(Epi)Genetic Analyses of Age-Related Macular Degeneration: Case-Control and Discordant Twin Studies

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Key Words

Allele-specific methylation · Age-related macular degeneration · Copy number variation · Epigenetic analysis · Monozygotic twin pairs ·

Abstract

Background/Aims: Phenotypic discordance in monozygotic (MZ) twin pairs can have an epigenetic or genetic basis. Although age-related macular degeneration (AMD) has a strong genetic component, few studies have addressed its epigenetic basis. **Methods:** Using SNP arrays, we evaluated differences in copy number variation (CNV) and allele-specific methylation (ASM) patterns (via methyl-sensitive restriction enzyme digestion of DNA) in MZ twin pairs from the US Twin Study of AMD. Further analyses examined the relationship between ASM and CNVs with AMD by both case/control analysis of ASM at candidate regions and by analysis of ASM

and CNVs in twins discordant for AMD. **Results:** The frequency of ASM sites differs between cases and controls in regions surrounding the AMD candidate genes *CFH*, *C2* and *CFB*. While ASM patterns show a substantial dependence on local sequence polymorphisms, we observed dissimilar patterns of ASM between MZ twins. The genes closest to the sites where discordant MZ twins have dissimilar patterns of ASM are enriched for genes implicated in gliosis, a process associated with neovascular AMD. Similar twin-based analyses revealed no AMD-associated CNVs. **Conclusions:** Our results provide evidence of epigenetic influences beyond the known genetic susceptibility and implicate inflammatory responses and gliosis in the etiology of AMD.

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Background

The US Twin Study of Age-Related Macular Degeneration (AMD), a population-based study of monozygotic (MZ) and dizygotic (DZ) twin pairs concordant and discordant for AMD, was established in 1988 to evaluate the relative effects of genetics and the environment on AMD [1]. The results of this study concluded that the heritability of AMD is between 46 and 71%, with more advanced cases having a higher heritability of between 67 and 71% [1]. As shown in our previous studies of twins, including the study of MZ twin pairs discordant for AMD, environmental factors such as smoking and diet also contribute to this disease, and together with genetic factors they are predictive of the onset and progression of the disease [2–4].

Since genetic susceptibility is a strong determinant of the development and progression of AMD, identical MZ twin pairs, in which one twin is affected with intermediate or advanced disease and the co-twin is free of disease or in the early stages of it, are a unique population to assess somatic differences and how they contribute to AMD. One unexplored source of these differences is post-twinning *de novo* copy number variation (CNV), and MZ twins have been reported to display different CNV profiles [5]. More commonly, differential environmental exposure is thought to cause MZ twin differences, as suggested in our previous report, which showed that smoking and dietary intake of betaine, methionine, and vitamin D differ between MZ twins discordant for AMD [3]. It is possible that environmental exposures exert their influence through molecular mechanisms such as DNA methylation [6], an epigenetic process that can influence gene expression [7].

Previous studies of DNA methylation in MZ twins have demonstrated DNA methylation differences between twins [8], particularly as the twins aged [9]. Studies of DNA methylation have traditionally focused on two types of genomic regions: (1) CpG islands which are statistically improbable clusters of CpGs occurring in gene promoters, and (2) differentially methylated regions associated with genomic imprinting, where methylation is associated with one allele in a parent-of-origin manner. Our recent studies [10, 11] as well as others [12, 13] have demonstrated a novel type of differential methylation where the methylation mark is consistently associated with one allele. Often termed allele-specific methylation (ASM), the phenomenon can be influenced to varying degrees by DNA sequence within a population, ranging from complete association of methylation and genotype

(i.e. cis-regulated ASM) [11–13] to more stochastic associations, where either allele may be associated with the methylation mark. As ASM is associated with expression changes in nearby genes, variation in differential methylation among individuals, including MZ twins, has the potential to affect phenotypic variation [12–15].

In this report, we examined whether differences in CNVs and ASM profiles correlated with the AMD phenotype between twins utilizing two sources of data: (1) a standard analysis of genome-wide CNV among 66 individuals (a total of 33 twin pairs: 7 case pairs, 16 control pairs, and 10 phenotypically discordant pairs) using Affymetrix 6.0 SNP arrays, and (2) an assay of ASM, using methyl-sensitive restriction enzyme (MSRE) digestions paired with Affymetrix 6.0 SNP arrays, at almost 100,000 genomic regions in a subset of these samples. The subset for differential methylation analyses included 11 MZ twin pairs (2 with phenotypically discordant AMD status, 4 case pairs and 5 control pairs) and 14 singleton samples.

Methods

For further details on methods development, see the online supplementary Material (for all online suppl. material, see www.karger.com/doi/10.1159/000362814).

Study Population

The study population for the US Twin Study of AMD was derived from the National Academy of Sciences – National Research Council World War II Veteran Twin Registry, which includes medical information for 15,924 white male twin pairs born between 1917 and 1927 and was used for both CNV and methylation analyses [16]. Details of the twin study methods have been previously described [1–3]. Briefly, among 12,126 individuals surveyed, 684 (5.6%) reported having been diagnosed with AMD, and 820 individual twins were enrolled in the Twin Study of AMD, including 381 MZ and DZ pairs and 58 singletons [1]. From this population, we selected MZ twin pairs discordant for AMD stage. We defined discordant pairs as each co-twin having a different Clinical Age-Related Maculopathy Staging System (CARMS) grade [17] at the last known assessment, with one twin being affected with intermediate AMD (large drusen and/or drusenoid retinal pigment epithelial detachment) or advanced AMD [either geographic atrophy (GA) or neovascular (NV) disease], and the other co-twin with no AMD or in the early stages. Blood samples were drawn, DNA was extracted and cell lines were established using standard procedures. Institutional review boards approved the research protocols and all participants signed consent statements. Research adhered to the tenets of the Declaration of Helsinki.

