Dark Matters in AMD Genetics: Epigenetics and Stochasticity

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Research strategies viewing age-related macular degeneration (AMD) as a complex genetic disease have achieved remarkable successes in the past decade. Classifying AMD in this fashion directly implies that disease risk is due to a combination of genetic and environmental effects. The first genetic risk factor identified for AMD was the $\varepsilon 2$ allele of the APOE gene. Two manuscripts were published in 1998 identifying this allele as a risk variant when compared to the $\varepsilon 4$ allele.[1,2] The Y402H variant of the complement factor H gene (*CFH*) was next independently identified by three groups.[3–5] Several new risk alleles have been added to this list, and these will be discussed here. AMD has the largest portion of genetic risk (>50%) to be explained among all common complex diseases.[6] This work has been so successful that it has been proposed as a paradigm for the study of complex disease in general.[7,8]

To investigate environmental factors, twin studies have been used in which the genetic model was based on additive genetic risk, common environmental risk, and unique environmental risk (the ACE model).[9] Follow-up studies found that a history of smoking and body mass index were positively associated with risk, whereas the consumption of fish and ω-3 fatty acids in the diet were protective.[10] These data were subsequently used to build a prediction model for the progression of early-grade AMD to intermediate and advanced grades.[11]

Much remains to be explained, however, concerning the origins of AMD at the molecular level. Although most of the genetic risk can be accounted for with known variant alleles, there is undetermined, or missing, heritability that is unexplained. This is the common *missing heritability* problem of complex disease,[6] often referred to as the *dark matter of the genome*. "One is sure that it exists, can detect its influence, but simply cannot see it."[6] Discordant phenotypes of monozygotic (MZ) twins with AMD must surely represent an example of this phenomenon.

What explains the remainder of the genetic and environmental risk for AMD? What are the best strategies for identifying variant alleles that are only infrequently found in the population but that may have significant effects on risk? How can gene-environment interactions such as a history of smoking be understood at a mechanistic level? What are the effects of gene-gene interaction (epistasis) and gene-environment interaction in building models of the genetic architecture of AMD? Finally, how can the association of chronological age with disease risk be understood at a mechanistic level?

These questions point to a view of complex disease in which genetics explain only part of the total risk. Epigenetics is a rapidly advancing area of research that may help to answer questions in complex disease that are beyond the domain of genetics. Epigenetics specifically provides a new means to investigate the molecular basis of nonheritable risk in complex disease.[12–19] Risk is viewed as having a genetic basis (multiple risk alleles and heritable epialleles), and an environmental basis (epigenetic modifications of the genome arising from environmental effects and stochasticity). The purpose of this review is to provide a basic background in epigenetics, to explore the current applications of epigenetics in the study of complex disease, and to provide some initial thoughts about its use in the study of AMD.

Epigenetics

Genetics can be defined at the molecular level as the study of differences in DNA sequence and the resultant effects on phenotype. All somatic cells in an organism are identical at the level of DNA sequence with the exception of special cases, such as B and T cells or cells with a somatic mutation. What then accounts for tissue specific differences in cellular structure and function within an organism? Why, for example, are rod photoreceptors and retinal pigment epithelial cells so clearly different when the nuclear genomes of each type of cell have identical DNA sequences?

Epigenetics is defined as the study of covalent modification of the genome that alters structure and function but does not alter gene sequence. The specification of all such modifications for a given genome is referred to as the epigenome,[12,20] and the same specification for an individual allele is termed an epiallele.[21] Epigenetic alterations that are associated with disease risk (i.e., an epimutation) can be present for an allele that is wild type with respect to DNA sequence. This alteration would not be obvious from DNA sequencing alone.

