Published in final edited form as:

Mech Ageing Dev. 2007 ; 128(5-6): 370–377.

A COMMON VARIANT OF THE p16^{INK4A} GENETIC REGION IS ASSOCIATED WITH PHYSICAL FUNCTION IN OLDER PEOPLE

David Melzer¹, Timothy M Frayling, TM¹, Anna Murray¹, Alison J Hurst¹, Lorna W Harries¹, Honglin Song², KayTee Khaw², Robert Luben², Paul G Surtees², Stefania S Bandinelli³, Anna-Maria Corsi⁴, Luigi Ferrucci⁵, Jack M Guralnik⁶, Robert B Wallace⁷, Andrew T Hattersley¹, and Paul D Pharoah²

¹ Peninsula Medical School, Exeter UK

² Department of Public Health and Primary Care, University of Cambridge, UK

³ Laboratory of Clinical Epidemiology, Italian National Research Council on Aging, Geriatrics Department, Florence, Italy

⁴ Tuscany Regional Health Agency, Firenze, Italy, I.O.T. and Department of Medical and Surgical Critical Care, University of Florence, Italy

⁵ Longitudinal Studies Section, Clinical Research Branch, Gerontology Research Center, National Institute on Aging, Baltimore, Maryland, USA

⁶ Laboratory of Epidemiology, Demography and Biometry, National Institute on Aging, Bethesda, Maryland, USA

⁷ Department of Epidemiology, University of Iowa, USA

Abstract

p16^{INK4a} is active in cell senescence, ageing and tumor suppression. Deletion of the small p16^{INK4a} / ARF / p15^{INK4b} region occurs in many cancers. We screened 25 common polymorphisms across the region and 3 related genes for associations with physical functioning in older people.

In an initial sample of 938 (aged 65 to 80yrs) from the EPIC study (Norfolk, UK) the rs2811712 SNP minor allele (located between the shared p16^{INK4a} / ARF locus and p15^{INK4b}) was associated with reduced physical impairment. This association remained after testing an additional 1319 EPIC-Norfolk samples (p-value=0.013, total n=2257), and on independent replication in the InCHIANTI Study (n=709, p=0.015), and at one sided significance in Iowa-EPESE (n=419, p=0.079). Overall (n=3372) the prevalence of severely limited physical function was 15.0% in common homozygotes and 7.0% in rare homozygotes (per minor allele Odds Ratio=1.48 95%CI: 1.17–1.88, p=0.001, adjusted for age, sex and study). This estimate was similar excluding screening set 1 (OR=1.45: 95% CI 1.09–1.92, p=0.010, n=2434).

These findings require further replication, but provide the first direct evidence that the p16^{INK4a} / ARF / p15^{INK4b} genetic region and the senescence machinery are active in physical ageing in heterogeneous human populations. The mechanism involved may be via greater cellular restorative activity and reduced stem cell senescence.

Address for correspondence: Prof. David Melzer, Peninsula Medical School, RD&E Wonford Site, Barrack Road, Exeter, EX2 5DW United Kingdom. Email: david.melzer@pms.ac.uk.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Conflicts of interest None

Introduction

Several recent findings suggest that cancer protection pathways leading to cell cycle arrest and cell senescence are important in ageing (Campisi, 2005;Beausejour and Campisi, 2006). A key protein in triggering cell senescence is p16^{INK4a}, which regulates the Rb cancer suppressor pathway (Sharpless, 2004;Herbig et al., 2006). p16^{INK4a} is coded for by the CDKN2a locus, situated on chromosome 9p21 in humans (Kim and Sharpless, 2006), but unusually this locus also codes for a second product, ARF (called p19^{ARF} in mice), which regulated the p53 cancer suppressor (Quelle et al., 1995).

The small region (<42kb in Hapmap II) including p16^{INK4a} / ARF and p15^{INK4b} is amongst the most frequent lost in human cancer (Kim and Sharpless, 2006) and germline mutations of p16^{INK4a}/ARF are linked to melanoma and other malignancies (Murphy et al., 2004;Lang et al., 2005).

p16^{INK4a} expression and cell senescence can be triggered by various major biological stresses including DNA damage and oxidative stress (Ben-Porath and Weinberg, 2005;Massague, 2004). Expression of p16^{INK4a} increases markedly with age (Nielsen et al., 1999;Zindy et al., 1997), including in many tissues in rodents (Krishnamurthy et al., 2004), in the skin of aged baboons (Herbig et al., 2006) and in human kidney tissue (Melk et al., 2004). In addition, transgenic models have recently shown that increased expression of p16^{INK4a} causes some aspects of ageing in several stem cell and self-renewing compartments, including neural stem cells (Molofsky et al., 2006), hematopoietic stem cells (Janzen et al., 2006) and pancreatic beta-cells (Krishnamurthy et al., 2006)).

In addition to p16^{INK4a} / ARF (CDKN2a) and p15^{INK4b} (CDKN2b) there are related Cyclin Dependent Kinase inhibitors involved at various points linked to the p53 or Rb pathways (Satyanarayana and Rudolph, 2004).

