely, aberrant telomere length homeostasis may disrupt the normal process of senescence and programmed cell death. Evidence is accruing that variation in constitutional telomere length plays an important role in the etiology of many diseases. Extremes of telomere length, either short or long, have been associated with cancer risk (2–24). Yet, across studies, peripheral blood leukocyte (PBL) telomere length has not been consistently associated with cancer risk, even within cancers of the same histopathology (25).

In meta-analyses of the association of telomere length and cancer risk (25), shorter telomere lengths have consistently been associated with the risk of bladder, esophageal, gastric, head and neck, ovarian, and renal cell cancers in all published studies. Results have not been consistent for non-Hodgkin lymphoma and breast, lung,

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and colorectal cancers. Differences in telomere measurement methods, interlaboratory variation, and influences of DNA extraction and storage procedures were identified as potential sources underlying inconsistent results (25).

The most frequently used technique to determine telomere length in cancer-risk association studies is quantitative PCR (qPCR), which measures the canonical region of the telomeres, the same region measured by the less common quantitative FISH (Q-FISH). In contrast, Southern blot analysis measurement includes the noncanonical (subtelomeric) portion of the telomeres. Reproducibility in reported studies remains an issue (26), although interassay variation (coefficient of variance, CV) may be improved with good quality control (27). Interassay variability for Q-FISH has been reported to be more than 5% (28), more than 6% for qPCR, and more than 2% for Southern blot analysis (26). Unlike Q-FISH, qPCR and Southern blot analysis require DNA extraction. PBL DNA is often extracted for using one of three methods: a column method, salting-out of the DNA or organic extraction with phenol/chloroform.

In our case–control study, assessing the association between PBL telomere length and the presence of colorectal cancer, we observed that relative telomere length (RTL) measured by qPCR was shorter in samples from two of six centers that comprise the Colon Cancer Cooperative Family Registry (Colon CFR) than for samples from the other four centers. Because each of the centers had used one or two of three different DNA extraction methods, we sought to determine, whether the DNA extraction methods might be driving the differences in the telomere lengths in both our cases and healthy controls.

Materials and Methods

Study population

All cases and controls were collected through Institutional Review Board (IRB)–approved protocols. The use of these specimens for this study was approved by the Mayo Clinic, IRB (Rochester, MN).

Colorectal cancer cases. Colorectal cancer cases were selected from two prospective collections. The Biobank for Gastrointestinal Health Research (BGHR) is an ongoing IRB-approved collection of biospecimens from participants with normal colonoscopic examinations, colon polyps or colorectal cancer seen at Mayo Clinic, from the year 2000 to the present. The second group of cases originated from the Colon CFR, an international consortium of six centers in North America and Australia described in more detail by Newcomb and colleagues (29). This collaborative resource collected colorectal cancer cases from family clinics; population-based sampling from incident cases of colorectal cancer; and population-based recruitment of patients with younger age of cancer onset (29). Overall, there were 1,033 cases available for telomere length assessment from two sources: BGHR ($n =$ 243; mean age $\pm$ SD $=$ 46.74 $\pm$ 8.73; 54% male); and Colon CFR ($n =$ 790; mean age $\pm$ SD $=$ 51.13 $\pm$ 8.05; 50% male).

Controls. Controls were collected from the (i) BGHR; (ii) Colon CFR; (iii) Mayo Clinic Biobank; and (iv) Mayo Clinic SPORE in Pancreatic Cancer (MCCSPC). Controls from the BGHR are consented subjects with normal colonoscopies and no prior polyp or cancer history and those from the Colon CFR are nonblood relative and/or spousal controls. Initiated in 2009, the Mayo Clinic Biobank controls are subjects from Olmsted County, MN, found to be healthy during a medical examination in the Department of Medicine divisions of Community Internal Medicine, Family Medicine and General Internal Medicine. A total of 962 healthy Caucasian controls recruited through the MCCSPC were from a Mayo Clinic–based control sample of patients with primary care having routine check-up visits (general medical exam) between May 1, 2004 and August 31, 2006.

