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Hypomethylation of the *IL17RC* Promoter in Peripheral Blood Leukocytes Is Not A Hallmark of Age-Related Macular Degeneration

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SUMMARY

Age-related macular degeneration (AMD) is a leading cause of visual impairment worldwide. Aberrant DNA methylation within the promoter of *IL17RC* in peripheral blood mononuclear cells has recently been reported in AMD. To validate this association, we examined DNA methylation of the *IL17RC* promoter in peripheral blood. First, we used Illumina Human Methylation450 Bead Arrays, a widely accepted platform for measuring global DNA methylation. Second, methylation status at multiple sites within the *IL17RC* promoter was determined by bisulfite pyrosequencing in two cohorts. Third, a methylation-sensitive quantitative PCR-based assay was performed on a subset of samples. In contrast to previous findings, we did not find evidence of differential methylation between AMD cases and age-matched controls. We conclude that hypomethylation within the *IL17RC* gene promoter in peripheral blood is not suitable for use as a clinical biomarker of AMD. This study highlights the need for considerable replication of epigenetic association studies prior to clinical application.

INTRODUCTION

Age-related macular degeneration (AMD) is the leading cause of irreversible loss of central vision in developed countries, affecting approximately 30–50 million people worldwide (Coleman et al., 2008). The disease severely impairs quality of life (Rung and Lövestam-Adrian, 2013) and is a substantial economic burden internationally. AMD is a complex disease involving an interaction between genetic and environmental risk factors. Numerous genes that confer a predisposition to AMD have now been identified, and together these account for 10%–30% of the variability in disease risk (Fritsche et al., 2013). Strong associations have also been established between AMD and cigarette smoking (Delcourt et al., 1998, 2011) as well as, to some degree, sun exposure (Sui et al., 2013), while increased dietary antioxidants and fish consumption appear to confer a protective effect (Tan et al., 2009; van Leeuwen et al., 2005).

The mechanisms underlying the observed interplay of genes and the environment in the pathogenesis of AMD are poorly understood; however, cumulative and stable epigenetic variation represents a plausible and attractive model. Epigenetic modification of the human genome is an important mechanism mediating gene-environment interactions by modulating environmental effects on gene expression. For example, cigarette smoking has been shown to be associated with widespread

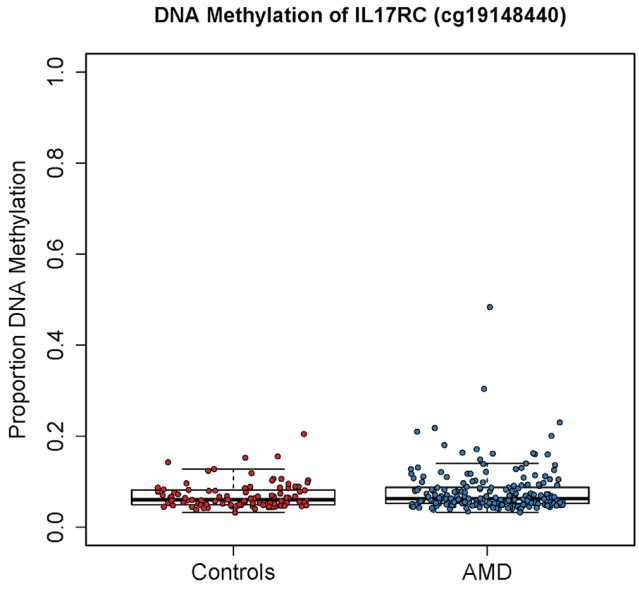

Figure 1. Methylation of the *IL17RC* Promoter in Peripheral Blood from the Michigan AMD-MMAP Cohort as Measured by the Illumina 450K Bead Array at Probe cg19148440

This region corresponds to the previously reported differentially methylated region in AMD (Wei et al., 2012). The proportion of DNA methylation from control (red, n = 99) and AMD (blue, n = 199) is plotted for each individual. There was no statistically significant difference in methylation between the AMD and control groups.

See also Figure S1 and Table S1.

changes in DNA methylation (Lee and Pausova, 2013). Thus, efforts have been begun to explore the role of epigenetics in AMD (Hunter et al., 2012; Baird and Wei, 2013).

It is widely accepted that highly expressed genes usually lack cytosine methylation within CpG-rich "islands" associated with many promoters; conversely, methylation within these regions is typically associated with suppression of transcription (Deaton and Bird, 2011). We have previously described the importance of DNA methylation in the regulation of retina-specific genes (Merbs et al., 2012; Oliver et al., 2013; Wan et al., 2013; Nasonkin et al., 2013). Aberrant CpG island hypermethylation leading to inactivation of tumor suppressor genes as well abnormal hypomethylation resulting in oncogene overexpression is characteristic of neoplasia (Jiang et al., 2013), including retinoblastoma (Choy et al., 2002) and uveal melanoma (Maat et al., 2007). Cytosine methylation may also influence the development of other age-related diseases (Adwan and Zawia, 2013; Miao et al., 2013), including AMD (Hunter et al., 2012; Wei et al., 2012). However, conclusive data in the field are scarce, primarily due to the lack of replication of original association studies. A recent report suggested that the promoter of the *interleukin 17 receptor C* (*IL17RC*) gene, which encodes a single-pass type I transmembrane protein involved in T cell activation (Martin et al., 2011), is hypomethylated in peripheral blood samples from patients with AMD compared to unaffected controls (Wei et al., 2012). In addition, this report also showed that *ILRC17* is overexpressed in the macula of AMD patients (Wei et al., 2012).