Physical ageing markers in human populations

While the most popular approach to identifying 'ageing genes' in humans involves comparisons of long lived with younger groups (Melzer et al., 2007), there is increasing interest in markers of age-related functioning (Barzilai and Shuldiner, 2001;Karasik et al., 2005;Martin, 2005), from early old age onward. Declines in functional abilities (e.g. slow walking speed, impaired ability to rise from the sitting position, etc) offer good summary measures of health in older people, and are predictive of disability progression and mortality (Guralnik et al., 1989;Guralnik et al., 1995;Guralnik and Ferrucci, 2003). Many aspects of physical functioning in older people show substantial heritability (Leinonen et al., 2005), including muscle strength (Reed et al., 1991;Frederiksen et al., 2002;Tiainen et al., 2004), and gait speed (Carmelli et al., 2000). Even the self reported SF36 health questionnaire physical functioning sub-score (based on limitations in walking, climbing stairs and certain activities of daily living) had an additive genetic component of 30% (95% CI 27%-45%) in middle-aged male twins in the USA (Romeis et al., 2005). Measured functional impairments have been shown to be a sensitive phenotype in older people for the ApoE e4 polymorphism (Melzer et al., 2005) and for a polymorphism in the inflammatory marker Interleukin 18 (Frayling et al., 2007).

In this study we tested the hypothesis that common genetic variants (minor allele frequency (MAF)>0.05) in the p16^{INK4a}/ARF/p15^{INK4b} region and three related Cyclin Dependent Kinase inhibitor genes involved in triggering cell senescence are associated with differences in physical functioning in older people.

Materials and methods

Three separate studies of white Europeans were used. An initial sample of 938 participants aged 65 to 80 years from the Norfolk site of the EPIC study (Day et al., 1999) was genotyped for the 25 single nucleotide polymorphisms (SNPs) chosen as tagging the known common variants (Table 1) in the genetic regions covering CDKN1a, CDKN1b, CDKN2a, CDKN2b and CDKN2c.

This initial screening set formed the older part of the control group for a breast cancer gene study (Anglian Breast Cancer Study Group, 2000): hence all were women. A SNP most closely associated with physical functioning was then genotyped in an additional 1319 EPIC-Norfolk participants aged 65 to 80 years in a second set (including 666 (48%) men). Seven hundred and twenty five participants (including 47% men) from the Italian InCHIANTI ageing study (Ferrucci et al., 2000) and 419 participants (including 48% men) from the Iowa site of the Established Populations for Epidemiologic Studies of the Elderly (EPESE) (Cornoni-Huntley et al., 1993) were available for further replication of the observed association. This staged design reduced genotyping costs and the need to adjust for multiple testing for statistical significance.

The Population studies

The Norfolk site of the European Prospective Investigation of Cancer study (EPIC-Norfolk) is a large population based cohort (Day et al., 1999). From 1993 to 1997 participants then aged 45 to 74 years were recruited through general practice registers in Norfolk, England. The sample was 98% white European. Blood for DNA extraction was collected during the second health check in 1998 to 2000. The study was approved by the Anglia and Oxford Multicenter Research Ethics committee and the Norwich Local Research Ethics Committee.

The InCHIANTI study (Ferrucci et al., 2000) is a population study of decline of mobility in late life. The sample is representative of the population of two small towns in Tuscany, Italy. Blood samples were taken at baseline. The participants were all of white European origin. To be consistent with the EPIC-Norfolk study, we have analysed data from those aged 65 to 80 years old only. The Italian National Institute of Research and Care of Aging Institutional Review Board ratified the study protocol.

The Iowa Established Populations for Epidemiological Study of the Elderly (EPESE) is also a population based cohort (Cornoni-Huntley et al., 1993). Between 1981 and 1983 the entire population aged ≥ 65 years living in two Iowa counties was surveyed, and follow-up data were collected yearly for seven years. Blood specimens were obtained from those re-interviewed for the sixth annual follow-up in 1988. Of the available sample genotyped for CDKN2a, $n=1042$ participants were aged 71 to 81 years at follow-up six. Loss to follow-up over the first six years of this study was strongly associated with disability at baseline, which would result in differential attrition of gene variants active in functioning. We therefore restricted the analysis to those who were free of functional impairments at baseline. 81% of the sample reported themselves to be of 'white' European origin, with the remainder mainly reporting unknown origins: the latter were excluded from the analysis, leaving 419 participants.

Measures of physical function

The details of the physical function measures available in the population studies varied, but the content covered was similar. In the EPIC-Norfolk study, the data were from the physical function subscale of the Short Form 36 (SF-36) (Ware et al., 1993) health survey questionnaire. The questionnaire was completed by post (Surtees et al., 2003) using the anglicised version (Jenkinson, 1999). SF36 subscales have been extensively validated (Kane and Kane RA,

2000) and, as mentioned, the physical functioning subscale has an additive genetic component of 30% (95% CI 27%-45%) (Romeis et al., 2005). Limitations elicited in the ten question subscale cover the range of physical activities from ability to perform strenuous sports, through moderate activities, climbing stairs and walking various distances, down to basic activities of daily living such as ability to bath and dress oneself.

In the InCHIANTI study, participants were asked during interviews about difficulties performing six Activities of Daily Living (ADL), including bathing, dressing, eating, grooming, toileting and continence, covering the more severe end of physical limitation. Responses were rated 1 'no difficulty', 2 'with difficulty but no help', 3 'with help' or 4 'unable'. Subjects were classified as having an ADL difficulty in InCHIANTI only if they required help or were unable to perform the task: this requirement identified a very disabled group with a low prevalence.