All control subjects were consented for recruitment into one of these registries; registry participation included collection of peripheral blood samples, and/or tissue samples, and/or completion of a self-administered clinical and family history questionnaire and food-frequency questionnaire. Controls had no previous diagnosis of cancer (except possibly nonmelanoma skin cancer) at the time of enrollment. Control subjects with a family history of Lynch Syndrome, FAP, Peutz-Jeghers syndrome, or Juvenile polyposis were excluded.

Overall, 2,952 controls were available for telomere length assessment from the four sources: BGHR ($n =$ 56; mean age $\pm$ SD $=$ 57.14 $\pm$ 2.73; 34% male); Colon CFR ($n =$ 1,400; mean age $\pm$ SD $=$ 50.85 $\pm$ 8.18; 47% male); Mayo Biobank ($n =$ 534; mean age $\pm$ SD $=$ 49.22 $\pm$ 8.84; 58% male); and MCCSPC ($n =$ 962; mean age $\pm$ SD $=$ 66.37 $\pm$ 9.78; 55% male). Controls were frequency matched to the cases' age ($\pm$5 years), gender, and race/ethnicity.

DNA extraction from PBLs for cases and controls

DNA extraction was conducted on all BGHR cases and controls, Mayo Biobank controls and MCCSPC controls using PureGene chemistries (a salting-out extraction) via the Gentra Autopure platform and quantified by UV absorbance; DNA quality was assessed by 260/280 optical density ratio. DNA extraction methods for the Colon CFR samples varied according to the origin site. Colon CFR sample DNA was extracted using one of three different methods: PureGene, phenol/chloroform, or QIAamp (Qiagen) column extraction (Table 1).

Assessment of telomere length by qPCR

DNA was quantitated with PicoGreen to ensure that the same amount of DNA was used for each PCR reaction. This PCR-based assay uses a set of primers to the telomeric hexamer repeats, thus amplifying the telomere (30). An average RTL for a sample is measured by determining the sample's telomere to single-copy gene ratio (T/S ratio) in comparison with the T/S ratio of a reference DNA sample.

Validation dataset

To determine whether the variation in RTL might be attributable to the specific DNA extraction method used,

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Table 1. Test set: extraction methods by study center

Study	Center no. (Site location)	Extraction method (column %)		
		Phenol/chloroform	Qiagen/QIAamp	PureGene
Colon CFR	11 (Ontario)	233 (43%)	252 (21%)	
	13 (Australia)	303 (57%)		
	14 (Hawaii)		101 (9%)	
	15 (Minnesota)			479 (21%)
	16 (Seattle)		822 (70%)	
Pancreas spore	21			962 (43%
BGHR	19			299 (13%)
Mayo Biobank	20			534 (24%)
Total		536	1,175	2,274

we extracted DNA on three samples from each of 25 new cases and 25 controls using each of the three extraction methods (PureGene, phenol/chloroform, and QIAamp from Qiagen). For six of the samples, RTL for one measurement was more than two SDs from the mean; these outliers were dropped, resulting in a final sample of 20 cases and 24 controls.

Systematic literature review

To evaluate whether the method of DNA extraction may be associated with a detected relationship between telomere length and cancer risk, we reviewed all case–control studies published through November 2012 that were designed to assess an association between cancer risk and peripheral blood or buccal DNA RTL measured by qPCR. We searched Medline via PubMed (U.S. Library of Medicine, Bethesda, MD) using the terms "cancer and telomeres" and "cancer and telomere length", and we cross-referenced these articles with those reported in two published meta-analyses of telomere length and cancer risk (25, 31). Reported ORs (below the median telomere length vs. above), 95% confidence intervals (CI), and DNA extraction methods were then sought from review of the primary article or calculated from the article as needed (ref. 2; Supplementary Table S1). In many studies, DNA had been used from long-standing registries and previously reported cohorts. Thus, the DNA extraction methods were identified in previously referenced articles; in situations in which the extraction method was still not clear, we contacted the originating site for the DNA used in those articles to determine the DNA extraction method.