Macular Characteristics

Fundus examination and standard fundus photography were performed to record the stage of AMD without knowledge of the zygosity of the twins. Photographs of the macula were graded using a grid according to the Wisconsin Age-Related Macular Degen-

Table 1. Sample AMD grades**a** CNV analysis of twins

		Twin-II: Affected			Unaffected		
		CARMS 5	CARMS 4	CARMS 3	CARMS 2	CARMS 1	total
<i>Twin-I</i>							
Affected	CARMS 5	4	3		6		13
	CARMS 4				3	1	4
	CARMS 3						0
Unaffected	CARMS 2						0
	CARMS 1					16	16
Total		4	3	0	9	17	33

b Methylation analysis of twins

		Twin-II: Affected			Unaffected		
		CARMS 5	CARMS 4	CARMS 3	CARMS 2	CARMS 1	total
<i>Twin-I</i>							
Affected	CARMS 5	1	3		2		6
	CARMS 4						0
	CARMS 3						0
Unaffected	CARMS 2						0
	CARMS 1					5	5
Total		1	3	0	2	5	11

c Methylation analysis of singletons

		Affected			Unaffected		
		CARMS5	CARMS4	CARMS3	CARMS2	CARMS1	total
Total		2	1		3	8	14

Grades of matched twins (**a** and **b**) and singletons (**c**) used for CNV analyses (**a**) and methylation analyses (**b** and **c**) using CARMS [17].

eration Grading System [18] for a detailed evaluation of macular drusen and retinal pigment characteristics. Study examination data and photographs were also graded by J.M.S. and classified into normal or no AMD (grade 1), early (grade 2), intermediate (grade 3), and advanced AMD stages of GA (grade 4) and NV disease (grade 5) according to the CARMS grading system (table 1 and online suppl. table 1) [17].

Zygosity Status and Genotyping

Each MZ twin pair was genotyped on the Affymetrix 6.0 (Affymetrix Inc., Santa Clara, Calif., USA) platform at the Broad Institute Center for Genotyping and Analysis (Cambridge, Mass., USA). The Affymetrix 6.0 has probe sets against 906,600 SNPs and 940,000 CNV probes. Data for each twin pair were analyzed using PLINK [19] for basic quality assurance and quality control (QAQC)

metrics. Samples that performed at acceptable QAQC levels in the SNP-scan part of our GWAS were used for the CNV part of our analysis. Zygosity was determined by an identity-by-descent (IBD) score, which looks at overall genotypic sharing as a function of relatedness. To pass zygosity checks, we required twin pairs to have IBD scores of >0.95. Known AMD genes were also genotyped and twins in each pair in the analysis had identical genotypes.

CNV Analyses

The initial study population consisted of 35 twin pairs. Two of these twin pairs failed the initial genotyping based zygosity checks, so 66 individuals (a total of 33 twin pairs: 7 case pairs, 16 control pairs, and 10 phenotypically discordant pairs) were passed through to the CNV twin pair analyses.

CNVs were identified using Birdseye [20], which identifies rare CNVs by integrating intensity data from neighboring probes using a Hidden Markov Model (HMM) on a per-individual basis. Performance is dependent on a number of factors including SNP and copy number probe density, mean intra-individual probe variance and CNV frequency. For each CNV, a logarithm of odds (LOD) score was generated that describes the likelihood of the CNV relative to no CNV over the given interval.

Within the 66 samples, we observed 297,345 unfiltered regions (i.e. without Birdseye baseline filters such as length of deletion event, number of probes identified in the region, contiguity of probes identified in a region) of copy number deletions that differed between twins. We restricted our search to deletions as they have much stronger signal-to-noise signal properties than duplications. A CNV duplication, unless there are multiple duplications, will only provide a 3:2 (one chromosomal duplication) or 4:2 (two chromosomal duplications) ratio, which is much harder to see than a 2:1 (one chromosomal deletion) or 2:0 (two chromosomal deletions) ratio. In order to obtain a high-quality data set, we restricted our analysis to the 307 CNVs with a LOD >10 and a physical length >100 kb within our analysis set. We imposed an approximately 1% frequency threshold by removing any CNV with >50% of its length spanning a region with more than 65 out of approximately 6,500 CNVs in the total sample [21]. We also removed events spanning regions of common CNV (>1%) in the CEU HapMap individuals identified using Affymetrix array data generated in the same laboratory and called with the same analytic pipeline. Additionally, we removed regions identified in the Database of Genomic Variants [22]. As a final step, we joined any CNVs that appeared to be artificially split by the HMM used in analysis, and also removed any CNVs that spanned known large gaps (>200 kb) in the hg18 reference genome or regions of known rearrangement (e.g. hg18: chr2:88695164–95087413; chr14:104644530–106268819; chr22:20797557–21512883) to yield novel CNV deletion events that differ between twins [23].

Methylation Analyses

Samples

DNA samples for methylation analyses were all derived from whole blood. Cell lines were not considered for these analyses since we found that, in agreement with recent reports [24], methylation patterns differ between lymphoblast cell lines and whole blood DNA derived from the same subjects. Among the 36 samples, 14 were singletons used only for case-control analyses and 22 were twins used in the twin-pair methylation analyses. Of the 14 singletons, 12 samples were derived from twins pairs for which one twin had only cell line DNA and 2 were derived from the two twin pairs which failed the zygosity checks in the CNV QAQC steps described above. The other 22 samples were from 11 twin pairs (table 1 and online suppl. table 1) consisting of 5 concordant control twin pairs (both twins grade 1), 4 concordant case twin pairs and 2 twin pairs discordant for AMD (1 pair with both twins grade 5 and 3 pairs with one twin grade 4 and the other twin grade 5), and 2 twin pairs discordant for AMD (one twin grade 5 and the other twin grade 2). These samples were used in both case-control and twin-pair methylation analyses. To adjust for technical variation, all methylation samples were analyzed as technical replicates on two independent Affymetrix arrays. Probe intensities used in further analyses represent the mean values from these technical replicates.

