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Expression of *p16*^{INK4a} in peripheral blood T-cells is a biomarker of human aging

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Summary

Expression of the *p16*^{INK4a} tumor suppressor sharply increases with age in most mammalian tissues, and contributes to an age-induced functional decline of certain self-renewing compartments. These observations have suggested that *p16*^{INK4a} expression could be a biomarker of mammalian aging. To translate this notion to human use, we determined p16^{INK4a} expression in cellular fractions of human whole blood, and found highest expression in peripheral blood T-lymphocytes (PBTL). We then measured *INK4/ARF* transcript expression in PBTL from two independent cohorts of healthy humans (170 donors total), and analyzed their relationship with donor characteristics. Expression of *p16*^{INK4a}, but not other *INK4/ARF* transcripts, appeared to exponentially increase with donor chronologic age. Importantly, *p16*^{INK4a} expression did not independently correlate with gender or body-mass index, but was significantly associated with tobacco use and physical inactivity. In addition, *p16*^{INK4a} expression was associated with plasma interleukin-6 concentration, a marker of human frailty. These data suggest that *p16*^{INK4a} expression in PBTL is an easily measured, peripheral blood biomarker of molecular age.

Keywords

INK4/ARF; *CDKN2a*; frailty; smoking; exercise; IL-6

Introduction

Human aging is characterized by a striking increase in frailty and age-associated diseases such as cancer, ischemic stroke, coronary artery disease and type 2 diabetes mellitus (T2DM). A biomarker that can faithfully measure molecular, rather than chronological,

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aging could be used to predict the future risk of these conditions. Work in model systems has suggested that some aspects of mammalian aging result from a decline in tissue replicative capacity. This decrease in tissue regeneration and repair in turn has been suggested to occur as the result of the activation of tumor suppressor mechanisms such as telomere dysfunction and $p16^{INK4a}$ activation (Campisi, 2003; Finkel et al., 2007; Kim and Sharpless, 2006).

The $p16^{INK4a}$ tumor suppressor originates from the *INK4/ARF* (or *CDKN2a/b*) locus on chromosome 9p21 which gives rise to two other anti-proliferative protein encoding transcripts ($p15^{INK4b}$ and *ARF*) as well as a recently described non-coding RNA, *ANRIL* (Broadbent et al., 2008; Pasmant et al., 2007). Expression of $p16^{INK4a}$ has been shown to markedly increase with aging in most rodent, baboon and human tissues tested (Edwards et al., 2007; Herbig et al., 2006; Krishnamurthy et al., 2004; Melk et al., 2003; Melk et al., 2004; Nielsen et al., 1999; Signer et al., 2008; Zindy et al., 1997), and caloric restriction, which retards aging in rodents, attenuates this age-induced increase in $p16^{INK4a}$ (Edwards et al., 2007; Krishnamurthy et al., 2004). In mice lacking $p16^{INK4a}$, an attenuated decline in the replicative capacity of several self-renewing compartments has been observed, including hematopoietic stem cells (Janzen et al., 2006), pancreatic β-cells (Krishnamurthy et al., 2006), neural stem cells (Molofsky et al., 2006) and B-lymphocytes (Signer et al., 2008). Moreover, inactivation of $p16^{INK4a}$, but not Arf, partially rescues several age-related phenotypes in a progeroid mouse strain (Baker et al., 2008). Provocatively, unbiased human association studies have demonstrated that the genotype of single nucleotide polymorphisms (SNPs) near the *INK4/ARF* locus is associated with human age-associated conditions such as frailty (Melzer et al., 2007), atherosclerotic diseases (Broadbent et al., 2008; Helgadottir et al., 2008; Helgadottir et al., 2007; Matarin et al., 2008; McPherson et al., 2007; Samani et al., 2007; The Wellcome Trust Case Control Consortium, 2007), and type 2 diabetes mellitus (T2DM) (Broadbent et al., 2008; Saxena et al., 2007; Scott et al., 2007; Zeggini et al., 2007). These observations suggest that the expression of $p16^{INK4a}$ is intimately associated with cell-intrinsic, molecular aging in humans, and could serve as a biomarker of this process.

