

Original Article

# Long-term Persistent Organic Pollutants Exposure Induced Telomere Dysfunction and Senescence-Associated Secretory Phenotype

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## Abstract

Environmentally persistent organic pollutant (POP) is the general term for refractory organic compounds that show long-range atmospheric transport, environmental persistence, and bioaccumulation. It has been reported that the accumulation of POPs could lead to cellular DNA damage and adverse effects of on metabolic health. To better understand the mechanism of the health risks associated with POPs, we conducted an evidence-based cohort investigation ( $n = 5,955$ ) at the Jinghai e-waste disposal center in China from 2009 to 2016, where people endure serious POP exposure. And high levels of aging-related diseases, including hypertension, diabetes, autoimmune diseases, and reproductive disorders were identified associated with the POP exposure. In the subsequent molecular level study, an increased telomere dysfunction including telomere multiple telomere signals, telomere signal-free ends, telomere shortening and activation of alternative lengthening of telomeres were observed, which might result from the hypomethylated DNA modification induced telomeric repeat-containing RNA overexpression. Moreover, dysfunctional telomere-led senescence-associated secretory phenotype was confirmed, as the proinflammatory cytokines and immunosenescence hallmarks including interleukin-6, P16<sup>INK4a</sup>, and P14<sup>ARF</sup> were stimulated. Thus, we proposed that the dysfunctional telomere and elevated systemic chronic inflammation contribute to the aging-associated diseases, which were highly developed among the POP exposure individuals.

**Keywords:** Human aging, Telomere dysfunction, Inflammation, Public health

Due to wide usage of consumer electronics, it has been estimated that approximately 20–50 million tons of electronic waste (e-waste) is disposed of every year in the world (1,2). The persistent organic pollutants (POPs), which could generate from inappropriate disposal of chemical products and electronics equipment in the e-waste, were linked to a large spectrum of adverse effects on human health and have attracted worldwide attention (1). POPs are organic compounds that include different kinds of hazardous substances, such as polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs), polycyclic aromatic hydrocarbons (PAHs), and so on, they

are hard to be metabolized in the natural environment and could be accumulated in the air, water, soil, and animal adipocytes. PCBs and PBDEs have similar structure and released from the flame retardants, whereas PAHs are mainly released from the incomplete combustion of organic materials (3,4). Due to the lipid solubility, POPs readily went through the lipophilic cell membranes and accumulated in tissue, finally played a negative role in human health (5–7). For example, long-term exposure to POPs was consistently associated with the risk of neuron system development, type 2 diabetes, reproductive disorders, and metabolic disorders (4,6,8,9).

Environmental or occupational pollutants exposure-induced oxidative stress was proposed to play a major role in the development of these POP-related diseases including cancer, diabetic complications, and cardiovascular disorders. Oxidative stress is interpreted as an imbalance between the production of oxidants and antioxidants, which results in increased level of free radicals, including reactive oxygen species and reactive nitrogen species. Environmental exposure-induced reactive oxygen species can attack DNA and lead to single- or double-strand DNA damage. And genes related to DNA damage response such as P53 and ATM were reported to be upregulated to response the exposure (10).

Telomeres were considered as a common fragile site of genome for DNA damage, due to the G-rich sequence and tandem repeat nature (11). They were favored targets for DNA damage recognized by oxidative stress (12). When the telomeres become critically short or sufficiently damaged, the deprotected telomeres trigger DNA damage signaling and lead to various senescence-related disorders (13), the telomeres shortening process can be accelerated by oxidative stress and chronic inflammation (14). Noxious environment factors could accelerate telomere loss and associated with the degenerative diseases (15). It has been observed that heavy metal pollution in the water, soil, exceeded PM<sub>2.5</sub>/PM<sub>10</sub> in the atmospheric particulate pollutants may cause the telomere-associated premature (16,17), and several PCBs congeners could downregulate telomerase activity and shorten telomere length in HaCaT cells (18,19). Therefore, telomere could be considered as a sensor of organic health maintenance under environmental stress.

In our previous studies, increased chromosomal aberrancy and DNA damage were detected along with high levels of POPs, including PCBs and PBDEs for the residents who were around the e-waste disposal area (20). Here, we report that POP exposure lead to telomere-specific dysfunction, including telomere shortening, telomere lost, and telomeric repeat-containing RNA (TERRA) overexpression, which may give explanation for the subsequent chronic inflammatory response and the occurrence of aging-related diseases. The aim of this study is to clarify the potential mechanism of aging induced by POPs in population research and systematically assess the human health risk that POPs may cause.

## Materials and Methods

### Samples

The e-waste recycling area in this study is located about 50-km southwest of Tianjin in Northern China, and the POP exposure individuals in this study were residents living in the region more than 20 years and the workers who had worked for average 6 years in manual dismantling and recycling with a crude and unsafe procedure in small family workshops. The control individuals were residents living in 40 km away from the POP exposure region, where people have similar overall environmental condition and personal lifestyle, but have not participated in any e-waste disposing from 2009 to 2016. All samples were used anonymously. Total 20-mL peripheral blood was collected from each participant into ethylenediaminetetraacetic acid-anticoagulant tubes and heparin-anticoagulant tubes, respectively. All the blood samples were kept on ice until the analyses within 4 hours. The detailed demographic information is listed in Supplementary Table 1. The study was approved by the Ethics Committee of Tianjin Medical University, Tianjin, China. Informed consent was obtained from all participants before taking any specimen.

