Carcinogenesis vol.31 no.2 pp.216–221, 2010
doi:10.1093/carcin/bgp278
Advance Access publication November 5, 2009

Shorter telomere length in peripheral blood lymphocytes of workers exposed to polycyclic aromatic hydrocarbons

Sofia Pavanello*, Angela-C.Pesatori[1], Laura Dioni[1], Mirjam Hoxha[1], Valentina Bollati[1], Ewa Siwinska[2], Danuta Mielzyńska[3], Claudia Bolognesi[4], Pier-Alberto Bertazzi[1] and Andrea Baccarelli[1]

Occupational Health Section, Department of Environmental Medicine and Public Health, Università of Padova, Via Giustiniani 2, 35128 Padova, Italy, [1]Center of Molecular and Genetic Epidemiology, Department of Environmental and Occupational Health, Università di Milano and IRCCS Maggiore Policlinico Hospital, Mangiagalli and Regina Elena Foundation, Via San Barnaba 8, 20122 Milan, Italy, [2]Department of Genetic Toxicology, Institute of Occupational Medicine and Environmental Health, Koscielna 13, 41-200 Sosnowiec, Poland, [3]Institute for Ecology and Industrial area, 6 Kossutha street, 40833 Katowice, Poland and [4]Environmental Carcinogenesis Unit, National Cancer Research Institute, Largo Rosanna Benzi, 10, 16132 Genoa, Italy

*To whom correspondence should be addressed. Tel: +390498216600; Fax: +390498212542;
Email: sofia.pavanello@unipd.it

Shorter telomere length (TL) in peripheral blood lymphocytes (PBLs) is predictive of lung cancer risk. Polycyclic aromatic hydrocarbons (PAHs) are established lung carcinogens that cause chromosome instability. Whether PAH exposure and its molecular effects are linked with shorter TL has never been evaluated. In the present study, we investigated the effect of chronic exposure to PAHs on TL measured in PBLs of Polish male non-current smoking cokeoven workers and matched controls. PAH exposure and molecular effects were characterized using measures of internal dose (urinary 1-pyrenol), effective dose [*anti*-benzo[*a*]pyrene diolepoxide (*anti*-BPDE)–DNA adduct], genetic instability (micronuclei, MN) and DNA methylation [*p53* promoter and *Alu* and long interspersed nuclear element-1 (*LINE-1*) repetitive elements, as surrogate measures of global methylation] in PBLs. TL was measured by real-time polymerase chain reaction. Cokeoven workers were heavily exposed to PAHs (79% exceeded the urinary 1-pyrenol biological exposure index) and exhibited lower TL ($P = 0.038$) than controls, as well as higher levels of genetic and chromosomal alterations [i.e. *anti*-BPDE–DNA adduct and MN ($P < 0.0001$)] and epigenetic changes [i.e. *p53* gene-specific promoter and global methylation ($P \leq 0.001$)]. TL decreased with longer duration of work as cokeoven worker ($P = 0.039$) and in all subjects with higher levels of *anti*-BPDE–DNA adduct ($P = 0.042$), *p53* hypomethylation ($P = 0.005$) and MN ($P = 0.009$). In multivariate analysis, years of work in cokery ($P = 0.008$) and *p53* hypomethylation ($P = 0.001$) were the principal determinants of shorter TL. Our results indicate that shorter TL is associated with chronic PAH exposure. The interrelations with other genetic and epigenetic mechanisms in our data suggest that shorter TL could be a central event in PAH carcinogenesis.

Introduction

The identification of markers predictive of lung cancer and the assessment of their frequency in subjects at risk are a priority for preventive medicine (1). Genomic instability and/or epigenetic changes are hallmarks of cancer, including that of the lung. However, the mechanisms through which lung carcinogens produce such alterations are still largely unknown (2).

Abbreviations: *anti*-BPDE, *anti*-benzo[*a*]pyrene diolepoxide; BEI, biological exposure index; BP, benzo[*a*]pyrene; CV, coefficient of variation; *LINE-1*, long interspersed nuclear element-1; MN, micronuclei; PAH, polycyclic aromatic hydrocarbon; PBL, peripheral blood lymphocyte; PCR, polymerase chain reaction; TL, telomere length.

Telomere dysfunction due to excessive shortening is a key element leading to chromosomal instability and an early event in carcinogenesis (3). Telomeres are DNA repeat sequences (TTAGGG)*n* that, together with associated proteins, form a sheltering complex that caps chromosomal ends and protects their integrity (4). Due to limitation of DNA polymerase (telomerase), genetic stability is gradually lost as telomeres shorten with each round of cell division (4,5). In addition, telomere erosion can even be further accelerated by external stressors. Telomeres, as triple-guanine-containing sequences, are highly sensitive to damage by oxidative stress (6), alkylation (7) or ultraviolet irradiation (8). The resulting damaged telomeric bases by double-strand breaks and/or interference with replication fork can induce a reduction in telomere length (TL) (9).

In the present study, we investigated cokeoven workers with high exposure to polycyclic aromatic hydrocarbons (PAHs), a class of lung carcinogens that are also a widespread exposure in the general population due to tobacco smoking, diet and environmental pollution (10). Benzo[*a*]pyrene (BP), which has been used as a tracer of PAH mixtures, after its metabolic activation forms stable DNA adducts prevalently at guanine nucleotides [*anti*-benzo[*a*]pyrene diolepoxide (*anti*-BPDE)–DNA)] (11). This specific stereoselective binding may result in genetic mutations, including mutational 'hotspots' in the pulmonary *p53* gene, that are critical to BP tumorigenesis (12). *P53*, a key factor in the DNA damage-signalling pathway (13), is also frequently epigenetically altered in smoking/PAH-associated lung cancer (for a review see ref. 14). *P53* hypomethylation is associated with DNA double-strand breaks and chromosomal instability (15,16). Global DNA hypermethylation following *in vitro* chronic exposure to BP was observed in mouse embryonic fibroblasts and has been associated to genetic mutations as a consequence of genetic instability (17,18).

