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GWAS in myopia: insights into disease and implications for the clinic

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GWAS in myopia: insights into disease and implications for the clinic

Summary

Myopia is the commonest eye trait worldwide and the prevalence is increasing. It is known to be highly heritable; total genetic variation explains up to 70-80% of variance. In an attempt to better understand the genetic architecture of myopia, with an ultimate view to better predict genetic risk and develop targeted treatments, several genome-wide association studies have been performed in the last 6 years. In this review we focus on what a genome-wide association study involves, what studies have been performed in relation to myopia to date, and what they ultimately tell us about myopia variance and functional pathways leading to pathogenesis. The current limitations of genome-wide association studies are reviewed and potential means to improve our understanding of the genetic factors for myopia are described.

Keywords

Myopia ; Refractive error ; Genetics ; GWAS ; GxE interactions

Introduction

Myopia is already the commonest eye condition and its prevalence is increasing across the world (1-4). Although myopia is strongly associated with a number of environmental factors, the most important risk factor in determining whether an individual develops the trait is having a family history of myopia, suggesting a genetic predisposition. The heritability of a trait is an estimate of how much phenotypic variation in a population is due to genetic factors. The heritability of refractive error, using spherical equivalent as a quantitative trait, has been determined in a number of family and, more credibly, twin studies [Figure 1]. These indicate the heritability of myopia is high at around 70% (5-15).

Figure 1 Heritability estimates for refractive error (Abbreviations: T = twin studies, F = family studies).

Myopia is a complex trait influenced by a complicated interplay of genetic and environmental factors. As with many complex traits there is a distribution of refractive error in the population, meaning the risk of ordinary or "simple" myopia developing is not determined by a classic Mendelian single gene mode of inheritance; there are likely many genes, each contributing a small effect to overall myopia risk. This may not be true for very high, familial or syndrome-associated forms of myopia, where a rare dominantly inherited mutation may be important in an individual family, but not important in the overall population risk. Up until the era of genome-wide association studies (GWAS), identification of disease-associated genes relied on family studies (using linkage analysis) or candidate gene studies. In myopia, these were singularly unsuccessful and prior

to 2009 there were no known myopia-associated genes, other than syndromes where myopia was a part of the phenotypic spectrum (eg Stickler's, Marfan syndromes). However, with the advent of GWAS a number of genes for myopia have been identified, providing new insight into how myopia develops with implications for future research into how this increasingly common eye trait might be treated.

Genome-wide association studies (GWAS)

Genome-wide association studies (GWAS) are approaches that allow a vast array of markers scattered across an individual's DNA or genome to be rapidly tested for association with a disease or trait. These 'markers' are variations in the base pair of nucleotides at specific points along the genome, commonly known as SNPs (single-nucleotide polymorphisms), and give an indication of what nearby genes may be associated with the trait.

In order for this analysis technique to be possible, all of the base pairs, namely adenine (A), guanine (G), thymine (T) or cytosine (C), forming the human DNA code had to be sequenced (ie. read and mapped). The human genome project, completed in 2003, was a major international scientific collaboration that identified all of the base pairs and genes that make up the human genome, approximately 20,500 genes in total (16, 17). This has enabled researchers to have access to a detailed resource on the structure, function and organization of the complete set of genes that make up the human species. However, to investigate the association between the human genome and disease, a 'map' of common patterns of genetic variation and inheritance was required, a 'haplotype

map'. This was firstly provided by the HapMap project, completed in 2005 (18); this international project compared the genetic sequences of individuals of African, Asian and European ancestry. Subsequently, the 1000 Genome Project that harnessed the increased speed, greater coverage and reduced cost of next-generation sequencing was launched. Released in 2012 this has provided the most detailed catalogue of human genetic variation to date with sequencing of over 1000 participants internationally (19). These maps of common inheritance patterns allow identification of what base pair is commonly at one position in the genome of a certain ethnic population, the 'common' allele, and what base pair tends not be at that position, the 'minor' allele. SNPs are generally termed a common polymorphism when the frequency of the minor allele, in a specific population, is greater than 1%.

GWAS rely upon the assumption that common complex traits are caused by common genetic variations in the population (the "common disease common variant" hypothesis). Therefore, in a GWAS the association between a trait and common genetic variants in the form of SNPs is examined. SNPs are not disease-causing mutations, as found in classical genetic studies of Mendelian rare diseases, and they rarely alter protein structure or function, but may relate to regulation of genes, or alterations in gene expression. In GWAS SNPs are used as markers, and indicate genes nearby or biological pathways that may be involved, allowing researchers to focus in on specific parts of the genome.

To perform a GWAS for a disease, an individual must be genotyped or sequenced; in large-scale genetic studies this is generally undertaken with the use of high-

throughput genotyping arrays or chips. These provide an output of somewhere between 500,000 and 2,500,000 SNPs for that individual, but obviously do not include all the common genetic variants (given there are around 3 billion base pairs in the human genome). The missing data is therefore imputed using reference haplotypes, either the HapMap or 1000 Genome data. Associations between these genetic variations, following extensive data cleaning (quality control), and disease status is examined in regression models either as a quantitative trait (eg. refractive error, as spherical equivalent) or as a categorical case-control trait (eg. 'myopia' or 'no myopia'). The output from such analyses is a list of associated SNPs with an indication of the strength of effect on myopia risk (the beta coefficient) and the confidence of the association (p-value). Significance thresholds are set at less than $p \leq 5 \cdot 10^{-8}$ to reduce the possibility of false positive associations, which may occur as result of correlation between SNPs and the high number of statistical tests involved. This means large studies of many thousands of individuals are required to identify statistically significant associations. Results are generally portrayed graphically as a Manhattan plot, which plots all the SNPs by chromosome position as a function of their association p-value; this plot resembles the Manhattan skyline with different SNPs reaching higher than others, like skyscrapers, in accordance with variations in significance. Results of putative genetic associations for a trait ('discovery stage') must then be verified through replication of associated variants in independent population samples, or through experiments that can examine the functional implications of the affected gene.

The first GWAS was performed in 2005 and since then there has been an exponential rise in the number of studies [Figure 2], reflecting the large reduction in time and cost of undertaking these types of analysis.

Figure 2 Studies, traits and SNP-trait associations from 2005-2013 reveal the growth in genome-wide association studies. Adapted from (20), Copyright obtained.

GWAS have now been successfully performed on a range of ophthalmic diseases (21, 22). The earliest and arguably the most 'successful' GWAS to date has been within the ophthalmic field; the discovery of the association of CFH with age-related macular degeneration was reported in three independent cohorts in 2005 (23-25), one of which was a GWAS, and has since been replicated in dozens of studies across the world. Subsequent meta-analysis involving large sample sizes (>17,100 cases and >60,000 controls) has identified 19 loci for AMD explaining 10-30% of the variance (26), which has an estimated heritability of 45-70%. These genetic associations explain a relatively high proportion of AMD variance, which disappointingly has proved to be fairly unusual in subsequent GWAS for other traits. Although GWAS had identified many variants for many diseases, relatively small effects on disease risk are conferred for the majority of variants and only a small proportional of familial clustering or heritability is explained. This issue of 'missing heritability' is a recurrent issue in GWAS and has prompted researchers to explore additional approaches to examine the genetic architecture of common complex diseases (27).

Genome-wide association studies in myopia

Refractive error and myopia have been examined using the full range of genetic methodologies. This initially included genome-wide linkage studies in related individuals, and candidate gene association studies. At least 17 loci have been identified through the former and although there was some success with the latter, results have proved poorly reproducible (28-30). The first GWAS study to examine myopia was performed in 2009 on a cohort with high, pathological myopia; subsequent studies have either been performed on myopia case-control cohorts, largely from East Asia where the prevalence of myopia and high myopia is greater, or refractive error as a quantitative trait. A database detailing all published GWAS for myopia, refractive error and other myopia endophenotypes is available at http://www.ebi.ac.uk/gwas/home.

The first published GWAS in myopia examined a Japanese population with 297 cases of pathological myopia (defined as axial length > 26mm) and 977 controls from the general population (31). The strongest association was located at 11q24.1, approximately 44kb upstream of the BLID gene, and conferred odds of higher myopia of 1.37 (95% confidence interval (CI) 1.21 - 1.54). Subsequently a meta-analysis of two ethnic Chinese cohorts, published in 2010, was performed for 287 cases of high myopia (defined as $\leq$ -6D) and 911 controls (32). The strongest association was an intronic SNP within the CTNND2 gene on 5p15.2. However neither of these initial associations met the conventional GWAS threshold ($p \leq 5\ 10^{-8}$) for statistical significance.

Li et al also studied an ethnic Chinese population inclusive of 102 high-grade myopia cases (defined as ≤ -8D with retinal degeneration) and 335 controls (33). The strongest association (p = 7.70 x 10^{-13}) was a high frequency variant located in a gene desert within the MYP11 myopia linkage locus on 4q25 (34). In a similar ethnic Han Chinese population of 419 high myopia cases (≤ -6D) and 669 controls, Shi et al identified the strongest association (p = 1.91 x 10^{-16}) at an intronic, high frequency variant within the MIPEP gene on 13q12 (35). Although the aforementioned studies attempted replication in independent cohorts, their results, published in 2011, have not been replicated in GWAS comprising of individuals of similar ethnic background, phenotypic definition or study design.