### Assessment of POP Level

The POPs in our study included PCB (25 congeners) and PBDE (11 congeners), and the lipid-standardized concentrations of serum POPs were detected as described previously (21). “Total PCBs” was defined as the sum of all measured all measured PCBs, and “Total PBDEs” was defined as the sum of all measured BDEs.

### Micronucleus Rate Assessment

The micronucleus rate was used to evaluate genotoxicity. Micronucleus rate assessment was performed as described previously (20).

### Immunofluorescence Microscopy

The number of lymphocytes nuclei containing foci of 53BP1, a marker of DNA double-strand breakage, was analyzed by immunofluorescence. Blood samples were lysed to remove red cells and washed twice with phosphate-buffered saline, then resuspended and centrifuged onto slides at 2,000 rpm for 10 minutes, washed twice in 0.1% Tween 20 in Tris-buffered saline (100 mM Tris-HCl, 150 mM NaCl; 0.1% TBST), fixed in freshly prepared 2% paraformaldehyde in phosphate-buffered saline (pH = 7.4), washed four times in 0.1% TBST, permeabilized in 0.5% Triton X-100 in phosphate-buffered saline for 20 minutes, washed three times, and left in blocking solution (5% goat serum, 1% gelatin, 4% bovine serum albumin, in 0.1% Tween 20, in Tris-buffered saline) for 2 hours. Cells were incubated for 1 hour at room temperature with primary antibodies against 53BP1 (NB 100-304, 1:5,000 dilution, Novus Biological, Littleton, CO), washed three times in 0.5% TBST, and incubated for 0.5 hour with secondary antibodies (A11008, 1:5,000 dilution, Life Technologies, Carlsbad, CA) at room temperature. Dehydrated in 70%, 95%, and 100% ethanol. Finally, the slides were mounted in the presence of 4',6-diamidino-2-phenyl-indole (DAPI, D3571, Life Technologies, Carlsbad, CA). Fluorescence was detected and imaged using fluorescence microscope (Nikon, Tokyo, Japan).

### Telomere Length Measurement by Quantitative Real-Time Polymerase Chain Reaction

Genomic DNA for telomere polymerase chain reaction (PCR) was extracted directly from blood samples with Blood Genomic DNA Miniprep Kit (Axygen, Tewksbury, MA) according to the manufacturer's instructions, diluted to the concentration of approximately 20 ng/ $\mu$ L. Quantitative real-time PCR was used to determine telomere length as previously proposed by Cawthon (22). PCR reactions were performed on the Applied Biosystems (ABI 7500, Carlsbad, CA) using reference control gene (36B4 single copy gene) and telomeric gene; primers and reaction system were shown in Supplementary Tables 2 and 3. The thermal cycling profile was as follows: Stage 1: 5 minutes at 95°C; Stage 2: two cycles of 15 seconds at 94°C, 15 seconds at 49°C; Stage 3: 40 cycles of 15 seconds at 94°C, 10 seconds at 62°C, 15 seconds at 74°C with signal acquisition.

### Telomere Restriction Fragment Analysis by Southern Blot

We confirmed telomere length measurement by Southern blot—a gold standard in measuring telomere length. The telomere restriction fragment (TRF) analysis was performed using a commercial kit (TeloTAGGG Telomere Length Assay, Roche Life Science, Mannheim, Switzerland), based on the instruction, with slight modifications. Briefly, genomic DNA was digested with Hinf I and Rsa I overnight at 37°C. Five hundred nanogram of digested DNA was

then electrophoresed on 0.8% agarose gels at 120 V for 2.5–3 hours at electrophoresed on 0.8% agarose; genomic DNA was depurination, denatured, neutralized, fixed as standard methods, and hybridized with a DIG-labeled (CCCTAA)<sub>4</sub> oligo probe (Sangon Biotech Co., Ltd, Shanghai, China) to the final concentration of 0.5 nM. The blotting membrane was washed and block, incubated with Anti-DIG-AP (Roche, 1:4,000 dilution, Mannheim, Switzerland) for 5–7 hours, washed, and exposed with CDP-star (Roche, Mannheim, Switzerland). After exposure of the blot to an X-ray film, an estimate of the mean TRF length can be obtained by comparing the mean size of the smear to the molecular weight marker with Telo Tool.

### 53BP1 Immunofluorescence-Telomere FISH and Quantitative-FISH

Quantitative-fluorescence in situ hybridization (Q-FISH) with the FITC-conjugated 18-mer peptide nucleic acid telomeric oligonucleotide probe (Panagene, Daejeon, South Korea) and immunofluorescence-Telomere fluorescence in situ hybridization (IF-FISH) were carried out as described by Kasbek and colleagues (23).

