Nevus Size and Number Are Associated with Telomere Length and Represent Potential Markers of a Decreased Senescence *In vivo*

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Abstract

Nevus counts represent one of the strongest risk factors for melanoma. They appear in childhood and adolescence and involute from middle age onwards. Recent evidence has shown that nevus cells undergo oncogene-induced senescence involving the p16/retinoblastoma pathway. However, telomere length also influences senescence in proliferative somatic cells and varies between individuals. This study explores whether telomere length measured in white cells is associated with nevus count and size in 1,897 Caucasian women ages 18 to 79 years. Total body nevus counts were positively correlated with white cell telomere length (mean, 7.09 kbp; range, 5.09-9.37) after adjustment for age (P = 0.0001). Age-adjusted telomere length was also associated with nevus count for nevi above 5 mm in diameter (P = 0.04). Subjects in the top category for nevus count had an average age-adjusted telomere length 150 bp longer than those in the lowest category. The positive correlation between white cell telomere length and nevi number and size may reflect an increased replicative potential (reduced senescence) in individuals with longer telomeres, which may not be melanocyte specific. Understanding mechanisms influencing the induction and involution of nevi will not only help in understanding the pathophysiology of melanoma but should also shed light on the complex relationship between aging and cancer. (Cancer Epidemiol Biomarkers Prev 2007;16(7):1499–502)

Introduction

Nevus numbers vary greatly in any Caucasian populations and undergo a significant reduction from middle age onwards (1, 2). Mean total body nevus count in the United Kingdom is 30 and remains the most powerful predictive marker of risk for melanoma (3-5). Nevus number is known to be significantly influenced by genetic factors, with twin studies showing that 60% of the variation in nevus number is explained by additive genetic factors (6-8). Furthermore, in families with a genetic susceptibility to melanoma, affected individuals often have large numbers of common and atypical nevi (9, 10). In these melanoma-prone families, nevi continue to appear in large numbers in adulthood and have a delayed involution with large numbers of nevi still present in older age groups (10, 11).

The melanoma suppressor gene *CDKN2A* is now known to encode the two cell cycle inhibitors p16 (INK4a) and ARF (alternative reading frame), with the related p15 (INK4b; ref. 12). Germ-line and somatic CDKN2A mutations and deletions have been linked respectively to familial and sporadic melanoma, and p16 carriers often show large numbers of nevi (10, 13). p16 inhibits cell proliferation by binding to cyclin-dependent kinases (cyclin-dependent kinases 4 and 6), preventing phosphorylation of the retinoblastoma protein. p16 is also known to be important in cell senescence in all cell types (14). Another well known effector pathway for human replicative cell senescence is attributed to telomere shortening and a DNA damage response through other growth inhibitors, such as p53 and p21 (15, 16).

Telomeres are specialized DNA-protein complexes that cap the end of eukaryotic chromosomes and are essential for maintaining genome stability and integrity. The human telomeric complex is composed of TTAGGG repeated DNA sequences with the enzyme telomerase where present and several associated proteins controlling telomere length and capping. Shortening of telomeres occurs naturally with successive divisions in somatic cells, whereas in the germ-line and most cancer cells, high telomerase activity allows cells to maintain long telomeres and avoid senescence (17-19). Twin and family studies have shown that telomere length in white cells is partly heritable, although environmental factors, such as inflammation, smoking, and obesity, are also important (20-22). Shorter white cell telomere length has also been linked to chronic diseases of aging, such as atherosclerosis, osteoarthritis, and diabetes (18, 23-26). Telomere attrition causes cell senescence via the p53 pathway, although p16 is also likely to be involved as senescent cells overexpress p16 (14, 15, 17). We speculate that telomere shortening may play a key role, as well as the p16 pathway, in limiting nevus growth and may be involved in the disappearance of nevi with age. The aim of this study was to explore this hypothesis by correlating leukocyte telomere length with total body nevus counts and nevus size in a large population-based sample of adults.