In 2013 two papers in Asian populations reported replicated loci for high myopia. Shi et al studied a Han Chinese population of 665 cases with high myopia (≤ -6D) and 960 controls (36). Following two-stage replication in three independent cohorts the most significantly associated variant (p = 8.95 x 10^{-14}) was in the VIPR2 gene within the MYP4 locus, and three further variants all reaching genome-wide significance were identified within the same linkage disequilibrium block in the SNTB1 gene (p = 1.13 x 10^{-8} to 2.13 x 10^{-11}). Secondly, Khor et al reported a meta-analysis of four GWAS of East Asian ethnicity totaling 1603 cases of severe myopia (based on either refractive error or axial length) and 3427 controls (37). After replication analysis, the aforementioned SNTB1 gene was confirmed and a novel variant within the ZFHX1B gene (also known as ZEB2) reached genome-wide significance (p = 5.79 x 10^{-10}).

In European populations, probably illustrating the lower prevalence of high myopia, there has only been one case-control GWAS from a French population, published in 2012. In this study of 192 high myopia cases (≤ -6D) and 1064 controls a suggestive association was identified within the MYP10 linkage locus, 3kb downstream of PPP1R3B, however this did not reach genome wide statistical significance and the study failed to replicate any of the previously reported loci (38).

Greater success has been achieved by considering refractive error as a quantitative treat, therefore inclusive of all data on the population studied. In 2010 the first two GWAS for refractive error were published, both in European populations; a British discovery cohort of 4270 individuals (39) and a Dutch discovery cohort of 5328 individuals (40), with replication in over 10,000 individuals from the two discovery cohorts and a smaller shared pool of replication samples. Two loci surpassing the GWAS threshold were identified near the RASGFR1 gene on 15q25.1 (p = 2.70 x 10^{-09}) and the other near GJD2 on 15q14 (p = 2.21 x 10^{-14}). Subsequently, in 2013, a relatively small meta-analysis was performed on 7280 individuals from five cohorts with refractive error, inclusive of various ethnic populations across different continents. Replication was then undertaken in 26,953 samples (41). A novel variant reaching the GWAS threshold was identified within the RBFOX1 gene on chromosome 16 was identified (p = 3.9 x 10^{-9}).

The field made a major breakthrough in 2013 when two major GWAS meta-analysis studies were published. The Consortium for Refractive Error and

Myopia (CREAM) is an international collaborative initiative between researchers studying cohorts of both European and Asian descent. A classic meta-analysis of the GWAS results for a linear regression between genotype and spherical equivalent of refractive error was performed for 35 participating centers, comprising 37,382 individuals of European descent and 12,332 of Southeast Asian ancestry (42). High statistical power was achieved by this large sample size, enabling replication of the two loci previously identified and identification of 22 novel loci [Figure 3]: BICC1, BMP2, BMP3, CACNA1D, CD55, CHD7, CHRNG, CNDP2, CYP26A1, GJD2, CRIA4, KCNJ2, KCNQ5, LAMA2, MYO1D, PCCA, PRSS56, RASGRF1, RDH5, RORB, SIX6, TOX, ZIC2 and ZMAT4.

Figure 3 Manhattan plot of genetic associations for refractive error in the CREAM combined GWAS meta-analysis. $-\log_{10}$-transformed P values for all SNPs. The upper horizontal line indicates the $p < 5.0 \times 10^{-8}$ threshold, the lower horizontal line indicates a p value $< 1 \times 10^{-5}$ (adapted from (42)).

A contemporaneous publication by the direct-to-consumer genomics company 23andMe (Mountain View, CA, USA) on a GWAS survival analysis was performed on 55,177 individuals of European descent using the phenotype of reported myopia and reported 'age of spectacle wear' as a proxy for myopia severity (43). The authors identified 20 novel loci: BMP3, BMP4, DLG2, DLX1, GJD2, KCNMA1, KCNQ5, LAMA2, LRRC4C, PABPCP2, PDE11A, PRSS56, RASGFR1, RBFOX1, RDH5, RGR, SFRP1, SHISA6, TJP2, TOX, ZBTB38 and ZIC2. Contrary to many researchers' expectations, the authors identified highly comparable genetic associations to those obtained using the carefully and expensively collected

refractive error data in population-based samples in the CREAM consortium. Of the 22 loci discovered by CREAM, 14 were replicated by 23andMe, whilst 16 of the 20 loci identified by 23andMe were confirmed by CREAM. Surprisingly the same 25 genetic loci were identified in both studies with consistent direction of effect despite analysis on different scales, namely dioptres for CREAM (more negative on the scale indicative of more myopia) and hazard ratios (higher positive hazard ratios indicative of more sever myopia) for 23andMe (44, 45).

Genome-wide association studies and myopia endophenotypes

The most common form of myopia is axial myopia and as such the axial length of the eye is a major determinant of the majority of myopia. A number of researchers have therefore used this proxy or 'endophenotype' for use in genetic association studies of myopia as a quantitative trait. The first of these, published in 2012, examined 4944 individuals of East and South East Asian ancestry (46). One locus on 1q41 containing the zinc-finger pseudogene ZC3H11B reached genome wide significance ($p = 4.38 \times 10^{-10}$), although replication was not performed.

A much larger GWAS meta-analysis for axial length comprising 12,531 Europeans and 8,216 Asians was published in 2013 (47). Eight, novel genome-wide significant loci were identified (RSPO1, C3orf26, LAMA2, GJD2, ZNRF3, CD55, MIP, ALPPL2) and the aforementioned ZC3H11B was confirmed. Relevantly, five of these loci had been previously associated with refractive error.

Shared determination of an individual's axial length and corneal curvature was identified in the Avon Longitudinal Study of Parents and Children (ALSPAC) and Singapore Chinese Eye Study, suggesting that genetic control of these two eye dimension parameters is by common genetic variants (48). A number of relatively small GWAS have been performed for corneal curvature with identified associations in individuals of varying ancestry, including FRAP1, PDGFRA (also associated with eye size), CMPK1 and RBP3 (49-52). More recently Miyake et al published a two-stage GWAS for three myopia-related traits: axial length, corneal curvature and refractive error (53). The study was performed on 9,804 Japanese individuals with trans-ethnic replication in Chinese and Caucasian individuals. A novel gene, WNT7B, was identified for axial length ($p = 3.9 \times 10^{-13}$) and corneal curvature ($p = 2.9 \times 10^{-40}$), whilst the previously reported association with GJD2 and refractive error was replicated.

Pathways implicated from genome-wide association studies in myopia

Identifying genes associated with myopia is just the first step in gaining the full utility from GWAS in improving our understanding of myopia etiology. Certain biological mechanisms are implicated from associated genes, whilst pathway analysis can enable a more comprehensive, systems biology approach to understanding how associated genetic variants can ultimately influence ocular growth. This analysis is of course reliant on what is already known about the functionality of certain genes.

Functional pathways (or ontological classifications) implicated by the large GWAS on myopia to date have been clear and reproducible (54). Interestingly,

they provide credible evidence that the genetic architecture is fairly consistent between two continental populations (European and Asian). As with many GWAS, the variants identified have not necessarily fallen within a gene but likely functional implications to proximal, relevant genes have been inferred. Although this is reasonable, there are other known factors, such as long-range distance equilibrium, which may mean alternate genes or pathways could equally be involved. Biological processes indicated from the CREAM meta-GWAS include neurotransmission (GRIA4), ion transport (KCNQ5), retinoic acid metabolism (RDH5), extracellular matrix remodeling (LAMA2, BMP2), and eye development (SIX6, PRSS56) (42). Whilst the 23andMe meta-GWAS similarly implied extracellular matrix remodeling (LAMA2, ANTXR2), the visual cycle (RDH5, RGR, KCNQ5), neuronal development (KCNMA1, RBFOX1, LRRC4C, NGL-1, DLG2, TJP2), eye and body growth (PRSS56, BMP4, ZBTB38, DLX1), and retinal ganglion cell projections (ZIC2, SFRP1) (43). Enrichment analysis has enabled confirmation that groups of genes implied remain remarkably significant between different cohorts. Hysi et al reported that plasma membrane, cell-cell adhesion, synaptic transmission, calcium ion binding and cation channel activity were significantly over-represented in association with refractive error in two British cohorts (54).

Whilst the biological processes implied by these genes may at first seem disparate, the protein products and end functions can be highly correlated. By examining known protein-protein interactions researchers have identified that in fact many of the genes implicated from the meta-GWAS in myopia are related to cell cycle and growth pathways such as the MAPK and TGF-beta/SMAD

pathways, as shown in Figure 4 (45). This network analysis can provide greater

insight into how refractive error develops and ultimately allow targeted,

molecular approaches for intervention to be developed by researchers using this

information.

Figure 4 Network connections of genes associated with myopia. Genes identified

in GWAS are in round grey nodes, linker elements in square nodes, MAPK & TGF-

beta/SMAD pathway elements are in orange, solid blue edges identify protein-

protein interactions and dashed blue edges symbolize corregulation

relationships. Adapted from (45).