### C-Circle Assay

C-circle assay is a specific, quantitative, and responsive indicator of alternative lengthening of telomere (ALT) activity levels, the protocol for C-circle amplification employed was slightly modified from that performed in Henson and colleagues (24). Briefly, 400-ng genomic DNA was digested with Hinf I and Rsa I restriction enzymes at 37°C overnight and purified by phenol-chloroform extraction. Genomic DNA from ALT-positive (U2OS) cells was used as a positive control. DNA was diluted in double distilled water. Samples (10 µL) were combined with 10 µL 1× Φ29 buffer (New England Biolabs, Beverly, MA), containing bovine serum albumin, 0.2 mM each d ATP, d GTP, d TTP, and incubated in the presence or absence of 5U ΦDNA polymerase (New England Biolabs, Beverly, MA) at 30°C for 12 hours and then at 65°C for 20 minutes. Added 60-ng reaction products to 200 µL 6× SSC and dot-blotted onto a 6× SSC-soaked nylon membrane. DNA was cross-linked onto the membrane and hybridized with a DIG-labeled probe (CCCTAA)<sub>4</sub> to detect C-circle amplification products. Blots were washed, exposed to Tanon 5200 (Tanon Science and Technology Co., Ltd, Shanghai, China), and quantified using ImageJ.

### Analysis of DNA Methylation

Global methylation of long interspersed nuclear element-1 (LINE-1) repeats was quantified using bisulfate-PCR and high-resolution melting curve. Genomic DNA was bisulfite-treated with Epi Tect kit (QIAGEN, Hilden, Germany) and diluted to the concentration of 5–10 ng/µL. The LINE-1 primer was the same as Tse (25), of which methylation levels were also significantly correlated to global DNA methylation. Then, the methylation level of bisulfited samples was detected by high-resolution melting according to the Eip Tect PCR Control DNA kit (QIAGEN, Hilden, Germany) introduction and analyzed with reference from a standard substance by software. The thermal cycling profile was as follows: Stage 1: 5 minutes at 95°C; Stage 2: 45 cycles of 10 seconds at 95°C, 30 seconds at 55°C, 10 seconds at 72°C; Stage 3: melt curve from 70°C to 95°C, every other 0.1°C with signal acquisition. Reduced representation bisulfite sequencing (RRBS) was used to analysis subtelomeric DNA methylation. RRBS sequencing and data processing were conducted as described previously (21). Analysis of methylation degree in

subtelomere was performed according to the sequence investigated by Riethman (26).

### Real-Time Reverse Transcription-PCR analysis of TERRA Level

The protocol of real-time reverse transcription-PCR (RT-PCR) analysis was slightly modified from that performed by Porro and colleagues (27). Total RNA of blood samples were isolated with the Trizol reagent, respectively (Invitrogen, Carlsbad, CA), and reverse transcribed with β-actin and telomere-specific oligonucleotides using HiScript SuperMix for qPCR kit (Vazyme Biotech Co., Ltd, Nanjing, China). cDNA was amplified using the SYBR green master mix (Vazyme Biotech Co., Ltd, Nanjing, China) with the primers listed in Supplementary Table 4 and analyzed using the Rotor gene Q 2000 (QIAGEN, Hilden, Germany) with the following thermal cycling profile: Stage 1: 2 minutes at 95°C; Stage 2: 40 cycles of 15 seconds at 98°C, 20 seconds at 60°C, 1 minute at 74°C with signal acquisition.

### Enzyme-Linked Immunosorbent Assay

The levels of interleukin (IL)-2, IL-6, IL-10, tumor necrosis factor-α (TNF-α), P53, RB, P16<sup>INK4a</sup>, and P14<sup>ARF</sup> in plasma were detected by enzyme-linked immunosorbent assay (ELISA; DAKEW Bioengineering, Shenzhen, China) according to the manufacturer's instructions.

### RT-PCR Analysis of mRNA Expression

Total RNA of blood samples were isolated with Trizol reagent (Invitrogen); 1-µg RNA was converted to poly A oligonucleotides, diluted 1:10; and 2 µL were used for RT-PCR. Primers were listed in Supplementary Table 5 and analyzed with the Eco system (Illumina, San Diego, CA) with a total reaction volume of 10 µL.

### Statistical Analyses

All data were analyzed using SPSS v.17.0 (SPSS Inc., Chicago, IL). The significance of differences between control and exposed individuals was analyzed with two-tailed Student's *t* test and  $\chi^2$  test. Spearman test was used to calculate correlation coefficient between general clinical characteristics and telomere length. Spearman test between serum concentrations of lipid-standardized POPs and telomere length was calculated. *p* Values below 0.05 were considered as statistically significant.

## Results

### POP Exposure Leads to Aging-Related Diseases

The previous studies have found that PCBs, PBDEs, PAHs, and Dioxin in the atmosphere, soil, river sediments, and groundwater samples increased in the e-waste disposal area, among which PAHs and PBDEs were 10–60 times of other contaminated areas. So we collected 5,955 questionnaires and most corresponding samples from the individual who live around the e-waste disposal area and control area. Statistical results (Supplementary Table 1) showed that the mean ages of the population in control and exposed group were 39.85 ± 0.20 (*n* = 3,349) and 39.55 ± 0.24 (*n* = 2,606) years, respectively; the body mass index of the population in control and exposed group was 23.48 ± 0.06 (*n* = 3,349) and 23.56 ± 0.07 (*n* = 2,606); the gender distribution was almost equal; besides, we calculated the percentage that individuals who had taken medicine during our investigation and shown as drug taking. The concentration of total 36 species of POPs was detected in the serum samples. The level of PBDEs and PCBs from POP exposure samples was identified much higher than

