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A Chemistry-Based Method To Detect Individual Telomere Length at a Single Chromosome Terminus

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Supporting Information

ABSTRACT: The understanding of telomeres is expected to provide major insights into genome stability, cancer, and telomere-related diseases. In recent years, there have been considerable improvements in the technologies available to determine the length of telomeres of human chromosomes; however, the present methods for measuring telomere length are fraught with shortcomings that have limited their use. Here we describe a method for detection of individual telomere lengths (DITL) that uses a chemistry-based approach that accurately measures the telomere lengths from individual chromosomes. The method was successfully used to determine telomere DNA by breaking in the target sequence and producing a "real telomere fragment." The DITL approach involves cleavage of the sequence adjacent to the telomere followed by resolution of the telomere length at the nucleotide level of a single chromosome. Comparison of the DITL method and the traditional terminal restriction fragment (TRF) analysis indicates that the DITL approach appears to be promising for the quantification of telomere repeats in each chromosome and the detection of accurate telomere lengths that can be missed using TRF analysis.

Human telomeres play a pivotal role in crucial life processes such as genome stability, cancer, and aging.[1−3] Telomere length has been linked to these biological events, and shortening it to a critical point triggers cells to senescence or death. Telomere length may also serve as a "molecular clock" for determining cellular lifespan. Short telomere length may induce cells to undergo cell cycle arrest and apoptosis. Recent studies have suggested that it is not the average telomere length but rather the individual lengths of critically short telomeres that trigger cellular responses to the loss of telomere function.[4] Accordingly, the detection of individual telomere lengths at a single chromosome terminus has been the focus of intense interest in both clinical and biological studies.

Several methods have been developed to measure telomere length, including terminal restriction fragment (TRF) analysis,[5−7] quantitative polymerase chain reaction (qPCR),[8,9] quantitative fluorescence in situ hybridization (Q-FISH),[10] slot blot or dot blot analysis, and single telomere elongation length analysis.[11] Despite their successful applications, these techniques have several major shortcomings that reduce their utility. TRF analysis is based on the use of restriction enzyme digestion of genomic DNA followed by Southern hybridization to measure the average size of TRFs. The measured size of the TRF includes not only true TTAGGG telomere repeats but also variants of both subtelomeric and telomeric repeats that lack functional restriction sites, thus leading to a considerable nontelomeric component within TRFs. Q-FISH uses fluorescently labeled probes to detect telomere length by measuring fluorescence arising from hybridization of the probes with metaphase chromosome preparations. The method does not directly measure the actual length of the DNA because it is hybridization-based, making detection below a telomere length threshold difficult. The requirement for metaphase chromosomes restricts its application to cells capable of growth, indicating limited use in the analysis of senescent cells for understanding the role of telomeres. Other PCR-based methods are unsuitable for the measurement of long telomeres (lengths of 10−50 kbp).

To overcome these limitations, a simple, specific, and sufficiently precise method for measuring telomere length for research and clinical purposes is desired. Moreover, improvements in the resolution of telomere length analysis are needed to understand the role of telomere loss in the human aging process and telomere-associated anticancer agents. For this purpose, we have developed a chemistry-based method for accurate detection of individual telomere lengths (DITL) at a single chromosome terminus.

Our DITL strategy involves the use of a chemistry-based artificial DNA cutter (ARCUT) that combines two pseudocomplementary peptide nucleic acid (pcPNA) strands (Figure 1, blue lines) for sequence recognition and a Ce(IV)/EDTA complex, a catalyst for DNA hydrolysis (Figure 1, yellow balls), that acts as molecular scissors.[12−14] The target sites of the two pcPNA strands of ARCUT in the invasion complex (Figure 1) are laterally shifted with respect to each other, forcing some of the nucleotides at predetermined sites in both strands to remain in the single-stranded state (Figure 1, red lines). These single-stranded portions are selectively hydrolyzed by the Ce(IV)/EDTA complex, which efficiently hydrolyzes single-stranded DNA (ssDNA) but not double-stranded DNA (dsDNA). The scission site and site specificity of ARCUT