Methylation of DNA

The most common epigenetic modification of DNA is methylation of cytosine in the 5′ position.[22] This epigenetic "mark" is reversible but can be stable to the process of cell division. Remarkably, cytosine methylation can also be passed from parents to offspring. In most (but not all) cases in which methylation is present on cytosine, it is part of a CG dinucleotide. This feature is called a CpG to differentiate it from CG base pairs. The haploid human genome contains 29 million CpG residues. Half of these are found in repetitive sequences.[23,24] A large number of CpG residues concentrated in a single region of DNA sequence is called a *CpG island*.[25,26] A high degree of methylation in CpG islands silences the expression of associated genes and repetitive sequences. CpG island methylation also regulates the expression of a large number of microRNAs, which, in turn, regulate the translation of mRNAs

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and in some cases the transcription of genes.[27,28] More widely spaced CpGs that are near, but not part of, a CpG island are also functional in regulating gene expression. These regions have been labeled *CpG island shores*.[29] Methods for the genome-wide identification of methyl CpG residues (the methylome) have recently been reviewed.[30–32]

Posttranslational Modifications of Chromatin

The second type of epigenetic modification present in the genome is the covalent modification of histones. An octomer of histones together with DNA form nucleosomes as the primary structural unit of chromatin. Epigenetic modifications of histones include methylation, acetylation, phosphorylation, and suomylation, among others.[33] Methylation of cytosine and posttranslational modification of chromatin work together in the regulation of gene expression.[33–35]

Epigenetic Regulation of the Genome

The current model of epigenetic regulation focuses on changes in the structure of chromatin. In a simple view, chromatin exists in two states that are dynamically related. Highly condensed chromatin (heterochromatin) is a compact state in which the molecular machinery for the transcription of DNA cannot gain access to gene promoters to initiate this process. Euchromatin is a "loose" structure that allows access of transcription factors to initiate transcription. Transitions between euchromatin and heterochromatin can occur over fairly short distances (i.e., within a single gene) and very long distances in the genome (i.e., loops of DNA extending from the chromosomal matrix). The latter, which are higher order structures, clearly play an important role in epigenetic regulation. Chromatin is attached to DNA through matrix attachment regions, which regulate the probability that the chromatin in the loops will be in either an active or a silent state.[36] Local events in the genome can even lead to silencing in regions on multiple chromosomes. When estrogen binds to the alpha form of the estrogen receptor (ER alpha), 11 different regions within the human genome are condensed to form heterochromatin. These regions range from 0.4 Mbp to 5.3 Mbp and occur on several different chromosomes.[37] This phenomenon is referred to as *long-range epigenetic silencing* (LRES). For a recent review of chromatin looping and LRES, see the article by Deng.[38]

Biological Processes Involving Epigenetic Regulation

Epigenetic mechanisms are found in a large number of biological processes. These include the regulation of gene expression,[20,33,35,39] imprinting,[33,40,41] development,[42] differentiation,[43–45] and X-inactivation.[46] In eye research, epigenetics approaches have been used to explore the differentiation of precursor cells into either rods or cones. This is, in part, achieved by the epigenetic silencing of whole groups of genes at the terminal step of differentiation.[47] This topic has recently been reviewed.[48]

Changes in the epigenome are directly attributable to genetics, environment, stochasticity (random processes), or all three. Each of these topics is substantial; therefore, only examples will be given.

Allele-Specific Methylation. Allele-specific methylation (ASM) is a clear example of genetic regulation of DNA methylation.[49,50] This phenomenon occurs when there is unequal methylation of alleles for a given gene. ASM is widespread throughout the genome and is both tissue and age specific.[51] ASM explicitly refers to methylation of individual alleles that are not imprinted or silenced as a result of X-inactivation. In the simplest case, polymorphisms in the noncoding DNA sequence that directly regulate the extent of CpG methylation are found within 1 Mbp of the gene. This effect is referred to as regulation in *cis* of allele-specific methylation. Examples of regulation in *trans* of ASM are less common but have also been documented.[49,50]

Environmental Regulation of Methylation. Epigenetics is a general mechanism through which the environment produces long-term functional effects on the genome.[52,53] These types of changes can occur during development, the perinatal period, and throughout the entire lifetime of the organism.[53] The range of environmental influences that have epigenetic effects includes the effects of toxic substances on the genome,[54] nutritional effects,[42] and exposure to carcinogens.[55]