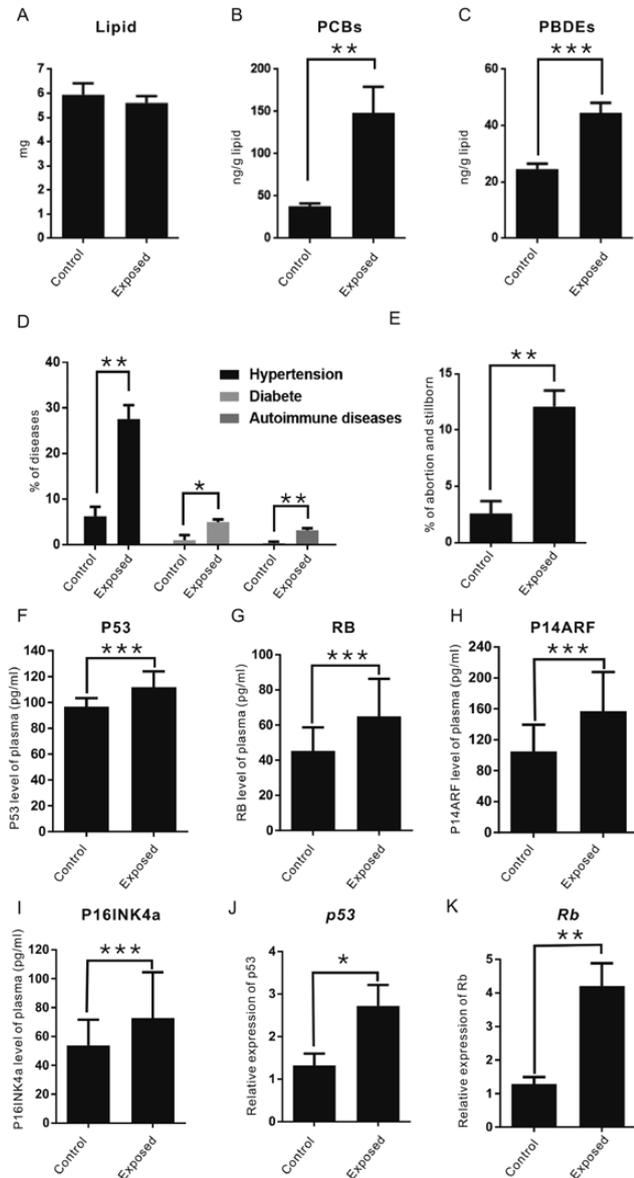
the control (Figure 1B and C). To evaluate the health condition of people historical exposure to POPs here, the incidence rates of cancer, type 2 diabetes, hypertension, cardiovascular disease, and autoimmune disease were compared between POP exposure and control residents. Statistical results showed that percentage of individual with hypertension, diabetes, and autoimmune diseases was significantly increased in POP exposure population, but not for cardiovascular (Figure 1D). Strikingly, the productive disorders including infertility, premature maternal delivery, stillbirth, and congenital disability were

much higher than control (Figure 1E). The aging-associated disease is a disease that is most often seen with increasing frequency with increasing senescence, including type 2 diabetes, cardiovascular, and reproductive issues because they could arise and develop from senescence (28). Our current data suggested that exposure to POPs can lead to the increased risk of premature senility-linked diseases.

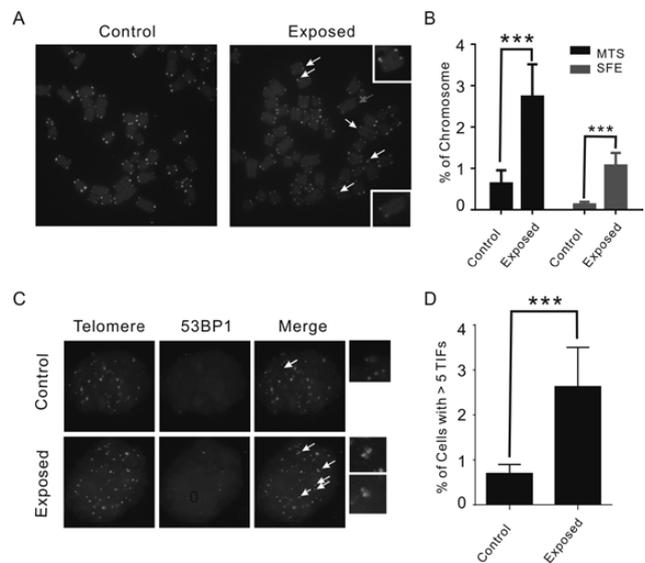
To further confirm the activation of the POPs led aging response signal pathway, the levels of P53, RB, P16<sup>INK4a</sup>, and P14<sup>ARF</sup> in the plasma were detected by ELISA assay (Figure 1F-I) and the relative RNA levels of p53 and Rb of blood were detected by RT-PCR (Figure 1J and K). Consistent with our speculation, the levels of P53, Rb, P16<sup>INK4a</sup>, and P14<sup>ARF</sup> in the plasma were dramatically increased in the exposed individuals, suggesting that persistent POP exposure may influence cell cycle and eventually induce senescence. However, the cellular and molecular mechanisms between POPs and aging are not well understood and deserved further investigation.