In addition to self reports, the InCHIANTI study included measures of tested physical performance summarized into the highly validated Short Physical Performance Battery Score (Guralnik et al., 2000). Tests included balance, four-meter walks and chair stands. For the balance tests, each subject was asked to stand for 10 seconds with feet in three different stances, side-by-side, semi-tandem and tandem. The gait speed result was defined as the best time (in seconds) of two 4-m walks at usual pace along a corridor during the medical examination. For the chair-stand test, the participants were asked to rise and sit five times as quickly as possible with their hands folded across the chest. The chair-stand and gait speed results were divided into quartiles and participants scored 4 points if their time was within the first quartile, 3 points for the second quartile etc. Those participants who were unable to complete the task were scored 0.

In the Iowa study, Activity of Daily Living questions were similar to InCHIANTI and included difficulties with bathing, grooming, dressing, eating without aid, transferring from bed to chair and using the toilet. Scoring was similar to that in the InCHIANTI study but included all grades of difficulty, not just those involving help. For tested performance in Iowa, the three components of the Short Performance Score were similar to those in the InCHIANTI study, except that the walk test was relatively short (over only 8 feet) and timings were carried out by stop watch, rather than electronic sensors.

Tag SNP selection—For EPIC-Norfolk set 1, we aimed to identify a set of tagging SNPs (tSNPs) that efficiently capture all the known common variants in each gene (Minor Allele Frequency MAF>0.05). Resequencing data from the NIEHS Environmental Genome Project (EGP) were used, and where resequencing data were not available we used data from the International HapMap Project to select tagging SNPs (21-06-2005: HapMap last public release used for SNP selection). We aimed to define a set of tagging SNPs such that the correlation between each SNP and a haplotype of tagging SNPs (r_s^2) was >0.8.

Genotyping—EPIC-Norfolk samples were genotyped using the ABI PRISM 7900 sequence detection system or 'Taqman' (Applied Biosystems, Foster City, CA, USA). Similarly, genotyping in InCHIANTI and Iowa-EPESE was by a TaqMan PCR assay: 20ng of genomic DNA was amplified in each assay, with specific probes designed by Applied Biosystems. Following 40 cycles of PCR, fluorescence was measured on a Pherastar plate reader and compared with an internal control ROX dye standard. Genotypes were assigned blind to phenotype using Kluster caller software (Kbioscience). Approximately 10% of samples were duplicated and scored blind, with low error rates: for example, there were 0/232 duplicate errors in InChianti and Iowa-EPESE.

Statistical Analysis—Deviation of genotype frequencies from Hardy-Weinberg equilibrium was assessed by a χ^2 test with one degree of freedom (1df). The power of a SNP in linkage disequilibrium with a second SNP to detect associations due to that second SNP was estimated using the r^2 statistic, which is the square of the conventional correlation coefficient between alleles at the two loci of interest (Cordell and Clayton, 2005).

Analyses in EPIC-Norfolk and InCHIANTI were on participants aged 65 to 80 years old (71 to 80 in Iowa EPESE), as disability rates of younger participants were too low and in older participants too high to yield useful estimates. These age limits were fixed before replication. In all analyses sex (except set 1) and years of age were included as covariates: inclusion or exclusion of these variables resulted in only small differences in estimates.

The available measures of functioning are most accurate in identifying poor performance and are skewed, with the majority of people scoring in the ‘normal functioning’ end of the scales. We therefore dichotomised scores for all follow-up association testing across the three studies, identifying a physically ‘limited’ group to compare to the rest. In the large EPIC-Norfolk dataset, dichotomization aimed to identify the most limited 15% and in the smaller InCHIANTI and Iowa EPESE studies aimed to identify the most limited 25%, although the exact size of groups varied a little depending on the frequency distributions of the original scores. A sensitivity analysis altering these cut-points was done, to check that associations were robust. To calculate the pooled estimates for all the data, dichotomised SF36 and performance measure data (with cut-points identifying the most impaired approximately 15%) were combined, and logistic regression models were adjusted for years of age, sex and study set (EPIC-Norfolk coded 1 & 2, InCHIANTI = 3 and Iowa = 4).

Results

We examined associations between 25 selected SNPs tagging variation in five CDK inhibitors and the SF36 physical performance subscale (SF36-PF), in an initial set of 938 EPIC-Norfolk participants. Genotyping frequencies did not deviate significantly from Hardy-Weinberg equilibrium. Of the SNPs tested, two (rs2811712 and rs3218005) were associated with the continuous SF36-PF score. The rare alleles of each SNP (G allele in both cases) were associated with higher SF36 PF scores, reflecting better function and less limitation. Alleles from these two SNPs tend to be present together (linkage disequilibrium $r^2 = 0.83$ in hapmap II; r^2 in EPIC-Norfolk set 1 = 0.88) and mark the same haplotype: we therefore selected the rs2811712 SNP as our marker of the shared haplotype for further study, given the marginally stronger statistical significance.

We then analysed an additional 1319 samples from EPIC-Norfolk respondents (Set 2, Table 2 & 3) to replicate the screening result. In set 2, disability levels were lower than set 1 (e.g. 13.4% of common homozygotes scoring less than 20 on the SF36 subscale vs. 19.9% in the set 1 common homozygotes). Lower prevalence of disability reduced power to detect differences and perhaps because of this a smaller odds ratio in the same direction as set 1 was found (OR=1.38: 95%CI: 0.91–2.08) which did not reach nominal significance on its own. Based on all the EPIC-Norfolk data identifying the 15% most disabled respondents, the overall odds ratio per rare allele was 1.46 (95% CI 1.08–1.97; $p=0.013$).