One of the most fascinating illustrations of gene-environment interactions can be found in studies of how neonatal stress in response to maternal care epigenetically programs the long-term stress response in the neonate.[56] The original experimental setting involved the care of rat pups by "good" and "bad" mothers.[56] Good mothers were defined on the quantitative basis of licking and grooming, as well as the frequency of arched back nursing. The first studies established that good mothers raised female pups that became good mothers, whereas bad mothers raised female pups that became bad mothers. When the pups of good mothers were switched to bad mothers for grooming and feeding, the females developed as bad mothers and vice versa. This result could not be genetic, and it was subsequently discovered that the phenotypes being observed were connected to the methylation state of the glucocorticoid receptor in the hypothalamus, the major receptor responding to cortisol as a stress hormone.[57]

Stochasticity in Epigenetics. Stochasticity is defined as randomness or noise. A discrete process has a defined result given the initial conditions, whereas a stochastic process has some element of noise or randomness; as a result, different outcomes may arise from a given set of initial conditions.

At the level of gene expression, stochasticity refers to the phenomenon of phenotypic variation.[58–63] Stochasticity in gene expression accounts for the nonidentity of the expression of a single gene in a population of genetically identical cells. Variation of the expression of single genes in the retinal pigment epithelium is a documented example of this phenomenon.[59,64–66] Feinberg[13] and Martin[67] have commented on the roles epigenetic phenotypic variation might play in evolution. It is useful to think about stochasticity in dividing cells and nondividing cells in different ways. The error rate for the replication of nuclear DNA in dividing cells is approximately 1 in 1,000,000 bases. This represents one mechanism by which to produce genetic diversity. The error rate for replicating epigenetic marks in the genome during cell division is 1 in 1,000, a much more frequent event. These numbers are taken from in vitro studies in which the methylation of discrete regions of the genome in dividing cells were compared.[68]

Epigenetic drift is the term used to describe spontaneous changes in the methylome of cells as a function of time. The best experimental evidence for epigenetic drift in humans comes from longitudinal studies of the methylomes of MZ twins.[69–74] In the study by Wong,[74] methylation across the promoters of three genes (dopamine receptor D4, serotonin transporter, and X-linked monoamine oxidase) was quantified in a large number of MZ and dizygotic (DZ) twins at ages of 5 and 10 years. Methylation scores for each promoter were calculated as the average methylation density for all CpG residues in the sequence examined. The results show that even genetically identical organisms (MZ twins) show evidence of epigenetic drift with age.

Twins, however, have cells that divide in many different tissues, so it is not entirely correct to infer that epigenetic drift occurs only in nondividing cells. The sources for genomic DNA

in these experiments are frequently either buccal swabs or lymphocytes isolated from whole blood. Both these sources are subject to continuing cell division. Perhaps an indication of epigenetic drift in nondividing cells can be found in a study of patients with late-onset (sporadic) Alzheimer's disease.[75] The tissue sampled in this study came from the central nervous system and would therefore represent mostly nondividing cells.

The assessment of epigenetic drift has also been investigated in the mouse.[50,76] Imprinted genes and X-inactivation were studied to quantify the loss of methylation as a function of age in the mouse. These results demonstrated that loss of methylation is a tissue- and age-related phenomenon that can be quantified. The net result is the loss of silencing of many genes with age.

How could the methylation profile of a gene change with time in a nondividing cell? This may depend on the dynamic methylation and demethylation of individual DNA sequences. Stochasticity or noise in these two reactions could cause the epigenetic drift seen in MZ twins and the mouse studies cited.

Complex Disease and Polygenic Inheritance

The traditional definition of complex disease includes disease phenotypes that do not exhibit Mendelian inheritance, multiple susceptibility genes with variant alleles, environmental effects, and increased incidence with age. In 1990, Risch[77–79] presented three models by which to characterize the inheritance of phenotypes arising from multiple genes. The first model included genes acting independently in an additive fashion to generate a phenotype. The second model included epistatic (i.e., gene-gene) interactions. Although many (if not most) models of phenotypes consider additive effects of individual genes, a growing body of work integrates gene-gene interaction into the genetic architecture of complex traits. Experimental evidence for several complex physiological phenotypes gathered with the use of congenic mouse strains indicates that epistatic interactions are the norm rather than the exception.[80–82] The inclusion of epistatic interactions in a model of sporadic Alzheimer's disease is a good example in human disease.[83] Phenotypes resulting from multiple genes that do not fit either the first or the second model are referred to as complex. Well-known complex diseases include cancer, type 2 diabetes, lupus erythematosus, bipolar disease, major depression, and schizophrenia, among many others. AMD fits this classification as well.[7,8,84]