A limitation of these observations for human studies, however, has been the need to measure $p16^{INK4a}$ in tissues (e.g. brain, bone marrow and pancreas) that are difficult to obtain. In this work, we therefore sought to develop an easily assessable biomarker of human molecular age that can be reliably measured longitudinally. Toward that end, we measured *INK4/ARF* transcript expression in the cellular fractions of human whole blood. In two independent cohorts of healthy adults, expression of $p16^{INK4a}$ in peripheral blood T lymphocytes (PBTL) strongly correlated with donor chronological age, demonstrating an apparent exponential increase with aging. Additionally, PBTL expression of $p16^{INK4a}$ was positively associated with tobacco use and physical inactivity, and with serum IL-6 levels, a marker of human frailty. These data suggest that measurement of $p16^{INK4a}$ in PBTL has the properties of a useful biomarker of human molecular age.

Results

Expression of $p16^{INK4a}$ in human peripheral blood cells

To develop a peripheral blood biomarker of human molecular age, we first determined the expression of $p16^{INK4a}$ in different peripheral blood compartments. We isolated six, highly-enriched cellular fractions from whole blood by Magnetic Activated Cell Sorting (MACS): T cells ($CD3^+$), B cells ($CD19^+$), monocytes ($CD14^+$), NK cells ($CD56^+$) and granulocytes ($CD15^+$ or $CD16^+$) (Supp. Fig. 1A). Of these fractions, expression of $p16^{INK4a}$ was highest in $CD3^+$ T cells (Fig. 1A), and was found to be stable in subjects who consented to re-testing more than 4 weeks after the initial analysis (Supp. Fig. 1B). Given these results, we decided

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to focus on PBTL for further study as these cells are relatively abundant in the peripheral blood, demonstrate easily measured expression of *p16^{INK4a}*, and are of undisputed importance with regard to immunologic aging.

Increased p16^{INK4a} expression is associated with chronological age

We next examined p16^{INK4a} mRNA and protein expression in PBTL from two independent, unselected cohorts of healthy donors aged 18-80 (cohorts described in Supp. Table 1). In general, associations noted in the exploratory cohort were confirmed in an independent validation cohort (Supp. Table 1), and aggregate data are shown except where indicated. In accord with work in other mammalian tissues (Edwards et al., 2007; Herbig et al., 2006; Krishnamurthy et al., 2004; Melk et al., 2003; Melk et al., 2004; Nielsen et al., 1999; Signer et al., 2008; Zindy et al., 1997), we noted a strong association between *p16^{INK4a}* mRNA expression and chronologic age (Table 1 and Fig. 1B). While protein expression of p16^{INK4a} also increased with age (Fig. 1C), several technical issues (e.g. importance of sample handling, ease of measure in large scale) limited the utility of protein quantification. Therefore, we elected to focus on mRNA quantification for the remainder of the studies. We found that the correlation (Pearson) with chronologic age was considerably stronger for $\log_2$-transformed *p16^{INK4a}* (r=0.63) than untransformed *p16^{INK4a}* expression (r=0.47). In accord with this result, Sedivy and colleagues have also reported an exponential increase of other biomarkers of aging in non-human primates (Herbig et al., 2006). These observations are consistent with the model that molecular age, as estimated by PBTL *p16^{INK4a}* expression and other aging biomarkers, increases exponentially with chronologic age.

To address the possibility that the increase of *p16^{INK4a}* expression in PBTL with age could be due to age-related changes in T cell differentiation or subset composition, we examined the expression of memory T cell markers (bcl-2 (Grayson et al., 2000), bcl-X_L (Zhang and He, 2005) and IL-7 receptor α (Huster et al., 2004)) and the ratio of $CD4^+$ versus $CD8^+$ T cells. Expression of bcl-2, bcl-X_L and IL-7R increased minimally, if at all, with age and no correlation between CD4/CD8 ratio and chronologic age was observed (Supp. Fig. 2A, B, C, and D, Left panels). Change in the mean expression of all three markers was less than two-fold across the entire examined age range (Supp. Fig. 2A, B, and C, Left panels). These markers of T cell differentiation also correlated minimally or not at all with *p16^{INK4a}* expression (Supp. Fig. 2A, B, C, and D, right panels). These observations, coupled with the very large observed changes in *p16^{INK4a}* observed with aging, suggest that the increased *p16^{INK4a}* expression with age is unlikely the result of the reported modest alterations in PBTL differentiation or composition with age (De Paoli et al., 1988; Naylor et al., 2005; Utsuyama et al., 1992).