To confirm the association in an independent population, we next used the InCHIANTI study. In this study data from tests of physical performance were combined into the well validated Short Physical Performance Battery score (see methods). This score is most accurate for poor function, and for a binary outcome of very limited function (selecting <25%) vs the rest (Tables 2 & 3) the per allele logistic regression odds ratio was 1.80 (95%CI 1.12–2.89; $p=0.015$). For the available measure of self-reported number of Activities of Daily Living disabilities

requiring help from others (which identified 5% of the sample as disabled), the per allele odds ratio was also significant (OR=7.38, 95%CI 1.01–53.7, $p=0.049$).

In the final but relatively small dataset, the Iowa EPESE study, the association with incident reported Activity of Daily Living disabilities reached one sided significance in the same direction as above (per allele OR 1.61; 95%CI 0.94–2.74, $p\text{-value}=0.079$). For tested performance (on less stringently measured tests than in InCHIANTI) the point estimate was in line with the previous estimates, but the confidence intervals were wide and the association was not significant ($p > 0.05$).

There are no established cut-points for identifying poor function on the physical impairment scales, as the underlying trait follows a continuous (but skewed) distribution. We therefore performed a sensitivity analysis using different cut-points classifying participants as most disabled. In the InCHIANTI study, dichotomising the Short Physical Performance measure between scores of 8 and 9 (out of 12) classified 12.5% of participants as impaired, and yielded an odds ratio for rs2811712 of 1.80 (95%CI 0.91–3.56, $p=0.089$). This odds ratio was the same as for the cut-point between scores of 10 and 11, identifying approximately 26% of participants as impaired (Table 3, OR=1.80: 95%CI 1.12–2.89; $p=0.015$). For a cut-point in-between (at 9 / 10) that classified approximately 20% of participants as impaired, as used in a previous paper (Frayling et al., 2007), the association was OR=1.42 (95% CI 0.88 – 2.29, $p=0.150$). For the small Iowa-EPESE study, altering cut points (of 15, 20 or 25%) for physical impairment did not alter the non-significant association reported in Table 3.

Although there are differences in the details of the functioning measures, the domains covered are similar. To provide an approximate overall summary of the results, we therefore pooled the results based on individual data, identifying the most severely impaired approximately 15% of participants across all three studies. In the sample total of 3372 participants the overall per minor allele odds ratio for the absence of severe physical limitation was 1.48 (95%CI 1.17–1.88, adjusted for age, sex and set/study), which was highly significant ($p=0.001$). Excluding the screening set 1 (which suffered from multiple statistical testing) produced a similar estimate (OR=1.45: 95%CI 1.09–1.92, $p=0.010$, $n=2434$). Fig. 1 shows the estimated adjusted prevalence of limited physical function from the overall logistic regression models. The strong ‘protective’ effect of the minor allele for physical limitation is evident: the overall prevalence of severely limited function declined from 15.0% in the common homozygotes through 10.6% in the heterozygotes to 7.0% in the rare homozygous group. Rates of limitation rise sharply across the 65 to 80 year age range, and the difference in prevalence of severe limitation is approximately equivalent to a four-year difference in age per allele: i.e. those carrying two rare alleles have rates of physical limitation broadly equivalent to the common homozygous group aged eight years younger.

As a secondary outcome, simple measures of cognitive function were analysed in the InCHIANTI and Iowa studies, but no associations were found. As the rare allele of rs2811712 marks variation in the cancer related p16^{INK4a} / ARF / p15^{INK4b} (CDKN2a and CDKN2b) locus, we also examined cancer registrations and total mortality for the EPIC-Norfolk cohort. Genotypes for the rs2811712 SNP were available for a total of 7850 respondents in EPIC-Norfolk from age 45 to 80 years. Allele frequencies were not significantly different by age. An analysis of all those reported as having any cancer from hospital registries found no association with rs2811712 allele count (OR=1.0007; 95%CI 0.81–1.23 $p=0.995$), and there was no association with total mortality ($n=370$ deaths, OR=1.16 95%CI 0.90–1.51, $p=0.255$). The numbers of respondents with physician diagnoses of specific diseases were small in the available data and association testing with SNP status was underpowered.

The p16^{INK4a}/ARF/p15^{INK4b} locus operates to modulate the expression of many genes including in the Insulin like Growth Factor 1 (IGF1) signalling system. Measures of free and total IGF1 levels were available in the InCHIANTI study only. In logistic regression models adjusted for age and sex (age-group 65 to 80 years) there was a strong association between the rs2811712 rare allele and higher free IGF1 levels (regression coefficient 0.11, $p = 0.004$). There was no significant association with total IGF1 levels or with fasting insulin levels.

Discussion

In a survey of 25 SNPs tagging variation in five Cyclin Dependent Kinase Inhibitors, we identified the rs2811712 SNP (which lies 3.5 kb proximal to the p16/ARF locus) as significantly related to physical function in a heterogeneous older human population. Our screening findings were then supported by further samples from the EPIC-Norfolk study, and from the independent InCHIANTI and Iowa-EPESE population studies. Using either the replication samples alone ($p=0.010$ excluding the multiply tested screening set 1) or all the data ($p=0.001$), there were significant associations between the minor allele and a lower prevalence of limited physical functioning. Overall the prevalence of severely limited function declined from 15.0% in the common homozygotes through 10.6% in the heterozygotes to 7.0% in the rare homozygous, in the 65 to 80 year age group studied. This difference is approximately equivalent to a four-year delay in this key aspect of ageing, per rare allele.