To further investigate the role of epigenetic modifications on gene regulation in AMD, we explored the methylation status of the CpG rich region within the *IL17RC* promoter in peripheral blood obtained from three independent cohorts of AMD patients and unaffected controls: the Michigan subset of the AMD-MMAP cohort, a Baltimore cohort and an Australian cohort. Initially, we examined the methylation status of probes adjacent to *IL17RC* from our ongoing genome-wide methylation analysis (using the Illumina Infinium Human Methylation450 [450K] Bead Array) in the Michigan AMD-MMAP cohort, comparing the peripheral whole blood profiles in patients with diagnosed AMD (either geographic atrophy [GA, dry AMD] or choroidal neovascularization [NV, wet AMD]) to that of healthy controls. We then specifically targeted the identical cytosine residues reported to be differentially methylated (Wei et al., 2012) and analyzed the methylation status of these residues in the Baltimore and Australian cohorts by direct bisulfite pyrosequencing and the methylation-sensitive, restriction-enzyme-based EpiTect Methyl II PCR assay. Combined, our analysis of the three cohorts revealed no evidence of disease association at this locus, with all peripheral whole blood samples examined showing very low levels of *IL17RC* promoter methylation. Hypomethylation at *IL17RC* was also evident in peripheral blood mononuclear cells (PBMCs) and granulocytes from control subjects with no evidence of any change in AMD individuals. Our results demonstrate that *IL17RC* hypomethylation in peripheral blood is unlikely to serve as a clinically useful biomarker for AMD.

RESULTS

Illumina Infinium Human Methylation450 Bead Arrays

We performed an epigenome-wide association study (EWAS) on peripheral whole blood from 100 AMD (GA and NV) case-control trios (a subset of the Michigan AMD-MMAP cohort; dbGaP phs000182) using the HM450 platform (V.F.O., A.E.J., K.E.B., M.O., J.R.H., A.S., J.Q., D.J.Z., and S.L.M., unpublished data). Each trio (one patient with bilateral GA, one patient with bilateral NV, one control) was matched for sex and for age (<1 year difference among the three individuals in each trio). The sample group comprised 63% females, with a mean ± SD age of 79.3 ± 5.6 years. There was no significant difference in age between the AMD (79.3 ± 5.5 years, n = 200) and control (79.3 ± 5.7, n = 100) groups (p = 0.93; unpaired Student's t test). We utilized this data set to examine the methylation of *IL17RC* in a cohort independent of that previously described (Wei et al., 2012). The Illumina Infinium Human Methylation450 (450K) array included 35 probes located within 26 kb downstream and 8 kb upstream of the *IL17RC* transcription start site (TSS; chr3:9958758; hg19) (Table S1). The region interrogated using the Methyl-Profiler assay assesses methylation within a 80 bp region (chr3: 9957001-9957080), ~1.7 kb upstream from the *IL17RC* TSS (Wei et al., 2012) and within the region covered interrogated in our analysis. One probe on the 450K array was contained within this region (probe cg19148440), located at chr3:9957031. This probe did not show a statistically significant difference in DNA methylation levels between the AMD and control groups (Figure 1 and Table S1). Specifically, the AMD population (n = 199) had a mean methylation level of 7.9% and the control population