### POP Exposure Induces Genomic DNA Damage

Increased reactive oxygen species activity was observed in the POP-exposed individuals in our previous studies (20,21); to further investigate the genotoxicity of POPs, the DNA damage and genomic instability were detected. The immunofluorescence data showed observable increased 53BP1 foci (five or more dots, 35.1%) in the exposed individuals compared to control (five or more dots, 8.6%; Supplementary Figure 1A and B). Serious DNA double-strand breaks may lead to genomic fragments during subsequent cell division, and some of the fragments could be kept in the cytoplasm, finally forming the micronucleus (29). Thus, micronucleus was detected by lymphocyte cytokinesis-block micronucleus cytome assay, and we observed that the incidence rate (Supplementary Figure 1C and D) in the exposed individuals was significantly elevated for the residents living around the e-waste disposal site (20.62% vs. 7.21%,  $p < .01$ ). These results indicated that exposure to POPs can lead to serious DNA damage and genomic instability.



**Figure 1.** Persistent organic pollutant (POP) exposure leads to aging-related diseases. (A) Lipid quality in serum used for POPs testing. (B and C) Concentration of PCBs and PBDEs in the blood of control ( $n = 29$  versus 39 respectively for PCBs and PBDEs) and exposed population ( $n = 53$  versus 66 respectively for PCBs and PBDEs). (D) Statistical data on aging-related diseases including diabetes, high blood pressure (HBP), and autoimmune diseases in control ( $n = 3,349$ ) and exposed ( $n = 2,606$ ) individuals. (E) Percentage of abortion and stillborn in both control ( $n = 1,239$ ) and exposed ( $n = 1,215$ ) individuals. (F-I) Abundance of P53, RB, P14ARF, and P16INK4a in the plasma was detected by ELISA assay in the control ( $n = 80$ ) and POP-exposed individuals ( $n = 170$ ). (J and K) Relative expression of p53 and Rb mRNA was confirmed by RT-PCR. Data represent mean  $\pm$  SEM. \* $p < .05$ ; \*\* $p < .01$ ; \*\*\* $p < .001$ .

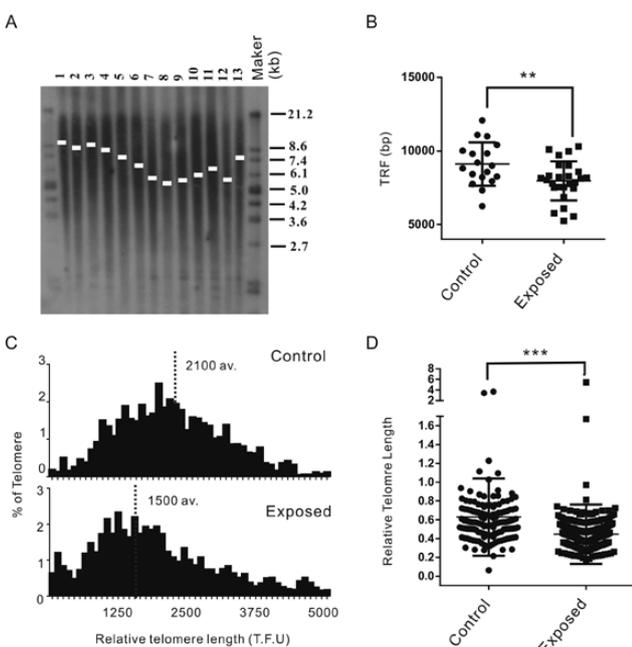


**Figure 2.** Persistent organic pollutant (POP) exposure causes telomere dysfunction. (A) Representative telomere images of control ( $n = 60$ ) and exposed individuals ( $n = 80$ ). Blue, chromosomes stained with 4',6-diamidino-2-phenyl-indole (DAPI); green, telomeres. Red arrow indicates telomere signal-free ends (SFE), and white arrow indicates multiple telomere signals (MTS). (B) Frequency of SFE and MTS. (C) Representative telomere damage images in control ( $n = 60$ ) and exposed individuals ( $n = 80$ ). (D) Frequency of telomere damage signal of lymphocytes. Data represent mean  $\pm$  SEM. \*\*\* $p < .001$ .

## POP Exposure Causes Telomere Dysfunction

It has been well established that dysfunction of the telomere caps leads to genomic instability and cell senescence. To directly investigate whether telomere was affected by POP exposure or not, we analyzed the telomere status on the metaphase spread by telomere fluorescence in situ hybridization (FISH). In general, telomere FISH signals should be presented on each sister chromatid end, and the intensity of the FISH signal is comparable. But here, we detected a high number (1.24% vs. 0.10%) of chromosomes with undetectable telomere FISH signal on the end (signal-free end), indicative of telomere loss (Figure 2A and B) for the exposed individuals. In addition, we also detected a high incidence (2.76% vs. 0.69%) of fragile telomeres in exposed individuals with multiple separated telomere FISH signals (Figure 2A and B). These results indicate that POP exposure can lead to telomere abnormalities and disturb telomere protective caps.

To further investigate, we measured telomere dysfunction-induced DNA damage focus (TIFs), by detecting the colocalization of the telomeric signals and DNA damage marker 53BP1. TIFs with more than five colocalized telomere-damaged signals were counted. A fourfold induction of DNA damage response at telomere was observed in exposed individuals (Figure 2C and D), indicating the DNA repair response activation at the dysfunctional telomeres sites. Moreover, 30%–45% of the newly induced 53BP1 signals were localized on telomere, suggesting a critical telomere-specific DNA damage was elevated by POP accumulation.