Expression of other *INK4/ARF* transcripts with aging

We sought to determine the relationship of other *INK4/ARF* transcripts (*ARF, p15^{INK4b}*, and *ANRIL*) with aging. We and others have previously noted a strong co-correlation between *p16^{INK4a}* and *Arf* expression in rodents, and expression of *Arf* increases sharply in most murine tissues with aging (Krishnamurthy et al., 2004; Signer et al., 2007; Zindy et al., 1997). Importantly, *p16^{INK4a}* and *ARF* start with different first exons but share common second and third exons translated in alternate reading frames (Supp. Fig. 3, reviewed in (Kim and Sharpless, 2006)). We therefore also measured aggregate *INK4a/ARF* transcript levels using primer/probes that span shared exons 2 and 3 (Exon2/3) which detect total *p16^{INK4a}* and *ARF* transcript levels. While strong positive correlations were noted among all the *INK4/ARF*-associated transcripts, only *p16^{INK4a}* expression was associated with chronological age (Table 1 and Fig. 2). The fact that the observed correlations are partial (R^2 ~0.40, with R^2=1.0 being complete correlation) explains how it is possible that *p16^{INK4a}* expression can be significantly associated with both chronologic age and the expression of

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other 9p21 transcripts, while age and *p15^INK4b^/ARF/ANRIL* expression are uncorrelated. Moreover, aggregate $p16^{INK4a}$ and *ARF* expression measured with exon 2/3 primers did not correlate with chronologic age; presumably because *ARF* is the more abundant transcript in this tissue, at least in donors of younger ages (not shown). Additionally, although *ANRIL* is believed to result from a spliced RNA that is anti-sense to $p15^{INK4b}$ (Broadbent et al., 2008; Pasmant et al., 2007), we did not observe a negative correlation between *ANRIL* levels and $p15^{INK4b}$; but rather, expression of $p15^{INK4b}$ and *ANRIL* were positively correlated (r=0.56, p<0.0001). Together, these observations suggest that the expression of $p16^{INK4a}$ in human PBTL is influenced independently by factors that globally control expression of at least four transcripts from the *INK4/ARF* locus, as well as aging-specific factors that influence only $p16^{INK4a}$. Donor genetics appears to be a factor that contributes to the global control of *INK4a/ARF* expression as we have recently described a strong correlation of all four *INK4a/ARF* transcripts with donor genotype of a nearby single nucleotide polymorphism that has also been associated with atherosclerosis (Liu et al., 2009).

Expression of $p16^{INK4a}$ is associated with cigarette smoking and physical inactivity

Tobacco use is positively associated with increased risk of many age-related diseases, particularly atherosclerosis and multiple cancers, and we therefore sought to determine the relationship between cigarette smoking and $p16^{INK4a}$ expression in PBTL. In linear regression analysis, expression of $p16^{INK4a}$ increased more rapidly (slope ~2-fold greater) with advancing age in current smokers compared to non-smokers, with an intermediate effect observed in former smokers (Fig. 3A). Moreover, we observed evidence of a dosage effect as $p16^{INK4a}$ expression was associated with cumulative exposure as estimated by tobacco pack-years (Fig. 3B, Supp. Fig. 4A and Table I). In accord with compelling *in vitro* and murine studies (reviewed in ref. (Finkel et al., 2007;Kim and Sharpless, 2006)), these findings are consistent with the view that exposure to DNA damaging agents and/or other mutagens such as those present in tobacco smoke accelerate molecular aging.