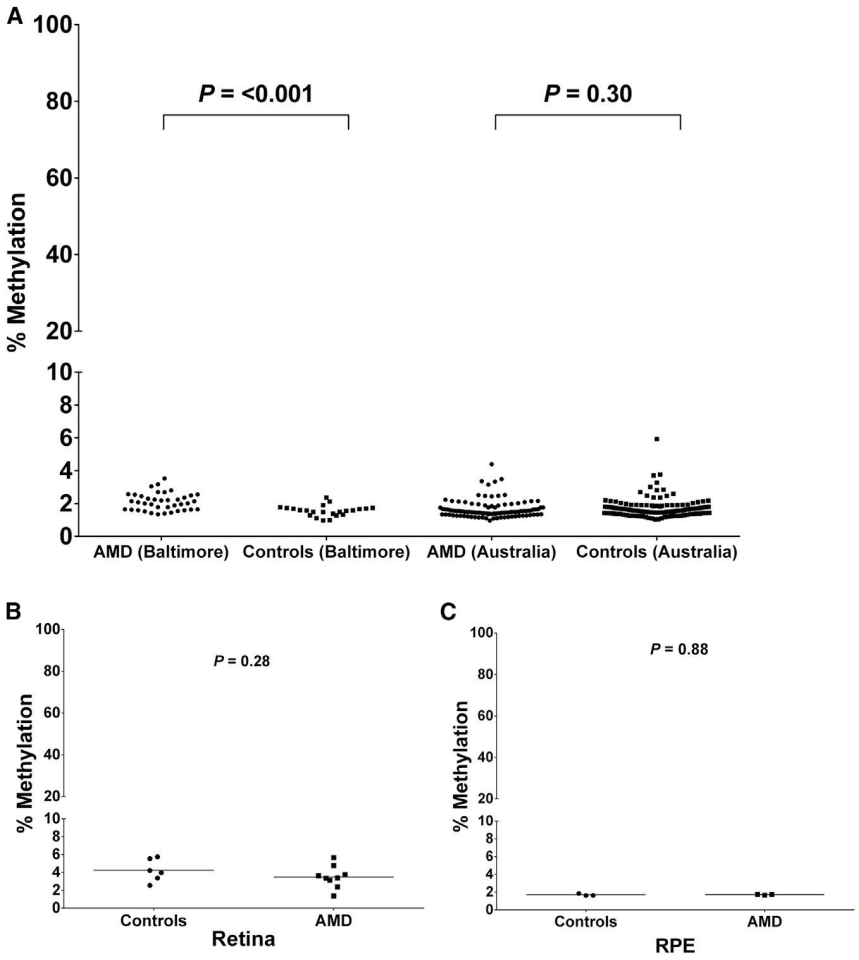

Figure 2. Comparison of DNA Methylation Levels within the *IL17RC* Promoter in Patients with AMD and Age- and Gender-Matched Controls with No Current, Past, or Family History of Any Ocular Disease

(A) DNA methylation of *IL17RC* in peripheral blood DNA. Each data point represents the mean percentage methylation within individual AMD (circles; Baltimore n = 39, Australia n = 100) and control (squares; Baltimore n = 23, Australia n = 116) subjects. Methylation was measured across 33 CpG sites (Baltimore cohort) or 8 CpG sites (Australian cohort), which overlapped the region previously interrogated (Wei et al., 2012). All samples showed <6% methylation by bisulfite pyrosequencing. The Baltimore cohort indicated small yet significant hypomethylation of the control cases ($p = < 0.0001$). There was no significant difference in mean methylation between cases and controls in the Australian cohort ($p = 0.30$). Note the split y axis. See also Table S2.

(B and C) Comparison of DNA methylation levels within the *IL17RC* promoter in donor retina/RPE tissue from individuals with AMD and controls with no reported history or pathological evidence of ocular disease. Each data point represents the mean percentage methylation within the individual tissue samples from AMD (circles) and control (squares) donors overlapping the region interrogated by Wei et al. (Wei et al., 2012). (B) Methylation was measured across 19 CpG sites in retina from AMD (n = 9) and control (n = 6) donors. (C) Methylation was measured across 33 CpG sites in RPE/choroid from AMD (n = 3) and control (n = 3) donors. Hypomethylation was observed in both tissues at all CpG sites examined.

(n = 99) had a mean methylation level of 6.9% ($p = 0.18$) (Figure 1). A vast majority of the peripheral whole blood samples showed low levels of methylation of < 10%, with only seven samples (one case and six controls of 198 total) having methylation levels >20% (Figure S1). Although 5 out of the 35 probes within 34 kb of *IL17RC* reached marginal statistical significance prior to multiple testing correction ($p < 0.05$), these differences had an absolute difference in methylation levels of <1% between the case and control groups and represented a mix of both relative hypo- and hypermethylation of *IL17RC* in AMD cases; the remaining 30 probes did not show a difference in methylation between the AMD cases and controls (Table S1).

Pyrosequencing of the *IL17RC* Promoter Region in Two Independent Cohorts

To explore the *IL17RC* methylation status in more detail, DNA methylation was measured using bisulfite pyrosequencing from samples derived from peripheral blood collected at Johns Hopkins University, Baltimore. The final cohort comprised 24 patients with GA (11 females), 15 patients with NV (10 females), and 23 controls (13 females). There was no significant age difference between the Baltimore control population (80.0 ± 7.4 years, mean ± SD; n = 23) and the Baltimore AMD population (76.6 ± 10.2 years; n = 39; unpaired Student's t test, $p = 0.17$). A total of 33 CpG sites were interrogated encompassing the region chr3:9956901-9957135, specifically including the region identified as hypomethylated in AMD (Wei et al., 2012). In accordance with the 450K data, both the AMD and the control samples from the Baltimore cohort showed very low levels of methylation overall (controls: 1.55% ± 0.07% [SEM], n = 23; AMD: 2.11% ± 0.08%, n = 39) (Figure 2). This lack of methylation at the promoter region of *IL17RC* differed from the levels previously observed in peripheral blood mononuclear cells of 30% in AMD cases and 60% in controls (calculated as the average of 96 control individuals and 202 individuals with AMD from Table S4 in Wei et al., 2012). We observed no significant hypomethylation of the promoter region of *IL17RC* in AMD cases relative to controls at any of the 33 CpG sites interrogated (Table S2). In fact, a slight but significant relative hypermethylation of AMD samples was observed (unpaired Student's t test, $p < 0.001$).