Genome-wide association studies and gene-environment interactions

Although myopia is a highly heritable trait, it is known that environmental

factors are highly influential in determining myopia risk and must be driving the

recent epidemic rise in prevalence (1). One of the most influential and highly

replicated factors is education (4, 55-58); research suggest that those going onto

higher education have double the myopia prevalence than those who leave

school after primary education (4). Education has therefore been the primary

environmental choice for gene-environment (GxE) interaction analyses in

myopia. GxE studies acknowledge that individuals of a differing genotype may

respond to environmental variation in differing ways; for example in some

individuals an environmental exposure may trigger a certain gene to be

unregulated whilst in others there is no effect. This method of analysis therefore

has the potential to show how existing significantly associated variants are

modified by environmental exposure, but may also identify variants that were previously only suggestively associated with the disease of interest.

Two research groups have examined this phenomenon by using the myopia-associated variants from the CREAM meta-GWAS analysis. In the first, individuals of European descent were firstly categorized as having completed a primary, intermediate or higher education, and then assigned a polygenic risk score based on the 26 myopia-associated variants from the CREAM meta-GWAS (59). The effect of higher education and high genetic predisposition was far higher than the risk of myopia in those with high genetic risk completing only a primary education; the odds ratio for those with high genetic risk completing higher education was 51.3 (95% CI 18.5 - 142.6) compared to an odds ratio of 7.2 (95% CI 3.1 - 17.0) if only primary education was achieved. The combined effect of the two risk factors was far greater than the sum of the separate factors (synergy index = 4.2, 95% CI 1.9-9.5), providing evidence that an interaction effect between an environmental factor and an individual's genotype was occurring. A similar analysis was performed on five Singaporean cohorts; this analysis identified three genes (DNAH9, GJD2 and ZMAT4-SFRP1) that were strongly associated with myopia in individuals achieving higher secondary or university education but that were either borderline or not statistically significant in individuals achieving lower secondary education or below (60).

Implications from genome-wide association studies in myopia

GWAS have enabled considerable progress in our understanding of what genetic variants are associated with myopia; the number of variants identified in the

recent meta-GWAS far exceeds those identified by linkage and candidate gene studies. However the high heritability of refractive error and myopia, between 70-80% (5-15), is only nominally explained by the variants so far identified. In a European cohort the variants identified by the CREAM meta-GWAS explain only 3.4% of the variance of refractive error (42). This means approximately 75% of the expected heritability is 'missing', a recurrent problem in GWAS studies of complex diseases (27).

In an attempt to identify missing variants for complex diseases, sample sizes need to be maximized. It is well known that small sample sizes reduce power and accuracy in capturing genetic associations. Since the publication of the major meta-GWAS in refractive error two studies, of relatively small size (less than 1,900 individuals), have failed to fully replicate results (61, 62). Conversely, results from high-grade GWAS in refractive error were not replicated by the meta-analysis of CREAM; this may be due to phenotypic or genetic heterogeneity, or, more likely, lack of statistical power (63, 64). It must be acknowledged that underpowered GWAS may produce spurious or false-positive results.

GWAS have confirmed that myopia is highly polygenic with significant variation in the allelic spectrum of identified loci; that is to say the minor allele frequency, indicative of how common the polymorphism is within a population, varied extensively within both the CREAM and 23andMe GWAS (45). However, the majority of variants had only a small effect on phenotypic variants with the highest effect sizes limited to the variants with the lowest minor allele frequency [Figure 5]. GWAS, in its current form, is limited to assessing associations

between a phenotype and common genetic variants. This means variants of lower allelic frequency (rare variants) but potentially large effect sizes have not been investigated.

Figure 5 Minor allele frequency against effect size for the significant variants identified in the CREAM GWAS (adapted from 45).

We can therefore infer that GWAS will never fully explain all the expected heritability from twin studies. A better means of estimating how much variance can potentially be explained by common genetic variation is to perform a genome-wide complex trait analysis or SNP-based heritability (65-67). This technique allows estimation of how much inter-subject variation of a trait can be explained by all the available SNPs. The number of SNPs that have been genotyped or imputed for that individual limits the method, and therefore the SNP-based heritability corresponds to a lower-bound estimate. In a pediatric, British cohort SNP-based heritability was found to remain stable over childhood and, after adjustment for the lack of cycloplegia on the study participants, the SNP heritability, averaged over childhood, was 0.35 (standard error=0.09) (68). Whilst this would suggest that common genetic variants could explain 35% of variance, approximately half of the estimated heritability from twins studies. For comparison the authors point out that the variance explained by non-genetic risk factors, such as time indoors and time spent reading, explain less than 1% of the variance in myopia. It therefore remains possible that more common variants of small effect could be found using common SNP-based association techniques and that there is good merit in continuing to use the technique with ever larger

sample sizes in attempt to capture more genetic variants. Rarer variants (in the order of MAF = 1% to 5%), with potentially greater effect on phenotypic variation, may be identified with improved accuracy using the greater coverage conferred with the 1000 genomes haplotype map and larger sample sizes.

One of the key questions for clinicians is can our current, genetic understanding of myopia allow prediction of future myopia status for patients. Predicting disease risk is most commonly performed using receiver-operating characteristic (ROC) curves (69). This is a plot of the sensitivity of a test against 1-specificity of a test using all possible thresholds of high risk versus low risk. The area under the curve (AUC) is equal to the probability that a randomly identified individual with the disease has a higher risk than a randomly selected healthy individual. An AUC, or C statistic, is given as a fraction with a perfect test yielding an AUC of 1 and a test with no discriminatory power having an AUC of <0.5. The predictive accuracy of genetic-risk models varies extensively between diseases but to date confer little benefit over non-genetic risk prediction models (70). Age related macular degeneration has been a somewhat exception, with an AUC of 0.82 for the full combination of associated genetic variants identified through GWAS (71). The utility of prediction models for age-related macular degeneration in clinical practice has been further tested by adding in phenotypic and demographic information, such as age and smoking, which increases the AUC to 0.87 (72). However, in the majority of disease phenotypes an AUC of 0.5 to 0.7 is more commonly achieved (70), which confers little predictive value, and this is true for myopia at our current level of understanding of the genetic architecture.

To increase the potential for predicting genetic risk entails greater understanding of the genetic architecture of myopia. As discussed, we estimate there are more common genetic variants to be identified and given that very low frequency variants are unlikely to contribute greatly to population variance, we can be optimistic that most of the phenotypic variation in myopia could be explained by common genetic variants (66). However, there are other genetic factors contributing to heritability. Genetic risk is a complex result of common genetic variation, rare genetic variation, gene-environment interactions, gene-gene interactions, epigenetics, and a host of other variations in our genetic make-up. Rare genetic variation requires new analysis techniques and more detailed sequencing of the genome of study participants. Fortunately next-generation sequencing has enabled reduced costs of high-throughput, high coverage genotyping, also enabling whole exome and whole genome examination. Higher-density SNP chips have also been developed, either for higher coverage of the genome or exome-specifc. This means greater coverage of the genome but also increased accuracy as the reliance on imputation, typically poor for rare SNPs, is reduced. As methods for analyzing these vast datasets are refined, this will dramatically increase the potential for identification of rare variants and has already proved successful (73, 74). Interactions between our environment and our genome have already proved informative in myopia, whilst interactions between genes and other genetic architectural analysis techniques hold promise for the future.

Expert commentary

Genome-wide association studies in myopia have undoubtedly transformed our understanding of the genetic architecture of this complex trait. This is very relevant as myopia, already the most common eye condition, is increasing in prevalence throughout world. In light of the fact that myopia is a highly heritable trait, deeper understanding of how genetic variation leads to development of myopia is increasingly necessary.

The genetic variants identified from the major GWAS in myopia have been clear and reproducible, providing credible evidence for their association. Biological processes indicated by the identified associations include neurotransmission, ion transport, retinoic acid metabolism, extracellular matrix remodeling, eye development, the visual cycle, neuronal development, eye and body growth, and retinal ganglion cell projections. Enrichment analysis suggests plasma membrane, cell-cell adhesion, synaptic transmission, calcium ion binding and cation channel activity appear to be significantly over-represented in association error. Whilst these biological processes may seem disconnected, protein products and end functions do appear correlated in myopia risk with many of the genetic associations related to cell cycle and growth pathways such as the MAPK and TGF-beta/SMAD pathways.

However, only around 3% of myopia variance is explained by the genetic variants identified to date. SNP-based heritability analysis suggests common genetic variation accounts for approximately 35% of myopia variance. Therefore, there is more work to be done in an effort to capture all associated common genetic variants. This requires larger samples and improved genotyping to

reduce the burden on imputation, which ultimately can lead to poor ability to capture associated variants or conversely false-positive results. Alternate analysis techniques and proxy endophenotypes are being explored in an effort to further increase our ability to identify these variants. The interplay between genes, and genes and environment is being examined in relation to myopia with some success, shedding new light on how genetic variation may be modified and ultimately lead to myopia development in different individuals. It also important to acknowledge that twin-based estimates of heritability are much higher, at 70-80%, and suggest that genetic factors other than common genetic variation may play a role.

This paper provides a review of our current understanding into the genetics of myopia. There is much work still to be done, and this will be required before our ability to predict future development of myopia becomes a reality. GWAS provides the first step in our ability to identify novel loci and functional pathways. This must then be built upon with other genetic association modalities and the use of both animal models, although notably to date there are few genetic animal models for myopia, and pharmacological studies. Only then can researchers begin to target the development of myopia and reduce the burden from this common, sight-threatening disease.