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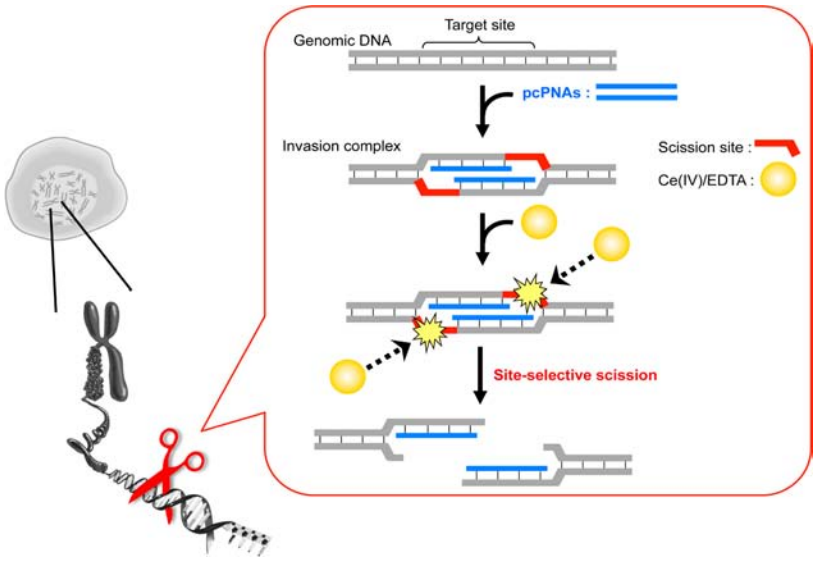

Figure 1. Overview of site-selective scission of a single chromosome by ARCUT.

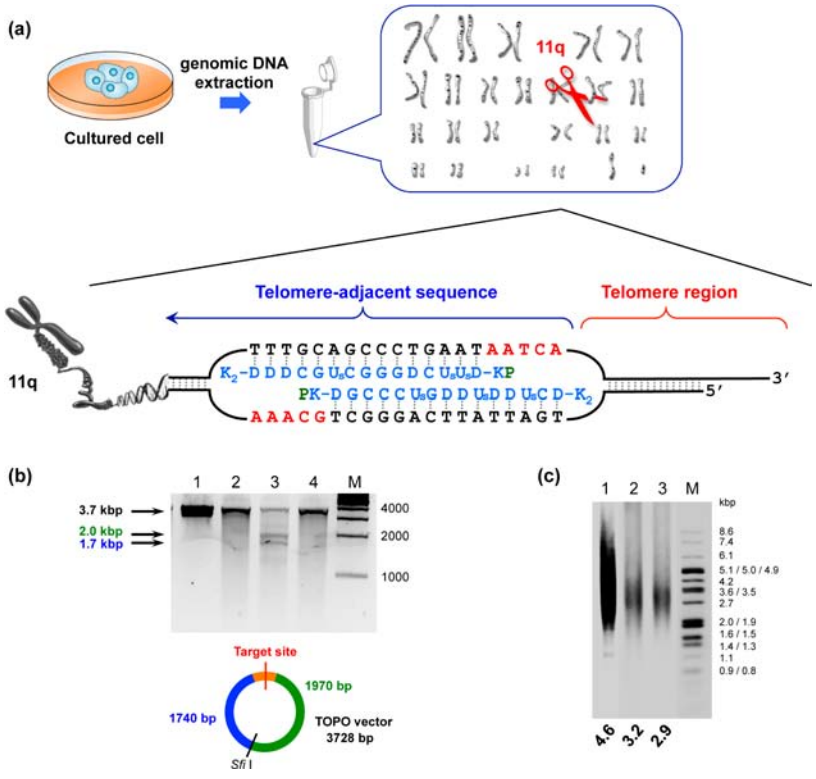

Figure 2. (a) Detection of individual telomere lengths by ARCUT. The whole human genomic DNA is isolated from cultured cell lines. Two pcPNA strands (15-mer) invade the telomere-adjacent duplex to form an invasion complex for ARCUT scission. (b) Site-selective scission of dsDNAs (TOPO vector products) containing the telomere-adjacent sequence of 11q chromosome as shown by agarose gel electrophoresis patterns: lane 1, TOPO vector + *Sfi* I; lane 2, TOPO vector + Ce(IV)/EDTA treatment; lane 3, TOPO vector + pcPNAs 1 and 2 + Ce(IV)/EDTA treatment; lane 4, TOPO vector + mismatched pcPNAs 5 and 6 + Ce(IV)/EDTA treatment; lane M, 1 kbp marker. Conditions: [TOPO vector] = 4 nM, [pcPNA] = 200 nM, [HEPES (pH 7.0)] = 5 mM, [NaCl] = 100 mM, [Ce(IV)/EDTA] = 200 μM. The mixture was incubated at 50 °C for 2 h to form a double-duplex invasion complex. The Ce(IV)/EDTA complex was then added, and the scission reaction was carried out at 50 °C for 16 h. (c) Southern blot analysis of individual telomere lengths of the 11q and Xp/Yp chromosomes in HeLa cells. The telomere lengths are expressed in kilobases. Lane M, marker; lane 1, traditional TRF method; lane 2, 11q targeting (DITL analysis); lane 3, Xp/Yp targeting (DITL analysis). Conditions: [pcPNA] = 200 nM, [Ce(IV)/EDTA] = 100 μM, [NaCl] = 100 mM. pcPNAs 1 and 2 were used for 11q and pcPNAs 3 and 4 for Xp/Yp.