**Figure 3.** Persistent organic pollutant (POP) exposure induces telomere shortening. (A) Telomere length was determined by Southern blot, and the results were calculated by TeloTool and shown with mean telomere restriction fragment (TRF). Representative image of results, lane 1–6 were control individuals, lane 7–13 were exposed individuals. (B) Scatter plot of TRF for control ( $n = 18$ ) and exposed individuals ( $n = 26$ ). (C) Histogram displays distribution of relative telomere length as T.F.U by quantitative-fluorescence in situ hybridization (Q-FISH). (D) Relative telomere length of the exposed ( $n = 316$ ) and control ( $n = 136$ ) individuals detected by real-time PCR. Data represent mean  $\pm$  SEM. \* $p < .05$ ; \*\* $p < .01$ ; \*\*\* $p < .001$ .

## POP Exposure Induces Telomere Shortening

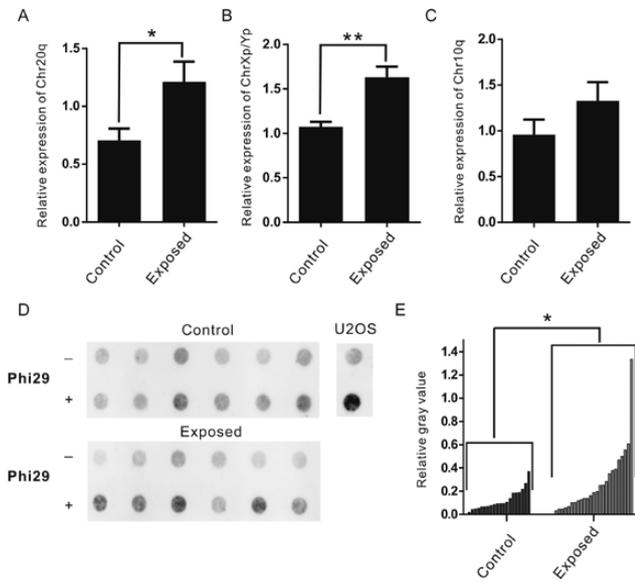
TIFs are thought to arise from the critical short length and uncapping of telomere ends (30). To determine whether high incidence of telomere loss could contribute to telomere shortening or not, the telomere length was detected by Southern blot, Q-FISH, and quantitative real-time PCR. The TRF length analysis by Southern blot revealed a significant telomere shortening for POP exposure individuals (Figure 3A and B) and the mean TRF length decreased from  $\sim 9.2$  kb for control samples to  $\sim 7.4$  kb for the exposed individuals. In the meanwhile, the Q-FISH and RT-PCR analysis confirmed that POP exposure leads to telomere length shortening (Figure 3C and D). The shortened telomere could trigger a cell-cycle arrest and cell senescence or apoptosis.

Furthermore, Spearman correlation coefficients of clinical characteristics and telomere length were displayed in Supplementary Table 6, and none of the demographic variables were associated with telomere length. In addition, Spearman analysis between various POPs and telomere length was shown in Supplementary Table 7. In general, PBDE 184 showed statistically significant correlation coefficients, whereas there was no significant association between the other serum concentrations of POP species and telomere length. These results hinted that telomere shortened in POP exposure individual cells, which may directly contribute to aging and aging-related diseases.

## POP Exposure Elevates TERRA Transcription and Stimulates Alternative Lengthening of Telomeres

Telomere-binding proteins, telomerase, and TERRA are three main components in telomere length regulation (31–33). However, our RNA-seq results showed that there was not any difference in the transcription of any telomere-binding protein or telomerase between two groups. TERRA transcription cannot be easily detected by the current RNA-seq assay, due to their repetitive nature. To investigate whether TERRA is affected by POPs, we determined the TERRA transcription from different chromosome ends by RT-PCR (Figure 4A–C). Our  $\beta$ -actin normalized data revealed that the overall TERRA transcription levels were notably increased in the individuals with the POP exposure, especially on 20q and Xp/Yp chromosome, suggesting transcription of TERRA could be upregulated by POP exposure. Moreover, we found a significant negative correlation between the expression of TERRA transcription levels and the telomere length in the same exposed individuals (Supplementary Figure 2A–D), suggesting TERRA transcription played a negative role for telomere elongation, which consistent with the previous report (34).