Exercise likewise has been associated with lower risk of several age-related diseases such as atherosclerosis and T2DM, while obesity has been associated with higher risk of these diseases. We noted negative correlations between several reported measures of exercise habits and $p16^{INK4a}$ expression (Table 1, Fig. 3C and Supp. Fig. 4B). This negative association was seen with both frequency and amount of exercise and remained significant after adjusting for age and smoking status (Table 2). A weak but significant Spearman correlation was observed between $p16^{INK4a}$ expression and obesity as estimated by categorized body-mass index (BMI, Supp. Fig. 4C). In contrast, $p16^{INK4a}$ expression did not significantly correlate with absolute, uncategorized BMI values (Supp. Fig. 4D), and no association with BMI was found in multivariate analysis (Table 2, see also multiple regression section below). These data suggest that increased molecular aging in PBTL is associated strongly with reduced exercise, but weakly, if at all, with BMI.

Expression of $p16^{INK4a}$ is associated with IL-6, a serologic marker of aging and frailty

In an effort to determine the functional consequences of increased $p16^{INK4a}$ expression in PBTL with aging, we investigated its correlation with plasma levels of IL-6, which has been reproducibly associated with cellular senescence (Coppe et al., 2008; Kuilman et al., 2008) *in vitro* as well as frailty and aging *in vivo* (reviewed in (Ershler and Keller, 2000)). While no correlation was noted between IL-6 and chronologic age in this small sample (Supp. Fig. 4E), a significant association was seen between levels of IL-6 and $p16^{INK4a}$ expression (Table 1 and Fig. 3D). As the expression of this marker of human frailty is associated more strongly with $p16^{INK4a}$ than chronologic age, this observation suggests the intriguing possibility that molecular age, as estimated by PBTL $p16^{INK4a}$ expression, is a better predictor of frailty than chronological age.

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Analysis of validation sample and multiple regression analysis

To control for chance associations resulting from the comparison of multiple genes and parameters in the exploratory cohort, we re-tested significant associations noted in the initial analysis in an independent validation cohort comprised of 90 subjects (Supp. Table 1). Comparably strong and statistically significant associations were seen in the validation cohort (Table I), suggesting that the observed relationships of *p16^INK4a^* expression with age, smoking, exercise, plasma IL-6, and other *INK4/ARF* transcripts are unlikely to represent chance associations.

A multiple regression model was first developed using only subjects from the aggregate cohort for which there was full clinical data (Table 2, N=99). In this model, expression of *p16^INK4a^* was independently associated with chronologic age, smoking pack-years, and minutes per month of exercise after adjusting for all other covariates. Expression of *p16^INK4a^* was not independently associated with BMI (p=0.91) or gender (p=0.33, see also Supp. Fig 4F), nor was there a significant effect due to study source (exploratory vs. validation, p=0.99). Expression of *p16^INK4a^* was best described by the model with age, pack-years, and exercise (min/mon) using the model selection methods described. The relative effect size of each covariate on *p16^INK4a^* and p-values are shown in Table 2. Age had the most significant effect on *p16^INK4a^* expression compared to the other covariates. Significant results were then confirmed in regression analysis incorporating missing values (total N=147), yielding similar results to those shown in Table 2.

Discussion

Here, we show that *p16^INK4a^* expression in PBTL has the properties of a useful peripheral blood biomarker of human molecular age. The test is an easily measured, low-cost assay that can be performed on a small specimen of whole blood with a short turnaround time. The test demonstrates low intra-individual variability and is highly dynamic with human age. Data from rodents systems suggest that p16^INK4a^ expression is not merely epiphenomenally associated with aging, but may also play a causal role in the process in diverse tissues (Baker et al., 2008; Janzen et al., 2006; Krishnamurthy et al., 2006; Molofsky et al., 2006; Signer et al., 2008). We further show that *p16^INK4a^* expression not only correlates with chronological age, but is associated with other aging modifying factors and markers of aging and frailty. These observations suggest that *p16^INK4a^* expression may provide an integrated measure of the effects of chronological age, environmental exposures and host genetics on molecular aging. Along these lines, it is worth noting that human *p16^INK4a^* expression in zero-hour kidney biopsies prior to transplantation has been recently shown to be a better predictor of long-term renal allograft function than other donor variables including chronologic age and telomere length (Koppelstaetter et al., 2008; McGlynn et al., 2008). In aggregate, these observations suggest that measurement of *p16^INK4a^* expression in a given tissue connotes information regarding intrinsic molecular age which is independent from chronologic age.