Peripheral blood lymphocytes (PBLs), which can be easily and non-invasively obtained from human subjects, have been shown to undergo several of the genetic and molecular modifications induced by PAHs in the lung. In particular, PAH(BP)–DNA adducts and DNA methylation levels in PBLs are correlated with levels in the lung (19–21). High levels of DNA adducts (22) and micronuclei (MN) (23), shorter TL (24,25) and *p53* hypomethylation (26) in PBLs have all been shown to predict lung cancer risk. DNA methylation changes of the *p53* promoter or of global methylation content [estimated in *Alu* and long interspersed nuclear element-1 (*LINE*-1) repeats] detectable in PBLs have been related to *anti*-BPDE–DNA adduct and MN formations in subjects exposed to high levels of PAHs (27). Additionally, high PAH exposure through metabolic activation—via aldo-keto reductase and/or manganese superoxide dismutase (MnSOD) (28)—produces oxidative stress (reactive oxygen species) that forms high levels of oxidized guanine in PBLs (29) and can represent another source of damage to telomeres. However, no studies have addressed whether PAH(BP) exposure alters telomeres length in PBLs from individuals at high risk of lung cancer due to high levels of PAH exposure.

In this study, we examine the effect of PAH(BP) exposure on TL in PBLs of non-current smoking Polish male cokeoven workers and matched controls to identify novel molecular pathways, which may mediate lung cancer risk. This study population has been characterized with measures of internal dose (urinary 1-pyrenol), effective biological dose (*anti*-BPDE–DNA adduct), early biological effects (MN) and DNA methylation (*p53* promoter methylation and global methylation estimated in *Alu* and *LINE-1* repeats). We hypothesized that individuals with high effective biological dose, as reflected in high levels of *anti*-BPDE–DNA adduct, would show higher genomic instability measured by TL and MN formation. Since DNA methylation modifications, mainly global DNA and *p53* promoter methylation, have been proposed as additional mechanisms associated with

genetic instability induced by PAHs (27), we also evaluated whether [PAH(BP)]-induced TL was in turn associated with these epigenetic changes in PBLs.

Materials and methods

Study design

The study subjects ($n = 92$) were all males non-current smokers and included 48 cokeoven workers from three polish cokeries and 44 controls matched by gender and ethnicity, who had been part of a group of 94 study subjects examined in our previous work (30) for which DNA was still available (enrolment from January through May 2006). Cokeoven workers all had tasks (i.e. charging, coking and pushing operations at the cokeoven battery section) involving exposure to high levels of PAHs. We excluded individuals whose work involved exposure to benzene (i.e. workers in by-product operations). Controls were clerks recruited during their periodic check-ups at the Institute of Occupational Medicine and Environmental Health in Sosnowiec (Poland). All subjects were non-current smokers, defined as either never-smokers or former smokers who had stopped smoking at least 1 year before sample collection. Urine analysis of nicotine and its metabolites (31) confirmed that all subjects were non-exposed to tobacco smoke as they had urine nicotine concentrations <0.01 mg/mmol creatinine.

PAH exposure levels were assessed by measuring 1-pyrenol in a urine sample (50 ml) collected from each of the workers at the end of their work shift (after at least 3 consecutive working days) and in the late afternoon from controls. At the same time as urine was collected, blood samples were taken and placed in ethylenediaminetetraacetic acid-containing tubes (20 ml) and evacuated heparin-containing tubes (10 ml) for adduct and MN analysis, as described previously (30,32). At the Institute of Occupational Medicine and Environmental Health in Sosnowiec: (i) PBL cultures for MN detection were prepared and MN analyses performed; (ii) PBLs for adduct analyses were isolated in Ficoll separating solution (Seromed, Berlin, Germany) within 4 h after blood collection and kept frozen at −80°C until they were sent to the Department of Environmental Medicine and Public Health in Padova (Italy), where DNA was extracted. Data on other possible elevated non-occupational PAH exposure (diet and environment) and consumption of fruit and vegetables were collected by means of a questionnaire, as described previously (33). Subjects with high dietary intake of PAHs were those who reported consumption of PAH-rich meals (charcoaled meat and pizza) more than once a week; subjects with indoor exposure to PAHs were those who used wood or coal heating at home; subjects with environmental exposure to PAHs were those identified by a high index based on the location of their residence that was based on intense local traffic and presence of industries in the area. This study was reviewed by the appropriate Ethics Committee of the Institute of Occupational Medicine and Environmental Health in Sosnowiec. All participants gave their written informed consent.

Analysis of exposure to PAHs

1-Pyrenol in urine samples was determined by high-performance liquid chromatography–fluorescence as described previously (34), following the original method of Jongeneelen (35). 1-Pyrenol levels in each urine sample were expressed per mole urinary creatinine, determined according to the Boehringer Mannheim (Mannheim, Germany) colorimetric test, based on the reaction of creatinine with picrate in an alkaline medium. *Anti*-BPDE–DNA adduct formation was detected, after DNA isolation with a Promega Wizard genomic DNA purification kit (Promega, Milano, Italy), by high-performance liquid chromatography/fluorescence analysis of BP-tetrol-I-1 (*r*-7,*c*-10,*t*-8,*t*-9-tetrahydroxy-7,8,9,10-tetrahydro-benzo[*a*]pyrene) released after acid hydrolysis of DNA samples, as described previously (33). The detection threshold of BP-tetrol-I-1 was 0.25 pg (signal/noise > 3) so that, with 100 μg DNA, this assay can measure 0.25 adducts per 10^8 nucleotides (1 fmol/μg DNA = 30 adducts per 10^8 nucleotides) (33). The mean coefficient of variation (CV) for analyses of standard curve repeated five times on five different days was 10%. The highest CV value was 5.70% for those samples ($n = 8$) with >200 μg DNA repeated twice. MN analysis was performed on coded slides scored by light microscopy at ×400 magnification, as described previously (30,32). To exclude artefacts, the identification of MN was confirmed at ×1000 magnification in 10% of samples. The scoring of bi-, tri- and tetra-nucleate cells and MN was done and the cytokinesis block proliferation index was calculated as being equal in both groups ($P = 0.599$). MN levels were verified on 20 slides (two slides per subject) previously scored at the Institute of Occupational Medicine and Environmental Health in Sosnowiec by blind scoring at the Environmental Carcinogenesis Unit, Istituto Nazionale per La Ricerca sul Cancro (IST, Genova, Italy). The results of this intercalibration exercise demonstrated a good correlation between Mn levels scored in the two laboratories (analysis of variance, $F = 20.9, P < 0.001$). Intraclass correlation coefficient was 0.79 (95% confidence interval = 0.55–0.91).

