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Short Communication

Effects of cigarette smoking and nicotine metabolite ratio on leukocyte telomere length.

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ABSTRACT

Studies of the effects of smoking on leukocyte telomere length (LTL) using cigarettes smoked per day or pack years smoked (PYS) present limitations. Reported high levels of smoking may not increase toxin exposure levels proportionally. Nicotine metabolism ratio (NMR) predicts total cigarette puff volume and overall exposure based on total N-nitrosamines, is highly reproducible and independent of time since the last cigarette. We hypothesized that smokers with higher NMRs will exhibit increased total puff volume, reflecting efforts to extract more nicotine from their cigarettes and increasing toxin exposure. In addition, higher levels of smoking could cause a gross damage in LTL. The urinary cotinine, 3-OH cotinine and nicotine levels of 147 smokers were analyzed using a LC/MS system Triple-Q6410. LTL and CYP2A6 genotype was determined by PCR in blood samples. We found a significant association between NMR and CYP2A6 genotype. Reduction in LTL was seen in relation to accumulated tobacco consumption and years smoking when we adjusted for age and gender. However, there were no significant differences between NMR values and LTL. In our study the higher exposure was associated with lower number of PYS. Smokers with reduced cigarette consumption may exhibit compensatory smoking behavior that results in no reduced tobacco toxin exposure. Our results suggest that lifetime accumulated smoking exposure could cause a gross damage in LTL rather than NMR or PYS. Nevertheless, a combination of smoking topography (NMR) and consumption (PYS) measures may provide useful information about smoking effects on health outcomes.

1. Introduction

Telomere length is considered a biomarker for aging (Harley et al., 1990; Zakian, 1995). Telomeres are deoxyribonucleic acid (DNA)–protein complexes that cap and stabilize the ends of chromosomes, maintaining genome integrity and protecting from damage. Telomere length is not only related to the basic biology of aging as a trigger of cellular senescence but also reflects the balance between cytotoxic stressors and antioxidant defense mechanisms (Aviv and Susser, 2013; Houben et al., 2008; von Zglinicki et al., 1995). Thus, telomere attrition in circulating white blood cells has been proposed as a marker for cumulative oxidative stress and inflammation and, therefore, biological aging.

Several cross sectional and prospective studies have associated short telomere length with increased risk of cardiovascular disease, pulmonary disease or cancer (Lan et al., 2013; Savale et al., 2009; Wolkowitz et al., 2011). At the same time there are other lifestyle factors such as smoking, obesity, physical inactivity and

Abbreviations: ROS, reactive oxygen species; COT, cotinine; 3´-HCOT, trans-3´-hydroxicotinine; CYP2A6, Cytochrome P450 2A6; NMR, nicotine metabolized ratio; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; LTL, leukocytes telomere length; CPD, cigarettes per day; PYS, pack years smoked; FTND, Fagerstrom Test for Nicotine Dependence; d3-COT, d3-cotinine; LOQ, limit of quantification

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alcohol intake that could be related to short telomere length although some data are conflicting (Chen et al., 2014; Cherkas et al., 2008; Huzen et al., 2014; Weischer et al., 2014).

Human health effects caused by smoking are a potential public health problem. Cigarette smoke contains a large amount of compounds (nicotine, acrolein, formaldehyde, carcinogens), including many oxidants and free radicals that are capable of initiating or promoting oxidative damage (Esterbauer et al., 1991; Voulgaridou et al., 2011). Oxidative damage may result from reactive oxygen species (ROS) generated by the increased and activated phagocytes following cigarette smoke. Cigarette smokers have a higher risk of developing several chronic disorders as cardiovascular diseases, several types of cancer or pulmonary diseases. Therefore, smoking increase levels of oxidative stress, a molecular mechanism for telomere attrition (Huzen et al., 2014; Valdes et al., 2005).

Smokers can extract varying levels of nicotine and other cigarette compounds depending on their smoking topography (puff volume or number of puffs), which reflects the level of toxine exposure (Djordjevic et al., 2000; Strasser et al., 2006).

Nicotine is metabolized to cotinine (COT), and then to trans-3′-hydroxicotinine (3′-HCOT). Mainly, the CYP2A6 enzyme catalyzes the conversion of nicotine to 3′-HCOT. COT has a half-life of 15–20 h compared to nicotine of 0.5–3 h, therefore COT is a better marker to exposure (Benowitz, 1996; Benowitz et al., 2009; Hukkanen et al., 2005a). The ratio of 3′-HCOT/COT, referred as nicotine metabolized ratio (NMR), reflects CYP2A6 activity, environmental factors influencing CYP2A6 and nicotine clearance in vivo (Benowitz et al., 2009; Benowitz, 2010). The ratio has been routinely measured in plasma and urine. The NMR is highly reproducible and independent of time since the last cigarette (Dempsey et al., 2004; Lea et al., 2006). Faster metabolizers of nicotine have higher smoking rates and therefore increased the risk for lung cancer (Boffetta et al., 2006; Derby et al., 2008; Joseph et al., 2005a, 2005b; London et al., 1999; Tyndale and Sellers, 2001; Zhu et al., 2013a, 2013b). Strasser et al., showed that the NMR predicts total cigarette puff volume and carcinogen exposure measured as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) levels (Strasser et al., 2011). Previous studies have shown that telomere length is shorter in peripheral blood leukocytes of smokers compared to nonsmokers (Morla et al., 2006; Rode et al., 2014; Valdes et al., 2005). Most of these assays analyzed leukocytes telomere length (LTL) in lung disease population, reporting an additive association between LTL and smoking on cancer development (Broberg et al., 2005; Wu et al., 2003). On the other hand, the interaction between LTL and smoking history is not clear (Broberg et al., 2005; Walters et al., 2014).