These data identify an intriguing difference between human and rodent aging. In mice and rats, a marked increase in *Arf* expression, comparable in magnitude to that of *p16^INK4a^*, is noted with aging (Krishnamurthy et al., 2004; Signer et al., 2008; Zindy et al., 1997). In human tissues (PBTL (present work) and kidney (Melk et al., 2004)), however, no association of chronologic age and ARF expression has been noted. Similarly, expression of murine p16^INK4a^ and Arf increases markedly with *in vitro* senescence in cultured murine fibroblasts (Kamijo et al., 1997; Sharpless et al., 2001), but only p16^INK4a^, and not ARF, appears to increase during the senescence of human cells (Munro et al., 1999; Wei et al., 2001). The molecular basis for this difference in the regulation of the *INK4/ARF* locus between species is unknown, but it is of interest that similar differences between human and

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murine cells in the patterns of *INK4/ARF* repression by Polycombgroup (PcG) proteins (e.g. BMI-1) have been recently reported (Bracken et al., 2007; Kotake et al., 2007). Most recently, loss of EZH2 expression with concomitant *p16*^{INK4a} activation has been reported in pancreatic β-cells *in vivo* with ageing in humans and mice (Hainan Chen, 2009). These observations suggest the model that a decrease in PcG repression occurs in all mammals with aging, with an attendant increased expression of only *p16*^{INK4a} in humans, but both *p16*^{INK4a} and *Arf* in rodents.

An important consideration in the interpretation of these results is the reliance on PBTL for sampling. It is likely that the rate of molecular aging will differ across human tissues, and that donor characteristics will influence molecular aging to a different degree in different tissues. For example, in a rat model (Krishnamurthy et al., 2004), we noted the greatest effects of caloric restriction on expression of *p16*^{INK4a} in the kidney; with little or no effect on certain other tissues such as the uterus. This observation suggests that the effects of an age-retarding stimulus (e.g. caloric restriction) on the rate of molecular aging may differ in distinct tissues of a given individual. Therefore, assays on components of peripheral blood may or may not be a useful surrogate for molecular age in other tissues or the organism *in toto*.

Another limitation of these analyses is that the correlative design does not establish an arrow of causality between *p16*^{INK4a} expression and other subject characteristics such as smoking and exercise. While evidence from model systems combined with the present observations can be most parsimoniously explained by the model that cigarette smoking potently accelerates molecular aging, interpreting the inverse correlation of PBTL *p16*^{INK4a} expression and exercise is more complex. This observation might signify that exercise retards the expression of p16^{INK4a} with aging; that individuals with lower p16^{INK4a} are more able to exercise; or that exercise and PBTL p16^{INK4a} expression co-correlate because they are both casually related to a third, unknown variable (e.g. certain dietary habits). Properly designed prospective studies will be needed to understand the relationships between molecular aging and behaviors such as smoking and exercise.