Statistical analysis

T/S ratio was used for the comparison of telomere length among the three DNA extraction methods. As the comparison of arithmetic means of the T/S ratio may be misleading because of violation of the normality assumption, the T/S ratio was log-transformed to compare geometric means of the T/S ratio, which enables meaningful statistical evaluations. RTL was also summarized for a descriptive purpose with mean, SD, and range for each DNA extraction method in cases and controls. For the case–control data (test set), an analysis of covariance (ANCOVA) was used for the comparison of the log (T/S ratio) among independent samples after controlling for age at blood draw. For the validation set, a mixed-effect model was used for the comparison among correlated samples.

Results and Discussion

RTL by DNA extraction methods in our case–control study was summarized in Table 2 (test set) and the interassay CV was 6%. A shorter RTL was observed in QIAamp, compared with phenol/chloroform and PureGene (Fig. 1A). This remained similar in the comparison of the T/S ratio in a fixed effects model: after controlling for age differences, DNA extracted using QIAmp still had significantly shorter telomeres than DNA extracted using phenol/chloroform or PureGene ($P < 0.001$).

However, there was uncertainty as to whether the difference was mainly due to different patient populations rather than DNA extraction methods. To reduce confounding effects from different patient characteristics and verify our findings from the test set, we selected an additional set of 20 colorectal cancer cases and 24 noncancer controls, and compared their RTL as measured using the three DNA extraction methods (phenol/chloroform, PureGene, and QIAamp; Fig. 1B). These samples were extracted in the same laboratory by all three methods. Consistent with the original test set of cases and controls, the range of RTL measured by qPCR from PBL DNA extracted by the QIAamp column method was less than that detected by either the PureGene or phenol/chloroform extraction methods (T/S ratio ranges, 0.17–0.58, 0.04–2.67, and 0.32–2.81, respectively). Table 3 shows the results of the mixed effects model to compare the T/S ratio among the three extraction methods for the same patients. Telomere length from DNA extracted by the phenol/chloroform and

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Table 2. Test set: differences in peripheral blood RTL by DNA extraction method

			T/S ratio			Log-transformed T/S ratio	
Sample	**Extraction method**	***n***	**Mean (SD)**	**Min**	**Max**	**Estimated difference (95% CI)**	***P***
All	Phenol/chloroform	536	0.78 (0.73)	0.01	6.54	0.52 (0.44–0.60)	<0.001
	PureGene	2,274	0.75 (0.79)	0.00	12.33	0.58 (0.52–0.63)	<0.001
	QIAamp	1,175	0.38 (0.31)	0.02	3.69	Reference	
Cases	Phenol/chloroform	190	1.09 (0.88)	0.04	6.26	0.86 (0.73–0.98)	<0.001
	PureGene	408	0.76 (0.53)	0.02	3.19	0.52 (0.42–0.62)	<0.001
	QIAamp	435	0.41 (0.37)	0.05	3.33	Reference	
Controls	Phenol/chloroform	346	0.61 (0.57)	0.01	6.54	0.33 (0.23–0.43)	<0.001
	PureGene	1,866	0.75 (0.84)	0.00	12.33	0.62 (0.55–0.69)	<0.001
	QIAamp	740	0.37 (0.27)	0.02	3.69	Reference	

Abbreviation: *n*, number of samples tested.

PureGene methods was significantly more than telomere length from DNA extracted by QIAamp ($P < 0.0001$ and $P = 0.0016$, respectively). Statistical significance was not found in the difference in telomere length between PureGene and phenol/chloroform extraction methods (data not shown). The residual variances indicate that RTL from QIAamp was less variable than either PureGene or phenol/chloroform (likelihood ratio test, $P < 0.0001$).

Telomeres protect eukaryotic chromosomes from degradation and DNA repair activity. In most normal cells, there is no continually compensating elongation mechanism and telomeres become shorter with each cell division. Telomere length has been widely studied in relation to disease risk and progression, particularly in cancer (25, 32–38). qPCR is the most common method used to estimate telomere length in studies with high sample numbers, as it is rapid, sensitive, and requires relatively small amounts of DNA. Although there is considerable variation in reproducibility with CV ranging between 0.37% and 28% (6% in our study), there are a growing number of reports using this approach (2, 3, 5, 6, 12–17, 19, 21, 23, 24, 34, 39–48).