Analysis of DNA methylation

DNA methylation was quantified using bisulphite-polymerase chain reaction (PCR) and pyrosequencing (27,36). In brief, the samples were bisulphite treated by EZ-96 DNA Methylation-Gold Kit™ (Zymo Research, Orange, CA) and PCR amplified. Analyses of DNA methylation in *Alu* and *LINE-1* repetitive element and *p53* gene were performed using previously published methods (27,36). For each reaction, a 50 μl PCR was conducted in 50 μl of GoTaq Green Master mix (Promega, Madison, WI), 1 pmol of the forward primer, 1 pmol of the reverse primer, 50 ng of bisulphite-treated genomic DNA and water. One of the forward or reverse primers was biotin labelled depending on the proximity to CG sites to be quantified and used to purify the final PCR product using Sepharose beads. The PCR product was bound to Streptavidin Sepharose HP (Amersham Biosciences, Uppsala, Sweden) and the Sepharose beads containing the immobilized PCR product were purified using the Pyrosequencing Vacuum Prep Tool (Pyrosequencing, Westborough, MA). Methylation quantification was performed using the provided software. The degree of methylation was expressed for each considered DNA locus as percentage of methylated cytosines divided by the sum of methylated and unmethylated cytosines. We used built-in controls to verify bisulphite conversion efficiency. Every sample was tested three times for each marker to confirm the reproducibility of our results. CV obtained from duplicate runs was 0.7, 1.6 and 1.8% for *LINE-1*, *Alu* and *p53*, respectively.

TL measurement by quantitative PCR

TL was measured in PBL DNA using the real-time quantitative PCR method developed by Cawthon (37). This method measures the relative TL in genomic DNA by determining the ratio of telomere repeat copy number (T) to single copy gene (S) copy number (T:S ratio) in experimental samples relative to a reference pool sample (38). The single copy gene used in this study was human β-globin (*hbg*). The analysis was conducted as described previously (39), with some minor modifications, mainly regarding the use of a MICROLAB STARlet Robot (Hamilton Life Science Robotics; Hamilton Bonaduz AG, Bonaduz, Switzerland) for transferring in a 384-well plate a reduced reaction mix (5 μl) with 2 μl (2 ng/μl) DNA and an 'eight-point standard curve'. This standard curve was generated from a serially diluted DNA pool (obtained from 20 DNA samples randomly selected from the samples tested in the present study), ranging from 30 to 0.234 ng in each plate, so that relative quantities of T and S (in nanograms) could be determined from it. The average of the three T measurements was divided by the average of the three S measurements to calculate the average T:S ratio.

As we already described (39), to increase PCR reproducibility (40) for every reaction, we prepared a 25 μl mixture of DNA sample (2 ng/μl) containing *Escherichia coli* DNA (15 ng/μl) used as a DNA carrier; these mixtures were heated to 96°C for 10 min and cooled to room temperature. PCR primer sets for telomere and *hbg* and PCR mix composition were described previously (39). The PCR runs were performed in triplicate on a 7900HT Fast Real Time PCR System (Applied Biosystems, Foster City, CA). The thermal cycling profile for both amplicons began with a 95°C incubation for 3 min to activate the hot-start iTaq DNA polymerase. The telomere PCR conditions were 25 cycles at 95°C for 15 s and 54°C for 60 s. The *hgb* PCR conditions were 35 cycles at 95°C for 15 s, 58°C for 1 s and 72°C for 15 s. After PCR amplification, the specificity of the product was confirmed by dissociation curve analysis. To test the reproducibility of TL measurements, we amplified telomere (T) and hbg (S) in 15 samples that were replicated three times on each of three different days. The within-sample CV for the average T:S ratio over the 3 consecutive days was 8.7%, which was similar to the CV reported for the original protocol (37).

Statistical analysis

Statistical comparisons were made between various groups using the non-parametric Mann–Whitney *U*-test or the chi-square test. Univariate linear regression analysis was used to test the influence of years of work, age, PAH internal dose (urinary 1-pyrenol), *anti*-BPDE–DNA adduct and *p53* and global DNA methylation levels (independent variables) on TL (dependent variable) of cokeoven workers. Multivariate linear regression analysis was used to assess the influence of age, years of work, urinary 1-pyrenol, *anti*-BPDE–DNA adduct and *p53* and global DNA methylation levels (independent variables, all fitted in the same model) on TL (dependent variable) of cokeoven workers and the entire study population. All statistical tests were two sided and were performed with Statsdirect Statistical software (Ashwell, Herts, UK).