Given that comprehensive analysis in the Baltimore cohort revealed consistent hypomethylation of the *IL17RC* promoter in both AMD cases and normal controls, a second, independent cohort from Australia was examined. Bisulfite pyrosequencing

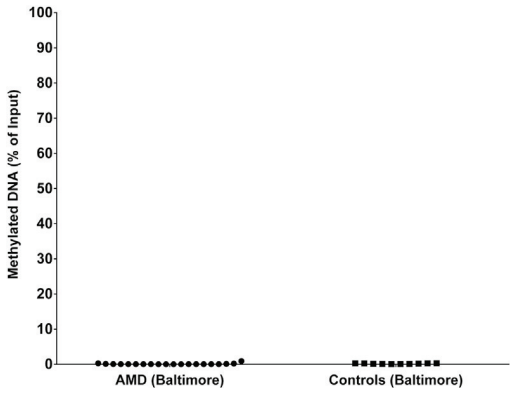

Figure 3. EpiTect Methyl II PCR Assay Results for the *IL17RC* Promoter CpG Island in Peripheral Blood from a Subset of the Baltimore Cohort

Each data point represents the percentage methylation from AMD (circles, n = 20) and control (squares, n = 10) subjects at chr3:9956961-9957116 (hg19). Data are plotted as the percentage of methylated DNA relative to the input. All samples showed hypomethylation by this assay. There was no significant difference in mean methylation between cases and controls using the EpiTect Methyl II PCR assay for *IL17RC* (p = 0.34). See also Table S3.

was performed on 100 AMD patients and 118 controls (Figure 2A). The mean age of the AMD patients (61 females) was 72.1 ± 6.3 years and of the controls (54 females) 71.0 ± 7.8 years (Student's t test, p = 0.25). Following bisulfite pyrosequencing of eight CpG sites within the previously interrogated region (chr3:9957024-9957056) (Wei et al., 2012), we did not detect any significance differences in the mean methylation in AMD patients (1.66% ± 0.06%, n = 100) compared to controls (1.75% ± 0.06%, n = 118; p = 0.30). The bisulfite pyrosequencing results indicated low levels of methylation (<6%) in all samples and were consistent with the results from the Baltimore cohort (Figure 2A). Only one individual (an AMD case) showed mildly elevated methylation (16%) of a CpG site (chr3:9957047) relative to the remainder of the cohort (Table S2).

Pyrosequencing of the *IL17RC* Promoter Region in Ocular Tissue

Retina samples were obtained from nine individuals with AMD (six men, 87.8 ± 5.8 years [mean age ± SD]; three women, 91.7 ± 0.6 years) and six controls (one man, 91 years; five women, 88.4 ± 3.6 years). Retinal pigment epithelium (RPE) samples were obtained from three AMD (three men, 86.3 ± 8.0 years) and three control (three men, 85.3 ± 5.5 years) donors. Bisulfite pyrosequencing was performed on the region interrogated by Wei et al. (2012), which showed low levels of methylation (<6%) in all samples examined (Figures 2B and 2C). There was no significant difference in the mean methylation of the retina samples between the control (4.23% ± 0.51%, n = 6) and AMD (3.49% ± 0.41%, n = 9; p = 0.28, Student's t test) groups. The RPE samples also showed a lack of methylation and no difference between the control (1.68% ± 0.08%, n = 3) and AMD (1.69% ± 0.03%, n = 3; p = 0.88) donors. The level of methylation within the ocular tissue samples was consistent with that observed in peripheral blood (Figure 2A).

EpiTect Methyl II PCR Assay of *IL17RC* Promoter Methylation in the Baltimore Samples

To determine whether the difference in overall peripheral blood methylation levels resulted from technical or biological differences, the commercial EpiTect Methyl II PCR assay for *IL17RC* (Qiagen) was performed on a subset of age/sex-matched Baltimore samples (AMD n = 20, 12 women; controls n = 10, six women). There was no significant age difference between the AMD samples (78.2 ± 5.7 years; mean ± SD) and the control samples (77.9 ± 6.2 years; unpaired Student's t test p = 0.89). This assay measures the amount of methylated DNA in a 156 bp region centered around chr3:9957039 using methylation-sensitive and methylation-dependent restriction digests followed by quantitative PCR (qPCR) as utilized previously (Wei et al., 2012). We did not detect any differences in the percentage of methylated DNA between the AMD (0.10% ± 0.05 [SEM]) and control groups (0.17% ± 0.03%; p = 0.34; unpaired Student's t test) using this restriction-enzyme-based approach (Figure 3). Furthermore, all samples examined showed <1% methylated DNA using the EpiTect Methyl II PCR Assay for *IL17RC*. These results were entirely consistent with our bisulfite pyrosequencing data from this region. Incomplete digest by the methylation-dependent enzyme was excluded as a potentially confounding factor in the observation of hypomethylation by the inclusion of the methylation-dependent control assay (Table S3).