Five-year view

Despite significant progress in recent years, we still can only explain a very small proportion of myopia variance by genetic factors. In the next five years new approaches to try and capture more of the genetic variance will be employed. Firstly the simple approach of 'bigger is better' should be employed; ever-larger meta-analysis of GWAS studies from across the globe must be utilized in a collaborative format to increase the research community's ability to find genes. This may involve using phenotype data that extends beyond the traditional modality of spherical equivalent into combining GWAS performed on proxy phenotypes and endophenotypes.

Secondly a more detailed interrogation of the genome is required to identity rare genetic variants, and notably these variants may play a more significant role in myopia risk. This can be brought about through a number of existing methods. Using currently genotyped data the improved imputation capacity conferred by haplotype maps such as 1000 genomes should be employed to reduce imputational errors leading to false-negative and false-positive associations; notably both of the major GWAS studies on myopia to date are based on HapMap imputed data. An alternate method is employment of the improved genotyping ability that can be achieved with high-density chips and next-generation sequencing. These modalities achieve greater coverage of the genome, reduced genotyping errors and a reduced reliance on imputation. Although there are many obstacles to overcome such as data storage requirements for these vast files, refinement of analysis techniques, and establishment of how results are

interpreted, they do provide a means to attempt to capture the known missing

heritability in myopia.

Finally alternate means of understanding the genetic architecture of myopia

should be employed - extending beyond simple association methods to explore

interactions and the effect of other 'omics'. This may include incorporation of

transcriptomics or metabolomics, for example, with existing association methods

to allow a more systems biology based approach to understanding how genetic

variation ultimately leads to myopia development.

Key issues

1. Myopia is the most common eye condition worldwide and the prevalence is increasing.

2. Myopia has a complex trait with strong environmental risk factors such as education and lack of time spent outdoors, and a high heritability of 70-80%.

3. GWAS studies have enabled rapid association of common genetic variants with disease since 2005 in various diseases, most successfully in age-related macular degeneration.

4. Case-control high myopia GWAS studies have been largely performed in Asian populations with a number of genetic variants identified.

5. The largest identification of variants for myopia was performed in two GWAS, by the CREAM consortium and 23andMe, published in 2013; the 26 genetic loci by CREAM identified explain less than 5% of myopia variance.

6. Functional pathways implicated by the genetic variants identified for myopia include plasma membrane, cell-cell adhesion, synaptic transmission, calcium ion binding and cation channel activity, with many of the genetic associations related to cell cycle and growth pathways.

7. Gene by environment analyses suggest interaction effects do occur between the currently identified genetic variants and higher education, one of the strongest risk factors for myopia.

8. In attempt to capture more of the genetic variants for myopia, with the ultimate of aim of enabling risk prediction and developing targeted

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interventions, larger sample sizes are required with deeper coverage of

the genome.

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Figure 1
254x254mm (72 x 72 DPI)
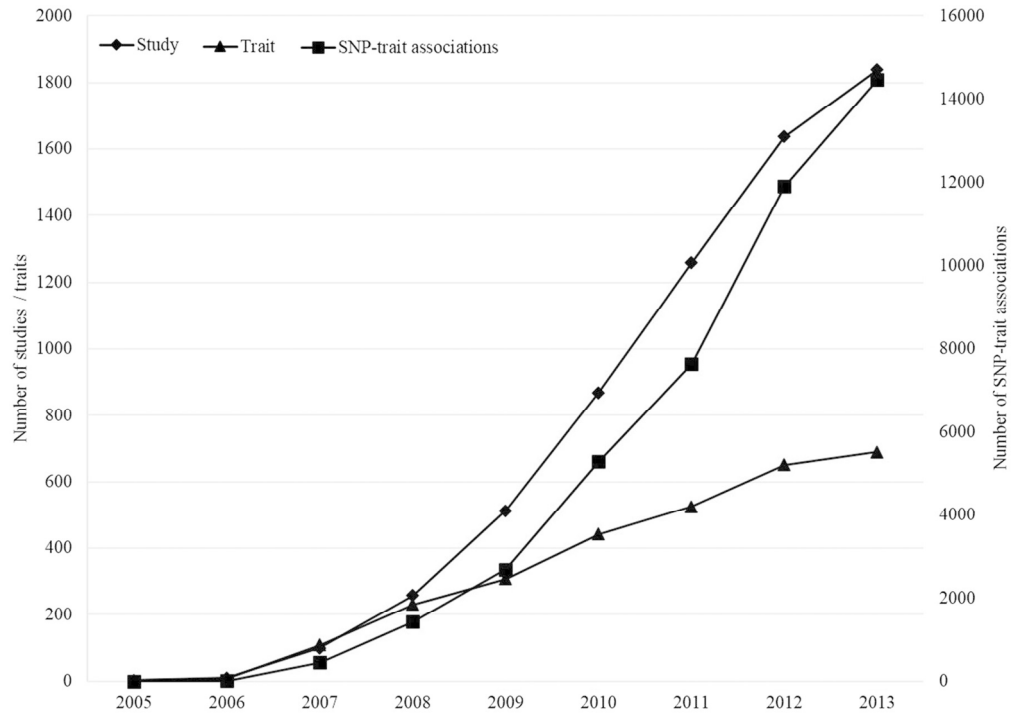
Figure 2
152x107mm (300 x 300 DPI)
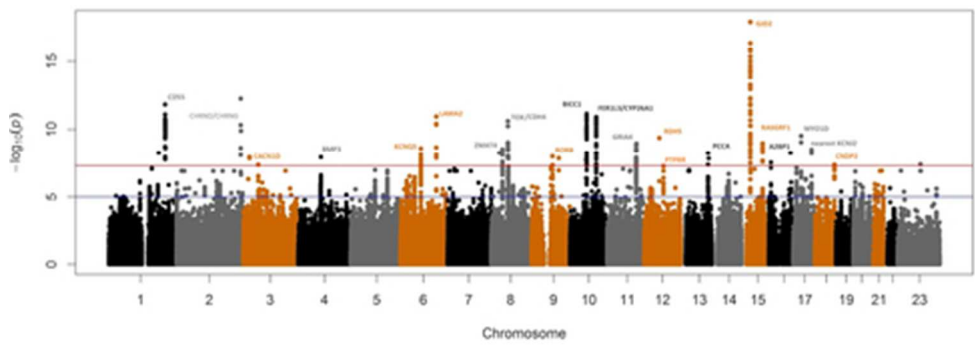
Figure 3
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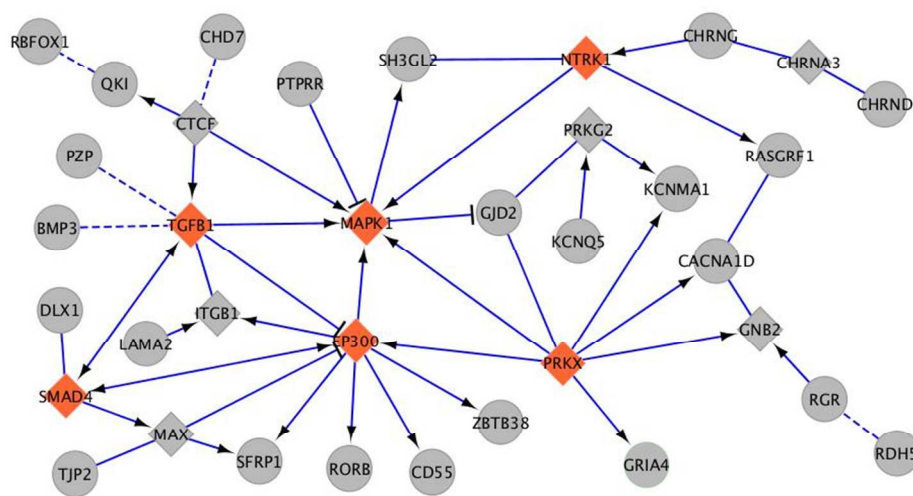
Figure 4
348x191mm (72 x 72 DPI)
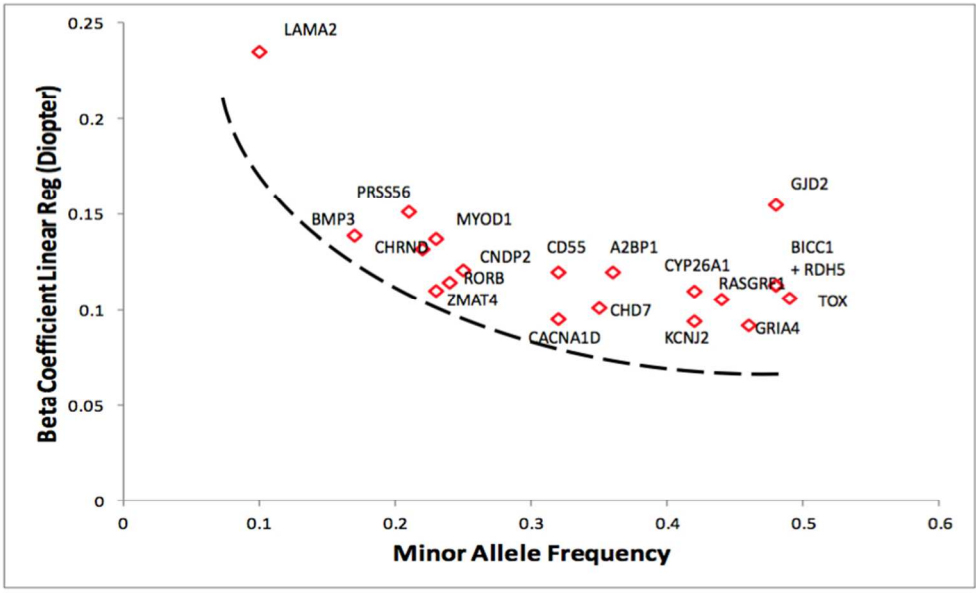
Figure 5
334x201mm (72 x 72 DPI)