A determination of telomere length in unfractionated peripheral blood has also been suggested to be a biomarker of human molecular aging. While both approaches have merit, we think several aspects of *p16*^{INK4a} testing are advantageous. Setting aside the biologic rationale for each assay, *p16*^{INK4a} expression is more dynamic, and therefore more reliably measured, with chronologic age. In our sample, untransformed *p16*^{INK4a} expression changes on average nearly 10-fold over six decades of adult aging, whereas decreases in telomere length reported for the same age range are typically less than 2-fold (Allsopp et al., 1995; Frenck et al., 1998; Rufer et al., 1999; Son et al., 2000; Valdes et al., 2005; Vaziri et al., 1993). Moreover, although telomere length can be assayed by several methodologies of varying complexity, we believe the relative ease, low cost and reproducibility of qRT-PCR are strengths of the PBTL *p16*^{INK4a} methodology. Lastly, telomere length, at least when measured by multicolor flow FISH in large human cohorts, appears to change modestly during young adulthood (e.g. from ages 20-60), with an acceleration in the rate of shortening after the sixth decade (see for example (Alter et al., 2007; Armanios et al., 2007)). In contrast, log_2-transformed p16^{INK4a} expression significantly changes with aging in young adults (e.g. compare mean expression in 25 year olds versus 45 year olds in Fig. 1B), suggesting that this form of molecular `aging' is apparent well before one is `aged'. Relative disadvantages of PBTL *p16*^{INK4a} testing are the need for MACS processing and the reliance on RNA rather than DNA for analysis. While both of these features could limit the utility of p16^{INK4a} testing on retrospectively collected samples, neither is an important limitation to prospective testing; for example, for clinical indications.

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In summary, our study suggests that PBTL expression of *p16^{INK4a}* but not other *INK4/ARF* transcripts is a robust biomarker of human molecular age. Expression of this surrogate marker of molecular age reflects donor chronologic age as well as gerontogenic behaviors (e.g. smoking, physical inactivity). We believe this biomarker could be used to predict a donor's risk of future age-related adverse outcomes, to measure the efficacy of anti-aging therapies, and to examine the influences of human germline genetics on the rate of molecular aging.

Experimental Procedures

Study subjects

This study was approved by the University of North Carolina Institutional Review Board. Participants were recruited from a central site on the campus of the University of North Carolina Hospitals in Chapel Hill, NC. After providing informed consent, each participant completed a brief questionnaire about their health, health behaviors such as smoking and exercise, and demographics. Each participant also provided a 10-15 ml sample of whole blood. The exploratory cohort (N=80, Supp. Table 1), consisted of samples obtained from healthy subjects recruited in Chapel Hill, NC from Sep 27, 2007 to Feb 29, 2008. The exploratory cohort was also supplemented with whole blood samples from twenty anonymous donors from the Gulf Coast Regional Blood Center in Houston. These were shipped overnight at 4 degrees for processing. Extensive testing (not shown) indicated that this handling did not affect *INK4/ARF* expression. These samples lacked questionnaire information and were only included in tests for association with age, sex, and other transcript expression. A validation cohort (N=90, Supp. Table 1) was recruited in Chapel Hill, NC from May 19 to May 30, 2008 using the same recruitment and sample handling techniques. Seven participants consented to a repeat blood draw more than 4 weeks after initial testing to assess intra-subject variability of testing.

Flow cytometry, cell sorting, western analysis and ELISA

Multiple-color flow cytometry was performed on a CyAn™ ADP flow cytometer (Dako Cytomation). To exclude subjects with acute illness and chronic condition, all samples were first examined for T-cell activation by flow cytometry using predefined criteria (> 2% of $CD3^+CD25^+CD69^+$ cells in total T cells); one of 171 subjects was excluded by this criteria. $CD3^+$ PBTL and other peripheral blood cells were enriched directly from whole blood using AutoMACS™ pro separator (Miltenyi Biotec) after labeling with corresponding magnetic microbeads (Miltenyi Biotec) and then assessed for purity and viability by flow cytometry after CD3 and 7AAD staining (Supp. Fig. 1A). All $CD3^+$ T cells were enriched to greater than 90% purity. Fluorescent-conjugated antibodies used for flow cytometry were from BD Bioscience. Antibodies used for Western blotting were anti-$p16^{INK4a}$ (BD Bioscience) and anti-tubulin (Sigma-Aldrich). Quantification was achieved by using a fluorescent secondary antibody (Alexa Fluor 680, Invitrogen) and an infrared Western quantification system (Odyssey Infrared Imaging system by Li-Cor Bioscience). Plasma IL-6 concentration was quantified using ELISA kits (Invitrogen) according to manufacturer's instructions.