A recent article by Olsen and colleagues (27) indicated that quality control is critical in qPCR telomere length studies. In this report, we note that the methods used to extract DNA influence measurements of RTL. While DNA isolated by salting-out (PureGene) or organic extraction (phenol/chloroform) produced similar RTL results, DNA extracted using the silica-gel membrane QIAamp column technology rendered shorter estimates for RTL as well as narrowed the range of RTL variance in the 44 subjects who had DNA extracted by all three methods. The method of DNA isolation may explain these differences because column methods have been associated with shearing of DNA leading to lower molecular weight DNA. Column-based techniques involve more mixing and vigorous vortexing with a greater potential for shearing DNA as it passes through the column, allowing longer strands that cannot stick to the column media to be spun off and not retrieved.

It is possible that some factor other than the average size of the DNA fragments is responsible for the differences in RTL we observed by extraction method. Measurement by qPCR is susceptible to the influence of residual inhibitors if organic solvents such as phenol or inorganic residual salts are not completely removed from the DNA before qPCR amplification. However, even when telomere length was assessed by Southern blot analysis conducted on unamplified DNA extracted from the same subjects by PureGene and by QIAamp (supplementary Tables S2 and S3), telomere lengths of the column based–extracted DNA were significantly less than those extracted by the PureGene method, indicating that the telomere length may be related to the DNA quality and not related to a qPCR inhibitor that is the sequela of the salting-out DNA extraction method.

A possible consequence of the apparent alteration in measured RTL using QIAamp extraction may increase false-negative (type II error) rates. Indeed, a systematic review of the literature indicates that DNA extraction method may, in part, contribute to the discrepancies between studies evaluating associations between telomere length and the risk of cancer or other diseases. Figure 2 presents a Forest Plot of the ORs and CIs from the studies identified in the systematic review grouped by DNA extraction method. The majority of studies used column methods (column, $n = 20$; phenol/chloroform, $n = 8$; salting-out, $n = 7$). Overall, studies that used QIAamp-extracted DNA were the least likely to find an association between PBL telomere length and cancer risk (column, 20%; phenol/chloroform, 50%; salting-out, 71%). We note that Q-FISH does not require a DNA extraction step, and most studies that used Q-FISH–reported associations between telomere length and cancer risk. This suggests that studies using QIAamp for DNA extraction may fail to find an association when one may truly exist, whereas studies using phenol/chloroform or salting-out methods, or those using methods that do not require DNA extraction (i.e., Q-FISH) may be better able to detect such associations.

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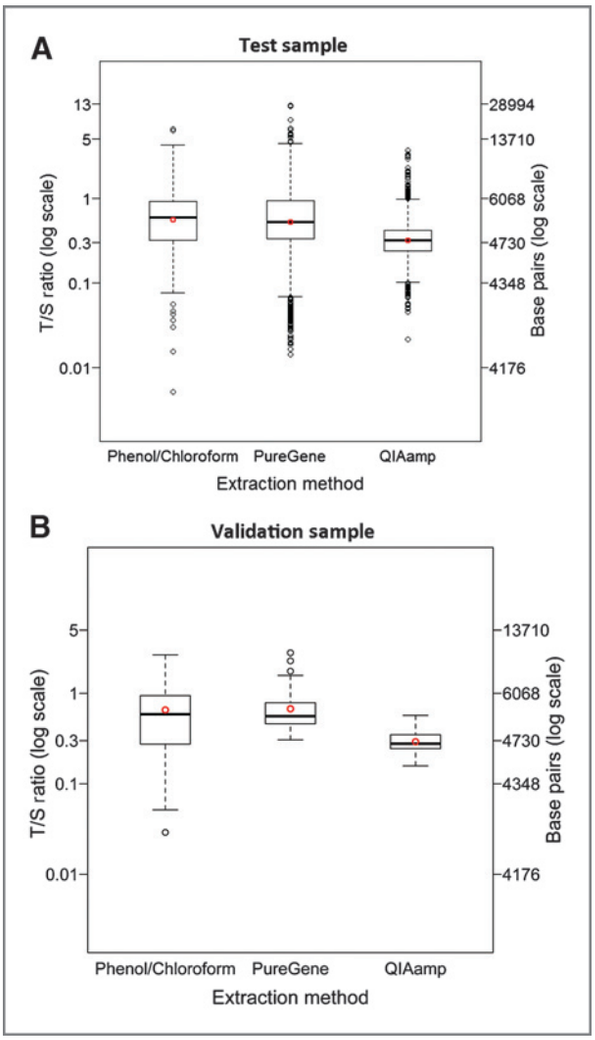