GWAS in myopia: insights into disease and implications for the clinic

Summary

Myopia is the commonest eye trait worldwide and the prevalence is increasing. It is known to be highly heritable; total genetic variation explains up to 70-80% of variance. In an attempt to better understand the genetic architecture of myopia, with an ultimate view to better predict genetic risk and develop targeted treatments, several genome-wide association studies have been performed in the last 6 years. In this review we focus on what a genome-wide association study involves, what studies have been performed in relation to myopia to date, and what they ultimately tell us about myopia variance and functional pathways leading to pathogenesis. The current limitations of genome-wide association studies are reviewed and potential means to improve our understanding of the genetic factors for myopia are described.

Keywords

Myopia ; Refractive error ; Genetics ; GWAS ; GxE interactions

Introduction

Myopia is already the commonest eye condition and its prevalence is increasing across the world (1-4). Refractive error is the term used to describe an error in the accurate focusing of light onto the retinal plane. M~~In m~~yopia, or ~~short~~near-sightedness, ~~there is~~ typically results from axial elongation of the eyeball and this results in an image forming anterior to the retinal plane, ~~whilst in~~ hyperopia ~~the reverse occurs with~~results when an image ~~forming~~lies posterior to the retinal plane. ~~Refractive error is the term used to describe an error in the accurate focusing of light onto the retinal plane, encompassing both myopia and hyperopia.~~ Although myopia is strongly associated with a number of environmental factors, the most important risk factor in determining whether an individual develops the trait is ~~having~~ a family history of myopia, suggesting a genetic predisposition. The heritability of a trait is an estimate of how much phenotypic variation in a population is due to genetic factors. The heritability of refractive error, using spherical equivalent as a quantitative trait, has been determined in a number of family and, more credibly, twin studies [Figure 1]. These indicate the heritability of myopia is high at around 70% (5-15).

Figure 1 Heritability estimates for refractive error (Abbreviations: T = twin studies, F = family studies).

Myopia is a complex trait influenced by a complicated interplay of genetic and environmental factors. As with many complex traits there is a distribution of refractive error in the population, meaning the risk of ordinary or "simple" myopia developing is not determined by a classic Mendelian single gene mode of

inheritance; there are likely many genes, each contributing a small effect to overall myopia risk. This may not be true for very high, familial or syndrome-associated forms of myopia – in these cases, where a rare dominantly inherited mutation may be important in an individual family, but not important in the overall population risk. Up until the era of genome-wide association studies (GWAS), identification of disease-associated genes relied on family studies (using linkage analysis) or candidate gene studies. In myopia, these were singularly unsuccessful and prior to 2009 there were no known myopia-associated genes, other than syndromes where myopia was a part of the phenotypic spectrum (eg Stickler's, Marfan syndromes). However, with the advent of GWAS, a number of genes for myopia have been identified, providing new insight into how myopia develops with implications for future research into how this increasingly common eye trait might be treated.

Genome-wide association studies (GWAS)

Genome-wide association studies (GWAS) are approaches that allow a vast array of markers scattered across an individual's DNA or genome to be rapidly tested for association with a disease or trait. These 'markers' are variations in the base pair of nucleotides at specific points along the genome, commonly known as SNPs (single-nucleotide polymorphisms), and give an indication of what nearby genes may be associated with the trait.

In order for this analysis technique to be possible, all of the base pairs, namely adenine (A), guanine (G), thymine (T) or cytosine (C), forming the human DNA code had to be sequenced (ie. read and mapped). The human genome project,

completed in 2003, was a major international scientific collaboration that identified all of the base pairs and genes that make up the human genome, approximately 20,500 genes in total (16, 17). This has enabled researchers to have access to a detailed resource on the structure, function and organization of the complete set of genes that make up the human species. However, to investigate the association between the human genome and disease, a 'map' of common patterns of genetic variation and inheritance was required, known as a 'haplotype map'. This was firstly provided by the HapMap project, completed in 2005 (18); this international project compared the genetic sequences of individuals of African, Asian and European ancestry. Subsequently, the 1000 Genome Project, whichthat harnessed the increased speed, greater coverage and reduced cost of next-generation sequencing, was launched. Released in 2012 this has provided the most detailed catalogue of human genetic variation to date with sequencing of over 1000 participants internationally (19). These maps of common inheritance patterns allow identification of what base pair is commonly at one position in the genome of a certain ethnic population, the 'common' allele, and what base pair tends not be at that position, the 'minor' allele. SNPs are generally termed a common polymorphism when the frequency of the minor allele, in a specific population, is greater than 1%.

GWAS rely upon the assumption that common complex traits are caused by common genetic variations in the population (the "common disease common variant" hypothesis). Therefore, in a GWAS the association between a trait and common genetic variants in the form of SNPs are is examined. SNPs are not disease-causing mutations, as found in classical genetic studies of rare Mendelian

diseases, and they rarely alter protein structure or function, but instead they may relate to regulation of genes or alterations in gene expression. In GWAS SNPs are used as markers, and indicate nearby genes nearby or biological pathways that may be involved, allowing researchers to focus in on specific parts of the genome.

To perform a GWAS for a disease, an individual must be genotyped or sequenced; in large-scale genetic studies this is generally undertaken with the use of high-throughput genotyping arrays or chips. These provide an output of somewhere between 500,000 and 2,500,000 SNPs for that individual, but obviously do not include all the common genetic variants (given there are around 3 billion base pairs in the human genome). The missing data is therefore imputed using reference haplotypes, either the HapMap or 1000 Genome data. Associations between these genetic variations, following extensive data cleaning (quality control), and disease status is examined in regression models either as a quantitative trait (eg. refractive error, measured byas spherical equivalent) or as a categorical case-control trait (eg. 'myopia' or 'no myopia'). The output from such analyses is a list of associated SNPs with an indication of the strength of effect on myopia risk (the beta coefficient) and the confidence of the association (p-value). Significance thresholds are set at less than $p \leq 5 \ 10^{-8}$ to reduce the possibility of false positive associations, which may occur as a result of correlation between SNPs and the high number of statistical tests involved. This means large studies of many thousands of individuals are required to identify statistically significant associations. Results are generally portrayed graphically as a Manhattan plot, which plots all the SNPs by chromosome position as a

function of their association p-value; this plot resembles the Manhattan skyline

with different SNPs reaching higher than others, like skyscrapers, in accordance

with variations in significance. Results of putative genetic associations for a trait

('discovery stage') must then be verified through replication of associated

variants in independent population samples, or through experiments that can

examine the functional implications of the affected gene.

The first GWAS was performed in 2005 and since then there has been an

exponential rise in the number of studies [Figure 2], reflecting the large

reduction in time and cost of undertaking these types of analysis.

Figure 2 Studies, traits and SNP-trait associations from 2005-2013 reveal the

growth in genome-wide association studies. Adapted from (20), Copyright

obtained.

GWAS have now been successfully performed on a range of ophthalmic diseases

(21, 22). The earliest and arguably the most 'successful' GWAS to date has been

within the ophthalmic field; the discovery of the association of CFH with age-

related macular degeneration was reported in three independent cohorts in

2005 (23-25), one of which was a GWAS, and has since been replicated in dozens

of studies across the world. Subsequent meta-analysis involving large sample

sizes (>17,100 cases and >60,000 controls) has identified 19 loci for AMD

explaining 10-30% of the variance (26), which has an estimated heritability of

45-70%. These genetic associations explain a relatively high proportion of AMD

variance, which disappointingly has proved to be fairly unusual in subsequent

GWAS for other traits. Although GWAS ha~~ve~~_ved_ identified many variants for many diseases, relatively small effects on disease risk are conferred for the majority of variants and only a small proportion~~al~~ of familial clustering or heritability is explained. This issue of 'missing heritability' is a recurrent issue in GWAS and has prompted researchers to explore additional approaches to examine the genetic architecture of common complex diseases (27).

Genome-wide association studies in myopia

Refractive error and myopia have been examined using the full range of genetic methodologies. This initially included genome-wide linkage studies in related individuals, which have identified at least 17 loci, and candidate gene association studies . At least 17 loci have been identified through the former and although there was some success with the latter, results have proved poorly reproduciblewhich were rarely replicated (28-30). The first GWAS study to examine myopia was performed in 2009 on a cohort with high, pathological myopia; subsequent studies have either been performed on myopia case-control cohorts, largely from East Asia where the prevalence of myopia and high myopia is greater, or on cohorts with refractive error measured as a quantitative trait. The GWAS catalog A database detailing all published GWAS for myopia, refractive error and other myopia endophenotypes was used to identify articles for this review (is available at http://www.ebi.ac.uk/gwas/home). Articles included are, summarized in Table 1.