Results

The study population of 48 cokeoven workers and 44 controls showed in Table I were all non-current smoking males, who were in the same age range, similar in dietary behaviour (PAH-rich meals and daily consumption of fruit and vegetables), as well as for their

Table I. Characteristics of Polish male cokeoven workers and non-exposed controls

	Cokeoven workers	Controls	*P*-values	
Number of subjects	48	44		
Age (years)				
Median (range)	36 (20–59)	38 (21–58)	0.81	$Z = 0.25$
Diet[a]	4	1	0.22	
Fruit and vegetables[b]	27	21	0.44	
Environmental exposure[c]	16	15	0.99	
Indoor exposure[d]	25	18	0.31	
Nicotine plus metabolites (mg/mmol creatinine)				
n (%) positive subjects[e]	0 (0)	0 (0)		
Years of work in the cokery (years)				
Median (range)	11 (1–40)	0 (0)		
PAH exposure (1-pyrenol μmol/mol creatinine)[f]				
Median (range)	3.09 (0.41–7.48)	0.09 (0.01–0.40)	<0.001	$Z = 8.25$
n (%) with urinary post-shift excretion of 1-pyrenol ≥2.28 μmol/mol creatinine	38 (79)	0 (0)	<0.001	
Genotoxic alterations				
Anti-BPDE–DNA (adducts per 10^8 nucleotides)				
Median (range)[g]	5.06 (0.90–12.24)	0.21 (0.125–5.56)	<0.0001	$Z = 8.13$
MN per 1000 binucleate cells				
Median (range)	4 (1–11)	1 (0–4)	<0.0001	$Z = 6.47$
Epigenetic alterations				
Global				
LINE-1 (%mC)[h]	80.3 (56.9–83.7)	75.7 (55.7–85.7)	<0.0001	$Z = 4.42$
Alu (%mC)	23.6 (21.8–24.5)	22.9 (20.6–24.4)	0.001	$Z = 3.29$
Gene specific				
p53 (%mC)[h]	11.8 (5.63–25.1)	18.6 (6.85–46.2)	0.0004	$Z = 3.55$
Relative TL				
Median (range)	0.99 (0.31–3.00)	1.20 (0.43–2.12)	0.038	$Z = 2.08$

Statistical comparison: Mann–Whitney *U*-test between cokeoven workers versus controls; age, 1-pyrenol μmol/mol creatinine, *anti*-BPDE–DNA (adducts per 10^8 nucleotides) and MN (MN per 1000 binucleate cells). Chi-square test, cokeoven workers versus controls: diet, fruit and vegetables, environmental exposure, indoor exposure and 1-pyrenol ≥2.28 μmol/mol creatinine.
[a]Number of subjects with charcoaled meat consumption more than or equal to once a week.
[b]Number of subjects with daily consumption of fruit or vegetables.
[c]Number of subjects with high environmental exposure from residence in town, intense traffic and presence of industries near home (see Materials and Methods).
[d]Number of subjects with wood- or coal-based heating at home.
[e]Subjects with values higher than threshold limit of assay (0.01 mg/mmol creatinine).
[f]PAH exposure evaluated by urinary excretion of 1-pyrenol.
[g]A value of 0.125 adducts per 10^8 nucleotides was assigned to subjects with non-detectable adducts.
[h]Methylated cytosine percentage.

environmental or indoor exposure to PAHs (Table I). Cokeoven workers had a median of 11 working years and were heavily exposed to PAHs, as their urinary 1-pyrenol concentrations were ∼30 times higher than in controls and in 79% of the workers versus 0% of the controls urinary 1-pyrenol exceeded the biological exposure index (BEI) of 2.28 μmol/mol creatinine proposed by Jongeneelen (35). Cokeoven workers exhibited significantly lower TL in PBLs compared with controls, as well as significantly higher levels of genotoxic alterations as reflected in higher *anti*-BPDE–DNA adduct and MN. DNA methylation levels (global and *p53* promoter methylation changes) were also significantly different between workers and controls. Decrease in TL in PBLs of cokeoven workers was significantly associated, in univariate linear regression analysis (data not showed), with years of work as cokeoven workers ($P = 0.039$), but not with age, urinary 1-pyrenol and *anti*-BPDE–DNA adduct. Table II shows the distribution of TL in the whole study population ($n = 92$) according to increasing quartile levels of 1-pyrenol, *anti*-BPDE–DNA adduct and DNA methylation. Individuals with higher *anti*-BPDE–DNA adduct and *p53* hypomethylation revealed significantly shorter TL. Likewise, frequency of subjects with TL >1.08 (TL median value of all subjects) significantly decreased with increasing *anti*-BPDE–DNA adduct and *p53* hypomethylation levels. These associations did not reach statistical significance when evaluated separately in the groups of 48 exposed and 44 non-exposed workers.

Linear multiple regression analysis (Table III) conducted on data from the entire study population ($n = 92$) showed that years of work and *p53* hypomethylation were the only independent significant determinants of lower TL. In cokeoven workers ($n = 48$), the decrease in TL was significantly associated with years of work but not with age, urinary 1-pyrenol and *anti*-BPDE–DNA adduct, *p53* promoter and global (*LINE-1* and *Alu*) DNA methylation. Linear multiple regression analysis (data not showed) showed that in controls ($n = 44$), there was a negative association of TL with *p53* promoter hypomethylation ($P = 0.009$), but not with age ($P = 0.489$), urinary 1-pyrenol ($P = 0.753$) and *anti*-BPDE–DNA adduct ($P = 0.954$) and global (*LINE-1* and *Alu*, $P = 852$ and $P = 0.560$) DNA methylation. However, the overall test of significance (analysis of variance) did not reach statistical significance ($F = 1.173$ and $P = 0.343$).

Table IV shows MN levels categorized according to the median value of TL (≤1.08 versus >1.08 TL) in PBLs of workers, controls and all subjects. Higher MN levels were found in subjects with shorter TL. In addition, the percentage of subjects with MN >2 (median value of all subjects) was significantly higher in workers and all subjects with shorter TL than in those with longer telomeres.