In evaluating these results there are a number of study strengths and weaknesses that need to be considered. The presence of data from three independent representative population studies all tending to show the same trends with genotype is clearly a strength. However, the physical functioning measures varied between studies, although all were designed to identify overlapping aspects of physical functioning. Different measures of functioning are likely to classify milder grades of disability in slightly different ways, but are also likely to have high levels of agreement on severe disability: therefore our analysis focussed on the most impaired group. Both self reported Activity of Daily Living difficulties and performance tests are predictive of mortality, with the self reports performing particularly well in more physically limited people (Reuben et al., 2004). Performing a meta-analysis of the data across measures (with dichotomisation identifying very limited functioning) is therefore unlikely to have been problematic, and has the advantage of providing a quantitative summary of effects across the studies.

A second point is that in the original 'screening' sample (Set 1) in EPIC-Norfolk, multiple statistical tests were undertaken: in addition to the 25 CDKN SNPs, over 600 other SNPs have been tested in this genetic association study program. To deal with multiple testing, the study design involved hypothesis driven replication in further samples and in independent datasets. The finding of similar trends or significant associations in the same direction in the independent EPIC-Norfolk set 2, InCHIANTI and Iowa samples increases the chances that the association is real, as testing of these was based on the *a priori* hypothesis from set 1, and did not suffer from multiple testing. Importantly, the overall replication effort showed the same association and was statistically significant ($p=0.010$) excluding the screening set 1.

Finally, the EPIC-Norfolk set 2 data and the Iowa-EPESE performance measures did not reach one sided statistical significance on their own, but central estimates in both cases were in the same direction as the overall effects. The low prevalence of impairment in set 2 and the small sample size in the Iowa-EPESE dataset are sufficient to explain the lack of nominal significance, although it is clear that the overall trend in all the data is in the same direction. The absence of nominal significance in an individual sample is not, of course, evidence of lack of association, and meta-analysis of all the available data provides the most robust estimate of overall significance.

Although the rs2811712 polymorphism was selected because it tags a variant in CDKN2a, rs2811712 is physically located between CDKN2a (p16^{INK4a}/ARF) and CDKN2b (p15^{INK4b}). There are 16 further SNPs in linkage disequilibrium with rs2811712 at $r^2 > 0.8$ in HapMap II. These spans a distance of 86,157 bases from position 21958199 to 22044356 on chromosome 9, including five SNPs in CDKN2a, three between CDKN2a and CDKN2b, two in CDKN2b and seven upstream (5' end) of CDKN2b, with none near other known genes. The 'upstream' SNPs include the proposed regulatory domain for both CDKN2a and CDKN2b (Gonzalez and Serrano, 2006). There are 8 SNPs recorded in dbSNP in this CDKN2 a&b region which cause non-synonymous changes in the coding sequence of either p16^{INK4a} or ARF and a further 24 in transcribed, but untranslated regions of p16^{INK4a}, ARF and p15^{INK4b}. The majority of these SNPs either do not have genotype frequencies listed, are monomorphic in Caucasians or are not in HapMap II.

Rs11515 is in linkage disequilibrium with rs2811712 ($r^2=0.85$) in Hapmap II and is in the 3'UTR of CDKN2a and hence is expressed in the p16^{INK4a} and ARF transcripts, making it a strong candidate for being the functional variant. However rs11515 was not associated with physical functioning ($p=0.715$) in EPIC-Norfolk set 1 (Table 1) or in the InCHIANTI study (data available from authors). Also, the linkage disequilibrium between rs2811712 and rs11515 in EPIC-Norfolk was less than the HapMap estimate ($r^2=0.55$ with a similar estimate in InCHIANTI) and therefore rs11515 is unlikely to be the SNP generating our association. Clearly, further work is needed to identify the active site.

As noted, the biological evidence for ageing effects linked to the p16^{INK4a}/ARF / p15^{INK4b} region implicates p16^{INK4a} as the likely active protein. The recent evidence from transgenic models that increased expression of p16^{INK4a} leads to some aspects of ageing in neural stem cells (Molofsky et al., 2006), hematopoietic stem cells (Janzen et al., 2006) and pancreatic beta-cells (Krishnamurthy et al., 2006) indicates that p16^{INK4a} is an active factor in ageing in laboratory models. Our findings of a common variant of the p16^{INK4a}/ARF / p15^{INK4b} region associated with less physical ageing is consistent with the p16^{INK4a} models. As far as we are aware our finding is the first direct evidence that the p16^{INK4a}/ARF / p15^{INK4b} region does have an active role in ageing processes in the heterogeneous older human population. More work is now needed to demonstrate each of the steps linking the polymorphism through gene expression to cell senescence, effects on organ function and to the overall reduction of physical impairment.

Clearly, more work is also needed to replicate and confirm the associations of the identified haplotype with poor physical functioning in independent study populations. Replication efforts should preferably include groups other than Caucasians. If confirmed, the identified haplotype needs to be characterised further, identifying the mechanisms of its effect. Larger epidemiological studies will be needed to identify any effects on specific disease incidence and overall mortality.

Conclusions

Increased p16^{INK4a} activity plays a pivotal role in triggering cell senescence and protection from malignancy, but such protection is also associated with acceleration of some aspects of ageing in laboratory models. We have shown that a common inherited (germline) variant of the p16^{INK4a}/ARF / p15^{INK4b} genetic region is strongly associated with reduced limitation in physical function in people aged 65 to 80 years, although further replication is needed in independent populations. These findings provide the first direct evidence for an active role for the p16^{INK4a}/ARF / p15^{INK4b} region in the physical ageing processes in heterogeneous human populations.

