IL-6 gene variation is not associated with increased serum levels of IL-6, muscle, weakness, or frailty in older women

J. Walston\textsuperscript{a,}\*, D.E. Arking\textsuperscript{a}, D. Fallin\textsuperscript{a}, T. Li\textsuperscript{a}, B. Beamer\textsuperscript{a}, Q. Xue\textsuperscript{a}, L. Ferrucci\textsuperscript{b}, L.P. Fried\textsuperscript{a}, A. Chakravartia

\textsuperscript{a}Johns Hopkins Medical Institutions, Baltimore, MD, USA
\textsuperscript{b}National Institutes on Aging, Baltimore, MD, USA

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Abstract

Elevated levels of the inflammatory cytokine IL-6 are associated with the development of disability, frailty, and mortality in older adults. These outcomes are likely mediated through inflammatory activity that alters hormones, skeletal muscle, and the immune system. Polymorphic variants in the IL-6 gene influence IL-6 expression. We hypothesized that IL-6 alleles associate with increased serum of IL-6, decreased muscle strength, and frailty, and tested this in the Women’s Health and Aging cohorts. We genotyped 463 participants age 70–79, and identified three common IL-6 haplotype blocks for the Caucasian (n = 363) and African American (n = 100) subsets. Using linear and logistic regression, and adjusting for age, BMI, race, and osteoarthritis, we identified no significant or clinically meaningful relationship between any single IL-6 single nucleotide polymorphism (SNP) or any IL-6 haplotype and serum IL-6 level, grip, knee, or hip strength, or frailty. Given that the promoter SNP (rs1800795) has been reported to influence IL-6 levels and health outcomes, we performed a similar association study in the In Chianti population (n = 266) and confirmed lack of association. These results suggest that IL-6 gene variation may not be an important factor in the determination of elevated IL-6 levels and related phenotypes found in older women.

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1. Introduction

Increased serum markers of inflammation, including the proinflammatory cytokine interleukin-6 (IL-6), have been identified as important correlates of altered body composition in older adults (Ershler and Keller, 2000), of a phenotype of frailty (Walston et al., 2002; Leng et al., 2002), and of increased disability, morbidity, and mortality in older adults (Ferrucci et al., 1999; Harris et al., 1999; Cohen et al., 1997). Chronic elevation of IL-6 contributes to anemia, glucose intolerance, osteopenia, and decreased hepatic protein synthesis (Ershler and Keller, 2000). In addition, loss of lean body mass and muscle strength, important components of frailty, are thought to be influenced by chronic elevations in serum IL-6 (Tisdale, 2001; Fujita et al., 1996). Thus, increasing evidence suggests a central role for IL-6 elevation in frailty and disability.

The etiology of chronically elevated IL-6 in older adults is likely multifactorial, with increased presence of disease states, declines in estrogen and testosterone and genetic variation all contributing to IL-6 level increases (Ershler and Keller, 2000). The IL-6 gene has been extensively surveyed for variation, and at least 50 SNPs and five common haplotypes have been identified to date (Osiri et al., 1999; Jordanides et al., 2000). Genetic variation found in non-coding promoter elements has been shown to strongly influence IL-6 expression (Osiri et al., 1999). Four polymorphisms (−597G→A, −572G→C, −373A→T, −174G→C) have been observed to segregate in human populations and have been shown to have a significant effect on IL-6 expression in an in vitro system...
(Osiri et al., 1999; Jordanides et al., 2000; Ota et al., 2001). In human population studies, individual IL-6 gene SNPs or haplotypes correlate with inflammatory conditions, including decreased bone mineral density (Ota et al., 2001), age of onset of rheumatoid arthritis (Pascual et al., 2000), and protection against severe forms of multiple sclerosis (Vandenbroeck et al., 2000). In addition, Hulkkonen et al. (2001) demonstrated an association between the rs1800795 promoter polymorphism and increased plasma IL-6 levels in groups of study patients with Sjögren’s syndrome. Importantly, subjects with this polymorphism and Sjögren’s syndrome had the highest levels of IL-6 and the highest rates of pulmonary fibrosis and other complications, highlighting the disease modification potential of this allele.