MSRE Digest and Validation

For the high-throughput methylation assay, genomic DNA was genotyped on the Affymetrix 6.0 SNP mapping arrays according to the manufacturer's manual (www.affymetrix.com). For methylation analysis, 3 µg of genomic DNA was digested at 37°C for 16 h with a MSRE cocktail including Aci I (60 units), BsaH I (3.9 units), Hha I (7.5 units), Hpa II (7.5 units), and HpyCH4 IV (30 units) (New England Biolabs), in a 200-µl reaction volume with 1% BSA and 10% NEB buffer No. 4 (New England Biolabs) and heat inactivated for 20 min at 60°C. Samples were ethanol precipitated, washed in 70% ethanol, and resuspended in reduced EDTA TE (5 mM Tris, 0.1 mM EDTA) at 50 ng/µl. After this pre-treatment, the DNA entered the mapping array procedure; it was further digested with the Nsp I and Sty I restriction enzymes, and fragments of 100–1,100 bp (containing the polymorphic sites to be assessed) were PCR amplified, with the resulting amplicons then end labeled and hybridized to the array. The efficacy of the independent MSRE digestions was determined by examining MSRE action on amplicons with solitary cut sites for a component MSRE in a control mix run alongside the AMD samples. Our results showed that amplicons with single MSRE cut sites, with the exception of HhaI, exhibited greatly reduced combined probe intensities compared to amplicons with no MSRE sites (MNRs) (online suppl. fig. 1). Any amplicons with HhaI sites only (6,132 in total) were ignored in further analyses.

Array Quality Control

To eliminate replicates with poor array hybridizations, the unnormalized probe intensities of the replicates of MSRE-digested samples were compared by linear regression. All replicate coefficients of determination were within 2.5 SD of the mean of the entire sample set (mean = 0.919, SD = 0.125). After normalization, the mean coefficient of determination was 0.94 with a SD of 0.028. All but two samples had coefficients within 2.5 SD of this mean (T39B: 0.86 and T27B: 0.87).

Array Normalization and ASM Detection

Briefly, invariant probe sets were first quantile-normalized between arrays and the normalized values for the variant probe sets were then interpolated from the normalized invariant probe sets derived from their respective arrays. After various levels of technical cleanup, including eliminating probe sets that performed poorly, ASM at heterozygous SNPs was detected as a sample and SNP-specific normalized deviation from the HapMap-derived heterozygote relative probe intensity (see online suppl. Methods for more details). The potential for artifacts arising from polymorphisms in MSRE recognition sites was eliminated as described previously [11]. Briefly, we excluded all SNPs on the Affymetrix array residing on amplicons containing any known polymorphism in an MSRE recognition site in the dbSNP129 database [25]. These filters were used for all such polymorphisms present at any frequency in the population. Unless otherwise noted, this filter was used in all analyses. Although this filter discards loci that may not be polymorphic for the individuals used in this study, we conservatively chose to ensure robust analyses. For the validation of these methods on known ASM regions, see the online supplementary Results section of the online supplementary Materials.

Independent Confirmation

We employed 454 large-scale parallel pyrosequencing to examine SNP and CpG site methylation status. Primers for bisulfite PCR (BSP) amplification were designed with Methprimer [26] and BiSearch [27] to uniquely amplify fragments of <500 bp encompassing the MSRE sites and target SNP. All amplifications were strand specific and designed to allow observation of the SNP status after bisulfite conversion and amplification; for C/T SNPs, the guanine strand was amplified. Primer sequences (against bisulfite-converted DNA) and amplicon genomic locations for all amplicons can be found in online supplementary table 2. DNA samples were bisulfite converted with the Qiagen EpiTect 96 Bisulfite Kit according to the manufacturer's instructions, quantitated by Nanodrop and amplified in 96-well plates for 20- μ l PCR reactions comprised of 10 ng converted DNA, 1 unit Qiagen HotStarTaq Plus, 50 μ M of each primer (2.5 μ M final concentration), 1 $\times$ PCR buffer, and 200 nM dNTPs (10 mM final concentration). All amplifications were run for 35 cycles. Product sizes were visually confirmed by agarose gel electrophoresis, quantitated by Picogreen, and equimolar amounts from each target's amplification products mixed for each individual before bar code-indexed 454 pyrosequencing.

After sequencing, pooled reads were deindexed and adapters and low-quality sequences were clipped; reads shorter than 100 were removed. Reads were nondirectionally aligned to amplicon sequences with Bismark [28] with the Bowtie 2 gapped. Bisulfite conversion rates >95% were confirmed, and reads not extending over both the amplicon's target SNP and MSRE sites were discarded. Genotypes at target SNPs were called in VCF format with SAMtools pileup [29] with extended BAQ, and a BAQ cutoff of 10.

All amplicons had a coverage depth of at least 20 reads and genotype qualities of at least 20 in a majority of the samples (>50%). SNPs did not significantly deviate ($p > 0.01$) from the expected Hardy-Weinberg equilibrium within the sample population. Agreement with the expected minor allele frequency, as defined by a relative mean difference <0.2 from the expected CEU minor allele frequency (HapMap release 27), was also confirmed for all amplicons. For the results of these validation experiments, see the online supplementary Results section of the online supplementary Materials. All unprocessed data are available by request from Dr. J.M. Seddon.

Results

CNV Analyses

After quality control and filtering, we observed a total of 307 CNVs that were not shared between the twins of a twin pair. These 307 unshared CNVs were distributed among the different twin pair types (e.g. case-case, control-control, case-control) rather than concentrated in any twin-pair type. While the majority of these 307 unshared CNVs were commonly observed deletions, 8 were novel CNV deletion events. None of these 8 events were seen in more than one twin pair, and they were also distributed throughout the twin-pair types. These 8 CNV

deletion events occur in genomic regions that are mostly devoid of genes, and where genes are present, they are neither implicated in AMD by previous association studies nor are they paralogs of the previously implicated genes.