Discussion

In this study, we found that cokeoven workers with very high levels of PAH exposure, as indicated by measures of internal dose (urinary 1-pyrenol) and effective biological dose (*anti*-BPDE–DNA adduct), had significant shorter TL in PBLs compared with controls. Cokeoven workers also exhibited higher genomic instability, as reflected in MN number, and DNA methylation alterations both in the global content and in the *p53* promoter. Moreover, telomeres significantly shortened

Table II. Distribution of TL ($N = 92$) according to increasing quartile levels of 1-pyrenol, *anti*-BPDE–DNA adduct and DNA methylation

	Subjects	TL		Chi^2 for trend; P^b
	n	Median (range)[a]	*n* (%) subjects with TL >1.08 (T:S)	
PAH exposure				
1-Pyrenol (μmol/mol creatinine)				
≤0.10	23	1.21 (0.43–2.12)	15 (65)	
>0.10 ≤ 0.58	23	1.17 (0.54–2.03)	14 (61)	
>0.58 ≤ 3.35	23	0.99 (0.42–3.01)	10 (43)	
>3.35	23	1.01 (0.31–1.79)	8 (35)	Chi^2 = 5.70; $P = 0.13$
Biological effective dose				
Anti-BPDE–DNA (adducts per 10^8 nucleotides)				
≤0.125	28	1.10 (0.54–2.03)	16 (57)	
>0.125 ≤ 2.47	18	1.43 (0.43–2.12)	13 (72)	
>2.47 ≤ 5.16	23	0.98 (0.40–1.52)	7 (30)	
>5.16	23	1.07 (0.31–3.01)*	11 (48)	Chi^2 = 7.65; $P = 0.054$
Global methylation (%mC)				
LINE-1				
≤75.0	23	1.13 (0.58–2.00)	15 (65)	
>75.0 ≤ 79.6	23	1.06 (0.43–2.03)	10 (44)	
>79.6 ≤ 80.8	23	1.10 (0.31–1.79)	13 (57)	
>80.8	22	0.99 (0.43–3.01)	9 (41)	Chi^2 = 3.54; $P = 0.32$
Alu				
≤22.7	24	1.27 (0.59–2.03)	15 (63)	
>22.7 ≤ 23.4	23	1.07 (0.32–1.96)	11 (48)	
>23.4 ≤ 23.8	21	0.99 (0.42–3.01)	7 (33)	
>23.8	23	1.18 (0.43–2.12)	13 (57)	Chi^2 = 4.26; $P = 0.24$
Gene-specific methylation (%mC)				
p53				
≤9.98	21	1.07 (0.43–1.95)	10 (47)	
>9.98 ≤ 13.95	24	0.98 (0.31–1.79)	8 (33)	
>13.95 ≤ 20.4	22	1.01 (0.40–1.96)	10 (46)	
>20.4	22	1.44 (0.43–2.12)**	17 (77)	Chi^2 = 9.43; $P = 0.02$

[a]Kruskal–Wallis test: *anti*-BPDE–DNA (adducts per 10^8 nucleotides).*$P = 0.042$ and *p53* methylation **$P = 0.005$.
[b]Chi-square (Chi^2) test for trend was performed on *n* (%)subjects with TL >1.08 (median value of all workers)

Table III. Linear multiple regression analysis of age, years of work, 1-pyrenol, *anti*-BPDE–DNA adduct and DNA methylation levels on TL in all subjects and cokeoven workers

Subjects	Age (years)	Years of work in the cokery (years)	1-Pyrenol (μmol/mol creatinine)	Adduct	DNA methylation		
				Anti-BPDE per 10^8 bases	*p53* (%mC)	*LINE-1* (%mC)	*Alu* (%mC)
All ($n = 92$)							
b[a]	0.006	−0.016	−0.002	0.037	0.020	−0.0019	0.009
SE(b)	0.005	0.006	0.345	0.025	0.006	0.009	0.054
r^2 (%)	1.59	8.53	0.004	2.75	12.3	0.06	0.03
P[c]	0.259	**0.008**	0.954	0.137	**0.001**	0.828	0.868
Cokeoven workers ($n = 48$)[b]							
b[a]	0.013	−0.027	0.013	0.032	0.022	−0.014	0.245
SE(b)	0.011	0.087	0.042	0.031	0.014	0.018	0.127
r^2 (%)	3.31	13.7	0.25	2.76	5.76	1.57	8.96
P[c]	0.260	**0.018**	0.757	0.304	0.130	0.441	0.061

Overall test of significance (analysis of variance) for all and cokeoven workers: $F = 3.56$, $P = 0.002$ and $F = 2.20$, $P = 0.054$.
[a]Coefficient of regression (b), standard error (SE(b)) and partial explained variance (r^2) were estimated for each term included in the model.
[b]TL as ln T/S.
[c]Significant *P* values are bold.

with years of work in exposed workers. Shorter telomeres in PBLs have been shown to predict the risk of lung and other cancers (24,25). Our findings extend these observations by demonstrating that shorter telomeres may also occur in healthy subjects who are occupationally exposed to high levels (high BEI) of carcinogenic PAH(BP) and are, therefore, at higher risk for lung cancer. In our study, 79% of the PAH-exposed workers exceeded the BEI proposed by Jongeneelen (35) for urinary 1-pyrenol. At this value (2.28 μmol/mol creatinine), corresponding to the post-shift excretion value with environmental exposure to the airborne threshold limit value of coal tar pitch volatiles [i.e. 0.2 mg/m^3 of 'benzene soluble matter', American Conference of Governmental Industrial Health (41)], cokeoven workers have been shown a 30% increased risk of lung cancer (35). Since telomeres play a key role in the maintenance of chromosome integrity and stability, our results indicate that decrease in TL of PBLs may represent an additional indicator of genetic instability induced by chronically high PAH exposure and confirm, with this new marker, that workers are at higher lung cancer risk than controls.