The Genetic Basis of Complex Disease

For any given complex disease, the genetic basis of the phenotype is derived (in part) by a set of variant alleles that alter disease risk. Allelic effects can include both increasing disease risk and decreasing risk. How these alleles alter disease risk is often not readily understood by examining the function of each gene.

The number of variant alleles of susceptibility genes associated with complex diseases is often large. As of the publication of an important review on missing heritability and complex disease in 2009,[6] the given numbers of susceptibility genes for various complex diseases were as follows: AMD, 5; Crohn's disease, 32; systemic lupus erythematosus, 6; type 2 diabetes mellitus, 18.

Alzheimer's disease has recently been estimated to have at least 40 risk alleles.[85] AMD may represent an unusual case of complex disease, however, because the genetic basis of the disease is largely determined by a small number of genes that explain a large portion of genetic risk.[6] As of the publication of the review cited above, 5 susceptibility genes were identified for AMD that accounted for greater than 50% of the genetic risk.

Susceptibility Genes for AMD. The first study linking the risk for AMD to a specific locus (ARMD1) was published in 1998.[86] In the same year, the epsilon allele of the *APOE* gene was identified as having a protective effect with respect to risk and AMD.[1,2] In 2005, three independent groups identified the Y402H variant allele of the *CFH* gene within the ARMD1 locus on chromosome 1q.[3,4,87] *CFH* is a key inhibitor of the alternative complement pathway, and several genes in this pathway have allelic variants associated with risk for AMD. Anderson et al.[88] have summarized the detailed studies of the alternative complement pathway, and Gehrs et al.[89] have also recently presented an overview of this work. The risk alleles in the alternative complement pathway carry a significant portion of the total genetic risk for AMD.

The next locus to be identified was chromosome 10 q26.[90–95] Recent studies indicate that this locus is complex and have identified haplotypes that either are protective or confer risk.[94]

Toll-like receptors 3 and 4 (TLR3 and TLR4) are pattern recognition receptors in the innate immune system that have also been proposed to have risk alleles, but the initial assertions could not be validated in more comprehensive studies.[96–98] A survey of 13 genes involved with extracellular matrix turnover found that two intronic SNPs in the tissue inhibitor of metalloproteinase 3 (*TIMP3*) gene were associated with AMD risk.[99] Earlier studies identified this locus.[100] The Mn superoxide dismutase (*SOD2*) gene has an allelic variant that alters transport of the MnSOD nascent peptide into mitochondria.[101,102] The status of this allele as a risk variant is not resolved.[103] Several genes related to lipoprotein metabolism have also been investigated. These include the hepatic lipase gene (*LIPC*),[104] paraoxonase 1 (*PON1*)[105–109] and *APOE*.[1,110–114] Vascular endothelial growth factor (VEGF) and the VEGF receptor (KDR) regulate angiogenesis, and both genes have SNPs associated with risk for AMD.[115] *XPD* and *XRCC1* are DNA repair genes recently proposed as risk alleles for AMD.[116] More comprehensive studies, however, could not substantiate this claim.[117] Finally, T2 haplotypes of mitochondrial DNA also appear to be associated with AMD risk.[118–120]

Gene Networks. The simplest genetic architecture given a set of susceptibility genes assumes no genetic or environmental interactions and is thus an additive model of risk. One interpretation of gene networks is simply a graphic representation of genes that constitute a pathway.