can be tuned simply by changing the sequences and lengths of the pcPNAs. We recently achieved site-selective DNA scission at desired sites in human genomic DNA.[15] The site-selective DNA scission achieved using ARCUT proceeds with high specificity for the target site; thus, ARCUT directly detects individual telomere lengths at the single chromosome terminus.

The DITL technique described here was developed for the telomere at the end of the 11q and Xp/Yp chromosomes from

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human embryonic kidney (HEK) 293 and HeLa cells, as shown schematically in Figure 2. pcPNA strands were designed to enable the recognition of the target site in the telomere-adjacent sequences of the 11q and Xp/Yp chromosomes (Figure 2; also see Table S1 and Figure S1 in the Supporting Information) and further synthesized in order to achieve the detection of individual telomere lengths at the single chromosome. In principle, this technology could also be adapted to other human telomeres. At the outset, the scission ability of ARCUT was confirmed using as a model a short PCR product (209 bp dsDNA) having its target site just before the telomere region in the 11q chromosome (Figure S2). Strand invasion and scission of the dsDNA were observed as expected (Figure S3, lane 3). A cut at the desired site in the dsDNA was further confirmed by examining the scission products of cloned plasmid DNA (Figure S4) and observing the target site of the telomere region in the 11q chromosome. The invasion complex of the cloned vector containing the target site with the pcPNAs was treated with Ce(IV)/EDTA and then digested with *Sfi* I. The agarose gel electrophoresis pattern of the digest displayed only two scission bands (Figure 2b) at around 2.0 and 1.7 kbp in addition to the band for the substrate DNA at 3.7 kbp, as expected for the site-selective scission (Figure 2b, lane 3). However, when fully mismatched pcPNAs were used as as a control, no scission bands were observed (Figure 2b, lane 4). Remarkably, ARCUT distinguished the alteration of the base pairing between the pcPNA and DNA and hydrolyzed only the ssDNA region at the target site. These results suggested that perfect Watson−Crick complementarity between pcPNA and DNA is essential for the scission, indicating that the targeting approach to the DNA sequence is site-specific.

Encouraged by the above-mentioned data, we tried to detect individual telomere lengths at a single chromosome. The whole human genomic DNA was treated as the substrate and isolated from cultured HEK 293 and HeLa cells. Upon incubation of the genomic DNA and pcPNAs at 50 °C for 4 h, a double-duplex invasion complex was formed. The reaction mixture was desalted and subjected to pulsed-field gel electrophoresis. Telomere fragments were detected by Southern blotting and analyzed on a bioimaging analyzer. Under the analysis conditions, the telomeres appeared as bands centered around 3.2 and 2.9 kbp for HeLa cells (Figure 2c) and 2.6 and 4.1 kbp for HEK 293 cells (Figure S5), which is consistent with the size of single telomeric molecules. TRF analysis of all of the telomeres showed a smear calculated at 4.6 kbp. Comparisons between the DITL and traditional TRF analyses showed that the gel of DITL was less smeared and that the DITL estimates were more precise than those determined by TRF. The difference between the two analyses was not unexpected. TRF analysis is based on the use of restriction enzyme digestion of genomic DNA, and includes not only true TTAGGG telomere repeats but also varying amounts of subtelomeric and telomere repeat variants, leading to a considerable nontelomeric component. It could also be explained by the presence of varying amounts of subtelomeric sequences for all of the telomeres containing recognition sites of restriction enzymes along with the inability to detect individual telomeres. For the accurate determination of telomere length, an exact scission array at the start of the telomere repeat sequences is required. The DITL approach provides a more accurate measurement because it involves cleavage of the telomere-adjacent sequence of a single chromosome at the nucleotide level.

