Short Technical Report

Chemiluminescent Measurement of Telomere DNA Content in Biopsies

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ABSTRACT

Telomeres are nucleoprotein complexes that protect the ends of chromosomes from fusion and degradation. They are typically shorter in tumor cells than in paired normal cells, and shorter telomeres are associated with poor outcome in cancer. We previously described a slot blot-based method for measuring telomere DNA content, a proxy for telomere length. Although this method represented an improvement over existing methods, its 30-ng limit of sensitivity was insufficient for use with biopsy or other scant tissues. Here we describe a chemiluminescent slot blot assay for telomere DNA content that has the sensitivity required for use with biopsy materials. The results obtained with DNA derived from human placental, HeLa, human peripheral blood lymphocytes, sham-needle core prostate biopsies, and archival prostatectomy tissues demonstrated that telomere DNA content can be reliably and reproducibly measured in 5 ng, and sometimes as little as 2 ng, genomic DNA. Sham-needle core prostate biopsy and prostatectomy specimens processed in parallel produced comparable results. The contribution of truncated telomeres in admixtures containing as much as 75% normal placental DNA could be established. We also demonstrated that the treatment of tissue with formalin before DNA purification does not decrease the efficacy of the assay.

INTRODUCTION

Telomeres are nucleoprotein complexes that protect the ends of chromosomes from fusion and degradation (1, 3, 6, 11, 12). Because telomeres are shortened each time a cell divides (1, 3, 11, 15), tumor cells typically have shorter telomeres than paired normal cells (1, 3, 6, 16). Reduced telomere DNA content (or length) has been associated with aneuploidy, metastasis, grade, disease recurrence, and survival (2, 4, 5, 7–10, 13, 14, 17). To validate the potential prognostic significance of telomere DNA content, it is necessary to measure telomere DNA content in relevant clinical specimens, particularly in biopsies.

We previously described a slot blot method for measuring telomere DNA content, a proxy for telomere length (5). In contrast to the commonly used Southern blotting method of measuring terminal telomere restriction fragments (1, 10, 13, 16), the slot blot assay required less than 0.1 µg genomic DNA and was insensitive to DNA breakage (5). Nevertheless, it lacked the sensitivity required for use with biopsy specimens and microdissected or other very scant tissues, took 5–8 days to complete, and relied on hybridization of a second centromere-specific oligonucleotide to normalize for DNA quantity/load and reactivity. In addition to these limitations, the effects of formalin fixation and normal cell contamination in tumors were unknown. These factors limit the potential clinical utility of the slot blot assay for use with biopsy specimens.

To address these limitations, we developed a new chemiluminescent assay for measuring telomere DNA content

that is both more sensitive and faster than previous methods. We also tested the assay's suitability for use with DNA purified from formalin-fixed tissue and admixtures of normal and tumor cells.

MATERIALS AND METHODS

DNA Isolation

DNA was purified from human placental tissue and cultured HeLa cells as previously described (5). Waste frozen human prostate tissue was obtained from the University of New Mexico Solid Tumor Facility. Sham-needle core biopsies were produced from the frozen prostate tissue using a 2-cm-long, 18-gauge needle core. Pieces (0.2–0.4 g) of human placental tissue were submerged in formalin (Sigma, St. Louis, MO, USA) for 0, 15, 30, 60, 120, 240, and 480 min at room temperature. The tissue was washed twice in 30 mL PBS (Sigma) to remove any residual formalin before DNA purification. DNA was purified from sham-needle core biopsies and formalin-treated placenta using the DNeasy® Kit and the manufacturer's protocol (Qiagen, Valencia, CA, USA). The average yield of DNA from a 2-cm-long, 18-gauge frozen needle core was 150 ng ($n = 9$; ± 15.9 SD). The DNA yield was not affected by formalin fixation. Archival paraffin-embedded prostatectomy tissue was obtained from the New Mexico Tumor Registry as approved by the Human Research Review Committee of the University of New Mexico. DNA was purified from archival paraffin-embedded tissue as described above.

Quantification of DNA

Placenta, HeLa, sham-needle core biopsy, and paraffin-embedded prostatectomy DNAs were quantitated with the fluorescent dye, PicoGreen® (Molecular Probes, Eugene, OR, USA), using a commercially available human genomic DNA purified from peripheral blood lymphocytes (Promega, Madison, WI, USA) as a standardized control. One milliliter of a 1:200 dilution of PicoGreen stock was added to both the standard and purified DNAs and excited at 480 nm, according to the manufacturer's instructions. Fluorescence emission intensities were measured at 520 nm using a Luminescence Spectrometer LS50 (Perkin Elmer Life Sciences, Gaithersburg, MD, USA). Concentrations of the DNA samples were calculated from the equation describing the best-fit line generated with the standardized control DNA.