As an example, when the Y402 variant was identified as a risk allele for AMD, it was logical to next examine all the genes in the alternative complement pathway. After finding several risk alleles with functional links to serum lipid levels, it also made sense to use this information to extend the search for candidate genes along these pathways. Serious attention is now being given to modeling phenotypes arising from multiple genes as gene networks.[121,122] These networks can specifically account for gene-gene interactions (epistasis) and gene-environment interactions. The construction of this type of network usually depends on bioinformatics approaches, which use large data sets of gene expression values.[123] The strengths and weaknesses of varying network inference methods have recently been reviewed.[124] A newer trend is to include epigenetic modification of the network models.[121,125] These studies claim to make subtle inferences of important regulatory mechanisms not available in simple gene network models.

Ultimately, building a gene network model that accounts for the effect of variant alleles, including epigenetic information and the stochasticity associated with these phenomena, among which are protein-protein and protein-gene interactomes, and can be exercised as a function of elapsed time is a problem

with a high number of dimensions. Approaches for these types of problems have been discussed in the literature,[126] but a complete solution for this level of complexity is not yet available.

Sporadic Disease. Sporadic and familial forms of a disease with identical phenotypes are frequently found in complex disease.[15,85] "Sporadic" indicates that no disease is present in any first-, second-, or third-degree relative. What explains the presence of the disease phenotype in patients who have no family history of the disease? Yang[85] has speculated that sporadic cases are the rule rather than the exception in complex disease. He proposes that this phenomenon is derived from a relatively large number of susceptibility alleles, with minor individual contributions to genetic risk in combination with nongenetic factors. This is certainly the case for Alzheimer's disease. Sporadic cases are far more frequent than familial cases.[75] What would constitute a case of sporadic AMD? Personal discussions between the author and several others in the field have not produced an answer to this question.

Sporadic disease can also be associated with somatic epimutation, a process that converts wild-type alleles to epialleles associated with disease risk. Examples of this phenomenon can be found in the literature on breast cancer.[127,128]

Transgenerational or Heritable Epimutations. Most epigenetic marks in the mammalian genome are erased and then reestablished early in development.[129] Some epigenetic marks are, however, stable to this process and may thus be inherited by offspring.[20,128,130–132] These marks are present throughout the entire organism and are thus more properly called *constitutive germline epimutations*.

Hereditary nonpolyposis colorectal cancer is usually an autosomal dominant syndrome involving heterozygous loss-of-function mutations in one of several mismatch repair genes (*MLH1*, *MSH2*, *MSH6*, or *PMS2*).[131,133] In one third of all clinical cases, however, no mutations are present. In a number of these cases, an epimutation of the *MLH1* gene, which silences expression, has been found.

How might epimutations affect the development or progression of complex disease? Constitutive germline epimutations should display normal heritability if they persist for a large number of generations (e.g., eight). For this reason, such epimutations should be in linkage disequilibrium with nearby SNPs and would therefore be discoverable by genomewide association studies.[134] If the effects of an individual epimutation were regulated by a separate locus, then the latter would be discoverable by GWAS. For these reasons, epimutations are considered to be unlikely as a major source of undiscovered heritable risk but more likely as a source of nongenetic effects.[134]

Environmental Effects in Complex Disease

Environmental effects also account for a large portion of risk in complex disease. The role epigenetics plays in the origins of cancer is the best studied example of gene-environment interaction. The initial observations of Issa,[135] among others, have led to the generalization that epigenetic silencing of tumor suppressor genes is a common mechanism in the formation of tumors. The methylation of CpG islands in this fashion might be either passive or active.[136] The passive mechanism involves stochastic methylation of CpG residues followed by clonal expansion of cells with highly methylated tumor suppressor genes. Limiting the expression of tumor suppressor genes in an individual cell gives this cell a selective growth advantage. Many CpG islands appear to be hypermethylated in an age- and tissue-specific fashion.[137]

Active mechanisms involve methylation at specific promoters by macromolecular complexes, which include proteins and sometimes noncoding RNAs. A recent study of the mechanism by which FAS is silenced by the RAS oncogene determined that a group of 28 proteins was recruited to the FAS promoter, leading to methylation. This experiment was conducted using an RNI screen which identified gene products necessary to achieve silencing. For a detailed discussion see the review by Muthusamy.[136]