ASM at Regions Implicated in AMD

A χ^2 analysis of individual MPRs between individuals classified as cases (13 individuals; CARMS grades 3–5) [17] and controls (23 individuals; CARMS 1 and 2) (online suppl. table 1) did not yield any significant results after correction for multiple hypothesis testing (online suppl. fig. 2; Bonferroni adjusted $p > 0.05$; online suppl. table 3). As ASM changes can correlate across larger genomic regions than assayed by a single MPR [14], we also examined the grouped ASM status of all MPRs in the 1-Mb windows around each of the 12 genetic variants previously implicated in AMD:

(1) rs1061170, or *CFHY*402H, affecting exon 9 of the *CFH* gene on chromosome 1q32, resulting in a substitution of histidine for tyrosine at codon 402 of the CFH protein [32–34];

(2) rs1410996, an independently associated SNP variant within intron 14 of *CFH* [35];

(3) rs10033900, an independently associated SNP located in the linkage peak region of chromosome 4, 2,781 bp downstream of the 3' untranslated region of *CFI* [36];

(4) rs9332739, or *C2* E318D, a non-synonymous coding SNP variant in exon 7 of *C2* resulting in a substitution of aspartic acid for glutamic acid at codon 318 [35, 37];

(5) rs641153, or *CFB* R32Q, a non-synonymous coding SNP variant in exon 2 of *CFB* resulting in the substitution of the amino acid glutamine for arginine at codon 32 [35, 36];

(6) rs4711751, a non-coding variant downstream of *VEGFA* [38],

(7) rs1999930, a non-coding variant near *FRK/ COL10A1* [38],

(8) rs10490924, a non-synonymous coding SNP variant in exon 1 of *ARMS2* on chromosome 10 resulting in a substitution of the amino acid serine for alanine at codon 69 [39, 40];

(9) rs10468017, a promoter variant of the Hepatic Lipase C gene *LIPC* on chromosome 15q22 [21];

(10) rs3764261, a non-coding variant approximately 2.5 kb upstream of *CETP* on chromosome 16 [21, 38]

(11) rs2230199, or *C3* R102G, a non-synonymous coding SNP variant in exon 3 of *C3* resulting in the substitution of the amino acid glycine for arginine at codon 102 [41, 42], and

Table 2. Analyses of allele-specific methylation in cases and controls in regions surrounding known AMD variants**a** Allele-specific methylation in cases and controls

	Cases		Controls		p value	Percen- tile
	MPRs	ASM MPRs	MPRs	ASM MPRs		
<i>5e5-bp window size</i>						
rs1061170	37	4	59	0	0.0199	2.02
rs1410996	37	4	60	0	0.0191	1.97
rs10033900	127	5	149	16	0.0404	4.07
rs9332739	128	3	158	16	0.0085	0.81*
rs641153	137	3	166	16	0.0082	0.76*
rs4711751	213	17	266	15	0.3586	32.15
rs1999930	133	12	140	11	0.8286	75.00
rs10490924	188	14	263	25	0.4994	45.23
rs10468017	391	27	496	32	0.7879	71.50
rs3764261	181	18	274	26	0.8726	80.09
rs2230199	151	7	213	10	1.0000	99.93
rs9621532	274	19	379	40	0.1285	12.12
<i>1e6-bp window size</i>						
rs1061170	84	11	145	2	0.0004	0.06**
rs1410996	84	9	144	2	0.0025	0.28**
rs10033900	350	28	430	39	0.6103	57.83
rs9332739	346	16	425	35	0.0574	5.95
rs641153	350	16	439	36	0.0438	4.58
rs4711751	331	25	431	20	0.2052	20.24
rs1999930	333	31	460	33	0.2924	28.83
rs10490924	382	45	567	64	0.8359	79.34
rs10468017	561	35	664	41	1.0000	99.98
rs3764261	389	30	511	37	0.7989	75.64
rs2230199	302	9	434	19	0.4340	42.06
rs9621532	477	49	644	66	1.0000	99.98

For the two window sizes shown, 500 kb and 1 Mb centered on the AMD-associated variant, ASM counts were summed for all MPRs within the window in both cases and controls and Fisher's exact tests performed. These values were compared to values derived from 10,000 identically sized, randomly located windows drawn from the same data. One region surrounding two variants (rs1061170 and rs1410996 located approx. 38 kb apart on chromosome 1) showed more differences between cases and controls than 99% of randomly drawn windows of 1 Mb in size and more than 95% of randomly drawn windows of 500 kb in size. Another region surrounding two variants (rs9332739 and rs6411536 located approx. 10 kb apart on chromosome 6) showed more significant differences between cases and controls than more than 99% of randomly drawn windows of 500 kb in size and almost 95% of randomly drawn windows of 1 Mb in size.

* Significant at $p < 0.1$ after multiple testing adjustment. ** Significant at $p < 0.05$ after multiple testing adjustment.

b Known AMD variants

SNP	Proximal gene	Chromosome	Position
rs1061170	CFH	1	194925860
rs1410996	CFH	1	194963556
rs10033900	CFI	4	110878516
rs9332739	C2	6	32011783
rs641153	CFB	6	32022159
rs4711751	VEGFA	6	43936560
rs1999930	FRK/COL10A1	6	116493827
rs10490924	ARMS2/HTRA1	10	124204438
rs10468017	LIPC	15	56465804
rs3764261	CETP	16	55550825
rs2230199	C3	19	6669387
rs9621532	TIMP3	22	31414511

(12) rs9621532, a variant upstream of *TIMP3* [21, 43].

While the majority of windows (8/12) showed no significant association of ASM occurrence with either case or control status, 2 largely overlapping windows centered on the *CFH* locus (rs1061170 and rs1410996) had significant differences (Bonferroni adjusted p values < 0.05 at 0.0072 and 0.0336, respectively). Two overlapping windows centered on SNPs within the *C2* and *CFB* loci (rs9332739 and rs641153) also showed significant differences (Bonferroni adjusted p values < 0.1 at 0.0972 and 0.0912, respectively) in ASM levels between cases and controls. To account for any correlation structure within our samples which might confound these results, we compared them to results from identically structured tests of 10,000 randomly drawn windows of equal base pair size and equal or higher MPR counts; the AMD SNP-containing windows represented genomic regions with some of the highest differences in ASM levels between cases and controls (table 2, fig. 1). Taken together, these results and the large overlap of the two windows within each genomic region indicate the presence of 2 genomic regions with differential levels of ASM in patients with a differential AMD status.