Preparation and Hybridization of Slot Blots

Slot blots were prepared as previously described (5), with the following modifications. DNA was applied to Tropilon-Plus™ membranes (Tropix, Bedford, MA, USA), air-dried, and cross-linked with 1200 mJ (UVP, San Gabriel, CA, USA). Following cross-linking, each blot was submerged in 0.25 M sodium phosphate buffer, pH 7.2. Each blot was prehybridized in a 200-mL glass hybridization bottle (Bellco Glass, Vineland, NJ, USA) for 1 h at 60°C in 50 mL hybridization buffer (7% SDS, 0.25 M sodium phosphate buffer, pH 7.2, 0.001 M EDTA). Following prehybridization, the buffer was replaced with 50 mL fresh hybridization solution containing 300 pmol telomere-specific fluorescein 3'-end-labeled probe [5'-TTAGGG-3']₄ (IDT, Coralville, IA, USA). Each blot was hybridized at 60°C for a minimum of 12 h and a maximum of 16 h. Following hybridization, the blot was washed twice at room temperature for 5 min in 30 mL 2× SSC and 1% SDS. The initial wash was followed by two high-temperature washes at 60°C for 15 min in 30 mL preheated 1× SSC and 1% SDS. Finally, the blot was washed twice at room temperature for 5 min in

30 mL 1× SSC. All washes were performed in glass hybridization bottles with constant agitation in an AutoBlot Hybridization Oven® (Bellco Glass).

Detection and Quantification of Telomere DNA

After hybridization, telomere DNA was quantitated using the Southern Star™ Chemiluminescent Kit, as described by the supplier (Tropix). The blots were equilibrated for 5 min in two successive 20-mL washes of blocking buffer [PBS, 2% I-Block™ Reagent (Tropix), and 0.1% Tween® 20 (Sigma)]. Each blot was then incubated separately for 45 min in 30 mL blocking buffer at room temperature with constant agitation. The blocking buffer was discarded and replaced with 50 mL blocking buffer containing 2 µL alkaline phosphatase-conjugated, anti-fluorescein antibody (Tropix). The blot was incubated for 30 min at room temperature with constant agitation, washed in 30 mL fresh blocking buffer, and then washed three times for 5 min at room temperature in 30 mL wash buffer (PBS and 0.1% Tween 20) with constant agitation.

The pH of the blot was optimized for alkaline phosphatase activity by incubating the blot twice for two min in 20 mL 1× assay buffer (Tropix). The surface of the blot was completely covered with approximately 4 mL CDP-Star® Chemiluminescent Substrate (Tropix) and incubated for 5 min at room temperature (Figure 1A). Following incubation, CDP-Star was wicked away, and the blot was sealed in a plastic bag and exposed to Hyperfilm™ (Amersham Biosciences, Little Chalfont, UK) for 2 or 5 min.

The films were developed using a medical film processor (model QX-70; Konica Medical Imaging, Wayne, NJ, USA) and scanned using a ScanJet™ ADF (Hewlett Packard). The intensity of the telomere hybridization signal was determined from the digitized images with the Nucleotech Gel Expert Software® 4.0 (Nucleotech, San Mateo, CA, USA). Telomere content was determined by comparing the slope of the equation generated from DNA input versus the relative signal from the placental standard to the samples at known concentration (Figure 1B).

RESULTS AND DISCUSSION

Sensitivity and Specificity

There was a linear relationship ($R^2 = 0.98-0.99$) between the telomere DNA content and inputs of 5–120 ng in human genomic DNAs purified from placenta, HeLa cells, and peripheral blood lymphocytes. Telomere contents in the placenta and the peripheral blood lymphocyte DNAs, representative of normal somatic cells, were nearly identical (slopes = 923 vs. 942, respectively). In contrast, the telomere content in HeLa DNA, representative of tumor cells, was significantly decreased (slope = 353) (Figure 1B). Telomere DNA contents in genomic DNA isolated from sham-needle core biopsies ranged 48%–100% of the placental control (Figure 1C). There was no signal when equivalent amounts of a nonspecific plasmid DNA (pGEM®-easy vector; Promega) were substituted for genomic DNA in the assay (data not shown).