The conditions for specificity and efficiency of the scission reaction were optimized. For this purpose, the dependence of site-selective scission by ARCUT on the pcPNA and Ce(IV)/EDTA concentrations were tested (Figure 3a). The most

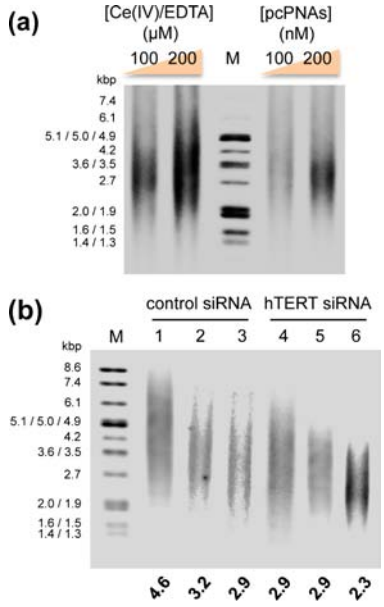

Figure 3. (a) Dependence of the site selectivity of the scission on the concentrations of the pcPNAs and the Ce(IV/EDTA) complex. Conditions 1: [pcPNA] = 100 or 200 nM, [Ce(IV)/EDTA] = 100 μM, [NaCl] = 100 mM. Conditions 2: [pcPNA] = 200 nM, [Ce(IV)/EDTA] = 100 or 200 μM, [NaCl] = 100 mM. (b) Southern blot analysis of 11q and Xp/Yp telomeres in HeLa cells using siRNA and control siRNA to inhibit hTERT expression. Lane M, marker; lanes 1 and 4, traditional TRF method; lanes 2 and 5, 11q targeting (DITL analysis); lanes 3 and 6, Xp/Yp targeting (DITL analysis). Conditions: [pcPNA] = 200 nM, [Ce(IV)/EDTA] = 100 μM, [NaCl] = 100 mM.

suitable scission conditions for the DITL detection were determined to be concentrations of 200 nM and 100 μM for the pcPNAs and the Ce(IV)/EDTA complex, respectively.

Furthermore, the DITL analysis was used to examine the shortening of telomere repeats in vitro by suppressing the human telomerase-reverse transcriptase (hTERT) activity (knockdown of hTERT). hTERT-specific small interfering RNA (siRNA) was used to inhibit its expression and induce telomere shortening in HeLa cells.[16] HeLa cells transfected with hTERT-specific siRNA (Figure S6) were cultured for 3 weeks, after which the genomic DNA was isolated and the telomere length was determined by DITL and TRF analyses. In the siRNA-treated cells, the individual telomere lengths of the 11q and Xp/Yp chromosomes were found to be 2.9 and 2.3 kbp (Figure 3b, lanes 5 and 6 for 11q and Xp/Yp chromosome). The mean TRF sizes obtained by the traditional TRF method (Figure 3b, lane 4) using restriction enzymes were found to be 2.9 kbp. Comparison of the DITL and TRF analyses of the Xp/Yp telomere showed that as expected, the Xp/Yp value (2.3 kbp) was lower than that estimated from the TRF analysis. Notably, there were no significant differences between the results obtained by the two methods in the length of 11q telomere (both 2.9 kbp). When HeLa cells treated with control siRNA containing mutated sequences (non-sequence-specific control), we did not observe a reduction in the TRF sizes of the 11q and Xp/Yp telomeres in comparison with the

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siRNA-treated cells (Figure 3b). These observations indicated that the DITL approach detects telomere lengths more accurately than the TRF analysis. Chromosome-specific telomere length differences were revealed with more clarity by DITL when telomere shortening was induced by using ligands to inhibit hTERT expression. Because of the rising interest in telomere function from the perspective of the development of anticancer agents and aging,[17] improvements in the resolution of telomere length analysis are essential for understanding both the extent and role of telomeres. The determination of accurate lengths of individual short telomeres will lead to a better understanding of telomere function. Significantly, a previous study suggested that only chromosomes with the shortest telomeres are involved in fusions and dysfunction of telomeres.[4] The possibility of detecting the presence of such telomeres lends great value to the DITL analysis method.

We utilized the unique feature of DITL analysis to detect individual telomere lengths at a single chromosome terminus. The method involves easily prepared PNAs based on a site-selective scission approach with high target site specificity and no restrictions on target sequences. The approach appears to be promising for quantification of telomere repeats in each chromosome and for the detection of accurate telomere lengths that can be missed when using traditional analysis methods. The improvement in the resolution of telomere length analysis provides a useful tool for obtaining a better understanding of the role of telomere loss in the human aging process and in the development of telomere-associated anticancer agents.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details and characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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