MZ Twins Exhibit Different ASM Patterns

We examined the overall level of disagreement between twins for ASM and observed 2 types of methylation scenarios: (1) allelic differences, where both twins showed ASM at an MPR, but for different alleles, and (2) ASM occurrence differences, where one twin exhibits ASM at an MPR and the other twin exhibits biallelic methylation (fig. 2). We observed very few cases of the first scenario;

Table 3. Analyses of allele-specific methylation allele preference agreement between twins

Twin pair	Informative events	Twin-I		Twin-II		Expected		Observed		p value (Fisher's exact test)
		A-ASM	B-ASM	A-ASM	B-ASM	agree	oppose	agree	oppose	
T04	53004	301	308	617	486	6	6	132	5	2.58E-05
T12	53386	536	593	585	569	12	12	644	0	1.81E-19
T14	53367	273	283	341	386	4	4	268	4	1.87E-05
T16	52897	1,091	1,046	842	687	31	31	952	22	4.74E-28
T20	51969	1,278	1,345	482	427	23	23	270	32	2.77E-09
T26	51923	430	502	925	1,032	18	17	438	0	7.27E-22
T27	53600	341	336	444	407	5	5	274	4	1.88E-06
T29	53116	1,548	1,415	422	423	24	24	351	30	5.80E-12
T30	52707	661	657	804	769	20	20	695	27	4.73E-16
T38	51995	644	664	574	546	14	14	782	0	7.48E-23
T39	52639	705	785	384	359	10	11	190	2	3.18E-11

Results are shown for ASM allele preferences for informative MPRs (heterozygous genotype, above intensity cutoff) in Twin-I and Twin-II of a twin pair; the number of ASM events associated with either allele A (A-ASM) or allele B (B-ASM) for each twin are shown (the remaining informative MPRs show biallelic methyla-

tion in at least one twin). The observed numbers of matching (agree) or opposite (oppose) allele preference between twins are shown as compared to the null hypothesis of random preference by Fisher's Exact tests.

Fig. 1. ASM differences between cases and controls in the regions surrounding the genetic variants with known AMD associations. Distributions of p values from Fisher's exact tests of ASM counts in cases and controls are derived from randomly drawn MPR-containing windows of 500 kb (**a**) and 1 Mb (**b**). Vertical lines indicate

the positions of p values derived from the windows surrounding the known AMD variants found to be within the 5th percentile of the distribution. The respective variant identities and percentiles are indicated to the left of the lines.

Fig. 2. Differential ASM scenarios in MZ twins. Potential modes of differing ASM calls between MZ twins are shown. In the first model (a), the identity of the allele associated with methylation distinguishes the twins: Twin-I or Twin-II exhibits methylation associated with the A allele (upper row) and the other twin exhibits methylation associated with the B allele (lower row). Alternatively, in a second model (b), the differential occurrence of ASM distinguishes the twins: Twin-I or Twin-II shows ASM (associated with either one of the A or B alleles), and the other twin exhibits biallelic methylation.

less than one would expect were allele preference random (table 3). This supports our prior work showing the prevalence of SNP-influenced ASM across the genome [11]. The second scenario is more prevalent; while most ASM events are present in both twins, we did observe instances where, for up to 5.2% of MPRs, one twin exhibits ASM and the other does not. To account for the possibility of these instances resulting from technical variation, we leveraged the two technical replicate arrays, comparing the level of disagreement found between all replicates between twins (4 comparisons) to that found when comparing replicates from the same twin (2 comparisons). We observed that for the majority of whole blood samples examined, biological ASM differences exceed technical noise, i.e. the percentage of ASM disagreements between twins at these MPRs (median 2.5% of MPRs) exceeds the percentage of ASM disagreements observed when comparing replicates from an individual twin (median 2.05% of MPRs) (fig. 3). Thus, while most of the disagreements are due to technical noise, some of the differences reflect stable differences between the twins or, alternatively,

could reflect differences we would have observed if we had compared samples taken at different time points from the same individual.

ASM Differs in Twins with Discordant AMD Status at Regions Proximal to Genes Implicated in Gliosis

We extended these results to search for genomic regions with consistent ASM differences between the discordant twins. For these twins, we did not observe any instances where both twins showed ASM at an MPR, but for different alleles (fig. 2a; e.g. regions where, for both discordant twin pairs, the cases exhibited methylation associated with the A allele and the controls exhibited methylation associated with the B allele). Instead, we observed the differential occurrence of ASM between the discordant twins (fig. 2b), where at 42 MPRs, for both discordant twin pairs, one twin showed biallelic methylation and the other had ASM (e.g. regions where, for both discordant twin pairs, the cases showed either ASM-A or ASM-B and the controls exhibited biallelic methylation) (table 4). To determine how likely we were to see this

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Fig. 3. ASM patterns between MZ twins. Each circle represents the percentage of regions found to disagree for ASM between the two sample comparisons. These results are derived from either technical replicates drawn from an individual twin or comparisons between the replicates from each of the different twins. Results are shown for an approach in which ASM calls were more extreme than a Z-score cutoff excluding 99% of MNRs (1% false positive rate) and non-ASM calls less extreme than 95% of MNRs (i.e. included within 95% of the MNR distribution). In this approach, SNPs with Z-score values more extreme than the 99% Z-score cut-off in one twin (i.e. ASM) and between 95 and 99% in the other twin (i.e. neither ASM nor non-ASM) were excluded as indeterminate. Biological differences between twins that cannot be explained by technical noise are consistent with the inter-twin-pair comparisons having greater values than both intra-twin-pair comparisons. The first five twin pairs are concordant controls, the next four are concordant cases and the final two twin pairs are discordant twins.

number of discordant ASM MPRs at random, we performed a permutation test of the data set, shuffling the discordant and concordant assignments of the twin pairs; 2,217 of the 10,000 of the permuted data sets resulted in at least the same number of methylation differences between the discordant twin pairs to yield a p value of 0.2217. To determine if any biological functions might be associated with these particular MPRs, we performed a gene ontology analysis of the genes closest to these 42 MPRs (as determined by the Affymetrix SNP 6.0 hg19 annotations). Our results showed this list of genes to be enriched for genes involved in the process of gliogenesis.