Given the recent elucidation of the potential importance of elevated IL-6 in poor health outcomes in older adults, and the knowledge that higher serum levels of IL-6 can negatively impact a wide variety of tissues and physiologic systems, identification of clinically relevant IL-6 genotypes would be important targets for the development of interventions. We hypothesized specific IL-6 genotypes and haplotypes would contribute to sub-phenotypes of inflammation as measured by serum IL-6 to muscle weakness, and, ultimately, to the phenotype of frailty in older adults. We sought to test this hypothesis in two companion population based cohorts of older women and validated the findings in a second cohort of Caucasian older Italian women.

2. Materials and methods

2.1. Population

The Women’s Health and Aging Studies (WHAS) I and II are companion prospective, observational studies of the causes and consequences of disability in older women. WHAS I is a study of 1002 women 65 years and older who represent the 1/3 most disabled women living in eastern Baltimore City and County, MD. WHAS II is a study of 436 women 70–79 years at baseline who were recruited from among the 2/3’s least disabled residents of the same geographic area. The combination of the studies are therefore representative of the community dwelling population of older women. WHAS I participants were extensively examined at baseline to characterize the presence and severity of 16 major chronic diseases and impairments, and were examined every 6 months for 3 years and then interviewed by telephone annually for two more years to determine change in function over time and mortality (Simonsick et al., 1997). In WHAS II, participants have been examined every 18 months for three examinations, and a fourth examination was conducted after a 3-year hiatus to a total follow-up of 10 years (Fried et al., 2000). Data sets in the two studies are identical and include standardized, objective measurements of strength (e.g. grip, hip and knee using dynamometry). Reliability of methods has been established (Rantanen et al., 1999). Informed consent was obtained for all data collected, including physiologic measurements and for genetic studies.

InChianti participants were randomly sampled from persons 65 and older residing in Greve in Chianti and Bagno a Ripoli (Bandinelli et al., 1999; Cesari et al., 2004), and are therefore a representative community dwelling population. We analyzed data from the subset of women aged 70–79 in order to better match the combined WHAS I and II cohort. Measurement of muscle strength and of serum IL-6 was previously reported (Bandinelli et al., 1999; Cesari et al., 2004). We chose the three measurements that approximated those performed in the WHAS cohort. Demographic information for both cohorts is displayed in Table 1.

2.2. Measurement of muscle strength and frailty in WHAS I and II cohorts

Measurements of grip, hip, and knee strength were obtained at the baseline visit in both WHAS I and WHAS II cohorts and were utilized in these analyses. For grip strength measurements, a JAMAR hand dynamometer (model BK-74978, Fred Sammons, Inc., Burr Ridge, IL) was used. Maximal grip strength was measured three times in both hands. The strongest reading with the non-dominant hand (theorized to be least influenced by non-genetic factors such as exercise and trauma) was used for this analysis. Maximal knee extension and hip flexion strength was measured in two trials on each leg using a hand-held isometric dynamometer (model 01160, Lafayette Instruments, Lafayette, IN). The strongest measurement from the side that best retained strength at subsequent visits was used in these analyses. An average strength measure that is the sum of the centered values of the hip, knee, and grip strength was also used (Seibert et al., 2001). For the analysis of the relationship between IL-6 genotype and frailty, we utilized a recently validated phenotype of frailty that consists of five screening criteria: slow walking speed, weight loss, fatigue, low activity levels, and weak grip strength. (Fried et al., 2001). Details of the screening exam and validation results were published previously (Fried et al., 2001).

2.3. Measurement of IL-6

Whole blood was drawn at baseline visits in each study, and serum was extracted and frozen at $-80 \degree C$ until processing. For the WHAS 1 and 2 populations, IL-6 was measured in duplicate by ELISA (High Sensitivity Quantikine kit, R and D Systems, Inc., Minneapolis, MN) from the frozen serum samples drawn at the baseline visit. The lower detection limit was 0.10 pg/ml, and the interassay coefficient of variation was 7%. For the In CHIANTI population, serum IL-6 were quantified in duplicate by ELISA with immunoassay kits (BioSource Cytoscreen human IL-6 Ultra
Sensitive kits; BioSource International, Inc., Camarillo, CA). The minimum detectable concentrations were 0.10 pg/ml, and the interassay coefficient of variation was 7%.