Nucleic acid preparation and analysis

RNA was prepared from PBTL using RNeasy mini kit (Qiagen) according to manufacture's directions. One µg of purified RNA was used for reverse transcription with ImProm-II™ RT system (Promega) according to manufacture's instructions. Expression of all transcripts was determined by Taqman® qRT-PCR (Details in Supp. Methods). Except for *p16^{INK4a}*, *ARF* and *ANRIL*; expression of all transcripts was determined using commercially available assays from Applied Biosytems. Expression of *p16^{INK4a}* and *ARF* was determined as previously described (Shields et al., 2007). For *ANRIL*, we developed and validated a qRT-

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PCR assay using primers and probes describe in the Supp. Methods. Transcript expression levels normalized to *18s* ribosomal RNA (Hs03003631, Applied Biosystems) and β*2 microglobulin* (β*2M*, Hs99999907, Applied Biosystems) are presented as $\log_2$-transformed data except where indicated.

Statistical analysis

Variables were first modeled by simple linear regression (see supplemental statistical methods). Pearson's correlation and R-squared statistics were computed in these instances. Multiple linear regression was then used to model the relationship between $p16^{INK4a}$ and covariates such as age, pack-years, exercise minutes per month, body mass index (BMI) and gender in aggregate data. The subjects without any clinical data for above covariates were excluded from the analysis (N=147 analyzed). We performed model selection using stepwise selection, backward elimination as well as criterion based methods such as AIC (Akaike Information Criterion)(Akaike, 1973) and BIC (Bayesian Information Criterion) (Schwarz, 1978). We also added an indicator variable for study (exploratory vs. validation) to test whether there is a significant effect caused by different datasets. To account for missing values in the dataset, especially in the exercise and pack-years, we conducted multiple regression incorporating the cases with missing values using the EM (Expectation Maximization) algorithm (Ibrahim, 1990) in LogXact. A p-value less than 0.05 were considered statistically significant. In addition, p-values were adjusted for multiple testing using the Bonferroni method in the pair-wise comparisons and exact p-values were computed when the sample size was small. Computations were performed using SAS (v. 9.1.3, SAS Institute Inc), StatXact and LogXact (v. 7, Cytel Software Corporation).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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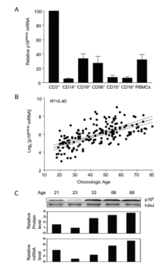

Figure 1. Expression of *p16^{INK4a}* in PBTL is associated with chronologic age

(A) Comparison of relative *p16^{INK4a}* expression in different cell types of the peripheral blood. Error bars indicate standard error of the mean (SEM). **(B)** Linear relationship between log_2-transformed *p16^{INK4a}* mRNA expression and chronological age. Aggregate data from the exploratory and validation cohorts are shown. The correlation coefficients and p-values of individual cohort are shown in Table 1. Dotted lines indicate the 95% confidence intervals (CI) of the fitted line. **(C)** Expression of p16^{INK4a} protein increases with chronologic age in a representative subset of patients.

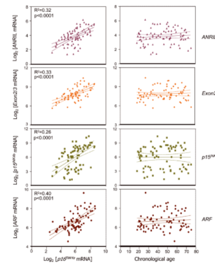

Figure 2. Expression of other *INK4/ARF* transcripts is associated with $p16^{INK4a}$ expression, but not age

Dotted lines indicate the 95% confidence intervals (CI). The correlations between the variables shown in the left panels are significant ($p<0.0001$) while those in the right panels are not significant ($p>0.4$). Data shown are from the exploratory cohort.