Specifically, 36 of the 42 genes had a gene ontology annotation, and 4 of these 36 genes were contained within the gliogenesis annotation, yielding an adjusted p value of 0.0045 (via the g:SCS algorithm, a multiple-test corrected p value which accounts for the hierarchical structure within gene ontology terms [44]).

Discussion

This work represents one of the first genome-wide analyses of differential ASM and DNA methylation within several MZ twin pairs participating in an AMD study. This work also represents the first evaluation of CNVs in MZ twin pairs with concordant and discordant AMD phenotypes. In addition to identifying smaller (approx. 0.1–1.1 kb) differentially methylated genomic regions in MZ twins discordant for AMD, we also demonstrate that larger genomic regions (0.5–1 Mb) containing known AMD-associated variants are differentially methylated according to the AMD status. Our results extend our previous AMD studies with this twin cohort and, while there is no incontrovertible evidence of an epigenetic signature for AMD, our results do suggest candidate epigenetic targets underlying the discordant phenotypes observed in twins [3].

There are few previous studies examining CNV differences between MZ twins. While Bruder et al. [5] claimed they could see many somatic differences between subjects, we took a more conservative approach to setting thresholds for calling these differential CNV events. Upon deeper examination of the underlying data, we found that frequently, when one twin reached the calling threshold, the other twin showed sub-threshold levels of signal. These results indicate that the differences in CNV deletions we do observe between twins are likely driven by small differences in signal between twins, and may not represent true CNV differences. In support of our conservative approach and the resultant low number of MZ twin-pair CNV differences, somatic mutations have historically been estimated to occur once every 100 Mb, yielding only about 30 events per individual across the genome. While it is difficult to determine when these events occur, we would expect any of the mutations occurring very early during embryogenesis prior to the ‘twinning’ event to be shared between MZ twins. Therefore, the expectation, which is fulfilled by our results, is for relatively few of these events to be seen between MZ twins.

Table 4. SNPs discordant for ASM incidence in monozygotic twins discordant for AMD

ProbeID	Discordant twin status		SNP			Closest gene		
	T30	T29	ID	Chr	position	relation to gene	distance from TSS (if not genic)	symbol
SNP_A-2083845	BI-B	BI-B	rs13411523	2	114454164	downstream	21527	ACTR3
SNP_A-2021856	A-BI	A-BI	rs11679258	2	241441734	upstream	15100	AGXT
SNP_A-2058335	B-BI	B-BI	rs1993329	6	68992781	upstream	409571	BAI3
SNP_A-2045223	B-BI	B-BI	rs12323921	14	98427236	downstream	278140	BCL11B
SNP_A-8484764	BI-B	BI-B	rs6140071	20	6654493	upstream	42251	BMP2
SNP_A-2210596	B-BI	B-BI	rs7909808	10	78062324	downstream	75194	C10orf11
SNP_A-8559771	BI-A	BI-A	rs9384701	6	109664235	downstream	72429	C6orf182
SNP_A-8717023	A-BI	A-BI	rs8063808	16	47795251	downstream	74460	CBLN1
SNP_A-2133676	A-BI	A-BI	rs10470437	3	133265423	upstream	28889	CPNE4
SNP_A-8573108	B-BI	B-BI	rs2693022	2	15842155	downstream	153479	DDX1
SNP_A-1896123	A-BI	A-BI	rs10930538	2	172813725	upstream	138001	DLX2
SNP_A-1793639	B-BI	B-BI	rs12691430	5	107116609	upstream	82114	EFNA5
SNP_A-1896576	A-BI	A-BI	rs11865876	16	13787369	upstream	134145	ERCC4
SNP_A-1888329	B-BI	B-BI	rs10988682	9	132099642	downstream	60238	FREQ
SNP_A-4282113	A-BI	A-BI	rs9309883	3	82097118	upstream	203478	GBE1
SNP_A-8392053	A-BI	A-BI	rs2017257	17	40352675	upstream	4235	GFAP
SNP_A-4284358	B-BI	B-BI	rs12478085	2	206766767	intron		GPR1
SNP_A-8321092	B-BI	B-BI	rs8076416	17	74822048	intron		hCG_1776007
SNP_A-1867638	A-BI	A-BI	rs2461056	8	80836175	downstream	2625	HEY1
SNP_A-8425957	A-BI	A-BI	rs11207325	1	59136207	upstream	113834	JUN
SNP_A-2246766	BI-B	BI-B	rs6985117	8	111591041	upstream	534906	KCNV1
SNP_A-8529083	A-BI	A-BI	rs10446676	4	36562606	upstream	360478	KIAA1239
SNP_A-4196589	B-BI	B-BI	rs10457178	6	108948401	intron		LACE1
SNP_A-8564552	A-BI	A-BI	rs130993	22	32897763	upstream	251347	LARGE
SNP_A-1855257	B-BI	B-BI	rs962885	17	41291420	intron		LOC100128977
SNP_A-2274687	A-BI	A-BI	rs4142502	6	143917664	exon		LOC285740
SNP_A-2162548	A-BI	A-BI	rs12361464	11	41476061	upstream	1203821	LRRC4C
SNP_A-8498579	BI-A	BI-A	rs4715127	6	49396584	downstream	110366	MUT
SNP_A-8338412	BI-A	BI-A	rs8134891	21	28888157	downstream	53610	NCRNA00161
SNP_A-2001046	A-BI	A-BI	rs7917091	10	55684541	intron		PCDH15
SNP_A-8682352	B-BI	B-BI	rs11949422	5	59292928	upstream	67550	PDE4D
SNP_A-1909129	A-BI	A-BI	rs532095	13	74708951	downstream	940	RP11-159J2.1
SNP_A-4276454	A-BI	A-BI	rs941655	14	94393085	upstream	55496	RPL15
SNP_A-4298178	B-BI	B-BI	rs1200470	13	31010348	upstream	201330	RXFP2
SNP_A-8634510	A-BI	A-BI	rs941597	14	93883114	upstream	23673	SERPINA6
SNP_A-8698392	A-BI	A-BI	rs7824948	8	14987154	intron		SGCZ
SNP_A-8607148	A-BI	A-BI	rs1731847	7	155348283	upstream	50555	SHH
SNP_A-8434018	A-BI	A-BI	rs13179872	5	128177775	upstream	151333	SLC27A6
SNP_A-4280941	BI-B	BI-B	rs454693	5	87579969	intron		TMEM161B
SNP_A-2065284	A-BI	A-BI	rs955336	1	100881570	upstream	76314	VCAM1
SNP_A-1788455	A-BI	A-BI	rs16963005	19	34756795	downstream	9729	VSTM2B
SNP_A-1953456	A-BI	A-BI	rs4796421	17	7320374	intron		ZBTB4