Figure 1. Distribution of telomere length by extraction method in the test and validation samples. A, in a test sample, telomere length was measured in 1,033 cases and 2,952 controls whose DNA had been extracted using either phenol/chloroform, PureGene, or QIAamp. B, in a validation sample, DNA was extracted from frozen buffy coats of 20 cases and 24 controls using all three extraction methods (phenol/chloroform, PureGene, and QIAamp). RTL was measured for each of the DNAs extracted by these different methods. The *y*-axis shows the telomere length in terms of the log-scale of the T/S.

Although other factors including the sample size, specific white blood cell subgroup source for DNA, DNA quality, storage and qPCR quality control may also affect telomere length, our results indicate that DNA extraction methods may be crucial to the proper assessment of telomere length. All three extractions methods use a proteinase K digestion but phenol/chloroform and salting-out techniques, which are both liquid-to-liquid phase methods differ significantly from a column extraction. Both liquid-to-liquid phase extraction methods typically have less protein contamination and typically result in higher molecular weight DNA. Deproteinization to enhance DNA purity occurs in the phenol/chloroform process because proteins have both hydrophobic and hydrophilic cores that draw them toward the organic phenol phase for precipitation, whereas the negatively charged nucleotides of DNA are preferentially attracted to the aqueous phase. Similarly, in salting-out methods, deproteinization results by dehydrating and precipitating out protein, leaving a supernatant of DNA that is then precipitated in ethanol. In the column-based methods, deproteinization occurs because only nucleic acids adsorb to a solid-phase component such as silica or resin beads in the face of adjustments of the salt and pH of the buffer. These changes in salt concentration and pH increase the negative charge of the solid-phase causing the DNA to stick and the other molecules including proteins to pass through the column. Column-based methods tend to shear DNA more than liquid-to-liquid phase extractions, likely because of vortexing differences. Column methods also result in lower molecular weight DNA during multiple washes to clean the column and these fragments are discarded in the filtrate that results from each wash. Only DNA that has remained on the column is finally removed during elution. Presumably, a portion of these low-molecular weight DNA fragments would include portions of the telomeres that cap every chromosome and the measurement of average telomere lengths would thus, be lower as portions of telomere base pairs are lost, along with other low-molecular weight DNA.

Table 3. Validation set: fixed effects and variance estimates of telomere length (log T/S ratio) by extraction method

Extraction method	Fixed effects (SE)	*P*	Residual variance
Intercept	−1.23 (0.04)		—
Phenol/chloroform	0.80 (0.08)	<0.0001	0.261
PureGene	0.49 (0.15)	0.0016	0.982
QIAamp	Reference		0.077

In our case–control study, including multiple covariates such as age, gender, and family history, we detected an association between colorectal cancer risk and RTL. This association was more evident in samples extracted by PureGene or phenol/chloroform than in samples extracted by QIAamp, where the range of RTL might be limited because the starting DNA itself has been truncated during extraction. An inability to detect the full range of RTL would presumably diminish the ability to reliably assess longer telomeres, leading to misclassification and less sensitivity for detecting associations between telomere length and cancer risk. We found that DNA extracted with either phenol/chloroform or PureGene methods yielded more accurate measurement of telomere length by qPCR than DNA extracted with QIAamp.