Table IV. MN levels according to TL in PBLs of study population

	MN			
	TL	*N*	Median (range)	*n* (%) with MN >2[a]
Cokeoven workers	>1.08	19	2 (1–11)	8 (42)
	≤1.08	29	4 (1–9)	22 (76)[c,*]
Controls	>1.08	28	0 (0–4)	3 (11)
	≤1.08	16	1 (0–3)	2 (13)
All	>1.08	46	1 (0–11)	11 (24)
	≤1.08	46	3 (0–9)[b,**]	24 (52)[c,**]

[a]Number and frequency (%) of subject with MN >2 median levels of MN.
[b]Mann–Whitney *U*-test: $Z = 2.59$, $^{**}P = 0.009$.
[c]Chi-square test: $Chi^2 = 5.58$; $^{*}P = 0.018$ and $Chi^2 = 7.79$; $^{**}P = 0.005$.

Loss of telomere sequence in circulating blood lymphocytes has been related with aging (42) and cumulative oxidative stress resulting from environmental exposures, including smoking (43,44), diet (43), life stress (45) and chronic inflammation (44). The chances that the association with shorter TL could depend on factors other than PAH exposure were minimized because no subjects were affected by chronic diseases, all study subjects were non-current smokers and exposed individuals were similar to controls for age, gender and ethnicity. In addition, we evaluated several other potential confounding factors, including dietary PAHs, indoor PAH exposure and environmental PAH exposure, that showed no differences between the two groups. However, we cannot exclude that different socio-economic status or different working conditions might have contributed, along with PAH exposure (for which we have supplied a measure of internal dose and biologically effective dose, i.e. the specific promutagenic *anti*-BPDE–DNA), in the reduction of TL that we observed in PBLs of cokeoven workers. Epel *et al.* (45) showed that individuals with high levels of psychological stress exhibited shorter telomeres. We cannot exclude that life stress related with differences in socio-economic and/or working conditions contributed to the decrease in TL of cokeoven workers. In our study, we did not find a statistically significant association between age and TL in PBLs. This lack of association is in line with other studies that used PBLs from healthy subjects (24,44,46), whereas investigations that used unfractionated blood leucocytes more consistently showed an age-related decrease in TL (39,42,47). The difference between studies using PBLs and those using unfractionated leucocytes could be ascribed to the longer half-life of blood lymphocytes, which therefore have less mitotic cycles over time compared with granulocytes, the other major white blood cell subtype that accounts for ~50 to 80% of circulating leucocytes. Moreover, loss in TL is most pronounced in childhood with a more gradual attrition in midlife (42). We have analyzed TL in subjects with a limited age span (20–59 years), which also limited our capacity to evaluate the association between age and TL. In addition, study subjects of the same age generally display a large variation in TL. Thus, a wider age range, as well as a larger sample size, might be necessary to detect a significant correlation between TL and age among both exposed and control subjects.

In our study, we found that telomeres length decreases in association with increasing levels of *anti*-BPDE–DNA adduct. This finding suggests that adduct generation may have a direct role in shortening TL. BP is a strong clastogen, primarily through the action of one of the four metabolically generated optical BPDE isomers, the (+)-*anti*-BPDE. (+)-*Anti*-BPDE shows stereoselective binding to the exocyclic N2 of guanine nucleotides, which is also considered the primary essential damaging event in BP carcinogenicity (48,49). Telomeres, as triple-G-containing sequences, may represent a sensitive target for damage by (+)-*anti*-BPDE. Double-strand breaks and interference with replication fork by bulky-damaged telomeric bases may directly induce telomere shortening (8,9). However, other genotoxic coexposure or other mode of action of PAH(BP) on telomere shortening may be hypothesized. Although PAHs are still considered the primary genotoxic carcinogens produced by coal combustion emissions (for a comprehensive review see ref. 10 and references therein), the presence of some metals in coke emissions, or rather reactive oxygen species, produced through PAH (28) or metal (50) metabolism, might have contributed along with BP exposure in determining the lower TL observed in our study. As an additional mechanism, alterations of proteins in the telomere-sheltering complex, induced by PAHs and/or by *anti*-BPDE adduct itself, could be considered as an alternative event accounting for shorter TL.

Our results also suggest a relevant role of *p53* promoter hypomethylation, which has been previously shown to induce activation of *p53* expression (51) in PAH-related attrition of PBL TL. *P53* has a key role in cellular responses to telomere dysfunction (13) and may be activated concurrently with telomere shortening and the associated genomic instability, leading to growth arrest and/or apoptosis (13). The *p53* hypomethylation we found in PBLs can be appreciated in the light of the findings of a previous study on smoking lung cancer patients showing that *p53* hypomethylation in PBLs was associated with lung cancer risk (26). Our finding adds further evidence indicating molecular mechanisms that may contribute to increase lung cancer risk in individuals occupationally exposed to high levels (>BEI) of carcinogenic PAH(BP).

We found that all study subjects and workers with shorter telomeres had significantly higher MN levels, which represent an efficient measure of chromosomal breakage and loss. Our data suggest a role for telomere erosion in MN formation, consistent with the known relation between telomere integrity and chromosomal protection. That kinetochore and spindle protein may be altered by *anti*-BPDE itself and/or PAHs could be an alternate hypothesis to explain increase in MN frequencies. Moreover, the concordant relationship between shorter telomeres and MN formation, both indicative of chromosome instability, suggests that these findings, taken together, may indicate higher cancer risk of the subjects carrying such alterations in PBLs.

The present study was carefully designed to minimize potential confounding and increase the capability to reveal PAH effects by selecting study subjects who were all non-current smoking males living in the same residential area. In addition, our study had reliable measurements of PAH(BP) internal and biological effective doses (27,30,34). By evaluating several other non-occupational potential sources of PAH exposure, we were able to confirm the specific association of genetic and epigenetic changes with occupational PAH exposure, thus excluding effects from other possible non-occupational genotoxic exposures. Finally, the results of this study appear to be biologically plausible and the direction of the effects is consistent with the available literature data on telomere shortening mechanisms.