Aging

The single largest nongenetic risk factor for most complex diseases is aging. Aging itself is a complex phenomenon that can be observed at the organismal, histologic, and molecular levels. Theories of aging have focused primarily on the roles of oxidative stress in the damage of macromolecules, especially the accumulation of mutations in the nuclear and mitochondrial genomes.[138–147]

DNA from the RPE of patients with AMD demonstrates evidence of elevated oxidative damage to both the nuclear and the mitochondrial genomes and elevated deletion at a common locus in the mitochondrial genome.[148,149]

Epigenetics and Aging

Newer models of aging include epigenetic effects.[23,24,137,150] The genome is progressively demethylated with age.[23,24,150,151] Methods for tracking global demethylation of the human and mouse genomes have been published.[150,152] These approaches focus on the family of Alu repetitive sequences, a specific subset of short interspersed DNA elements.[24,150] The mouse B family of repetitive sequence elements is related to the human Alu family, and demethylation of B2 repetitive sequences has also been correlated with age.[152] Demethylation of Alu sequences has functional consequences. These sequences can be transcribed by pol3 in response to cellular stress, and it is now clear that Alu transcripts have the capacity to suppress the transcription of specific genes.[153] A good example is presented in heat stress experiments on HEK293 cells in vitro. The cultures were subjected to heat stress; after that, Alu transcripts were shown to specifically inhibit the transcription of hexokinase II, acyl-CoA synthetase II, glutamate dehydrogenase-1, and heat shock protein 70.[154]

Some genes exhibit elevated expression with aging because of hypomethylation of the gene promoter.[155,156] This is essentially age-related loss of silencing. CpG islands are progressively methylated with age, leading to gene silencing effects.[137] This was first observed in a functional analysis of tumor suppressor genes in the development of many types of cancers.[29,55,135,157–159] Closer inspection revealed that many of the same genes are also methylated as a function of age in normal tissues.[137,155,156,160–166] These studies, along with a recent study of tissue and age behavior of CpG island methylation in the mouse,[137] have refined global statements made about DNA methylation and aging. Although it remains true that the genome is progressively hypomethylated with age, it now appears that CpG islands represent an exception to this behavior as they become progressively hypermethylated with age.[137]

Twin Studies

When complex disease is studied in outbred populations, the background genetics, environmental effects, and effects of aging must all be accounted for as sources of risk. It is possible to reduce the complexity of this problem by studying persons who have the same DNA sequence (identical twins). Discordance in the prevalence of a disease phenotype between MZ twins, and to a greater extent DZ twins, is a hallmark of

complex disease. Because each MZ twin is derived from a single zygote, MZ twins are genetically identical (with the exception of somatic mutations). It is possible, however, to have one MZ twin who exhibits a disease phenotype and one who does not. This is called MZ twin discordance. Differences between the MZ twins must be due to nongenetic factors. Gene environment models treat this nongenetic risk as either shared or unique environmental effects.

Estimates for the genetic portion of susceptibility to common complex diseases in MZ twins include rheumatoid arthritis, 15%; multiple sclerosis, 25%; type I diabetes, 30%; obesity, 37%; schizophrenia, 50%; asthma, 58%; Alzheimer's, 60%; type II diabetes, 63%; bipolar disorder, 68%; and autism, 70%. In each case, discordance accounts for the remaining risk (i.e., if the genetic risk for schizophrenia is 50%, then the discordant risk must equal 50% so that total risk adds up to 100%). These data were taken from a review by Schumacher and Petronis[18] and are approximate values. Analysis of a large set of male MZ and DZ twins has determined the extent of genetic and environmental risk for AMD.[9] The genetic portion of risk is 46% overall, 67% for intermediate grade disease, and 71% for advanced grade disease. AMD is therefore in the middle of the spectrum of complex disease when considering what portion of total risk is due to heritable sources. It is atypical in that relatively few genes explain most of the genetic portion of total risk.

Twin Studies Using Epigenetics Methodologies

MZ twin sets have been a natural choice as an experimental platform from which integrate epigenetics with more traditional approaches to complex disease. The first such study was published in 2003 and was conducted on a single MZ twin set discordant for schizophrenia and a second MZ twin set concordant for schizophrenia.[73] This initial study focused on several hundred base pairs of a DNA sequence in the 5′ region of the dopamine D2 receptor gene. The results showed significant intraindividual and interindividual variations in the patterns of methylation.