Results are shown for an approach in which ASM calls were based on Z-score cutoffs excluding 99% of MNRs (1% false positive rate) and any non-ASM calls less extreme than 95% of MNRs (i.e. included within 95% of the MNR distribution). In this approach, regions with Z-score values more extreme than the Z-score cutoff in one twin (i.e. ASM) and between 95 and 99% in the other twin (i.e. neither ASM nor non-ASM) were excluded as indeterminate. The 42 MPRs shown exhibit consistent ASM incidence discordance in twin pairs discordant for AMD, where the ASM call for the twins within the

twin pair are separated by a dash. BI = biallelic methylation; A = ASM of the A allele; B = ASM of the B allele; BI-BI = biallelic methylation in both twins; A-A = ASM of the A allele in both twins; B-B = ASM of the B allele in both twins. The code before the dash indicates the ASM status of the unaffected twin (control), and the code after the dash that of the affected twin (case). Affymetrix annotations for genomic location and relationship to the closest gene are also shown for each of these variants.

Previous attempts to associate methylation changes with phenotypic traits in discordant twins have demonstrated differences between MZ twins within a pair but have largely been limited in genomic scope. Multiple studies targeting CpG islands have shown suggestive results; Javierre et al. [45] observed widespread changes in the DNA methylation status of a significant number of CpG-containing promoters of genes associated with immune function in 5 MZ twin pairs discordant for systemic lupus erythematosus. Using the Illumina 27K platform (which largely targets CpG islands of cancer-associated genes), Dempster et al. [46] found a significant enrichment of epigenetic changes in biological pathways relevant to psychiatric disorder and neurodevelopment in 22 twin pairs discordant for major psychosis, while Wang et al. [47] observed methylation differences in 2 genes between 7 twin pairs discordant for obesity. In contrast, a study of 3 MZ twin pairs discordant for multiple sclerosis examined genome-wide DNA methylation using next-generation bisulfite sequencing and did not find any methylation changes associated with the phenotype [48]. Far fewer studies have examined the association of ASM changes with phenotypic traits and none have done so genome wide. Stepanow et al. [49] showed ASM of MCHR1 to be associated with body mass index. In cancer progression, Kang et al. [50] reported an association of p14ARF (CDKN2A) polymorphisms with the likelihood of methylation of this gene within colorectal cancers, and Bumber et al. [51] found an indel polymorphism in PDLIM4 which influences the methylation of this gene in leukemia and colon cancer. Wei et al. [52] examined methylation patterns in 3 twin pairs (1 MZ and 2 DZ) discordant for AMD, assaying methylation levels of promoter region genes in PBMCs using MeDip-Chip and NimbleGen Human DNA Methylation 2.1M Deluxe Promoter Arrays. In contrast to our results, they reported consistent differential methylation patterns upstream of IL17RC in these 3 twin pairs. Though other groups have failed to replicate these results [53], the differences between our results and those reported by Wei et al. are due to differing definitions of differential methylation. While Wei et al. define differential methylation as a binary difference in overall methylation between discordant twins (i.e. 0% methylation in one twin vs. >0% methylation in the other), we searched for changes in ASM patterns which present themselves as changes from 100% methylation (biallelic methylation) to 50% methylation (ASM). As such, Wei et al. would not have detected our loci. Furthermore, as we screened out loci with overall low intensity levels, we did not examine the unmethylated loci described by Wei et al. [52].

Given our focus on rare deletion events, it is perhaps not surprising that we found only 8 CNV differences between MZ twin pairs. Even though we were conservative in calling differential CNV events, we did find several instances of unique CNV events in one twin of a pair. However, due to the rarity of these events and the relatively large amount of the variance already explained for this disease, we found no statistically significant result showing any of these CNVs to be associated with AMD.

In contrast, our analysis of differential methylation revealed some methylation events associated with AMD. In particular, analyses of large genomic regions from 0.5–1 Mb in size near complement factor genes uncovered evidence of differential ASM in these regions between cases and controls. We also found smaller genomic regions with ASM differences between MZ twins discordant for AMD near genes involved in gliogenesis. Taken together, these results further implicate the role of inflammation and glial cell proliferation in AMD progression.

A recent study by Hunter et al. [54] used the Illumina 27K platform to assess methylation in a case-control study of postmortem retina pigment epithelium/choroid samples. In contrast to the results we report here, Hunter et al. did not report methylation changes upstream of either CFH or CFB. However, we report results for large regions (0.5–1 Mb) composed of multiple MPRs that only partially overlap CpGs assayed by both studies. As such, it is possible that (a) the signal we observe is driven by CpGs not located within the targets assayed by Hunter et al., or that (b) our signal represents an aggregate of signals from multiple amplicons that would not be detected as individual results from CpGs. This latter hypothesis is supported by our failure to find significant differences in ASM in individual MPRs (online suppl. table 3).