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Telomere Contents in Admixtures

Tumor and biopsy specimens typically contain mixtures of normal cells and tumor cells, whose telomeres differ in length. Thus, the observed telomere content of such a specimen reflects both the telomere content and relative proportions of normal and tumor cells. The observed telomere content (TC_{obs}) can be expressed quantitatively by the following equation:

$$TC_{obs} = (TC_N)(\%N) + (TC_T)(\%T) \quad [\text{Eq. 1}]$$

where TC_N and TC_T are the telomere contents in normal and tumor cells, respectively, and $\%N$ and $\%T$ are the percentages of normal and tumor cells in the specimen, respectively. Ideally, the telomere content of the tumor cells in the mixed samples could be calculated with the following equation:

$$(TC_T) = [TC_{obs} - (TC_N)(\%N)]/(\%T) \quad [\text{Eq. 2}]$$

where TC_{obs} , TC_N , and $\%T$ (defined by histopathological examination) are known. To validate this approach, telomere contents were measured in mixtures containing defined proportions of HeLa cell DNA, mimicking tumor cells, and placental DNA, mimicking normal cells (Figure 2). There was a linear and predictable relationship between the telomere content and the rel-

ative proportions of HeLa and placenta DNA in each of the admixtures. Identical results were obtained when telomere contents were assayed in defined admixtures containing 30, 40, 50, or 60

ng total genomic DNA (data not shown). Thus, the contributions of the truncated HeLa cell telomeres in admixtures containing as much as 75% normal placental DNA can be defined.

Figure 1. Sensitivity of telomere DNA content assay. (A) Representative slot blot showing placental, HeLa, and five archival paraffin-embedded prostate tumor samples. Placenta and HeLa DNA concentrations range 0–50 ng, and tumor DNAs were analyzed in triplicate (one replicate for sample 5 is not shown) at 40 ng. (B) Representative data demonstrating differential telomere DNA contents in normal somatic (human blood lymphocytes and placenta) and tumor (HeLa) cells. The slot blot was prepared and analyzed as described in the Materials and Methods section. (C) Representative data demonstrating telomere DNA contents in sham-needle core biopsies. Slopes, x-intercepts, and correlation coefficients were calculated by linear regression.

Figure 2. Telomere contents in DNA admixtures. HeLa and placenta DNAs were mixed in the following proportions: 0%, 25%, 50%, 75%, and 100% HeLa. Data shown represent telomere DNA contents measured in 60 ng total genomic DNA analyzed in triplicate. The regression line and correlation coefficient were calculated as in Figure 1. The error bars represent standard deviation of the means.

Figure 3. Effect of formalin exposure on telomere content assay. Human placenta was placed in formalin for 0–8 h at room temperature. Telomere DNA contents were measured in DNA that was purified following tissue treatment with formalin. Identical results were obtained from all time points, and data from 0–8 h are shown. See Figure 1 legend for additional details.

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Telomere DNA Content in Formalin-Fixed Tissue

It is not uncommon for surgical and biopsy specimens to be stored in formalin for as long as 8 h before embedding in paraffin. To determine whether formalin fixation affected the reliability of the telomere DNA content assay, human placenta was submerged in formalin for up to 8 h at room temperature before DNA purification. As shown in Figure 3, telomere DNA contents measured in genomic DNA isolated from tissue treated with formalin were not different from the untreated control (slopes = 768 and 804, respectively). Telomere DNA content could also be assayed with DNA purified from paraffin-embedded archival prostatectomy specimens (Figure 1A).

CONCLUSION

Although the reported associations between reduced telomere DNA content and several prognostic markers of cancer are provocative (2,4,5,7–10,13,14,17), prospective investigations with biopsy materials are necessary to establish the clinical relevance of telomere DNA content. Such investigations have not been possible with existing methods. We have developed a chemiluminescent assay for telomere DNA content that is particularly well suited for the analysis of biopsy or microdissected specimens. The assay, including DNA purification and quantification, can be completed in two days and requires as little as 2–5 ng genomic DNA, the equivalent of 285–715 diploid cells. This technique is at least 10 times more sensitive and five times faster than existing methods. In addition, the assay is unaffected by standard formalin treatment and is informative for tumor tissues that contain up to 75% normal cells.

It is important to emphasize that telomere DNA contents determined by this method reflect the average telomere content in cells comprising the sample. It does not provide information about the variability of the telomere DNA content on chromosomes within or among individual cells. However, with the assay's improved sensitivity,

telomere DNA content could be measured in microdissected or flow-sorted tumor cell subpopulations.

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