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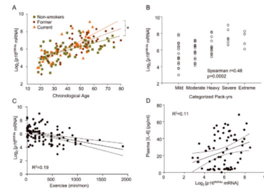

Figure 3. Expression of *p16^{INK4a}* is associated with smoking, exercise and plasma IL-6 concentrations

(A) The comparison of *p16^{INK4a}* -age linear regression among never smokers, former smokers and current smokers is shown for the combined cohort. Slope comparison: Current, $7.5 \pm 0.1 \times 10^{-2}$; Never, $3.9 \pm 0.7 \times 10^{-2}$. *p<0.05, adjusted for multiple comparisons. **(B)** Expression of *p16^{INK4a}* is associated with tobacco exposure. Categorized pack-years: Mild (>0, ≤5); Moderate (5-10); Heavy (11-30); Severe (31-50); Extreme (51 and up). **(C)** Decreased *p16^{INK4a}* mRNA expression is associated with increased exercise intensity. **(D)** Plasma IL-6 levels are associated with increased *p16^{INK4a}* expression. Aggregate data from the exploratory and the validation cohorts are shown in a, b, and c; whereas d represents data only from the validation cohort. The Pearson correlations and p-values in each cohort are shown in Table 1. Dotted lines in c and d indicate 95% CI.

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Table 1

Summary of tested correlations in both cohorts.

		All (N=170)				Exploratory (N=80)				Validation (N=90)***			
Response(Y)	Covariate(X)	N†	R^2	Correlation‡	p-value	N†	R^2	Correlation‡	p-value	N†	R^2	Correlation‡	p-value
Log_2[*p16^{INK4a}*]	Age	170	0.40	0.63	<.0001	80	0.37	0.61	<.0001	90	0.46	0.68	<.0001
	Pack-yrs	128	0.15	0.38	<.0001	44	0.20	0.45	0.0023	84	0.13	0.36	0.0008
	Extimeswk*	109	0.18	-0.42	<.0001	32	0.22	-0.47	0.0061	77	0.14	-0.38	0.0007
	Exminse*	105	0.14	-0.38	<.0001	28	0.30	-0.55	0.0024	77	0.10	-0.31	0.0054
	Exminmo*	104	0.19	-0.44	<.0001	28	0.28	-0.53	0.0037	76	0.14	-0.38	0.0007
	Serum IL-6**	145	N/A	N/A	N/A	69	0.08	0.27	0.02	76	0.11	0.33	0.003
	BMI	120	0.01	0.09	0.32	31	0.00	0.04	0.85				
	Log_2[*ARF*]	167	0.18	0.43	<.0001	77	0.40	0.63	<.0001	90	0.30	0.55	<.0001
	Log_2[*Exon2/3*]	165	0.17	0.41	<.0001	75	0.33	0.57	<.0001	90	0.32	0.57	<.0001
	Log_2[*p15^{INK4b}*]	167	0.25	0.50	<.0001	77	0.26	0.51	<.0001	90	0.34	0.59	<.0001
	Log_2[*ANRIL*]	167	0.31	0.56	<.0001	77	0.32	0.56	<.0001	90	0.38	0.62	<.0001
Log_2[*ARF*]	Age	167	0.00	0.03	0.67	77	0.01	0.08	0.47				
Log_2[*Exon2/3*]	Age	165	0.00	0.06	0.46	75	0.01	0.08	0.47				
Log_2[*p15^{INK4b}*]	Age	167	0.01	0.09	0.22	77	0.00	-0.01	0.93				
Log_2[*ANRIL*]	Age	167	0.01	0.11	0.15	77	0.01	0.09	0.45				

*Extimeswk=exercise times/week; exminse=exercise minutes/session; exminmo=exercise minutes/month

**Data were not combined due to batch effect caused by different ELISA measurement experiments.

***Only significant correlations in exploratory cohort were tested to confirm.

†For some variables, observations used in the analysis are less than total due to missing values

‡Pearson Correlation

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Table 2

Multiple regression model of $p16^{INK4a}$ expression and covariates.

Variable[*]	Label	Parameter estimate ±SE[**]	p-value
Age	Per chronological year	0.04±0.005	<.0001
Pack-yrs	0: Pack-yrs <= 5, 1: Pack-yrs > 5	0.36±0.17	0.0348
Exercise	0: min/month <= 240, 1: min/month > 240	-0.61±0.15	0.0001

[*] Estimated intercept: 4.21

[**] Parameter estimate indicates change in Log_2-transformed $p16^{INK4a}$ expression per unit change of covariant. SE: standard error