2.4. Genotyping

IL-6 SNPs were chosen based on known assays available through ABI’s Assay on Demand (Applied Biosystems, Foster City, CA). Adequate coverage of the gene was assessed via $D^2$ and $r^2$ statistics and additional SNPs were added to fill in linkage disequilibrium (LD) gaps. SNPs with a minor allele frequency (MAF) $<0.05$ were dropped from subsequent analyses.

2.5. Statistical analysis

Allele and genotype frequencies were estimated for each SNP. Quantitative muscle strength and serum IL-6 levels were evaluated for fit to a normal distribution and transformed, if necessary. Descriptive analyses for all quantitative phenotypes included means and box plots by each SNP genotype. SNP frequencies were also calculated within qualitative phenotype categories (frailty). Single-SNP genetic association analyses were performed via linear and logistic regression models, coding heterozygotes and homozygotes separately, such that two regression parameter estimates were obtained, with the most common homozygote genotype as baseline. All models were adjusted for age, BMI, race, and osteoarthritis when muscle strength was modeled. From these adjusted regression coefficient estimates for heterozygotes and homozygotes, constrained 1-parameter genetic models such as dominant, recessive, additive, or multiplicative were chosen if appropriate, and denoted ‘best’ model. These analyses were performed using the SAS v8.

Pair-wise LD estimates ($D'$ and $r^2$) and departures from the Hardy–Weinberg proportions were estimated separately for whites and non-whites. Haplotype blocks were estimated via the confidence interval method of Gabriel et al. (2002), and haplotype frequencies estimated via the EM algorithm. All descriptive haplotype and linkage disequilibrium calculations were performed and displayed graphically using the Haploview (V2.04) software (www.broad.mit.edu/personal/jcbarret/haplo/index.php). The haplotype assignments were then implemented into a GLM framework following the method of Schaid (2003), assuming an additive model (Schaid et al., 2002). This method estimates regression coefficients for each haplotype category, corresponding to a unit increase in the dependent variable proportional to the number of copies of a particular haplotype. Global $p$-values were obtained empirically, representing the overall impact of diplotype (haplotype pair) status on the dependent variable, rather than focusing on any particular haplotype a priori.

3. Results

We genotyped 14 SNPs encompassing the IL-6 gene and 70 KB of surrounding sequence. SNPs with MAF $<0.05$ were removed. Allele frequency, genotypes, and HWE for
combined WHAS I and WHAS II data sets are shown in Table 2. Genotype distributions were in agreement with Hardy–Weinberg equilibrium (HWE) for both Caucasians and African Americans at the \( p < 0.01 \) level. Final LD statistics are shown in Fig. 1A for Caucasians and Fig. 1B for African Americans.

3.1. Serum IL-6

Using log adjusted mean values for serum IL-6, we performed regression analysis with adjustment for age, BMI, and smoking status in order to evaluate whether there was an association between genotype and serum IL-6 level. We found no significant relationship between IL-6 SNPs and serum levels of IL-6 in the white subset of the cohort \( (n=363) \) including the rs1800795 C/G promoter variant (Fig. 2). This polymorphism has previously been associated with altered serum IL-6 levels and with altered disease related phenotypes (Hulkkonen et al., 2001; Brull et al., 2001). In the smaller African American subset \( (n=100) \), we identified modest but significant relationships between higher serum IL-6 and heterozygotes in three contiguous SNPs, including the rs1800795 SNP (Fig. 2). Because of the negative findings in the Caucasian subset of the WHAS population for the rs1800795 allele, we sought to reproduce these results in

<table>
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HWE, Hardy–Weinberg equilibrium; **Bold** indicates \( p < 0.05 \).
the In Chianti cohort. In the overall In Chianti population, of men and women age 65 and over no genotype effect was observed for the rs1800795 SNP (data not shown). In order to directly compare our WHAS findings to In Chianti, we analyzed data from the subset of women aged 70–79, and identified a modest but statistically significant difference in serum IL-6 adjusted for mean age, BMI and non-smoking status (CC 1.55 pg/ml, CG 1.40 pg/ml, GG 1.14 pg/ml; \( p < 0.05 \)). Although this was statistically significant, the very modest difference between genotypes is unlikely to be clinically meaningful, especially given that levels in this study are below the cut off value of > 1.80 pg/ml that correlates with decreased muscle strength (Visser et al., 2002).