Acknowledgements

This work is supported in part by NIH/NIA grant R01 AG24233-01, and by the Intramural Epidemiology Demography and Biometry Laboratory, National Institute on Aging. DM is supported by a NHS Executive National Public Health Career Scientist Awards, Ref: PHCSA/00/002

EPIC-Norfolk is supported by programme grants from the Medical Research Council and Cancer Research UK, with additional support from the Stroke Association, Research Into Ageing and British Heart Foundation.

The InCHIANTI study was supported as a “targeted project” (ICS 110.1\RS97.71) by the Italian Ministry of Health, by the U.S. National Institute on Aging (Contracts N01-AG-916413, N01-AG-821336 and Contracts 263 MD 9164 13 and 263 MD 821336) and in part by the Intramural Research Branch, NIA-NIH, USA

Reference List

- Anglian Breast Cancer Study Group. Prevalence and penetrance of BRCA1 and BRCA2 mutations in a population-based series of breast cancer cases. *Br.J Cancer* 2000;83:1301–1308. [PubMed: 11044354]
- Barzilai N, Shuldiner AR. Searching for human longevity genes: The future history of gerontology in the post-genomic era. *Journals of Gerontology - Series A Biological Sciences and Medical Sciences* 2001;56:M83–M87.
- Beausejour CM, Campisi J. Ageing: balancing regeneration and cancer. *Nature* 2006;443:404–405. [PubMed: 16957734]
- Ben-Porath I, Weinberg RA. The signals and pathways activating cellular senescence. *Int.J Biochem.Cell Biol* 2005;37:961–976. [PubMed: 15743671]
- Campisi J. Aging, tumor suppression and cancer: High wire-act! *Mechanisms of Ageing and Development* 2005;126:51–58. [PubMed: 15610762]
- Carmelli D, Kelly-Hayes M, Wolf PA, Swan GE, Jack LM, Reed T, Guralnik JM. The contribution of genetic influences to measures of lower-extremity function in older male twins. *J Gerontol A Biol Sci Med Sci* 2000;55:B49–B53. [PubMed: 10719763]
- Cordell HJ, Clayton DG. Genetic association studies. *Lancet* 2005;366:1121–1131. [PubMed: 16182901]
- Cornoni-Huntley J, Ostfeld AM, Taylor JO, Wallace RB, Blazer D, Berkman LF, Evans DA, Kohout FJ, Lemke JH, Scherr PA. Established populations for epidemiologic studies of the elderly: study design and methodology. *Aging (Milano.)* 1993;5:27–37. [PubMed: 8481423]
- Day N, Oakes S, Luben R, Khaw KT, Bingham S, Welch A, Wareham N. EPIC-Norfolk: study design and characteristics of the cohort. *European Prospective Investigation of Cancer. Br.J Cancer* 1999;80 (Suppl 1):95–103. [PubMed: 10466767]
- Ferrucci L, Bandinelli S, Benvenuti E, Di Iorio A, Macchi C, Harris TB, Guralnik JM. Subsystems Contributing to the Decline in Ability to Walk: Bridging the Gap between Epidemiology and Geriatric Practice in the InCHIANTI Study. *Journal of the American Geriatrics Society* 2000;48:1618–1625. [PubMed: 11129752]
- Frayling T, Rafiq S, Murray A, et al. An Interleukin-18 Polymorphism Is Associated With Reduced Serum Concentrations and Better Physical Functioning in Older People. *J Gerontol A Biol Sci Med Sci.* 2007In Press
- Frederiksen H, Gaist D, Petersen HC, Hjelmborg J, McGue M, Vaupel JW, Christensen K. Hand grip strength: a phenotype suitable for identifying genetic variants affecting mid- and late-life physical functioning. *Genet.Epidemiol* 2002;23:110–122. [PubMed: 12214305]
- Gonzalez S, Serrano M. A new mechanism of inactivation of the INK4/ARF locus. *Cell Cycle* 2006;5:1382–1384. [PubMed: 16855387]
- Guralnik JM, Branch LG, Cummings SR, Curb JD. Physical performance measures in aging research. *J.Gerontol* 1989.Sep.;1989 44:M141–M146. [PubMed: 2768767]
- Guralnik JM, Ferrucci L. Assessing the building blocks of function: Utilizing measures of functional limitation. *American Journal of Preventive Medicine* 2003;25:112–121. [PubMed: 14552934]
- Guralnik JM, Ferrucci L, Leveille SG, Pieper CF, Markides KS, Ostir GV, Studenski S, Berkman LF, Wallace RB. Lower-extremity function and subsequent disability: Consistency across studies, predictive models, and value of gait speed alone compared with the short physical performance