Table 1 Summary of published GWAS in myopia. † Associations not reaching conventional GWAS threshold (p ≤ 5 10^{-8}) for statistical significance

High Myopia GWAS

The first published GWAS in myopia examined a Japanese population with 297 cases of pathological myopia (defined as axial length > 26mm) and 977 controls from the general population (31). The strongest association was located at 11q24.1, approximately 44kb upstream of the BLID gene, and conferred odds of higher myopia of 1.37 (95% confidence interval (CI) 1.21 - 1.54). Subsequently a meta-analysis of two ethnic Chinese cohorts, published in 2010, was performed for 287 cases of high myopia (defined as ≤ -6D) and 911 controls (32). The strongest association was an intronic SNP within the CTNND2 gene on 5p15.2. However neither of these initial associations met the conventional GWAS threshold ($p ≤ 5\ 10^{-8}$) for statistical significance.

Li et al also studied an ethnic Chinese population inclusive of 102 high-grade myopia cases (defined as ≤ -8D with retinal degeneration) and 335 controls (33). The strongest association ($p = 7.70 \times 10^{-13}$) was a high frequency variant located in a gene desert within the MYP11 myopia linkage locus on 4q25 (34). In a similar ethnic Han Chinese population of 419 high myopia cases (≤ -6D) and 669 controls, Shi et al identified the strongest association ($p = 1.91 \times 10^{-16}$) at an intronic, high frequency variant within the MIPEP gene on 13q12 (35). Although these aforementioned studies attempted replication in independent cohorts, their results, published in 2011, have not been replicated in GWAS comprising of individuals of similar ethnic background, phenotypic definition or study design.

In 2013 two papers ~~in Asian populations~~ reported replicated loci for high myopia in Asian populations. Shi et al studied a Han Chinese population of 665 cases with high myopia (≤ -6D) and 960 controls (36). Following two-stage replication in three independent cohorts the most significantly associated variant (p = 8.95 x 10^{-14}) was in the VIPR2 gene within the MYP4 locus, and three further variants all reaching genome-wide significance were identified within the same linkage disequilibrium block in the SNTB1 gene (p = 1.13 x 10^{-8} to 2.13 x 10^{-11}). ~~Secondly,~~ Khor et al reported a meta-analysis of four GWAS of East Asian ethnicity ~~totaling~~totalling 1603 cases of "severe" myopia (based on either refractive error or axial length) and 3427 controls (37). After replication analysis, the ~~aforementioned~~ SNTB1 gene was confirmed and a novel variant within the ZFHX1B gene (also known as ZEB2) reached genome-wide significance (p = 5.79 x 10^{-10}).

In European populations, probably illustrating the lower prevalence of high myopia, there has only been one case-control GWAS from a French population, published in 2012. In this study of 192 high myopia cases (≤ -6D) and 1064 controls a suggestive association was identified within the MYP10 linkage locus, 3kb downstream of PPP1R3B, however this did not reach genome wide statistical significance and the study failed to replicate any of the previously reported loci (38).

Refractive Error Quantitative GWAS.

Greater success has been achieved by considering refractive error as a quantitative treat, ~~therefore~~ including ~~inclusive~~ all subjects in population-based

studies rather than a selected clinic-based sampleof all data on the population studied of highly affected individuals. In 2010 the first two GWAS for refractive error were published, both in European populations; a British discovery cohort of 4270 individuals (39) and a Dutch discovery cohort of 5328 individuals (40), with replication in over 10,000 individuals from the two discovery cohorts and a smaller shared pool of replication samples. Two loci surpassing the GWAS threshold were identified near the RASGFR1 gene on 15q25.1 (p = 2.70 x 10^{-09}) and the other near GJD2 on 15q14 (p = 2.21 x 10^{-14}). Subsequently, in 2013, a relatively small meta-analysis was performed on 7280 individuals from five cohorts with refractive error, inclusive of various ethnic populations across different continents. Replication was then undertaken in 26,953 samples (41). A novel variant reaching the GWAS threshold was identified within the RBFOX1 gene on chromosome 16 was identified (p = 3.9 x 10^{-9}).

The field made a major breakthrough in 2013 when two major GWAS meta-analysis studies were published. The Consortium for Refractive Error and Myopia (CREAM) is an international collaborative initiative between researchers studying cohorts of both European and Asian descent. A classic meta-analysis of the GWAS results for a linear regression between genotype and spherical equivalent of refractive error was performed for 35 participating centers, comprising 37,382 individuals of European descent and 12,332 of Southeast Asian ancestry (42). High statistical power was achieved by this large sample size, enabling replication of the two loci previously identified and identification of 22 novel loci at genome-wide significance [Figure 3]: BICC1, BMP2, BMP3, CACNA1D, CD55, CHD7, CHRNG, CNDP2, CYP26A1, GJD2, CRIA4, KCNJ2, KCNQ5,

LAMA2, MYO1D, PCCA, PRSS56, RASGRF1, RDH5, RORB, SIX6, TOX, ZIC2 and

ZMAT4.

Figure 3 Manhattan plot of genetic associations for refractive error in the

CREAM combined GWAS meta-analysis. -log$_{10}$-transformed pP values for all

SNPs. The upper horizontal line indicates the $p < 5.0 \times 10^{-8}$ threshold, the lower

horizontal line indicates a p value $< 1 \times 10^{-5}$ (adapted from (42)).

A contemporaneous ~~publication~~ GWAS by the direct-to-consumer genomics

company 23andMe (Mountain View, CA, USA) ~~on~~ using a ~~GWAS~~ survival analysis

was performed on 55,177 individuals of European descent using the phenotype

of reported myopia and reported 'age of spectacle wear' as a proxy for myopia

severity (43). The authors identified 20 novel loci: BMP3, BMP4, DLG2, DLX1,

GJD2, KCNMA1, KCNQ5, LAMA2, LRRC4C, PABPCP2, PDE11A, PRSS56, RASGFR1,

RBFOX1, RDH5, RGR, SFRP1, SHISA6, TJP2, TOX, ZBTB38 and ZIC2. Contrary to

many researchers' expectations, the authors identified highly comparable

genetic associations to those obtained using the carefully and expensively

collected refractive error data in population-based samples in the CREAM

consortium. Of the 22 loci discovered by CREAM, 14 were replicated by

23andMe, whilst 16 of the 20 loci identified by 23andMe were confirmed by

CREAM. Surprisingly the same 25 genetic loci were identified in both studies

with consistent direction of effect despite analysis on different scales, namely

dioptres for CREAM (more negative on the scale indicative of more myopia) and

hazard ratios (higher positive hazard ratios indicative of more severe myopia)

for 23andMe (44, 45).

Genome-wide association studies and myopia endophenotypes

The most common ~~form~~ type of myopia is axial myopia (lens-induced or lenticular myopia is seen in old age due to early nuclear cataract) and as such the axial length of the eye is a major determinant of refractive error~~the majority of myopia~~. A number of researchers have therefore used this myopia proxy or 'endophenotype' ~~for use~~ in genetic association studies ~~of myopia as a quantitative trait~~. The first of these, published in 2012, examined 4944 individuals of East and South East Asian ancestry (46). One locus on 1q41 containing the zinc-finger pseudogene ZC3H11B reached genome wide significance (p = 4.38 x 10^{-10}), although replication was not performed.

A much larger GWAS meta-analysis for axial length comprising 12,531 Europeans and 8,216 Asians was published in 2013 (47). Eight, novel genome-wide significant loci were identified (RSPO1, C3orf26, LAMA2, GJD2, ZNRF3, CD55, MIP, ALPPL2) and the ~~aforementioned~~ study also replicated the ZC3H11B ~~was confirmed~~gene. Relevantly, five of these loci had been previously associated ~~with~~ in refractive error GWAS.

Shared determination of an individual's axial length and corneal curvature was identified in the Avon Longitudinal Study of Parents and Children (ALSPAC) and Singapore Chinese Eye Study, suggesting that shared genetic variants ~~genetic~~ control ~~of~~ these two parameters which contribute to the eye's focus~~eye dimension parameters is by common genetic variants~~ (48). A number of relatively small GWAS have been performed for corneal curvature in individuals

of varying ancestry with identified associations ~~in individuals of varying ancestry~~ ~~,~~ including FRAP1, PDGFRA (also associated with eye size), CMPK1 and RBP3 (49-52). More recently Miyake et al published a two-stage GWAS for three myopia-related traits: axial length, corneal curvature and refractive error (53). The study was performed on 9,804 Japanese individuals with trans-ethnic replication in Chinese and Caucasian individuals. A novel gene, WNT7B, was identified for axial length ($p = 3.9 \times 10^{-13}$) and corneal curvature ($p = 2.9 \times 10^{-40}$), whilst the previously reported association with GJD2 and refractive error was replicated.

