NEOPLASIA
www.neoplasia.com

Volume 20 Number xx Month 2018 pp. 425–431 425

Development of a New Monochrome Multiplex qPCR Method for Relative Telomere Length Measurement in Cancer

Paige N. Dahlgren, Kanokwan Bishop, Shatovisha Dey, Brittney-Shea Herbert and Hiromi Tanaka

Department of Medical and Molecular Genetics, Indiana University School of Medicine, Indianapolis, IN

Abstract

Excess telomere shortening has been observed in most cancer cells. The telomere quantitative polymerase chain reaction (qPCR) assay has become an important tool for epidemiological studies examining the effects of aging, stress, and other factors on the length of telomeres. Current telomere qPCR methods analyze the relative length of telomeres by amplifying telomere sequence products and normalizing with single-copy gene products. However, the current telomere qPCR does not always reflect absolute telomere length in cancer DNA. Because of genomic instability in cancer cells, we hypothesized that the use of single-copy genes (scg) is less accurate for normalizing data in cancer DNA and that new primer sets are required to better represent relative telomere length in cancer DNA. We first confirmed that cancer cells had a different copy ratio among different scg, implying that DNA is aneuploid. By using the new primer sets that amplify multiple-copy sequences (mcs) throughout the genome, the telomere qPCR results showed that the mcs primers were interchangeable with the scg primers as reference primers in normal DNA. By comparing results from the traditional southern blotting method (as kilobases) and results from monochrome multiplex qPCR using the mcs primers (as T/M ratios), we verified that the T/M ratio is highly correlated with absolute telomere length from the southern blot analysis. Together, the mcs primers were able to represent the telomere lengths accurately in cancer DNA samples. These results would allow for analyses of telomeres within cancerous DNA and the development of new, less invasive diagnostic tools for cancer.

Neoplasia (2018) 20, 425–431

Introduction

The telomere quantitative polymerase chain reaction (qPCR) assay was originally developed by R.M. Cawthon for measuring relative average telomere length [1,2]. This telomere qPCR assay has been widely distributed because of its time efficiency and low DNA requirement (theoretically <100 ng); therefore, the technique is advantageous for studies using limited resources such as large-scale epidemiological studies in a variety of fields including aging, metabolic, and psychosocial research [3–8]. In this qPCR assay, DNA quantities (in nanograms) both from telomere and reference amplifications are determined by each standard curve created using a serial dilution of standard DNA (e.g., normal genomic DNA with known telomere length). The PCR primers for the reference are typically designed within single-copy gene (scg) sequences such as albumin (*ALB*) or gamma globin (*HBG*) genes [1,2]. The relative telomere length is expressed as the T/S ratio that is calculated by dividing the telomere quantity (T) by the reference quantity of a single-copy gene (S) to normalize the data. Each quantity is calculated by standard curves created using standard DNA with known concentrations. The telomere length of the standard DNA always indicates the T/S ratio is equal to 1.0.

Abbreviations: scg, single-copy genes; mcs, multiple-copy sequences; T, telomere quantity; S, scg quantity; M, mcs quantity; DNA, deoxyribonucleic acid; PCR, polymerase chain reaction; MMQPCR, monochrome multiplex quantitative PCR; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; TE, Tris-EDTA; DIG, digoxigenin; CV, coefficient of variation; UCSC, University of California, Santa Cruz; T_m, melting temperature; Eff, PCR efficiency; Ct, cycle threshold; ALT, alternative lengthening of telomeres; IDC, invasive ductal carcinoma; DCIS, ductal carcinoma *in situ*

Address all correspondence to: Hiromi Tanaka, 975 West Walnut Street IB130, Indianapolis, IN 46202.
E-mail: hirtanak@iu.edu

Received 29 November 2017; Revised 14 February 2018; Accepted 19 February 2018

1476-5586/18
https://doi.org/10.1016/j.neo.2018.02.007

The latest version of telomere qPCR being performed is monochrome multiplex quantitative PCR (MMQPCR) which uses a single fluorescent DNA-intercalating dye (i.e., SYBR green I) and adds both target and reference primer sets in a single reaction tube.[1] The MMQPCR method is feasible only when the number of target sequences is different from the number of reference sequences in a PCR template; for example, telomere repeat sequences are more abundant than single-copy genes such as *ALB* and *HBG* in genome. This difference in target sequences allows for the distinguishing between the two signals created by the separate primer sets. The multiplexing method further increases throughput and improves the accuracy by minimizing sample handling error while also saving the amount of starting materials and the cost of reagents, which are sometimes of critical value.

Regardless of whether the telomere qPCR is singleplexing or multiplexing, it is worth remembering that the current telomere qPCR method is optimized for diploid DNA samples. To normalize the data, the single-copy genes (scg) are used because diploid cells, in theory, always contain two copies (or alleles) of the single-copy genes, one per chromosome. The current telomere qPCR method is beneficial for most epidemiological studies which use peripheral blood DNA samples as a PCR template. However, when nondiploid DNA is the study subject, such as is the case in cancerous tissues, the current telomere qPCR may mislead the interpretations by reducing the data accuracy and integrity [9,10]. In other words, since tumor tissues or cells often form aneuploidy which represents an abnormal number of chromosomes in a cell, the scg may not reflect the amount of input DNA if the aneuploidy exists within the scg sequences.