The epigenetic modification of TERRA promoters plays an important role in TERRA transcription regulation (31,35). Thus, methylation level of the genomic DNA and subtelomeric DNA was determined. *LINE-1* DNA methylation, which can represent the global genomic methylation level, was detected by high-resolution melting assay. And we observed significantly decreased *LINE-1* DNA methylation in population lived in exposure region (Supplementary Figure 3A–C). The subtelomere methylation was determined by RRBS, and the results showed that the methylation of chromosomes 10 and 15 was repressed (Supplementary Figure 4D–E) and so were chromosomes 1q, 3q, 6q (Supplementary Figure 4A–C). We did not capture enough signals of chromosomes 20q and Xp/Yp. These results demonstrate that the TERRA transcriptional probably regulated via epigenetic modification, which resulted from POP exposure. As we know, at telomeres, TERRA competes with telomeric DNA for ATRX binding and suppresses ATRX localization and ATRX plays an important role in ALT suppression in mammalian cells. ALT

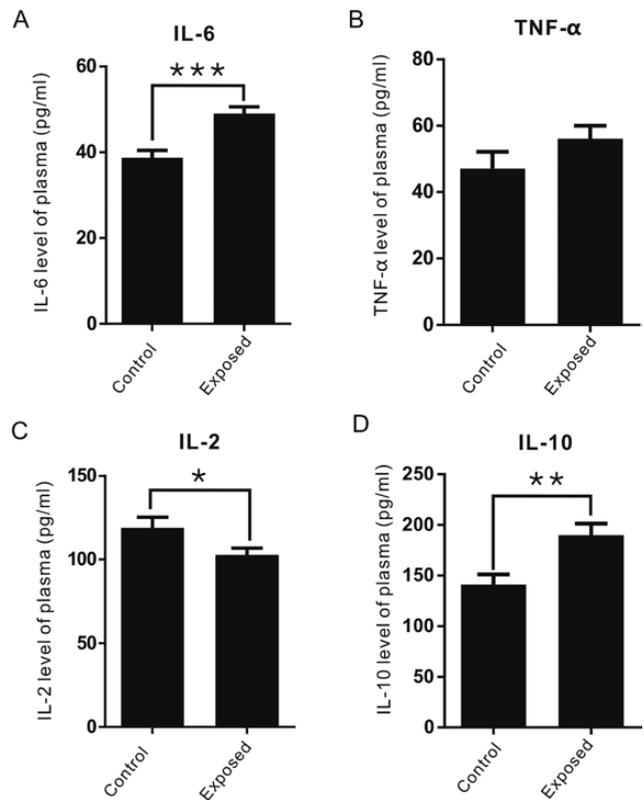


**Figure 4.** Persistent organic pollutant (POP) exposure elevates telomeric repeat-containing RNA (TERRA) transcription and stimulates alternative lengthening of telomeres. (A–C) Changes of relative TERRA level from chromosome 20q, Xp/Yp and 10q (chr 20q, chrXp/Yp and chr10q) in control ( $n = 20$ ) and exposed ( $n = 40$ ) individuals. (D) Representative dot blot of the C-circle assay. (E) Histogram showing distribution of C circles in 60-ng blood genomic DNA from control ( $n = 20$ ) and exposed individuals ( $n = 25$ ). Data represent mean  $\pm$  SEM. \* $p < .05$ ; \*\* $p < .01$ .

is a telomere maintenance mechanism that does not involve telomerase, which probably involves recombination and disrupts telomeric stability. The real-time PCR and Q-FISH data revealed a number of exposed individual samples with abnormally extended telomere. The rarely elongated telomere may indicate a promoted telomerase activity or activation of ALT. Thus, the telomerase activity was detected by TRAP assay for those long telomere samples ( $n = 25$ ) and no significant telomerase activity changes were observed (data not show). The ALT formation was detected by single-stranded telomeric DNA circles (C-circles) assay, and the ALT-positive U2OS cells were used as a positive control and quantitative criteria. Interestingly, a high level of single-strand telomere DNA was observed (Figure 4A and B), suggesting the POP-promoted TERRA overtranscription then induced an occasional homology DNA recombination at telomere. These results fit our previous identification that the genes involved in DNA homologous recombination repair including Rad51, Rad52, and DMC1 were upregulated by POP exposure.

### POP Exposure Alters Inflammatory Cytokine Secretion

Telomere dysfunction is furthermore associated with a state of decreased cellular metabolic activity. One consequence is that senescent cells secrete a predictable profile of cytokines, chemokines, and proteases into the environment, a phenotype known as the senescence-associated secretory phenotype (SASP), which is different from the transient DNA damage-induced inflammation. To evaluate SASP alteration, the plasma concentrations of several SASP cytokines including IL-6, IL-2, TNF- $\alpha$ , and anti-inflammatory cytokines IL-10 were determined by ELISA assay (Figure 5A–D). Compared to control, the production of IL-10 and IL-6 significantly increased in exposed individuals. In contrast, IL-2 was significantly reduced, and



**Figure 5.** Persistent organic pollutant (POP) exposure alters inflammatory cytokine secretion. (A–D) Secretion of inflammatory cytokines, including interleukin (IL)-6, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), IL-2, IL-10 in the plasma, detected by ELISA assay in both control ( $n = 80$ ) and POP-exposed individuals ( $n = 170$ ). Data represent mean  $\pm$  SEM. \* $p < .05$ ; \*\* $p < .01$ ; \*\*\* $p < .001$ .

no significant change but an increasing trend was observed in TNF- $\alpha$ . IL-10 is a well-known anti-inflammatory cytokine that inhibits production of proinflammatory cytokines, to keep the balance of the immune function and it could increase with rising age. TNF- $\alpha$  and IL-6 are considered as a hallmark of senescence; thus, our current data suggested that the POP exposure-induced telomere dysfunction can lead to aging through inflammatory cytokine secretion.