Materials and Methods

Caucasian female subjects (2,786) were recruited with collection of phenotypic skin data from the UK Adult Twin Registry (Twins UK) at St. Thomas Hospital (London, United Kingdom) between January 1997 and December 2003 (27). As well as

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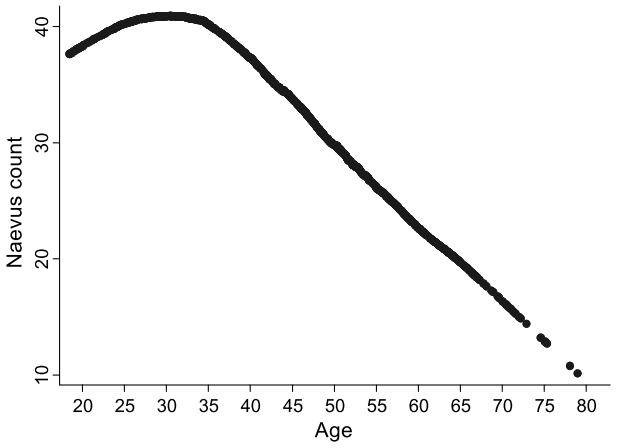

Figure 1. Nevus count in relation to age.

answering a comprehensive questionnaire on many diseases other than skin diseases, the women were given a validated questionnaire on sun and sunbed exposure. A skin examination was done by trained research nurses that included a record of skin type and freckle counts, as well as total body nevus counts. The standardized skin examination including a full body nevus count, divided into 17 body sites (excluding the genital area, breasts, and posterior scalp), was done by nurses trained by V.B. for 4 weeks before the start of the study. The nevus count protocol has been validated previously and found to be reproducible (5, 7). Nevi were recorded by size in three categories (>2 and ≤5 mm, >5 and ≤10mm, and >10mm). Nevi >5 mm were defined as atypical nevi for the purpose of this study. The total body nevus count was defined as the sum of all nevi >2 mm in diameter. Nevus density was defined as the ratio of nevus count over body surface area, which is a function of height and weight (28). Skin type was assessed according to the Fitzpatrick classification: type I, always burn and never tan; type II, often burn and tan lightly; type III, burn moderately and tan gradually; type IV, burn minimally and tan easily; and type V, never burn and tan deeply. Hair and eye color was also recorded for each subject.

DNA was extracted from isolated leukocytes after an overnight fast and the mean leukocyte terminal restriction fragment length (TRFL) was measured using the Southern blot method as described previously (24). Each DNA sample was resolved in duplicate (on different gels). If the difference between the duplicates was >5%, a third measurement was done and the mean of two results <5% apart was taken. This was only necessary in <5% of the assays. The coefficient of variation of the TRFL assay in this study was 0.92%. The laboratory conducting the TRFL measurements was blinded to all characteristics of the white cell donors. The data presented here are restricted to the 1,897 subjects on whom both telomere length data and nevus data were available. Approval for this study was from St. Thomas Hospital NHS Trust Ethics Committee (London, United Kingdom).

Statistical Methods. Associations between TRFL and nevus counts were done using single and multiple linear regressions with adjustment for age, body surface area, body mass index (kg/m^2), smoking, weeks of holiday abroad over a lifetime, sunburns, social class, and skin type. All results are shown as age-adjusted TRFL as all other potential confounders listed above did not affect the results. Nevus count and nevus density were log transformed to make them normally distributed. Test for trend for the association between age-adjusted TRFL across four categories of nevus counts was done using a nonparametric test (29). Robust regression was used to adjust for nonindependence of twin pairs. A discordant twin analysis within DZ pair to explore differences in nevus counts in a small subgroup of extreme twins for TRFL was also assessed to support the cross-sectional results. All analyses were carried out using Stata 9 (Stata Corp.).

Results

The mean age of the 1,897 Caucasian female subjects was 46 years (18-79 years). The mean number of nevi was 32 and the median was 20 (range, 0-324). The median nevus density was 12.4 (0.4-191.2). Thirty-three percent of the subjects had skin type I or II, whereas 39% and 18% had skin type III and IV, respectively. Nevus count was not correlated with skin type (data not shown). Nevus count and density in this age range were inversely correlated with age for subjects above 30 years ($P < 0.0001$; Fig. 1). Nevus counts were positively associated with mean number of weeks of holidays abroad [mean, 20 (0-277); $P = 0.001$] and mean number of severe lifetime sunburns [mean, 2.6 (0-68); $P = 0.003$] even after adjusting for age and skin type.