It is possible that the methylation changes we observe in whole blood directly influence AMD progression through this cell population's known effects on both the inflammatory and complement responses. Given previous observations of the lack of tissue specificity of ASM [11], it is possible that these methylation differences reflect a broader methylation status within multiple tissues that only manifests itself in combination with other factors within a particular susceptible cell type such as the retinal pigment epithelium. However, given the demonstrated existence of tissue-specific ASM [13, 55], it is possible that the observed methylation changes are the result of differences in the proportions of cell types in the samples examined. While this is unlikely the case in our case-control analyses, which involve multiple independent samples, the differences observed between discordant

twins could be the result of different cell type proportions within the twin blood samples. Lacking prior data on tissue representation, we resorted to examining whether any of the loci we identified in twin pairs with discordant ASM also showed tissue specific methylation. None of the 100 tissue-specific loci identified by Houseman et al. [56] or of the 679 tissue-specific loci identified by Glossop et al. [57] are located within the amplicons identified in our discordant twin study. Of course, this does not eliminate the possibility that these discordant MPRs are as yet unidentified tissue-specific loci, and our results should be interpreted with this in mind.

One smaller region of interest that was identified by our analysis of the discordant MZ twins was that of the MPR-encompassing rs2017257, located downstream of kinesin family member 18 B and approximately 4.2 kb upstream of the glial fibrillary acidic protein gene (*GFAP*). *GFAP* is an intermediate filament protein expressed in numerous cell types of the central nervous system and is a primary marker of astrocytes [58]. Gliosis, an inflammatory injury response involving astrocyte proliferation and glial scarring during wound healing, is associated with increased levels of *GFAP* expression [59]. Importantly, both gliosis and increased *GFAP* levels have been reported to be associated with AMD [58, 60–62]. In this context, the discordant ASM signal observed in the region encompassing rs11949422 is also interesting. This region overlaps some isoforms of the gene for cAMP-specific phosphodiesterase 4D (*PDE4D*). Rolipram, an inhibitor of phosphodiesterase 4, can attenuate gliosis [63] indicating a potential role for this gene in gliosis and AMD progression. While the exact molecular effect of the differential methylation event near *GFAP* awaits further research, ENCODE data do identify the MPR's genomic coordinates as a strong CTCF-based insulator in multiple cell types [64]; DNA methylation of a CTCF-binding site can disrupt its insulator function [65]. It is possible that the biallelic methylation observed at this MPR in discordant twin cases results in lower levels of CTCF binding as compared to that of the discordant twin controls which have ASM at this MPR. This in turn could result in a decreased CTCF insulator function and an increased expression of *GFAP* within the discordant case twins.

Findings of a genetic influence on differential methylation raise the possibility that ASM differences observed between cases and controls at larger genomic regions near known AMD variants originate from these variants themselves. In this respect, we note that the rs1410996 variant near *CFH* is noncoding [35], suggesting its potential regulatory role in AMD progression. The

risk allele frequencies of rs1410996 in cases (73.1%) and controls (50%) vary significantly ($p < 0.05$), raising the possibility of genetic influence on the observed ASM differences. In contrast, the risk allele frequencies for AMD variants near *C2* and *CFB* (rs9332739 and rs641153) are essentially equal between cases (92.3% and 100%, respectively) and controls (100% for both), suggesting a non-genetic origin for the observed ASM differences between cases and controls.

While the ASM differences observed in genetically identical MZ twins discordant for AMD do not appear to be the result of genetic differences, it is difficult to differentiate between the observed ASM changes as either a cause or effect of AMD (much like with expression changes). In this respect, as noted above, detection of these methylation differences in whole blood derived DNA could be interpreted as an organism-wide epigenetic response to an external stimulus that manifests within a susceptible cell type. Attempting to examine this possibility, we investigated whether the presence of external stimuli in our patients – such as smoking history and body mass index (BMI), known risk factors for AMD [2–4] – correlated with twin discordance and case/control differences in outcome and ASM patterns/levels. We were, however, unable to detect any statistically significant patterns or changes in ASM that correlated with these external events within our small sample set (data not shown).

This study has the advantages of a well-characterized population examined according to the rigorous standard protocols with longitudinal follow-up to obtain the most recent AMD grade. Although we took a very conservative approach towards CNV detection and calling, we did see unique events in one MZ twin and it remains possible that these early somatic mutations could give rise to some of the phenotypic discordance observed in MZ twins. Although our results do not explain AMD discordance between MZ twins, we have helped to develop a foundation on which the field can build its knowledge of somatic structural variation in genetically identical subjects. Further improvements upon previous studies include its genome-wide unbiased examination of ASM through the use of a platform specifically designed for differential allele detection. We observed many suggestive results, particularly those involving genomic regions proximal to loci involved in the inflammatory and wound-healing responses. Although this is the largest twin study of AMD done for these types of analyses, the sample size was small and some associations may have been missed.

Conclusions

In summary, we examined the role of CNV and differential methylation in AMD in a genetically constrained cohort of MZ twins and identified differentially methylated regions in discordant twins with biologically suggestive roles. Our results provide no definite evidence that epigenetics plays a central role in AMD, but they do provide some evidence of epigenetic influences beyond the known genetic susceptibility and further implicate inflammatory responses and gliosis in the etiology of AMD.

Larger studies of carefully selected twins or singletons targeted to these genomic regions may be an effective way of further assessing the effect of epigenetic modifications at these locations in AMD. Given the malleable nature of

epigenetic changes, these results may eventually provide therapeutic targets for this important and increasingly prevalent disease.

Acknowledgements

We thank Yi Yu and Lillian Merriam for their constructive criticism of the manuscript. This work was supported in part by grants R01-EY11309 from the National Institutes of Health, Bethesda, Md.; Massachusetts Lions Eye Research Fund, Inc.; unrestricted grant from Research to Prevent Blindness, Inc., New York, N.Y.; the American Macular Degeneration Foundation, Northampton, Mass.; donations to the Macular Degeneration Research Fund of the Ophthalmic Epidemiology and Genetics Service, New England Eye Center, Tufts Medical Center, Tufts University School of Medicine, Boston, Mass.

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