- battery. *Journals of Gerontology - Series A Biological Sciences and Medical Sciences* 2000;55:M221–M231.
- Guralnik JM, Ferrucci L, Simonsick EM, Salive ME, Wallace RB. Lower extremity function in persons over the age of 70 years as a predictor of subsequent disability. *N.Engl.J.Med* 1995;332:556–561. [PubMed: 7838189]
- Herbig U, Ferreira M, Condel L, Carey D, Sedivy JM. Cellular Senescence in Aging Primates. *Science* 2006;311:1257. [PubMed: 16456035]
- Janzen V, Forkert R, Fleming HE, Saito Y, Waring MT, Dombkowski DM, Cheng T, DePinho RA, Sharpless NE, Scadden DT. Stem-cell ageing modified by the cyclin-dependent kinase inhibitor p16INK4a. *Nature* 2006;443:421–426. [PubMed: 16957735]
- Jenkinson C. Comparison of UK and US methods for weighting and scoring the SF-36 summary measures. *Journal of Public Health Medicine* 1999;21:372–376. [PubMed: 11469357]
- Kane, R.; Kane, RA. *Assessing Older People*. Oxford University Press; Oxford: 2000.
- Karasik D, Demissie S, Cupples LA, Kiel DP. Disentangling the genetic determinants of human aging: Biological age as an alternative to the use of survival measures. *Journals of Gerontology Series A-Biological Sciences and Medical Sciences* 2005;60:574–587.
- Kim W, Sharpless NE. The Regulation of INK4/ARF in Cancer and Aging. *Cell* 2006;127:265–275. [PubMed: 17055429]
- Krishnamurthy J, Ramsey MR, Ligon KL, Torrice C, Koh A, Bonner-Weir S, Sharpless NE. p16INK4a induces an age-dependent decline in islet regenerative potential. *Nature* 2006;443:453–457. [PubMed: 16957737]
- Krishnamurthy J, Torrice C, Ramsey MR, Kovalev GI, Al-Regaiey K, Su L, Sharpless NE. Ink4a/Arf expression is a biomarker of aging. *J.Clin.Invest* 2004;114:1299–1307. [PubMed: 15520862]
- Lang J, Boxer M, MacKie RM. CDKN2A mutations in Scottish families with cutaneous melanoma: Results from 32 newly identified families. *British Journal of Dermatology* 2005;153:1121–1125. [PubMed: 16307646]
- Leinonen R, Heikkinen E, Rantanen T, Tolvanen A, Kaprio J, JylhΣ M, Koskenvuo M. Genetic influences underlying self-rated health in older female twins. *Journal of the American Geriatrics Society* 2005;53:1002–1007. [PubMed: 15935024]
- Martin GM. Genetic modulation of senescent phenotypes in *Homo sapiens*. *Cell* 2005;120:523–532. [PubMed: 15734684]
- Massague J. G1 cell-cycle control and cancer. *Nature* 2004;432:298–306. [PubMed: 15549091]
- Melk A, Schmidt BMW, Takeuchi O, Sawitzki B, Rayner DC, Halloran PF. Expression of p16INK4a and other cell cycle regulator and senescence associated genes in aging human kidney 2004;65:510–520.
- Melzer D, Hurst A, Frayling T. Genetic Variation and Human Aging: Progress and Prospects. *J Gerontol A Biol Sci Med Sci*. 2007In press
- Melzer D, Dik MG, Jonker C, Deeg DJ, Van Kamp GJ. The apolipoprotein E ε4 polymorphism is strongly associated with poor mobility performance test results but not self-reported limitation in older people. *Journals of Gerontology - Series A Biological Sciences and Medical Sciences* 2005;60:1319–1323.
- Molofsky AV, Slutsky SG, Joseph NM, He S, Pardal R, Krishnamurthy J, Sharpless NE, Morrison SJ. Increasing p16INK4a expression decreases forebrain progenitors and neurogenesis during ageing. *Nature* 2006;443:448–452. [PubMed: 16957738]
- Murphy JA, Barrantes-Reynolds R, Kocherlakota R, Bond JP, Greenblatt MS. The CDKN2A database: Integrating allelic variants with evolution, structure, function, and disease association. *Hum.Mutat* 2004;24:296–304. [PubMed: 15365986]
- Nielsen GP, Stemmer-Rachamimov AO, Shaw J, Roy JE, Koh J, Louis DN. Immunohistochemical survey of p16(INK4A) expression in normal human adult and infant tissues. *Laboratory Investigation* 1999;79:1137–1143. [PubMed: 10496532]
- Quelle DE, Sherr CJ, Zindy F, Ashmun RA. Alternative reading frames of the INK4a tumor suppressor gene encode two unrelated proteins capable of inducing cell cycle arrest. *Cell* 1995;83:993–1000. [PubMed: 8521522]
- Reed T, Fabsitz RR, Selby JV, Carmelli D. Genetic influences and grip strength norms in the NHLBI twin study males aged 59–69. *Ann.Hum.Biol* 1991;18:425–432. [PubMed: 1952800]

- Reuben DB, Seeman TE, Hirsch SH, Keeler E, Hayes RP, Bowman L, Sewall A, Wallace RB, Guralnik JM. Refining the categorization of physical functional status: The added value of combining self-reported and performance-based measures. *Journals of Gerontology - Series A Biological Sciences and Medical Sciences* 2004;59:1056–1061.
- Romeis JC, Scherrer JF, Fu Q, True WR, Heath AC, Eisen SA, Bucholz KK, Xian H, Goldberg J, Lyons MJ, Waterman B, Tsuang MT, Pedersen NL. Heritability of SF-36 among middle-age, middle-class, male-male twins. *Medical Care* 2005;43:1147–1154. [PubMed: 16224309]
- Satyanarayana A, Rudolph KL. p16 and ARF: activation of teenage proteins in old age. *J.Clin.Invest* 2004;114:1237–1240. [PubMed: 15520854]
- Sharpless NE. Ink4a/Arf links senescence and aging. *Experimental Gerontology* 2004;39:1751–1759. [PubMed: 15582292]
- Surtees PG, Wainwright NWJ, Khaw KT, Day NE. Functional health status, chronic medical conditions and disorders of mood. *British Journal of Psychiatry* 2003;183:299–303. [PubMed: 14519607]
- Tiainen K, Sipilä S, Alen M, Heikkinen E, Kaprio J, Koskenvuo M, Tolvanen A, Pajala S, Rantanen T. Heritability of maximal isometric muscle strength in older female twins. *Journal of Applied Physiology* 2004;96:173–180. [PubMed: 12959964]
- Ware, J.; Snow, K.; Kosinski, M. SF-36 Health Survey: Manual and Interpretation Guide. Nimrod Press; Boston: 1993.
- Zindy F, Quelle DE, Roussel MF, Sherr CJ. Expression of the p16(INK4a) tumor suppressor versus other INK4 family members during mouse development and aging. *Oncogene* 1997;15:203–211. [PubMed: 9244355]