Mean TRFL was 7.09 kb (range, 5.09-9.37) and was negatively associated with age with a decrease in length of 27 ± 1.5 bp per year ($P < 0.0001$). Age-adjusted TRFL was positively associated with log-transformed total body nevus count ($P = 0.0001$). Further adjustment for confounders, which could have affected either TRFL or nevus counts, such as smoking, weeks of holidays abroad, number of sunburns, skin type, body mass index, and body surface area, were also done. Body surface area affected the results by increasing the significance of the association. TRFL was also highly significantly associated with log-transformed nevus density, which accounts for body surface area ($P < 0.0001$). All data are shown below using age-adjusted TRFL and nevus count. Fig. 2 and Table 1 show the increase in age-adjusted TRFL with increasing numbers of nevi with a significant trend ($Z = 2.53$; $P = 0.011$). Subjects in the highest category of nevus counts had telomeres of an average 150 bp longer telomeres than those in the lowest category. The association between nevus counts and age-adjusted TRFL remained the same for individuals when stratifying for women below and above 50 years of age (Fig. 3).

The association between TRFL and nevi was the same on sun-exposed sites (nevi on face, neck, forearms, lower legs, and feet) and sun-protected sites (chest, abdomen, buttocks, upper arms, and thighs).

Only 29 DZ pairs (and no MZ pairs) were discordant for top and bottom tertiles for TRFL. The mean nevi difference within

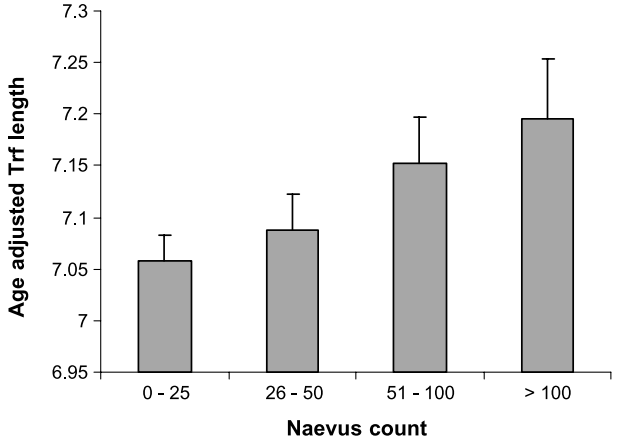

Figure 2. Age-adjusted means of TRFL (kb) and SE within each category of nevus counts.

DZ pairs discordant for TRFL was on average 6 extra nevi in the top TRF tertile group, which is ~20% of the mean number of nevi in our twin population. The difference within twin pairs is in the same direction as the cross-sectional results but did not reach statistical difference in view of the small number of discordant pairs.

Age-adjusted TRFL was also correlated with log-transformed number of atypical nevi ($P = 0.004$). This association reflects the strong association between high nevus number and atypical nevi. Mean numbers of atypical nevi was 1.8 in subjects with long telomeres (age-adjusted telomere length, ≥7 kb) compared with 1.2 in subjects with short telomeres (age-adjusted telomeres, <7 kb). Age-adjusted TRFL was not associated with skin type or freckle count (data not shown).

Discussion

This study is the first to show a correlation between high nevus counts and white cell telomere length. Furthermore, an association was found with nevus size, supporting the hypothesis that longer telomeres may be associated with increased proliferative potential of cells within a melanocytic nevus. The age-adjusted difference in telomere length between subjects with high nevus counts and low nevus counts was on average 150 bp and the average extrapolated attrition rate for telomere length per year is 22 bp per year from our cross-sectional data. Telomere length varies between individuals, decreases with age, and is, in part, influenced by genetic factors (20, 21). Although telomere length was measured in white cells, this is likely to reflect relative telomere length in other cell types, such as melanocytes, as telomere length tends to be well correlated in different tissues within one subject (19, 30-32). These findings provide evidence that telomere length influences the replicative potential within a nevus clone, despite evidence that p53 is not activated in benign nevi (16, 33). It is plausible that in melanocytes, telomere attrition or dysfunction may activate the p16/retinoblastoma pathway as reported in other human cells by Jacobs and de Lange (17). Oncogene-driven cell senescence was shown recently to be important in limiting the growth of a variety of benign lesion types including nevi in which BRAF and p16 are overexpressed (33, 34). Furthermore, telomere length may also affect apoptosis of nevi from middle age onwards with longer telomere causing a delayed disappearance of nevi with age.