Comparison of *IL17RC* Promoter Methylation between PBMCs and Granulocytes

Given that PBMCs were investigated previously (Wei et al., 2012), we sought to directly compare the methylation profile of the *IL17RC* promoter region between PBMCs, granulocytes and whole blood. Additionally, given that hypermethylation of this region was previously identified in individuals without AMD, we analyzed fresh samples from elderly control subjects (n = 18). Bisulfite pyrosequencing of the *IL17RC* promoter revealed hypomethylation of all individuals and cell populations examined (Figure 4 and Table S4). While there was a significant difference in mean percentage methylation among the PBMCs (2.34% ± 0.16% [SEM]), granulocytes (1.71% ± 0.05%), and whole blood (1.90% ± 0.05) ($F_{2,17} = 13.92$, p = 0.0006), the overall methylation levels were low and are likely to be below the sensitivity level of the pyrosequencing assay. No significant cellular heterogeneity or cell-type-specific DNA methylation differences potentially accounting for the discrepancy with the previously published results could be identified at this locus.

DISCUSSION

A recent study reported hypomethylation of the *IL17RC* promoter in PBMCs of AMD patients and a corresponding increase in *IL17RC* gene expression in macular tissue of AMD cadaver eyes (Wei et al., 2012). These results suggested *IL17RC* as a potential biomarker for AMD diagnosis. To explore these reported findings further, we examined the *IL17RC* promoter region in circulating peripheral whole blood (leukocytes), using three distinct techniques in three independent cohorts (summarized in Table 1). Our results indicate that *IL17RC* is essentially unmethylated in peripheral blood of both AMD and control

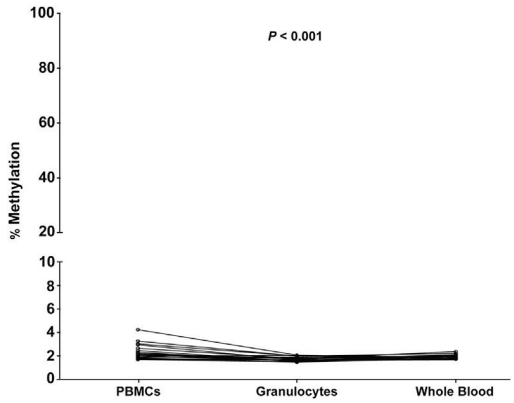

Figure 4. Comparison of DNA Methylation Levels within the *IL17RC* Promoter in Ficoll-Separated PBMCs and Granulocytes or Unsorted Whole Blood from Patients with No Current or Past Ocular Disease or Known Family History of AMD

Methylation was measured in 18 individuals across 14 CpG sites encompassing the region interrogated by Wei et al. (Wei et al., 2012). Each data point represents the mean percentage methylation across this region with the lines joining samples obtained from the same individual. No individual CpG sites had >6% methylation by bisulfite pyrosequencing in any sample. There was a small but significant difference in methylation between the cell populations ($p < 0.001$). Note the split y axis.

See also Table S4.

populations and that the methylation level of *IL17RC* is unlikely to serve as a useful clinical biomarker of AMD.

Using two methods that interrogate methylation changes at individual CpG dinucleotides (bisulfite pyrosequencing and the 450K methylation array), we were unable to reproduce the high percentage of methylated DNA (60%) in the *IL17RC* promoter previously seen in control individuals (Wei et al., 2012). To ensure that our findings did not reflect a population-specific effect, we bisulfite pyrosequenced the region in a second independent cohort from Australia, confirming a low level of methylation in both the AMD and control peripheral whole blood samples (1.7% and 1.8%, respectively). Unexpectedly, the difference in our results is not due to a difference in the clinical definition of the AMD phenotype, as our results differed with respect to the unaffected control population, which contained over two and a half times the number of controls compared with the Wei et al. study (263 versus 96) (Wei et al., 2012). The average age of our Michigan AMD-MMAP control individuals is 79 years and that of our Baltimore controls is 80 years. Although these values are not statistically different from the average age of our AMD patients (79 years and 75 years, respectively; $p = 0.93$), these individuals are slightly older than the average control subjects (70 years) in the study by Wei et al. (2012). However, it seems unlikely that the younger age in the National Eye Institute cohort (Wei et al., 2012) alone explains the higher level of methylation, as the average age of controls within the Australian cohort is 71 years and each of these individuals showed a methylation level of <6%. In the previous report, control individuals had methylation levels covering the entire spectrum of 0%–100% methylation (Wei et al., 2012).

The previous study (Wei et al., 2012) used an enzymatic method to measure methylation that relies on effective digestion of high-quality genomic DNA with methylation-dependent and methylation-independent restriction enzymes (Methyl-Profiler). It is designed to detect global methylation changes, rather than site-specific methylation changes. To verify that our observation did not result from differences in the methylation analysis techniques, we performed the same restriction enzyme-based analysis using the rebranded EpiTect Methyl Restriction kit (previously Methyl-Profiler) followed by the EpiTect Methyl II PCR Assay for *IL17RC* on a subset of the Baltimore peripheral whole blood samples. Using the commercially available *IL17RC* primers, we again found that the percentage of methylated DNA was very low in both cases and controls. Wei et al. used primers that differed from those provided in the commercially available EpiTect Methyl II PCR assay (see the Supplemental Information in Wei et al., 2012), although the same (but not identical) genetic location was targeted for amplification and interrogation by the enzyme digestion. This technique is susceptible to giving falsely high methylation measurements if incomplete digestion of unmethylated DNA with the methylation-dependent enzyme occurs. Sodium bisulfite conversion-based methods are widely considered the gold standard for measuring DNA methylation and enable quantitative measurements from a single CpG site. Both of the techniques utilized in our study (Illumina Infinium Human Methylation450 [450K] Bead Array and bisulfite pyrosequencing) are based on bisulfite-converted DNA.