In conclusion, cokeoven workers exposed to high levels of PAHs, in addition to higher levels of genotoxic alterations (i.e. *anti*-BPDE–DNA adduct and MN), exhibited significant shorter TL in PBLs. Subjects with shorter TL showed higher MN, a finding suggestive of chromosome instability. TL was independently associated with years worked as a cokeoven worker and to DNA methylation in the *p53* gene. These features were found in PBLs of subjects chronically exposed to PAHs, thus suggesting that these events could be determined to identify subjects at high cancer risk. However, whether shorter TL mediates the risk of lung cancer determined by high-level PAH exposure should be determined in future investigations.

Funding

CARIPLO Foundation (2007-5469); Italian Association for Research on Cancer 2008; Italian Ministry of University and Research (PRIN2007-2S2HT8).

Acknowledgements

Conflict of Interest statement: None declared.

References

1. Mulshine,J.L. *et al*. (2005) Clinical practice. Lung cancer screening. *N. Engl. J. Med.*, **352**, 2714–2720.
2. McKenna,E.S. *et al*. (2009) Epigenetics and cancer without genomic instability. *Cell Cycle*, **8**, 23–26.
3. Feldser,D.M. *et al*. (2003) Telomere dysfunction and the initiation of genome instability. *Nat. Rev. Cancer*, **3**, 623–627.
4. Chan,S.R. *et al*. (2004) Telomere and telomerase. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.*, **359**, 109–121.
5. Harley,C.B. *et al*. (1990) Telomeres shorten during ageing of human fibroblasts. *Nature*, **345**, 458–460.
6. Henle,E.S. *et al*. (1999) Sequence-specific DNA cleavage by Fe2+-mediated fenton reactions has possible biological implications. *J. Biol. Chem.*, **274**, 962–971.
7. Petersen,S. *et al*. (1998) Preferential accumulation of single-stranded regions in telomeres of human fibroblasts. *Exp. Cell. Res.*, **239**, 152–160.
8. Oikawa,S. *et al*. (2001) Site-specific DNA damage at the GGG sequence by UVA involves acceleration of telomere shortening. *Biochemistry*, **40**, 4763–4768.
9. von Zglinicki,T. (2002) Oxidative stress shortens telomeres. *Trends Biochem. Sci.*, **27**, 339–344.
10. Lewtas,J. (2007) Air pollution combustion emissions, characterization of causative agents and mechanisms associated with cancer, reproductive, and cardiovascular effects. *Mutat. Res.*, **636**, 95–133.
11. Baird,W.M. *et al*. (2005) Carcinogenic polycyclic aromatic hydrocarbon-DNA adducts and mechanism of action. *Environ. Mol. Mutagen.*, **45**, 106–114.
12. Denissenko,M.F. *et al*. (1997) Cytosine methylation determines hot spots of DNA damage in the human p53 gene. *Proc. Natl Acad. Sci. USA*, **94**, 3893–3898.
13. Chin,L. *et al*. (1999) p53 deficiency rescues the adverse effects of telomere loss and cooperates with telomere dysfunction to accelerate carcinogenesis. *Cell*, **97**, 527–538.
14. Risch,A. *et al*. (2008) Lung cancer epigenetics and genetics. *Int. J. Cancer*, **123**, 1–7.
15. Pogribny,I.P. *et al*. (1995) Breaks in genomic DNA and within the p53 gene are associated with hypomethylation in livers of folate/methyl-deficient rats. *Cancer Res.*, **55**, 1894–1901.
16. Kim,Y.I. *et al*. (1997) Folate deficiency in rats induces DNA strand breaks and hypomethylation within the p53 tumor suppressor gene. *Am. J. Clin. Nutr.*, **65**, 46–52.
17. Yauk,C.L. *et al*. (2008) Tandem repeat mutation, global DNA methylation, and regulation of DNA methyltransferases in cultured mouse embryonic fibroblast cells chronically exposed to chemicals with different modes of action. *Environ. Mol. Mutagen.*, **49**, 26–35.
18. Yauk,C. *et al*. (2008) Germ-line mutations. DNA damage, and global hypermethylation in mice exposed to particulate air pollution in an urban/industrial location. *Proc. Natl Acad. Sci. USA*, **105**, 605–610.
19. Wiencke,J.K. *et al*. (1995) Correlation of DNA adducts in blood mononuclear cells with tobacco carcinogen-induced damage in human lung. *Cancer Res.*, **55**, 4910–4914.
20. Gyorffy,E. *et al*. (2004) DNA adducts in tumour, normal peripheral lung and bronchus, and peripheral blood lymphocytes from smoking and non-smoking lung cancer patients, correlations between tissues and detection by 32P-postlabelling and immunoassay. *Carcinogenesis*, **25**, 1201–1209.
21. Russo,A.L. *et al*. (2005) Differential DNA hypermethylation of critical genes mediates the stage-specific tobacco smoke-induced neoplastic progression of lung cancer. *Clin. Cancer Res.*, **11**, 2466–2470.
22. Veglia,F. *et al*. (2008) DNA adducts and cancer risk in prospective studies, a pooled analysis and a meta-analysis. *Carcinogenesis*, **29**, 932–936.
23. Bonassi,S. *et al*. (2007) An increased micronucleus frequency in peripheral blood lymphocytes predicts the risk of cancer in humans. *Carcinogenesis*, **28**, 625–631.
24. Wu,X. *et al*. (2003) Telomere dysfunction, a potential cancer predisposition factor. *J. Natl Cancer Inst.*, **95**, 1211–1218.