Pathways implicated from genome-wide association studies in myopia

Identifying genes associated with myopia is just the first step in ~~gaining~~ maximizing information~~the full utility~~ from GWAS ~~in~~ to improve~~ing~~ ~~our~~ understanding of myopia etiology. ~~Certain~~ Some individual biological mechanisms ~~are~~ can be implicated from ~~associated~~ genes associated, ~~whilst~~ but pathway analysis ~~can~~ enables a more comprehensive, systems biology approach to understanding how associated genetic variants can ultimately influence ocular growth. Pathway analysis, however, does rely on previously published work on ~~This analysis is of course reliant on what is already known about~~ the functionality of certain genes.

Functional pathways (or ontological classifications) implicated by the large GWAS on myopia to date have been clear and reproducible (54). Interestingly, they provide credible evidence that the genetic architecture is fairly consistent between two continental populations (European and Asian). As with many

GWAS, the variants identified have not necessarily fallen within a gene but likely functional implications to proximal, relevant genes have been inferred. Although this is reasonable, there are other known factors, such as long-range distance equilibrium, which may mean alternate genes or pathways could equally be involved. Biological processes indicated from the CREAM meta-GWAS include neurotransmission (GRIA4), ion transport (KCNQ5), retinoic acid metabolism (RDH5), extracellular matrix remodeling (LAMA2, BMP2), and eye development (SIX6, PRSS56) (42). ~~Whilst T~~the 23andMe meta-GWAS similarly implied extracellular matrix remodeling (LAMA2, ANTXR2), the visual cycle (RDH5, RGR, KCNQ5), neuronal development (KCNMA1, RBFOX1, LRRC4C, NGL-1, DLG2, TJP2), eye and body growth (PRSS56, BMP4, ZBTB38, DLX1), and retinal ganglion cell projections (ZIC2, SFRP1) (43). ~~Enrichment analysis has enabled confirmation that groups of genes implied remain remarkably significant between different cohorts.~~ Hysi et al reported that plasma membrane, cell-cell adhesion, synaptic transmission, calcium ion binding and cation channel activity were significantly over-represented in association with refractive error in two British cohorts (54).

Whilst the biological processes implied by these genes may at first seem disparate, the protein products and end functions can be highly correlated. By examining known protein-protein interactions researchers have identified that in fact many of the genes implicated from the meta-GWAS in myopia are related to cell cycle and growth pathways such as the MAPK and TGF-beta/SMAD pathways, as shown in Figure 4 (45). This network analysis can provide greater insight into how refractive error develops, although it must be acknowledged

that the risk loci identified from GWAS have not been shown to be causative in functional studies and therefore any pathway analysis is speculative. ~~and ultimately allow targeted, molecular approaches for intervention to be developed by researchers using this information.~~

Figure 4 Network connections of genes associated with myopia. Genes identified in GWAS are in round grey nodes, linker elements in square nodes, MAPK & TGF-beta/SMAD pathway elements are in orange, solid blue edges identify protein-protein interactions and dashed blue edges symbolize corregulation relationships. Adapted from (45).

Genome-wide association studies and gene-environment interactions

Although myopia is a highly heritable trait, it is known that environmental factors are highly influential in determining myopia risk and must be driving the recent epidemic rise in prevalence (1). One of the most influential and highly replicated factors is education (4, 55-58); research suggest that those going onto higher education have double the myopia prevalence than those who leave school after primary education (4). Education has therefore been the primary environmental choice for gene-environment (GxE) interaction analyses in myopia. GxE studies acknowledge that individuals of a differing genotype may respond to environmental variation in differing ways; for example in some individuals an environmental exposure may trigger a certain gene to be unregulated whilst in others there is no effect. This method of analysis therefore has the potential to show how prior identified~~existing~~ ~~significantly associated~~

variants are modified by environmental exposure, but may also identify variants that were previously only suggestively associated with the disease of interest.

Two research groups have examined this phenomenon by using the myopia-associated variants from the CREAM meta-GWAS ~~analysis~~. In the first, individuals of European descent were ~~firstly~~ categorized as having completed a primary, intermediate or higher education, and then assigned a polygenic risk score based on the 26 myopia-associated variants from the CREAM meta-GWAS (59). The<u>re appeared to be an interaction between the</u> effect of higher education and <u>having a</u> high genetic ~~predisposition~~ <u>risk score</u>~~was far higher than the risk of myopia in those with high genetic risk completing only a primary education~~; the odds ratio <u>for myopia in</u> ~~for~~ those with high genetic risk completing higher education was 51.3 (95% CI 18.5 - 142.6) compared to an odds ratio of 7.2 (95% CI 3.1 - 17.0) if only primary education was achieved. The combined effect of the two risk factors was far greater than the sum of the separate factors (synergy index = 4.2, 95% CI 1.9-9.5), providing evidence that an interaction effect between an environmental factor and an individual's genotype was occurring. A similar analysis was performed on five Singaporean cohorts; this analysis identified three genes (DNAH9, GJD2 and ZMAT4-SFRP1) that were strongly associated with myopia in individuals achieving higher secondary or university education but that were either borderline or not statistically significant in individuals achieving lower secondary education or below (60).

Implications from genome-wide association studies in myopia

GWAS have enabled considerable progress in our understanding of what genetic variants are associated with myopia; the number of variants identified in the recent meta-GWAS far exceeds those identified by linkage and candidate gene studies. However the high heritability of refractive error and myopia which is, between 70-80% (5-15), is only ~~nominally~~ partly explained by the variants so far identified. In a European cohort the variants identified by the CREAM meta-GWAS explain only 3.4% of the variance of refractive error (42). This means approximately 75% of the expected heritability is 'missing', a recurrent problem in GWAS studies of complex diseases (27).

In an attempt to identify missing variants for complex diseases, sample sizes need to be maximized. It is well known that small sample sizes reduce power and accuracy in capturing genetic associations. Since the publication of the major meta-GWAS in refractive error two studies, of relatively small size (less than 1,900 individuals), have failed to fully replicate results (61, 62). Conversely, results from high-grade GWAS in refractive error were not replicated by the meta-analysis of CREAM; this may be due to phenotypic or genetic heterogeneity, or, more likely, lack of statistical power (63, 64). It must be acknowledged that underpowered GWAS may produce spurious or false-positive results.

GWAS have confirmed that myopia is highly polygenic with significant variation in the allelic spectrum of identified loci; that is to say the minor allele frequency, indicative of how common the polymorphism is within a population, varied extensively within both the CREAM and 23andMe GWAS (45). However, the majority of variants had only a small effect on phenotypic variants with the

highest effect sizes limited to the variants with the lowest minor allele frequency [Figure 5]. GWAS, in its current form, is limited to assessing associations between a phenotype and common genetic variants. This means variants of lower allelic frequency (rare variants) but potentially large effect sizes have not been investigated.

Figure 5 Minor allele frequency against effect size for the significant variants identified in the CREAM GWAS (adapted from 45).

We can therefore infer that GWAS will never fully explain all the expected heritability from twin studies. A better means of estimating how much variance can potentially be explained by common genetic variation is to perform a genome-wide complex trait analysis or SNP-based heritability (65-67). This technique allows estimation of how much inter-subject variation of a trait can be explained by all the available SNPs. The number of SNPs that have been genotyped or imputed for that individual limits the method, and therefore the SNP-based heritability corresponds to a lower-bound estimate. In a pediatric, British cohort SNP-based heritability was found to remain stable over childhood and, after adjustment for the lack of cycloplegia on the study participants, the SNP heritability, averaged over childhood, was 0.35 (standard error=0.09) (68). TWhilst this would suggest that common genetic variants could explain 35% of variance, approximately half of the estimated heritability from twin studies. For comparison the authors point out that the variance explained by non-genetic risk factors, such as time indoors and time spent reading, isexplain less than 1% of the variance in myopia. It therefore remains possible that more common

variants of small effect could be found using common SNP-based association techniques and that there is ~~good~~ merit in continuing to use the technique with ever larger sample sizes in <u>an</u> attempt to capture more genetic variants. Rarer variants (in the order of MAF = 1% to 5%), with potentially greater effect on phenotypic variation, may be identified with improved accuracy using the greater coverage conferred with the 1000 genomes haplotype map and larger sample sizes.

One of the key questions for clinicians is ~~can~~ <u>whether</u> our current~~, genetic~~ understanding of myopia <u>genetics</u> allow<u>s</u> prediction of future myopia status for ~~patients~~<u>children</u>. Predicting disease risk is most commonly performed using receiver-operating characteristic (ROC) curves (69). This is a plot of the sensitivity of a test against 1-specificity of a test using all possible thresholds of high risk versus low risk. The area under the curve (AUC) is equal to the probability that a randomly identified individual with the disease has a higher risk than a randomly selected healthy individual. An AUC, or C statistic, is given as a fraction with a perfect test yielding an AUC of 1 and a test with no discriminatory power having an AUC of <0.5. The predictive accuracy of genetic-risk models varies extensively between diseases but to date confer little benefit over non-genetic risk prediction models (70). Age related macular degeneration has been a<u>n</u> ~~somewhat~~ exception, with an AUC of 0.82 for the full combination of associated genetic variants identified through GWAS (71). The utility of prediction models for age-related macular degeneration in clinical practice has been further tested by adding in phenotypic and demographic information, such as age and smoking, which increases the AUC to 0.87 (72). However, in the

majority of disease phenotypes an AUC of 0.5 to 0.7 is more commonly achieved (70), which confers little predictive value, and this is true for myopia at our current level of understanding of the genetic architecture.