Cellular heterogeneity is widely regarded as a potential confounding factor in case-control studies aimed at linking epigenetic disruption to specific phenotypes. As such, we considered this as another potential explanation for the discrepant findings. Our collection of peripheral blood samples contained the entire leukocyte (white blood cell) component of blood, unlike the PBMC fraction previously interrogated, which lacks polymorphonuclear (granulocyte) cells (Wei et al., 2012). To address this issue, we isolated PBMCs and granulocytes from peripheral blood and compared the methylation status of 14 previously analyzed CpG sites in each cell type as well as in peripheral whole blood from the same control individual. All three cell populations had methylation levels of <5%, thereby eliminating the possibility that cell composition differences could account for the discrepancy. As Ficoll-separated samples were processed within 2 hr of phlebotomy and still yielded low methylation levels, we also excluded the possibility that processing time may have altered the DNA methylation in our cohorts.

Genes expressed in many different tissues typically have an unmethylated CpG island upstream from their TSS, and methylation of such islands is strongly associated with decreased gene expression (Jones, 2012). The region in question is ~1,700 bp upstream from the *IL17RC* TSS and located in a CpG island. Consistent with our finding of a low methylation level is the fact that *IL17RC* is widely expressed in a range of human tissues (Haudenschild et al., 2002). We have shown comparably low levels of methylation at *Rho* and *Rbp3* in expressing cells, while nonexpressing cells showed > 60% methylation (Merbs et al., 2012). Many factors other than DNA methylation could explain the expression difference between AMD patients and controls seen by Wei et al. Our bisulfite pyrosequencing also indicated

Table 1. Summary of Our Present *IL17RC* Promoter Methylation Results in Comparison to the Previously Published AMD Data Set

Cohort	Technique	Target Chromosome Location (hg19)	Mean Methylation Level (%)	
			Case	Control
Michigan AMD-MMAP	Illumina Infinium Human Methylation450 (450K) Bead Arrays	chr3:9957031	7.9	6.9
Baltimore	bisulfite pyrosequencing	chr3:9956901-9957135	2.1	1.6
	methylation-sensitive qPCR (EpiTect Methyl II PCR assay)	chr3:9956961-9957117	0.1[a]	0.2[a]
Australia	bisulfite pyrosequencing	chr3:9957024-9957056	1.7	1.8
National Eye Institute (Wei et al., 2012)	methylation-sensitive qPCR (Methyl-Profiler)	chr3:9957001-9957080	30.0[a]	60.0[b]

[a]Percentage of methylated DNA relative to input.
[b]Calculated from Table S4 in Wei et al. (2012).

that the *IL17RC* promoter is lowly methylated in the retina and RPE of both AMD cases and controls. Wei et al. previously reported increased expression of *IL17RC* in the macula of patients with AMD. While our data do not dispute these findings, they do suggest that the regulation of *IL17RC* expression is unlikely to be the result of differential methylation of the promoter region in AMD individuals. While we believe that the level of *IL17RC* DNA methylation is not a good marker for AMD, it is possible that the expression and protein levels of *IL17RC* might still be useful biomarkers.

In summary, our comprehensive analysis of DNA methylation of the *IL17RC* promoter in peripheral blood samples from three independent AMD cohorts was unable to replicate the reported differential methylation using three different techniques, including the gold standard of bisulfite pyrosequencing. Examination of retinal tissue also indicated low levels of methylation in all samples examined. Furthermore, we observed very little methylation in all of our control samples, compared to the relatively high levels previously reported. Consistent with our finding of a low methylation level is the fact that *IL17RC* is widely expressed in a range of human tissues (Haudenschild et al., 2002). The difference in the percentage of methylation previously reported is surprisingly large. A 30% methylation difference between cases and controls is much greater than methylation differences typically seen in other complex diseases such as autism (7%–16% in brain samples; Ladd-Acosta et al., 2013) and obesity (1%–16% in blood; Almén et al., 2012) and is also higher than many methylation differences seen between cancer and normal tissues (Hansen et al., 2011; Irizarry et al., 2009). Our findings suggest that hypomethylation within the *IL17RC* gene promoter is not suitable for use as a clinical biomarker of AMD and highlight the need for considerable replication of epigenetic association studies prior to clinical application.

EXPERIMENTAL PROCEDURES

Ethical Approval

All aspects of this project were conducted in accordance with the principles of the Declaration of Helsinki, with informed consent being obtained from all participants. This project was approved by the Human Research Ethics Committees of the Royal Victorian Eye and Ear Hospital and the University of Western Australia and with Institutional Review Board approval from the Johns Hopkins University School of Medicine.