Fig. 1. Linkage disequilibrium \((D')\) plot for white (A) and non-white (B) subsets of WHAS I and II populations. The top is a schematic of the IL-6 gene with the SNP locations. The value within each diamond represents the pair-wise LD (correlation, measured as \(D'\)) between the two SNPs defined by the top left and the top right sides of the diamond. For example, the LD between SNP 1 and 5 is \(D' = 0.85\). Diamonds without a number correspond to a \(D' = 1\); i.e. the LD between SNPs 1 and 3 is \(D' = 1\). Shading represents magnitude and significance of the pair-wise LD, with the red reflecting highest LD and white reflecting lowest. Please see the Haploview website referenced in text for further details.
3.2. Skeletal muscle strength and frailty

We next evaluated the relationship of each of the SNPs with hip, grip, knee and summary strength measurements and frailty as determined by a previously validated frailty screening exam in the WHAS cohorts (Fried et al., 2001). Of 120 single SNP regression models, we identified two with a nominal \( p \)-value of \(<0.05 \) (Fig. 3). However, considering the number of tests performed, and the inconsistency with other phenotypes on correlated SNPs, these two are unlikely

Fig. 2. Standard deviation range of (age + BMI) adjusted IL-6 serum levels by genotype for 12 IL-6 SNPs in African Americans and Caucasians in WHAS I and 2 women age 70–79.

Fig. 3. Schematic of negative log 10 of \( p \)-values for 120 tests of relationship between specific IL-6 SNPs, strength measurements and frailty. IL-6 SNPs are represented on X axis, negative log 10 of \( p \)-values for strength and frailty measures in WHAS populations are on the Y axis. Dotted lines represent \( p \)-value thresholds of 0.1, 0.05, and 0.01. (HOM, homozygote; HET, heterozygote).
other to represent true associations. This lack of association between rs1800795 and muscle strength was also reproduced in the entire In Chianti data set of community dwelling adults of both genders ranging in age from 20 to 100 (data not shown). In the subset of women age 70–79, we identified a modest difference between all three genotypes in hip strength (CC 15.5, CG 17.9, GG 16.6; \( p < 0.01 \)) and knee strength (CC 13.2, CG 14.5, GG 13.2; \( p = 0.05 \)), but none for grip strength. These results were contrary to our hypothesis, as the genotype with highest mean strength does not have the highest mean serum IL-6 level and the mean hip and knee strength is highest in the heterozygote groups.

3.3. Haplotype analysis

Three haplotype blocks in the IL-6 gene were identified in this data set. For Caucasians, Block 1 includes 2&3 SNPs, block 2 include 4&5 SNPs, and block 3 7–13 SNPs. SNPs 6 and 11 were deleted due to very low minor allele frequency. Haplotype blocks for the African American subset includes SNPs 7&8 for block 1, SNPs 9&10 for block 2, and SNPs 12 and 14 for block 3. SNPs 11 and 13 were deleted due to very low minor allele frequency (Fig. 1A and B). Association studies revealed no statistically significant relationship between any of the haplotype blocks and serum levels of IL-6, muscle strength, or frailty in either white or non-white population.

4. Discussion

Serum levels of IL-6 have been associated with poor health outcomes for older adults, including frailty, disability, and earlier mortality. These outcomes are likely mediated through declines in multiple physiologic systems, including inflammatory, endocrine, and skeletal muscle changes (Ershler and Keller, 2000). We sought here to determine the genetic influence on the IL-6 variability observed in older adults through a comprehensive IL-6 gene study.