To increase the potential for predicting genetic risk entails greater understanding of the genetic architecture of myopia. As discussed, we estimate there are more common genetic variants to be identified and given that very low frequency variants are unlikely to contribute greatly to population variance, we can be optimistic that most of the phenotypic variation in myopia could be explained by common genetic variants (66). However, there are other genetic factors contributing to heritability. Genetic risk is a complex result of common genetic variation, rare genetic variation, gene-environment interactions, gene-gene interactions, epigenetics, and a host of other variations in our genetic make-up. Rare genetic variation requires new analysis techniques and more detailed sequencing of the genome of study participants. Fortunately next-generation sequencing has providedenabled reduced costs of high-throughput, high coverage genotyping, also enabling whole exome and whole genome examination. Higher-density SNP chips have also been developed, either for higher coverage of the genome or exome-specifc. This means greater coverage of the genome but also increased accuracy as the reliance on imputation, typically poor for rare SNPs, is reduced. As methods for analyzing these vast datasets are refined, this will dramatically increase the potential for identification of rare variants and has already proved successful (73, 74). Interactions between our environment and our genome have already proved informative in myopia, whilst

interactions between genes and other genetic architectural analysis techniques hold promise for the future.

Expert commentary

Genome-wide association studies in myopia have undoubtedly transformed our understanding of the genetic architecture of this complex trait. This is very relevant as myopia, already the most common eye condition, is increasing in prevalence throughout world. In light of the fact that myopia is a highly heritable trait, deeper understanding of how genetic variation leads to development of myopia is increasingly necessary.

The genetic variants identified from the major GWAS in myopia have been clear and reproducible, providing credible evidence for their association. Biological processes indicated by the identified associations include neurotransmission, ion transport, retinoic acid metabolism, extracellular matrix remodeling, eye development, the visual cycle, neuronal development, eye and body growth, and retinal ganglion cell projections. Enrichment analysis suggests plasma membrane, cell-cell adhesion, synaptic transmission, calcium ion binding and cation channel activity appear to be significantly over-represented in ~~association~~ refractive error. ~~PWhilst these biological processes may seem disconnected, protein products and end functions do appear correlated in myopia risk,~~ with ~~m~~Many of the genetic associations are related to cell cycle and growth pathways such as the MAPK and TGF-beta/SMAD pathways.

However, only around 3% of myopia variance is explained by the genetic variants identified to date. SNP-based heritability analysis suggests common genetic variation accounts for approximately 35% of myopia variance. Therefore, there is more work to be done in an effort to capture all associated common genetic variants. This requires larger samples and improved genotyping to reduce the burden on imputation, which ultimately can lead to poor ability to capture associated variants or conversely false-positive results. Alternate analysis techniques and proxy endophenotypes are being explored in an effort to further increase our ability to identify these variants. The interplay between genes, and genes and environment is being examined in relation to myopia with some success, shedding new light on how genetic variation may be modified and ultimately lead to myopia development in different individuals. It also important to acknowledge that twin-based estimates of heritability are much higher, at 70-80%, and suggest that genetic factors other than common genetic variation may play a role.

This paper provides a review of our current understanding into the genetics of myopia. There is much work still to be done, and this will be required before our ability to predict future development of myopia becomes a reality. GWAS provides the first step in our ability to identify novel loci and functional pathways. This must then be built upon with other genetic association modalities and the use of both animal models, although notably to date there are few genetic animal models for myopia, and pharmacological studies. Only then can researchers begin to target myopiathe development of myopia and reduce the burden from this common, sight-threatening disease.

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Five-year view

Despite significant progress in recent years, we still can only explain a very small proportion of myopia variance by genetic factors. In the next five years new approaches to try and capture more of the genetic variance will be employed. Firstly the simple approach of 'bigger is better' should be employed; ever-larger meta-analysis of GWAS studies from across the globe must be utilized in a collaborative format to increase the research community's ability to find genes. This may involve using phenotype data that extends beyond the traditional modality of spherical equivalent into combining GWAS performed on proxy phenotypes and endophenotypes.

Secondly a more detailed interrogation of the genome is required to identity rare genetic variants, and notably these variants may play a more significant role in myopia risk. This can be brought about through a number of existing methods. Using currently genotyped data the improved imputation capacity conferred by haplotype maps such as 1000 genomes should be employed to reduce imputational errors leading to false-negative and false-positive associations; notably both of the major GWAS studies on myopia to date are based on HapMap imputed data. An alternate method is employment of the improved genotyping ability that can be achieved with high-density chips and next-generation sequencing. These modalities achieve greater coverage of the genome, reduced genotyping errors and a reduced reliance on imputation. Although there are many obstacles to overcome such as data storage requirements for these vast files, refinement of analysis techniques, and establishment of how results are

interpreted, they do provide a means to attempt to capture the known missing

heritability in myopia.

Finally alternate means of understanding the genetic architecture of myopia

should be employed - extending beyond simple association methods to explore

interactions and the effect of other 'omics'. This may include incorporation of

transcriptomics or metabolomics, for example, with existing association methods

to allow a more systems biology based approach to understanding how genetic

variation ultimately leads to myopia development.

Key issues

1. Myopia is the most common eye condition worldwide and the prevalence is increasing.

2. Myopia has a complex trait with strong environmental risk factors such as education and lack of time spent outdoors, and a high heritability of 70-80%.

3. GWAS studies have enabled rapid association of common genetic variants with disease since 2005 in various traits~~diseases~~, most successfully in age-related macular degeneration.

4. Case-control high myopia GWAS studies have been largely performed in Asian populations with a number of genetic variants identified.

5. The largest identification of variants for myopia was performed in two GWAS, by the CREAM consortium and 23andMe, published in 2013; the 26 genetic loci by CREAM identified explain less than 5% of myopia variance.

6. Functional pathways implicated by the genetic variants identified for myopia include plasma membrane, cell-cell adhesion, synaptic transmission, calcium ion binding and cation channel activity, with many of the genetic associations related to cell cycle and growth pathways.

7. Gene by environment analyses suggest interaction effects do occur between the currently identified genetic variants and higher education, one of the strongest risk factors for myopia.

8. In an attempt to capture more of the genetic variants for myopia, with the ultimate of aim of enabling risk prediction and developing targeted

interventions, larger sample sizes are required with deeper coverage of

the genome.

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Study	Year	Association count	Region of associations	Genes implicated
Nakanishi H et al. A genome-wide association analysis identified a novel susceptible locus for pathological myopia at 11q24.1.	2009	1 †	11q24.1	BLID
Li YJ et al. Genome-wide association studies reveal genetic variants in CTNND2 for high myopia in Singapore Chinese.	2011	1 †	5p15.2	CTNND2
Li Z et al. A genome-wide association study reveals association between common variants in an intergenic region of 4q25 and high-grade myopia in the Chinese Han population.	2011	2	4q25	MYP11 linkage locus
Shi Y et al. Genetic variants at 13q12.12 are associated with high myopia in the Han Chinese population.	2011	1	13q12	MIPEP
Meng W et al. A genome-wide association study provides evidence for association of chromosome 8p23 (MYP10) and 10q21.1 (MYP15) with high myopia in the French Population.	2012	64 †	8p23 10q21.1	MYP10 linkage locus MYP15 linkage locus
Shi Y et al. A genome-wide meta-analysis identifies two novel loci associated with high myopia in the Han Chinese population	2013	5	13q12.12 8q24.12	VIPR2 SNTB1
Khor CC et al. Genome-wide association study identifies ZFHX1B as a susceptibility locus for severe myopia.	2013	2	2q22.3 8q24.12	ZFHX1B SNTB1
Hysi PG et al. A genome-wide association study for myopia and refractive error identifies a susceptibility locus at 15q25	2010	1	15q25.1	RASGFR1
Solouki AM et al. A genome-wide association study	2010	1	15q14	GJD2

identifies a susceptibility locus for refractive errors and myopia at 15q14.				
Stambolian D et al. Meta-analysis of genome-wide association studies in five cohorts reveals common variants in RBFOX1, a regulator of tissue-specific splicing, associated with refractive error.	2013	1	16p13.3	RBFOX1
Verhoeven VJ et al. Genome-wide meta-analyses of multiancestry cohorts identify multiple new susceptibility loci for refractive error and myopia.	2013	26		BICC1, BMP2, BMP3, CACNA1D, CD55, CHD7, CHRNG, CNDP2, CYP26A1, GJD2, CRIA4, KCNJ2, KCNQ5, LAMA2, MYO1D, PCCA, PRSS56, RASGRF1, RDH5, RORB, SIX6, TOX, ZIC2, ZMAT4
Kiefer AK et al. Genome-wide analysis points to roles for extracellular matrix remodeling, the visual cycle, and neuronal development in myopia.	2013	22		BMP3, BMP4, DLG2, DLX1, GJD2, KCNMA1, KCNQ5, LAMA2, LRRC4C, PABPCP2, PDE11A, PRSS56, RASGFR1, RBFOX1, RDH5, RGR, SFRP1, SHISA6, TJP2, TOX, ZBTB38, ZIC2