Illumina Infinium Human Methylation450 Bead Array

The 450K methylation array is widely accepted as a robust platform to assess DNA methylation, showing a high concordance with bisulfite pyrosequencing ($r > 0.80$) (Roessler et al., 2012). DNA samples from peripheral blood of 298 age- and sex-matched samples (comprising 100 bilateral GA, 99 bilateral NV, and 99 controls) were obtained from a subset of the Michigan patients from the Age-related Macular Degeneration Michigan, Mayo, AREDS, Pennsylvania (AMD-MMAP) study cohort (dbGaP: phs000182). DNA was extracted from whole blood using the Puregene Blood Core Kit C (Qiagen). Both the AMD and the control groups were 63% female, with a mean ± SD age of 79.3 ± 5.7 years in the AMD group and 79.3 ± 5.5 years in the control group. All samples were derived from the Kellogg Eye Center (KEC) at the University of Michigan (Chen et al., 2010). From the original KEC cohort, those patients with bilateral GA or NV were selected. Control patients were examined and accepted if they had small drusen and pigment changes in one eye only with no family history of AMD. All cases and controls were ≥60 years of age. DNA samples (1.3 μg) were processed for the HM450 platform (Illumina) at the Center for Inherited Disease Research (CIDR; Johns Hopkins University) as per the manufacturers' protocols. All matched samples (GA-NV-control) were run within a single BeadChip array.

Illumina Infinium Human Methylation450 Bead Array Data Preprocessing

We normalized the intensity data using a modified version of quantile normalization in the *minfi* Bioconductor package (http http://bioconductor.org/packages/devel/bioc/html/minfi.html/), which, briefly, forces the distribution of type I and type II probes on the Illumina 450K microarray to be the same. Quantile normalization is performed on the type II probes and then interpolated to a reference distribution to which the type I probes are normalized. This normalization is performed separately on the methylated (M) and unmethylated (U) channel intensities. The logit-transformed DNA methylation (DNAm) is $y = \log2(M/U)$, and the proportion methylation ("beta" scale, as reported by Illumina) can be obtained by: $2^y/(1 + 2^y)$. The "beta" values for probes in and around *IL17RC* (n = 35) were selected for subsequent statistical analyses.

Sample Selection from the Baltimore Cohort

DNA samples derived from peripheral whole blood were obtained from a cohort collected at Johns Hopkins University in Baltimore as previously described (Yang et al., 2008, 2010). In short, diagnosis of advanced AMD was based on the presence of GA or CNV (equivalent to AREDS category 4 or 5). Controls were identified as >60 years of age and having fewer than five small drusen (<63 μm) and no RPE abnormalities. The final cohort comprised 24 patients with GA (mean ± SD age at interview of 78.5 ± 7.6 years; 13 men and 11 women; European = 24), 15 patients with NV (73.7 ± 13.1 years; five men and ten women; European = 14, Ashkenazi Jew = 1), and 23 controls (80.0 ± 7.4 years; 10 men and 13 women; European = 23). Genomic DNA was isolated from peripheral whole blood using Puregene Blood Kit chemistry

on an Autopure LS automated DNA purification instrument (Qiagen). DNA concentrations are determined by spectrophotometry using a DU 530 Life Science UV/Vis Spectrophotometer (Beckman Coulter).

Sample Collection in an Australian Cohort

Patients with AMD were recruited from ophthalmology clinics at Launceston Eye Institute, Australia. The clinical criteria for the diagnosis of AMD included the presence of extensive drusen and associated pigmentary abnormalities of the RPE layer or evidence of advanced disease by presence of GA or NV. Age- and sex-matched controls were recruited from adjunct genetic studies. All control subjects underwent a comprehensive examination including fundus photography as well as macular assessment using optical coherence tomography. To be included as a control, subjects required no evidence of ophthalmic pathology as well as no prior or known family history of ocular disease. In total, we recruited 100 patients with AMD and 120 age-matched individuals with no signs of AMD. Two control samples failed DNA extraction from whole blood. All participants were of reported Northern European ancestry. The mean ± SD age of the AMD patients and controls (passing whole blood DNA quality control) was 72.1 ± 6.3 years and 71.0 ± 7.8 years, respectively. A total of 39% of the AMD patients and 54% of controls were male. Approximately 20 ml EDTA peripheral whole blood was collected via venipuncture from all participants and genomic DNA was extracted from circulating leukocytes by the QIAcube platform (Qiagen) through the facilities at the Western Australian DNA Bank.

Bisulfite Pyrosequencing of the *IL17RC* Promoter

Bisulfite pyrosequencing is widely considered the gold-standard approach for quantitatively measuring DNA methylation across targeted regions (<400 bp) of the genome. Pyrosequencing assays were designed using algorithms built into the PyroMark Assay Design Software (version 2.0.1; Qiagen). Briefly, primers designed to target specific CpG sites within the *IL17RC* promoter were chosen from a list generated by the software on the basis of the algorithms' predicted assay quality. Genomic DNA (500 ng) was bisulfite converted using the EZ DNA Methylation Gold kit (Zymo Research) according to the manufacturer's instructions. Following bisulfite treatment, all previously unmethylated cytosine residues are converted to uracil, whereas methylated cytosine residues remain unconverted. DNA containing the segment of the *IL17RC* promoter analyzed by Wei and colleagues (Wei et al., 2012) was amplified from the bisulfite-treated DNA using PCR primer set 1 (Australian samples) and PCR primer sets 1 and 3 (Baltimore samples) as shown in Table S5.