By 2009, a study of 15 MZ twin pairs discordant for lupus erythematosus, rheumatoid arthritis, or dermatomyositis and 15 control MZ twin pairs was conducted.[167] Substantial epigenetic differences were found only for twins discordant for lupus erythematosus. This study increased the scope of the survey to 807 promoters of known genes; the candidate gene set for lupus erythematosus included 49 genes.

Two studies have appeared in 2010. In the first, a group from Europe reexamined the question of epigenetic changes in MZ twins discordant for lupus erythematosus, rheumatoid arthritis, or dermatomyositis.[167] Once again, substantial epigenetic differences were found only in the twin sets discordant for lupus erythematosus. This study included pathway analysis for genes that were differentially methylated and expression analysis to verify that these changes in methylation had functional consequences in gene expression.

The second study to appear in 2010 reported complete genome, epigenome, and transcriptome investigations for one MZ twin set discordant for multiple sclerosis and the additional investigation of three discordant MZ twin sets for differences in transcriptome or epigenome.[168] This study represents the most exhaustive methodological approach to the analysis of discordant twin sets that has been published to date. The authors concluded that there was no convincing evidence of differences between discordant MZ twin pairs in any of the results.

It is disappointing that only lupus, among the complex diseases studied, has given any indication of epigenetic differences in discordant MZ twins. The exhaustive approach used, however, still has limitations that the authors cite at the end of their article. The investigations of methylation focused primarily on gene bodies. Approximately 1 million CpGs were interrogated, but the total number of such residues for the haploid human genome is 29 million (i.e., 3% surveyed). Intergenic repetitive sequences were not studied. The transcription and possible function of Alu sequences has been mentioned. The authors also note day-to-day variation in the methylomics data, which sometimes exceeded the differences between MZ twin pairs. This suggests that the procurement of samples should be performed under identical conditions (e.g., time of day, nutritional status, presence of various forms of disease, physiological status) to the greatest extent possible. Perhaps more important, the study considered only a subset of lymphocytes ($CD4^+$) for investigation. A rationale for the selection of this subset was given, but the study lacks any investigation of affected tissue. Finally, a limited number of individuals were examined.

Isogenic Animal Strains

Studies with isogenic strains of animals constitute a complementary approach to twin studies with humans. Siblings all arise from individual zygotes. Because the parental genomes are from an inbred strain, however, siblings have a very high level of genetic uniformity. Wong et al. [169] have commented on the use of isogenic animals for studies of epigenetics and complex disease. In addition to eliminating diversity in the genetic background, studies on the mouse would allow the use of exacting environmental conditions, including light cycles and feed, as well as the availability of longitudinal sampling. Using the mouse would allow the direct harvesting of the most relevant tissues for analysis.

Isogenic strains of laboratory animals are also optimal for probing gene-environment and gene-gene interactions. Several groups have made mouse transgenics with human genes, and some of these used homologous recombination to remove the wild-type gene and replace it with the variant human allele. This approach evades the variegation of expression that typically occurs using random transgenesis techniques.[170,171] All three alleles of the human *APOE* gene, for example, have been placed into the mouse,[172] as have several variants of the *CFH* gene.[173] The major phenotypes examined in these studies were anatomic and physiological, but an epigenetic investigation of isolated genes or of the entire mouse genome would also be an interesting approach.

Finally, it makes sense to study the epigenetics of aging in isogenic animals. The environmental influences can be carefully controlled, and multiple individuals can be used for sample procurement at any age desired. Multiple animals allow such issues as epigenetic drift and the reproducibility of experimental approaches to be tested. Combining aging with the transgenesis of humanized genes provides an attractive experimental platform. See Ref. 174 for a current review.