This is the first large-scale genetic study that has rigorously screened the entire IL-6 gene in a population of older adults for genetic variants that may impact the phenotypes associated with frailty, which are themselves risk factors for disability. Using this comprehensive approach, we identified no significant associations of any individual IL-6 SNPs or haplotypes with the sub-phenotypes of increased inflammation as measured by serum levels of IL-6, altered skeletal muscle strength, and, ultimately, with the syndrome of frailty in the Caucasian component of the Women’s Health and Aging population. This negative finding between the 174 polymorphic variant (rs1800795) and the same phenotypes was further confirmed in the In Chianti cohort. Although there was a very modest association between this SNP and serum IL-6, the absolute mean differences between genotypes of 0.41 pg/ml were minimal and unlikely to have meaningful clinical impact. This lack of clinically meaningful association with serum IL-6 levels is further supported by total lack of association between IL-6 genotypes and muscle strength and with frailty.

There are several factors that may explain these negative findings. First the large number of co-morbid diseases in the WHAS cohorts, and in older adults in general, may have a much larger impact on the serum levels of IL-6 than any particular genotype, and hence overwhelm our ability to detect meaningful differences in this population. Although this rationale is contrary to the findings discussed previously in studies of IL-6 genotypes and specific inflammatory diseases, the bulk of co-morbid conditions in this population are not likely to be inflammatory disease states, and hence not trigger a strong IL-6 response.

Next, the effects of aging may overwhelm the modest genetic contribution to IL-6 levels. There is substantial evidence that the increase in serum levels of IL-6 with age results, in part from the loss of sex steroids such as estrogen, testosterone, and DHEA-S (Ershler and Keller, 2000; Pfeilschifter et al., 2002). These hormones play an important role in blocking IL-6 gene transcription. Thus, the differential loss of these hormones may play a stronger role in differential IL-6 gene transcription than genotype.

Finally, there may be survivorship bias in the study of older populations as those with higher levels of IL-6 are known to have earlier mortality than those with lower levels. If there are particular genotypes that strongly predict higher levels of IL-6, those individuals may drop out of the population in earlier age groups, and therefore may not be part of cohorts of older adults (Harris et al., 1999; Rea et al., 2003). Therefore, one might predict that genotype specific results would be better determined in populations of younger adults or in populations with specific inflammatory diseases as have been previously published. However, we did not observe significant different allele frequencies by age in the WHAS or In Chianti samples (data not shown).

Strengths of this study include the access to two large, well-characterized cohorts of older adults, and the comprehensive genotyping performed that provides excellent coverage of the gene. This study was also adequately powered for the dominant models, and is therefore strong evidence that these SNPs have minimal if any effect on the muscle strength phenotype. For SNPs with MAF=0.5, our power to detect a difference in strength measures by genotype at alpha=0.05 was 96% under a dominant model, and 86% under an additive model. At the other extreme, SNPs with MAF<0.10 provided power of 43% for an additive model and 93% for a dominant model. The power was not adequate to conclusively rule out specific SNP effects in the additive models, especially in the rarer alleles. However, there were no trends toward significance that would support further investigation.
Potential weaknesses of this study include the fact that the WHAS and In Chianti populations may not be directly comparable because of the differences in ethnic backgrounds among WHAS and Caucasian participants, and because disease frequencies were lower in the IN Chianti population. In addition, the high BMI observed in this population may overwhelm any genotype effect on IL-6. Finally these WHAS cohorts include a relatively small subset of African Americans. There are substantial allele frequency differences between Caucasians and African Americans in this gene, and few if any studies have documented gene expression differences between the two races based on genotype (Osiri et al., 1999). Genetic differences may, in part, explain the significant differences seen in the African American subset, and suggest that further study in larger African American cohorts may be needed to conclude that IL-6 gene variation has minimal impact on older African Americans.

In summary, we have performed the most comprehensive study of the IL-6 gene to date in older adults, and have identified no clinically significant relationships between IL-6 genotypes, haplotypes and phenotypes of serum IL-6, muscle strength or frailty in two populations. Our findings suggest that other etiologies of age-related increases in serum IL-6 such as disease states and declines in sex steroid levels may play a more important role than gene variation.

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