Cross-sectional data do not provide evidence of causality. However, it is unlikely that telomere length is increased by mechanisms that determine nevus number and size. Telomere shortening is a known stimulus for human cell senescence through the p16 and p53 pathways (14). Thus, longer telomeres (due to genetic or environmental mechanisms) would be expected to entail an increased replicative potential with delayed senescence in somatic cells including melanocytes. Nevus characteristics are likely to be influenced by many genes with complex interactions with environmental exposure. Our group and others using the twin model have shown that 60% of the variability in total body nevus counts was influenced by genetic factors and the remaining 40% by unique and common environmental factors (6-8). The number of common and atypical nevi is usually present in larger numbers in families with a susceptibility to melanoma (9, 10). These include those with *p16* mutations, showing that this gene is likely to be involved in nevus biology (10). Melanocytic senescence involves the p16 pathway as melanocytes from two rare patients found to be null for p16 were shown to have greatly increased replicative potential *in vitro* (35). Moreover disruption of this pathway in concert with telomerase activation is obligatory to immortalize melanocytes (16). *p16* is therefore not only a tumor suppressor gene but also a senescence gene with increased expression in senescent cells (14, 15). Senescence is important in protecting cells against malignant transformation with age and all benign tumors are likely to be controlled by senescence signals (12). The role of the telomere pathway in melanocyte senescence is also apparent from work showing that telomerase activity increases steadily from benign nevi to dysplastic nevi to melanoma (36, 37).

We propose that individuals with longer telomeres express a delayed cell senescence that is reflected in the skin by increased nevus size and numbers. Whether longer telomeres also influence other skin phenotypes remains to be determined. However, it has been observed that both the ''moley'' phenotype and the ''sun damage'' phenotype (with solar elastosis and solar keratoses), which are both associated with an increased risk of melanoma, are often mutually exclusive (38, 39). It is therefore possible that longer telomere length delays senescence in keratinocytes and fibroblasts so patients with higher nevus counts may display less photoaging. The data presented here need to be consolidated with studies done on other populations and in males. It would also be important to investigate whether longer white cell telomeres reflect a delayed senescence of melanocytes in culture, but this is likely to be technically difficult, requiring many cultures to fully assess variability in melanocyte senescence. Recent studies have suggested that shorter telomeres in white cells may be associated with an increased risk of cancer but these studies have been small and confounding factors, such as smoking, were an issue so the role of white cell telomere length in determining cancer risk remains controversial. Our group has not found any association between telomere length and common cancers, such as breast cancer.[5] Nevi are visible and easily accessible benign tumors reflecting variable replicative potential between individuals and results on melanocytes may

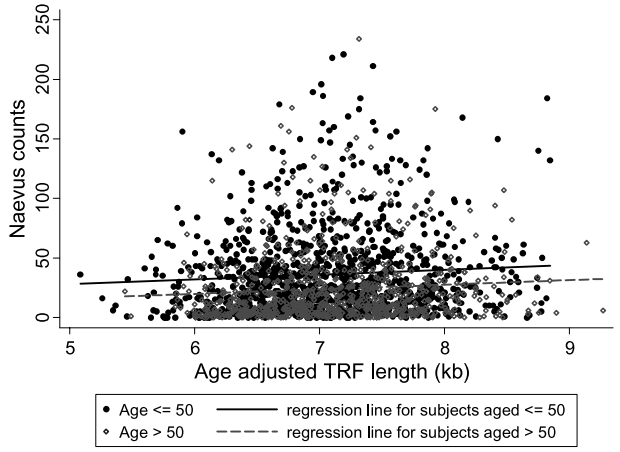

Figure 3. Nevus counts in relation to age-adjusted TRFL (kb) with regression line for individuals below and above the age of 50 y.

Table 1. Age-adjusted means (and SE) of TRFL within each category of nevus counts

TBNC	*n* (%)	Mean TRFL (kb)	SE	95% CI
0-25	1,097 (57.8)	7.05	0.025	7.00-7.10
26-50	432 (22.8)	7.09	0.034	7.02-7.15
51-100	259 (13.6)	7.15	0.045	7.06-7.24
>100	109 (5.8)	7.20	0.058	7.08-7.31

Abbreviation: TBNC, total body nevus count.

[5] S. Hodgson, unpublished data.

be applicable to less accessible tissue. This work is therefore likely to shed light on the complex interactions between the telomere unit and cell senescence offering potential insight into the complex relationship between aging and cancer.

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