25. Hosgood,H.D.3rd *et al*. (2009) Genetic variation in telomere maintenance genes, telomere length, and lung cancer susceptibility. *Lung Cancer*, **66**, 157–161.
26. Woodson,K. *et al*. (2001) Hypomethylation of p53 in peripheral blood DNA is associated with the development of lung cancer. *Cancer Epidemiol. Biomarkers. Prev.*, **10**, 69–74.
27. Pavanello,S. *et al*. (2009) Global and gene-specific promoter methylation changes are related to *anti*-B[a]PDE-DNA adduct levels and influence micronuclei levels in polycyclic aromatic hydrocarbon-exposed individuals. *Int. J. Cancer*, **125**, 1692–1697.
28. Palackal,N.T. *et al*. (2001) The ubiquitous aldehyde reductase (AKR1A1) oxidizes proximate carcinogen trans-dihydrodiols to o-quinones, potential role in polycyclic aromatic hydrocarbon activation. *Biochemistry*, **40**, 10901–10910.
29. Liu,A.L. *et al*. (2006) Elevated levels of urinary 8-hydroxy-2-deoxyguanosine, lymphocytic micronuclei, and serum glutathione S-transferase in workers exposed to coke oven emissions. *Environ. Health Perspect.*, **114**, 673–677.
30. Pavanello,S. *et al*. (2008) Micronuclei related to *anti*-B[a]PDE-DNA adduct in peripheral blood lymphocytes of heavily polycyclic aromatic hydrocarbon-exposed nonsmoking cokeoven workers and controls. *Cancer Epidemiol. Biomarkers Prev.*, **17**, 2795–2799.
31. Pavanello,S. *et al*. (2007) Mutagenic activity of overnight urine from healthy non-smoking subjects. *Environ. Mol. Mutagen.*, **48**, 143–150.
32. Siwinska,E. *et al*. (2004) Association between urinary 1-hydroxypyrene and genotoxic effects in coke oven workers. *Occup. Environ. Med.*, **61**, e10.
33. Pavanello,S. *et al*. (2006) Determinants of *anti*-benzo[a]pyrene diol epoxide-DNA adduct formation in lymphomonocytes of the general population. *Mutat. Res.*, **611**, 54–63.
34. Pavanello,S. *et al*. (2005) Reduced nucleotide excision repair and GSTM1-null genotypes influence *anti*-B[a]PDE-DNA adduct levels in mononuclear white blood cells of highly PAH-exposed coke oven workers. *Carcinogenesis*, **26**, 169–175.
35. Jongeneelen,F.J. (1992) Biological exposure limit for occupational exposure to coal tar pitch volatiles at coke ovens. *Int. Arch. Occup. Environ. Health*, **63**, 511–516.
36. Yang,A.S. *et al*. (2004) A simple method for estimating global DNA methylation using bisulfite PCR of repetitive DNA elements. *Nucleic Acids Res.*, **32**, e38.
37. Cawthon,R.M. (2002) Telomere measurement by quantitative PCR. *Nucleic Acids Res.*, **30**, e47.
38. Farzaneh-Far,R. *et al*. (2008) Prognostic value of leukocyte telomere length in patients with stable coronary artery disease, data from the Heart and Soul Study. *Arterioscler. Thromb. Vasc. Biol.*, **28**, 1379–1384.
39. Hou,L. *et al*. (2009)) Telomere length and gastric cancer risk. *Cancer Epidemiol. Biomarkers Prev.*, **18**, 3103–3109.
40. Nordfjäll,K. *et al*. (2007) hTERT (-1327)T/C polymorphism is not associated with age-related telomere attrition in peripheral blood. *Biochem. Biophys. Res. Commun.*, **358**, 215–218.
41. American Conference of Governmental Industrial Health. (2008) *TLVs and BEIs*. Signature publications, Cincinnati, OH.
42. Frenck,R.W.Jr *et al*. (1998) The rate of telomere sequence loss in human leukocytes varies with age. *Proc. Natl Acad. Sci. USA*, **95**, 5607–5610.
43. Valdes,A.M. *et al*. (2005) Obesity, cigarette smoking, and telomere length in women. *Lancet*, **366**, 662–664.
44. Morlá,M. *et al*. (2006) Telomere shortening in smokers with and without COPD. *Eur. Respir. J.*, **27**, 525–528.
45. Epel,E.S. *et al*. (2004) Accelerated telomere shortening in response to life stress. *Proc. Natl Acad. Sci. USA*, **101**, 17312–17315.
46. Broberg,K. *et al*. (2005) Constitutional short telomeres are strong genetic susceptibility markers for bladder cancer. *Carcinogenesis*, **26**, 1263–1271.
47. Hoxha,M. *et al*. (2009) Association between leukocyte telomere shortening and exposure to traffic pollution, a cross-sectional study on traffic officers and indoor office workers. *Environ. Health*, **8**, 41.
48. Huberman,E. *et al*. (1976) Identification of mutagenic metabolites of benzo(a)pyrene in mammalian cells. *Proc. Natl Acad. Sci. USA*, **73**, 607–611.
49. Kapitulnik,J. *et al*. (1978) Tumorigenicity studies with diol-epoxides of benzo(a)pyrene which indicate that (+/-)-trans-7beta,8alpha-dihydroxy-9alpha,10alpha-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene is an ultimate carcinogen in newborn mice. *Cancer Res.*, **38**, 354–358.
50. Salnikow,K. *et al*. (2008) Genetic and epigenetic mechanisms in metal carcinogenesis and cocarcinogenesis, nickel, arsenic, and chromium. *Chem. Res. Toxicol.*, **21**, 28–44.
51. Esteller,M. (2006) Epigenetics provides a new generation of oncogenes and tumour-suppressor genes. *Br. J. Cancer*, **94**, 179–183.

Received August 14, 2009; revised October 28, 2009; accepted October 30, 2009