### Discussion

Previous evidence-based epidemiological studies have established the adverse effects of POPs on metabolic health, even at low-dose exposure levels. Bioaccumulation of POPs has been linked to many human diseases, including cardiovascular disease (36), metabolic disorders (8), developmental disorders (37), endocrine disorders (38), and epigenetic alterations (39). We observed that the POP exposure was highly associated with the occurrence of the type 2 diabetes, autoimmune disorders, abnormal pregnancy, and adverse fetal growth, which could be clustered to premature metabolic disorders. As we know, somatic cells undergo cell senescence due to the continuous telomeres shortened. And, the abnormal telomere shortening or damage was associated with premature aging syndromes and tissue repair retard (40,41). Recently, increasing evidence showed that the outside environmental factors might play an important role in telomere length regulation for unclear reasons (42). The environmental and occupational exposures, including traffic-related air pollution (ie, particulate matter and black carbon), PAHs, N-nitrosamines,

and pesticides, were reported to induce telomere shortening (14,43). Here, we observed obvious telomere-specific dysfunction, including telomere loss and telomere shortening after long terms of POP exposure. Subsequently, the uncapped telomeres could activate cellular senescence following a DNA damage response, then lead to subsequent aging-related diseases. These identifications were opposite to the previous report that low-dose exposures to POPs were associated with longer telomere in an apparently healthy Korean population (44). The variation may due to the content of atmosphere and serum POP species contamination was different from one place to another. Additionally, telomere length was measured by real-time PCR in the previous study, which may not as reliable and reproducible as Southern blot and Q-FISH approach. Here, we determined the telomere by three different assays and identified that the long terms of POP exposure could induce telomere length shortening. Interestingly, we found the serum concentration of PBDE 184 showed statistically significant correlation with telomere shortening, among 36 POP species we analyzed.

TERRA, the long noncoding telomeric RNA, has been reported to play a protection role in the telomere length and stability regulation (45,46). The TERRA expression is directly controlled by DNA methylation at the CpG-rich gene promoters (35). It has been identified in our previous study that global DNA hypomethylation is associated with high serum POP concentration (K. Li, unpublished data, 2017), which may be due to the upregulation of TET1 and TET2 methylcytosine dioxygenases. A recent population study showed that high level of BDE-66 exposure was associated with decreased DNA methylation and in utero PBDEs exposure (39); this study is similar to our observation that POP exposure decreased *LINE1* methylation. Interestingly, *LINE-1* can also participate in the regulation of telomere maintenance (47), of which mechanism is not well understood. And here we identified a high level of TERRA expression, which may contribute to the dysfunctional telomere. Additionally, the demethylated DNA would be an ideal platform for DNA recombination, the frequency of gene rearrangement could be increased 10-fold after *DNMT1* knockout, and that chromatin exchange could be reduced more than 100-fold when the substrate is methylated (48). The increased level of ALT, which depends on the recombination between telomere repetitive DNAs, was identified for the POP exposure individuals. There are two possibilities, one is the demethylated subtelomere facilitated the recombination and the other one is the TERRA competed the binding of ALT repressor ATRX to telomeric DNA. Here, we did not observe obvious changes in the subtelomeric methylation level, which may be because the RRBS is not a good assay to determine methylation at repetitive sequence. Thus, detailed mechanisms of the TERRA overexpression and subtelomere demethylation under POP exposure condition need further elucidation.

Besides telomere dysfunction, chronic inflammation contributes to accelerate biological aging (49). And as we know, when exposed to environmental pollution, toxin damage, radiation exposure, or mental stress, the body will activate the stress response process that could produce intracellular protein and DNA through cascade amplification and lead to aging inflammatory response (50,51) known as SASP. SASP is different from acute inflammatory response that mount a strong and transient response upon invasion, and SASP is characterized by permanent state of mild activation and reduced ability to mount an effective defense (52). Nuclear factor NF- $\kappa$ B plays an important role in SASP signaling pathway, due to its induction expression of proinflammatory factors including cytokines, chemokines, and adhesion molecules (53). In the exposed group, we

observed a stimulation of IL-6 and IL-10. IL-6 is considered as the proinflammatory cytokines that are associated with aging. The actively stimulated SASP could further induce telomere dysfunction in a positive feedback manner and accelerate ageing (54). Interestingly, we observe an increase of IL-10, which mainly secreted by Th2 cells, and an decrease of IL-2, which secreted by Th1 cells in the exposed group, suggesting an activation of the chronic proinflammatory state and reduced ability to mount an effective defense, which could be referred to as immunosenescence.

Our study provides evidence that long-term exposure to POPs significantly increase the risk of aging-related diseases including hypertension, diabetes, autoimmune diseases, and abortion. Exposure increased the level of TERRA transcription and induced the telomere dysfunction, which may contribute to cell senescence and degenerative diseases by activation of p16<sup>INK4a</sup>-Rb and p14<sup>ARF</sup>-p53. Meanwhile, persistent POPs exposure could promote chronic systemic inflammation through NF- $\kappa$ B pathway, which accelerate aging incidence (Supplementary Figure 5). The telomere heterogeneity might be considered as a biological marker for occupational exposure assessment. Moreover, this achievement could provide appropriate guidance for occupational practitioner and further benefits the public health.

## Supplementary Material

Supplementary data is available at *The Journals of Gerontology, Series A: Biological Sciences and Medical Sciences* online.

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## Conflict of Interest

None reported.

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