Fig 1.

Percentage with severely limited physical function by rs2811712 genotype, all studies combined (participants aged 65 to 80 years, per minor allele adjusted logistic regression $p=0.001$)*

Note: * age, sex and set/study adjusted, logistic regression model based estimates

Table 1

Associations between 25 tagging SNPs and SF36 physical function subscore (ordinal logistic regression odds ratios) in 938 participants from EPIC-Norfolk (Set 1)

Gene regions tagged	rs number	odds ratio	SF36 physical score 95% confidence interval	p value
CDKN1a				
1	rs1801270	0.95	(0.70 – 1.30)	0.742
2	rs3176352	1.05	(0.87 – 1.26)	0.617
3	rs6457937	0.90	(0.68 – 1.18)	0.436
4	rs2395655	1.09	(0.94 – 1.27)	0.259
5	rs762624	1.00	(0.85 – 1.19)	0.956
6	rs3176336	1.11	(0.95 – 1.30)	0.192
7	rs3176343	0.85	(0.61 – 1.18)	0.340
CDKN1b				
1	rs34330	0.96	(0.79 – 1.17)	0.703
2	rs2066827	1.10	(0.90 – 1.34)	0.339
3	rs7330	0.99	(0.84 – 1.17)	0.950
CDKN2a / CDKN2b				
1	rs3731222	0.97	(0.77 – 1.21)	0.785
2	rs3731211	1.06	(0.89 – 1.26)	0.540
3	rs3218020	1.08	(0.92 – 1.26)	0.359
4	rs2811712	1.38	(1.06 – 1.79)	0.015*
5	rs3218005	1.36	(1.04 – 1.77)	0.027*
6	rs3217992	1.08	(0.92 – 1.26)	0.336
7	rs3731239	0.92	(0.79 – 1.09)	0.339
8	rs11515	1.04	(0.83 – 1.31)	0.715
9	rs3088440	0.81	(0.62 – 1.07)	0.138
10	rs3731249	1.08	(0.69 – 1.69)	0.727
11	rs3731257	1.10	(0.92 – 1.31)	0.296
12	rs1063192	0.91	(0.78 – 1.05)	0.200
13	rs3218009	1.02	(0.88 – 1.19)	0.760
CDKN2c				
1	rs12855	1.06	(0.81 – 1.40)	0.663
2	rs3176459	0.99	(0.84 – 1.17)	0.887

Notes: SNP status coded as 1 for common homozygous, 2 for heterozygous and 3 for rare homozygous in ordinal models

* significant at nominal $p < 0.05$

Unadjusted numbers by rs2811712 status and study, with unadjusted percentages of participants classified as physically limited on dichotomized measures in each group.

Table 2

Total		Common homozygous		Heterozygous		Rare homozygous	
		n	%	n	%	n	%
			limited		limited		limited
EPIC- Norfolk study	SF36 physical performance subscale	938	19.9	170	13.5	10	10.0
	Set 1	758	13.4	266	10.15	15	6.7
	Set 2	1038	16.2	436	11.47	25	8.0
	All Epic	1796					
InCHIANTI study	Requiring help with one or more Activities of Daily Living	581	4.3	144	0.7	16	0.0
	Short (tested) performance score	725	27.5	138	21.7	15	13.3
		709					
Iowa study	Difficulty with Activities of Daily Living	419	35.3	86	24.4	2	50.0
	Simplified short (tested) performance score	331	30.2	83	25.3	2	0.0
		406					

Note: *due to older mean age, dichotomization of the Iowa EPESE measures included a higher percentage of limitation.

Table 3

Numbers genotyped and odds ratios for associations of rs2811712 (per minor allele count) with physical function measures*

Study, measure and sample	number genotyped	odds ratio	95% Confidence intervals	p-value
EPIC-Norfolk Study				
SF36 physical performance score (rest vs most limited 15%)				
Set 1	938	1.56	(1.00–2.43)	0.049
Set 2	1319	1.38	(0.91–2.08)	0.120
All EPIC-Norfolk	2257	1.46	(1.08–1.97)	0.013
InCHIANTI Study				
Activities of Daily Living (help needed with 1 or more)	725	7.38	(1.01–53.7)	0.049
Tested physical performance: (vs most limited 25%)	709	1.80	(1.12–2.89)	0.015
Iowa Study				
Incident Activity of Daily Living disability (vs most limited 25%)	419	1.61	(0.94–2.74)	0.079
Tested physical performance (vs most limited 25%)	406	1.32	(0.77–2.28)	0.307

Notes:

* all models were age, sex and set/study adjusted as relevant;

** estimate differs slightly from Table 1 as functioning measure is dichotomized here.