To eliminate the abovementioned concern, we introduce an alternative telomere MMQPCR method which is applicable for cancer DNA. Using cancerous DNA, the results from our newly developed MMQPCR method is well correlated with those from traditional southern blot analysis, suggesting that the refined MMQPCR method is useful for human genomic DNA samples with abnormal ploidy levels.

Materials and Methods

Tissue and Blood Specimens

Tumor tissue specimens were obtained at the time of surgery, immediately frozen in liquid nitrogen, and stored in liquid nitrogen until use. Frozen tissues (100-150 mg) from colon cancer (n = 8), renal cell carcinoma (n = 7), and breast cancer (n = 2) were obtained from the tissue bank at the Indiana University Simon Cancer Center. All cases were reviewed by pathologists to assess tumor histology (Supplemental Table S1). The purity of each specimen was shown as at least 50% tumor. Whole blood DNA samples (n = 18, 23 ≤ age ≤ 70, mean age = 48.8) from healthy women were obtained from the Susan Komen Tissue Bank. Additional frozen breast tissues of ductal carcinoma *in situ* (DCIS) and invasive ductal carcinoma (IDC) were also obtained from the tissue bank at the Indiana University Simon Cancer Center (DCIS, 30-80 years, mean, 55 ± 12 years; IDC, 31-86 years, mean, 47.6 ± 13.3 years) [11]. The study was approved by the institutional review board of the Indiana University.

Cell Lines

A total of 21 human cell lines were used to isolate genomic DNA as follows: cervical carcinoma (HeLa), breast carcinoma (MCF7, T47D), renal cell carcinoma (RCC23) [12], lung adenocarcinoma (H1299), colorectal carcinoma (HT29, SW620, HCT116, and DLD-1), pancreatic carcinoma (PANC1, MIA PaCa-2, and AsPc-1), ovarian carcinoma (A2780, SKOV3), prostate carcinoma (PC-3), giant cell tumor (GCT), fibrosarcoma, (HT1080), preneoplastic mammary epithelial cells (MCF10A), and three ALT-positive cells (U-2 OS, Saos-2, and VA13). All cell lines were cultured in ambient oxygen, 5% CO_2 and maintained in appropriate medium according to ATCC (www.atcc.org).

DNA Extraction

Genomic DNA from both cell lines and tumors was extracted by a salt precipitation method as previously described [13]. Briefly, about 20 mg of each frozen tissue fragment was minced quickly in cold PBS (–) (which is PBS without divalent cations) or cells from 10-cm dish were trypsinized and washed with PBS(–). The PBS(–) was removed by centrifuging at 3500×*g*, and the pellets were resolved in lysis buffer (20 mM Tri-HCl/pH 8.2, 10 mM EDTA, 400 mM NaCl, 0.5% SDS, 0.05 μg/μl proteinase K) and incubated overnight at 56°C. The DNA solution was centrifuged for 15 minutes at 9600×*g* after adding one-fourth volume of saturated NaCl. The supernatant was transferred to a new tube, and the DNA was precipitated by adding the equal volume of isopropanol. After rinsing with 70% ethanol, the DNA was resolved in TE buffer.

DNA Quantitation

The DNA concentration was quantitated by Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific) as well as picogreen assay. Quant-iT TM PicoGreen dsDNA Assay Kit (Life Technologies) was used according to the manufacturer's instruction. Fluorescence intensity was measured with a Synergy 2 Multi-Mode Reader at emission wavelength of 520 nm and excitation wavelength of 480 nm.

Telomere Length Measurement: Southern Blot Analysis

Absolute mean telomere length was measured by TeloTAGGG Telomere Length Assay (Roche). Briefly, 4 μg of each DNA sample were digested overnight with two tetra-cutter restriction enzymes, *Rsa*I and *Hin*fI. Digested DNA samples were resolved on a 0.8% agarose gel, blotted to a hybond-N+ membrane (GE Healthcare) using capillary transfer and 20× SSC buffer. A digoxigenin-labeled probe was used for the hybridization. The results were scanned from Amersham Imager 600, and mean telomere lengths were calculated by ImageQuant and the Excel spreadsheet program TELORUN as described [14,15].

Telomere Length Measurement: qPCR

Telomere length qPCR was performed with a QuantStudio 6 Flex Real-Time PCR System and analyzed under either singleplex or multiplex conditions. The telomere primers were used for the telomere signal (telg and telc, final concentrations 900 nM) [1]. A set of single-copy gene (scg) signal primers within albumin or β-globin genes was used as reference (final concentrations 200 nM each) [1,2]. In the case of the new multiple-copy sequence (mcs) primer sets (final concentrations 800 nM each) [16], the telomere primers contained a GC clamp and were used for the telomere signal (GC-Telg and GC-Telc, final concentrations 150 nM). The PCR cycle for MMQPCR is as follows: Stage 1: 2 minutes at 95°C; Stage 2: 2 cycles of 15 seconds at 94°C, 15 seconds at 49°C; and Stage 3: 35 cycles of 15 seconds at 94°C, 10 seconds at 60°C, 20 seconds at 74°C with signal acquisition (for mcs amplification), 10 seconds at 84°C, 20 seconds at 88°C with signal acquisition (for telomere