Accurate assessment of telomere length is critical to the recognition of the impact of telomere length in disease and

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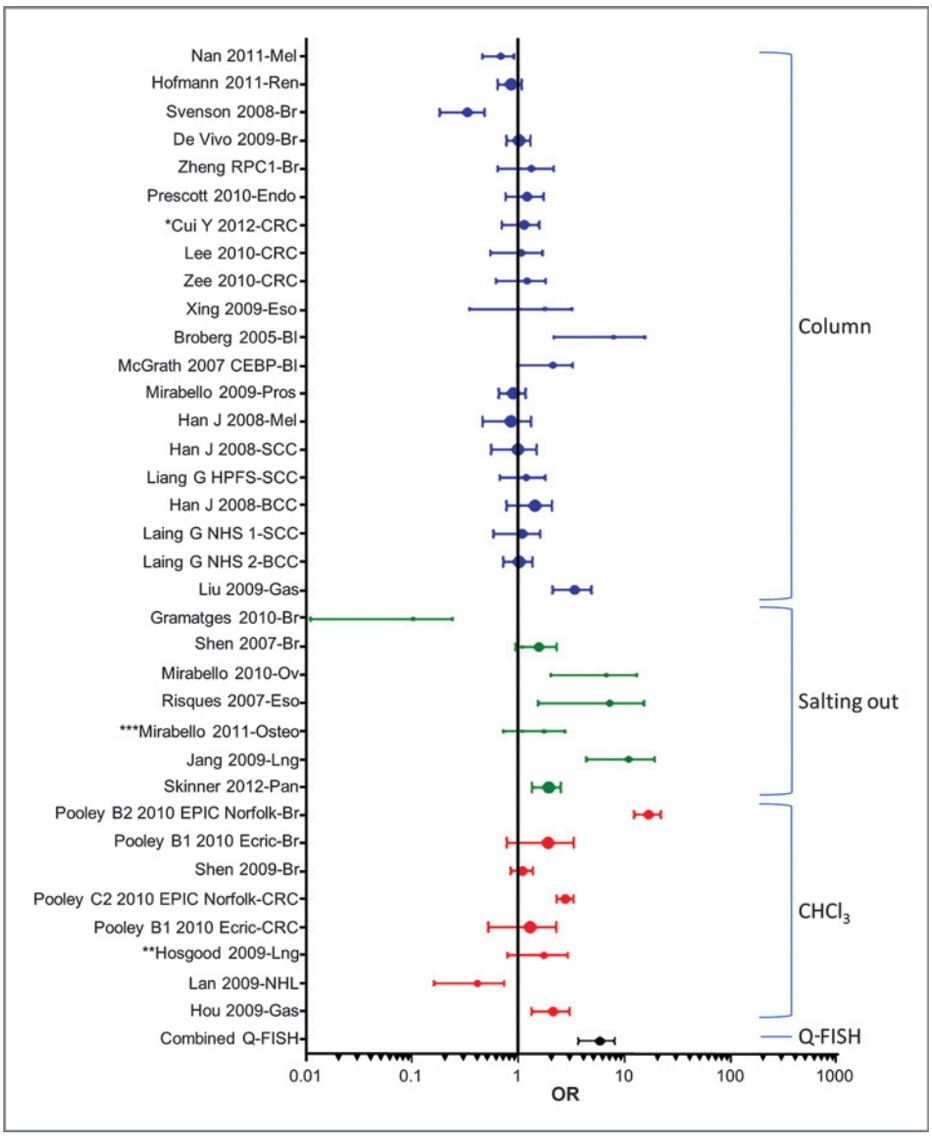

Figure 2. Association between RTL and cancer risk: forest plot clustered by DNA extraction methods. Colored bars indicate the ORs for studies using qPCR to measure RTL. The blue bars represent RTL results from DNA extracted using column methods, green bars from DNA extracted via salting-out methods, and red bars represent RTL from DNA extracted by phenol/chloroform. The black bar represents OR for studies using Q-FISH to measure telomere length directly and does not require DNA extraction. Br, breast cancer; CRC, colorectal cancer; Bl, urinary bladder cancer; Mel, melanoma; SCC, squamous cell skin carcinoma; BCC, basal cell carcinoma; Lng, lung cancer; Gas, gastric cancer; Eso, esophageal cancer; NHL, non-Hodgkin's lymphoma; Ren, renal cancer; Pan, pancreatic cancer; Osteo, osteosarcoma; Pros, prostate cancer; Ov, ovarian cancer; Endo, endometrial cancer. *, Compared quintiles; **, compared tertiles; and ***, compared dichotomized telomere length.

health. Here, we report that the method of DNA extraction may influence the ability to properly measure telomere length and should be considered in epidemiologic studies of telomere length.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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