In this study we explored the relationship between accelerating aging in smokers measured as LTL and NMR in addition to cumulative smoking. Telomere length may reflect cumulative exposure to cigarette smoke measured by these biomarkers.

2. Material and methods

2.1. Subject recruitment and sample collection

One hundred and forty-seven healthy smokers (all of Caucasian (Spanish) descent for ≥ 3 generations) between 25 and 65 years of age were recruited from the Occupational Medicine Committee of Banco Popular, Madrid (Spain) from 2010 to 2013. The present genetic analyses were restricted to Caucasian smokers to minimize population stratification as the frequency of these variants varies substantially between populations. Screening of smokers for study inclusion involved a medical history, clinical examination, electrocardiogram, blood and set of lung function tests. Exclusion criteria were not being a smoker and suffer from any illness related to smoking. In addition, we excluded smokers who suffered any disease which affects LTL as cardiovascular disease, cancer, obesity, diabetes, osteoporosis, infectious diseases or chronic psychological stress. Approval was obtained from the local Ethics Committee (Hospital Guadalajara, Guadalajara, Spain) and all patients provided written informed consent in accordance with the principles expressed in Helsinki Declaration.

All participants completed a questionnaire regarding demographic characteristics, smoking habits, self-reported cigarettes per day (CPD), the number of years the person had smoked and cumulative consume as pack years smoked (PYS). Nicotine dependence was assessed with the Fagerstrom Test for Nicotine Dependence (FTND) (Lessov-Schlaggar et al., 2008). In addition, CO levels and lung function (spirometry) were measured in each participant.

On the same day, for the analysis of nicotine metabolites, morning urine samples in sterile bottles were collected and stored at 4 °C until analysis. Peripheral blood samples were obtained by venipuncture and we extracted blood leukocyte DNA from the participants using a standard phenol chloroform protocol. The genotype analyzes were performed in the Biomedicine laboratory of the *Universidad Europea,* Madrid (Spain).

The study followed recent recommendations for replicating genotype–phenotype association studies (NCI-NHGRI Working Group on Replication in Association Studies et al., 2007). Genotyping was performed specifically for research purposes, blood and urine samples were tracked solely with bar-coding and personal identities were only made available to the main study researcher who was not involved in actual genotyping). The DNA samples were diluted with sterile water and stored at −20 °C until analysis.

2.2. Telomere length analysis

Telomere length was measured using a quantitative PCR-based method previously described (Cawthon, 2002; Martinez-Delgado et al., 2011). By this technique telomere length was calculated as a ratio between telomere repeat copy number and a single-copy gene, *36B4*. In order to amplify telomere repeats and the *36B4* gene, we used primers described before (Codd et al., 2010). All samples, for both telomere and *36B4* reactions, were analyzed in triplicate using a Step One Plus (Applied Biosystems, Foster City, CA), in 96 well format. For each sample the relative concentration of both telomere and *36B4* was calculated relative to the calibration sample and PCR efficiency to obtain the ratio, as previously described (Willeit et al., 2010).

2.3. CYP2A6 genotyping

Genotyping of *CYP2A6* (*CYP2A6**1 × 2, *CYP2A6**2 (1799T > A) [rs1801272], *CYP2A6**9 (−48T > G) [rs28399433], *CYP2A6**12) was carried out using genomic DNA isolated from blood samples as previously described (Verde et al., 2011). Subjects were classified into three phenotype groups (very slow, slow and normal-fast metabolizer) according to the *CYP2A6* genotypes, genetically faster metabolizers of nicotine (carriers of two *1 alleles or *1 × 2), slow metabolizers (carriers of *2, *9, and *12) and very slow metabolizers (carriers of two alleles *2, *9, or*12) (Lerman et al., 2007; Verde et al., 2014).

2.4. Biomarkers in urine

Urine (2 ml) was mixed with 2 ml of ammonium acetate buffer (15 mM, pH = 6) followed by the addition of 5 ul of 1000 ng/ml d3-cotinine (d3-COT) and 5 ul of 1000 ng/ml d4-nicotine (as internal