Integrating Epigenetics into AMD Research

Given the rationale presented, it might be possible that epigenetics could aid in the study of AMD. More specific questions, however, must be asked. Can epigenetics help to explain the unknown portion of genetic risk in AMD? Do epialleles of the risk alleles for AMD or genes associated with monogenic retinal degenerations make a contribution to genetic risk? Do any of these genes exhibit allele-specific methylation, and is this related to risk? Can gene networks be built that incorporate epigenetic states that will improve our general understanding of pathogenesis? Does adding epigenetics to the genetic model

provide a basis for understanding the effects of age in complex disease?

Epigenetic Investigations of Individual Candidate Genes

Perhaps the most direct test of the relevance of epigenetics to AMD would be the examination of individual candidate genes. In this approach, all the currently agreed on genes with risk alleles for AMD could be examined. In addition, genes for monogenic retinal degenerations could be selected, especially those that have phenotypes related to AMD.

Consider the *CFH* gene on chromosome 1. A study would include the bisulfite genomic sequencing of many individuals with AMD and appropriate controls. The study design would include enough power to find the most frequent epialleles. Sequencing would have to include all exons and introns as well as reasonable blocks of sequence upstream and downstream from the gene itself. The goals of such a study would be to identify the most frequent epialleles of the wild-type gene and to determine whether these epialleles were associated with risk. A second stage would include bisulfite sequencing of variant alleles with the same goals in mind. In association with the sequencing, it would also be important to identify any asymmetric expression of alleles. Consider the situation of an individual heterozygous for the Y402H allele. If the wild-type allele were epigenetically silenced, the patient would be functionally homozygous for the Y402H allele. If the Y402H allele were silenced, this heterozygote would be functionally homozygous for the wild-type allele, but the variant allele would still be found by sequencing.

Next-Generation Sequencing of Targeted Chromosomal Regions

With the advent of next-generation sequencing,[175] it is now feasible to sequence targeted regions of chromosomes. A more exhaustive study of *CFH* might examine the extended haplotypes on chromosome 1 having several complement genes that have been shown to be associated with risk for AMD.[176] These could include selective investigations of the transcriptome for the same region. As stated, studies of asymmetric allelic expression show that this phenomenon can be regulated in *cis* by SNPs, which may be somewhat distant from the gene promoter.[49,51]

Allele-Specific Methylation

As discussed, the unequal methylation of alleles can confound interpretations based on sequencing alone. The silencing of 1 of 2 alleles would amount to a gene dosage reduction for homozygotes and a loss of heterozygosity for heterozygotes at a given locus. It appears that loss of methylation with age is a specific phenomenon that can be quantified. One such study quantified the loss of methylation of imprinted genes and genes on the X chromosome silenced through X-inactivation.[76]

Combined Genetics/Epigenetics Analysis

The outlines of a comprehensive study can be obtained from current work on discordant MZ twins. A large group of MZ and DZ twins (including females) would be one choice for a study population. Each of the subjects could be genotyped for currently known risk alleles. A deeper look could be obtained by sequencing the entire genome for each subject. Next, it would be preferable to obtain a complete transcriptome or sets of transcriptomes for each subject under normalized conditions. Finally, the entire CpG methylome could be determined by next-generation sequencing coupled to genomic bisulfite conversion.

Conclusion

The scale of the last study considered is clearly beyond our current abilities, but how far beyond? The pace of technical advances in dealing with entire genomes or epigenomes is dazzling. This fact alone makes predicting the future a risky endeavor. Statements made by leaders in the field of computers provide a cautionary tale. Thomas Watson Sr., the president of IBM, stated in 1943: "I think there is a world market for maybe five computers." *Popular Mechanics* added in 1949, "Computers in the future may weigh no more than 1.5 tons." In 1977, Ken Olsen, the founder, president, and chairman of Digital Equipment Corporation, commented, "There is no reason anyone would want a computer in their home." Finally, Bill Gates, founder, chief executive officer, and chairman of Microsoft, stated in 1981 that "640 K ought to be enough for anybody." But new applications of computing, the microchip, the personal computer, and mass storage came along anyhow. If history is correct, we may only be at the beginning of a rich, new period of experimental work to determine not only the structure of the genome but also how it functions as a machine through our understanding of epigenetics and stochasticity.

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