PCR products were bound to Streptavidin Sepharose High Performance beads (GE Healthcare) and a single-strand template was isolated using the Pyrosequencing Vacuum Prep Tool (Qiagen). The beads were transferred to an optically clear, 24-well sequencing plate in 0.3 μM of the sequencing primer. Serial pyrosequencing was performed where multiple sequencing primers were contained within a single PCR amplicon (Tost et al., 2006; Tost and Gut, 2007). Pyrosequencing was performed on a PyroMark 24 Pyrosequencing System (Qiagen) as per the manufacturer's instructions. Data were analyzed on the PyroMark Q24 software (Qiagen). This software calculates the C peak as a percentage of the T plus C peak at each CpG site taking into account sequence length and signal strength. The mean methylation for each individual was calculated. Initially, eight CpG sites overlapping the region specifically targeted previously (Wei et al., 2012) were investigated in the Baltimore and Australian cohorts. To further explore additional regions at the locus, an additional 25 CpGs were sequenced in the Baltimore cohort alone. An unpaired Student's t test was used to compare the methylation difference between the matched case-control groups.

Collection of Retina/RPE from Cadaver Eyes

Cadaver eyes with a known medical history of AMD and unaffected controls were obtained from the National Disease Research Interchange (NDRI) with ethical approval from the Johns Hopkins University School of Medicine IRB. In brief, the anterior segment was removed, along with the pupil and lens. Four cuts were made down the eye cup to expose the macula. The flower-cut eye cup was exposed to a sucrose gradient as previously described (Hackler et al., 2012). The macula and optic nerve head were removed by 6 mm biopsy punches, and the remaining peripheral retina and RPE/choroid were stored at −80°C. AMD was confirmed by assessment of macula photos taken prior to the sucrose treatment. DNA was extracted from the retina/RPE using the DNEasy Blood & Tissue kit (Qiagen) as per the manufacturer's protocol. Retina samples were obtained from nine AMD (six men, 87.8 ± 5.8 years [mean age ± SD]; three women, 91.7 ± 0.6 years) and six controls (one man, 91 years; five women, 88.4 ± 3.6 years). RPE samples were obtained from three AMD (three men, 86.3 ± 8.0 years) and three control (three men, 85.3 ± 5.5 years) donors. The bisulfite conversion and subsequent pyrosequencing of *IL17RC* was performed as described above.

Application of the EpiTect Methyl II PCR Assay for *IL17RC* to Baltimore Samples

DNA samples from ten age/sex-matched GA/NV/control trios (derived from peripheral whole blood) were selected from the Baltimore samples previously used for bisulfite pyrosequencing. The mean age of the subset of Baltimore AMD samples was 78.2 ± 5.7 years (mean ± SD; n = 20, 12 women). The mean age of the Baltimore controls subgroup was 77.9 ± 6.2 years (n = 10, of whom six were female). The Methyl-Profiler restriction assay Kit (MeA-01, SABiosciences) has been rebranded as the EpiTect Methyl II Restriction Kit (335452, Qiagen). The EpiTect Methyl II restriction digest was set up according to the manufacturer's protocol. Briefly, 230 ng of DNA was used in each of the following restriction enzyme digest reactions: (1) methylation-sensitive, (2) methylation-dependent, (3) methylation-sensitive and methylation-dependent double digest, or (4) mock digest. qPCR was performed as per the manufacturer's protocol, using commercially available primers for *IL17RC* (EPHS110029-1A, Qiagen) in RT^2 SYBR Green qPCR Mastermix (330500, Qiagen) on a CFX96 real-time PCR machine (Bio-Rad). The commercially validated *IL17RC* EpiTect Methyl II PCR primers interrogated methylated DNA in a 156 bp region centered on chr3:9957039, targeting the same region reported by Wei et al., (2012). Methylation-sensitive (EPHS115450-1A) and methylation-dependent (EPHS115451-1A) digest control assays were performed on a subset (n = 7) of samples. Samples were analyzed as recommended by the manufacturer (http://www.sabiosciences.com/dna_methylation_data_analysis.php/).

Cell Separation for Comparison of PBMCs and Granulocyte Cell *IL17RC* Promoter Methylation Status

PBMCs and granulocytes were isolated from whole blood collected from 20 Australian controls (a subset of those described above; mean age 82 years, range 77–90 years, 65% male) using Ficoll-Plaque Premium (GE Healthcare Life Sciences) as per the manufacturer's protocol. Blood samples were all processed within 2 hr of phlebotomy. The granulocyte fraction was extracted from the bottom of the tubes, with the PBMC fraction being extracted from the serum/medium boundary. Genomic DNA isolated from each cell type was bisulfite treated as described above. Two samples failed DNA extraction from whole blood. The methylation status of 14 previously analyzed CpG sites within the *IL17RC* promoter was compared between PBMCs, granulocytes (polymorphonuclear leukocytes), and unsorted whole blood using bisulfite pyrosequencing primer sets 1 and 2 (Table S5). Groups of paired measurements were compared using repeated-measures ANOVA.

SUPPLEMENTAL INFORMATION

Supplemental Information includes one figure and five tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.11.042.

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