Genetic Dissection of Common Diseases

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Abstract

The complex genetic nature of many common diseases makes the identification of the genes that predispose to these ailments a difficult task. In this review we discuss the elements that contribute to the complexity of polygenic diseases and describe an experimental strategy for disease-related gene discovery that attempts to overcome these factors. This strategy involves a population-based case-control paradigm and makes use of a highly informative, homogeneous founder population, many of whose members presently reside in Israel. The properties of single nucleotide polymorphisms, which are presently the markers of choice, are discussed, and the technologies that are currently available for SNP genotyping are briefly presented.

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Everyone knows that many diseases run in families. What is often not appreciated is the reason why scientists have been able to identify the causative genetic mutations in Tay-Sachs, Gaucher and cystic fibrosis, but not in other common ailments such as ischemic heart disease, diabetes and asthma. The answer lies in the realization that the former disorders are inherited as simple Mendelian traits in which mutations in a single gene give rise to the disease phenotype, usually with either a dominant or recessive pattern of inheritance. By contrast, heart failure, diabetes, asthma, various cancer syndromes, Alzheimer disease and a long list of other common ailments are all complex genetic diseases. In these diseases, specific variants in any single gene are only contributory to the overall predisposition of a person to eventually suffer from that disease; hence the genotype-phenotype correlation, which is the basis for gene discovery, is small.

The challenge of complex diseases

There are multiple reasons for the complexity of complex diseases. The primary source of complexity is the polygenic nature of these diseases, i.e., the involvement of many genes. In order for a specific clinical end-result to be manifested, several different alleles must come together additively, or in specific combinations (and often in a particular environmental background). Thus, unlike in Mendelian disorders, the contribution of each allele is often small and difficult to detect. Furthermore, the specific genes involved may vary between two persons suffering from similar symptoms, a phenomenon known as locus heterogeneity. Since no single gene form is uniquely responsible for the symptoms presented, the contributing alleles are often common in the non-diseased population as well as in those suffering from the disorder, making them more difficult to identify.

An additional confounding factor in the study of complex diseases is variable expressivity. Variable expressivity refers to differences in the severity of the disease in different individuals with the same genotype. This situation points to the involvement of a large environmental component in the etiology of complex diseases. In fact, environmental factors may even cause "phenocopying," or mimicking, of a syndrome that is usually thought to be of genetic causation. Thus, while the genetic component of complex diseases translates, for example, into a significantly higher than average chance of one identical twin falling ill if his twin suffers from a complex ailment, contributory environmental factors ultimately determine whether he actually does. Consequently, in the case of complex disorders, "disease genes" are "susceptibility genes" rather than true causative agents.

In summary, the multiplicity of genes involved, the small impact of each gene on predisposition to the disease, and the camouflaging effect of environmental factors, all combine to make the identification of susceptibility genes a major challenge. The unarguable benefits to be gained in the areas of diagnostics, preventive medicine, drug discovery and personalized drug therapies make it a challenge worth undertaking. And the new tools that have become available for these studies in the "post-genome era" now make it feasible.

Paradigms for gene discovery

Linkage analysis

Linkage analysis is an approach that has been widely employed in the past. In this approach, families segregating for the disease are studied by various family-based designs. In linkage analysis, co-segregation of two loci, or a locus and a phenotypic trait, is examined in a family to see if they tend to be inherited together, implying that the two genes, or the locus and the gene responsible for the phenotypic trait in question, reside on the same chromosome in physical proximity to each other. The statistical procedure is based on estimating the excess of allele sharing, identical by

SNP = single nucleotide polymorphism

descent, in sibling pairs who are both affected by the disease. This approach has been very successful in mapping a large number of Mendelian disease genes, including cystic fibrosis and amyotrophic lateral sclerosis. There are also several examples of common genes whose roles in complex diseases have been detected by these approaches. One is human leukocyte antigen, which was found to play a role in type I diabetes when it was observed that affected sib pairs share the allele in 73% of cases (instead of the expected 50%) [1]. Another is apolipoprotein E, which is a major determinant in late-onset Alzheimer's disease [2].

However, it must be realized that these examples of genes, whose roles in complex diseases have been deciphered by linkage analysis, involve loci with a very high displacement, or large relative effect. In such cases, despite the polygenic nature of the disease, the inheritance pattern appears nearly Mendelian, since alternative genotypes show a large phenotypic difference and the phenotypic variation within each genotypic group is small. However, linkage analysis has not been successful in identifying genes of the kind that are likely to represent most gene effects in complex diseases. These are genes that have smaller displacements and greater overlap of genotype distributions among individuals with different phenotypes. The reason that small gene effects cannot be recognized by linkage analysis is that, in most family-based studies, a limited pedigree size severely limits the power to detect the underlying genes [3].

The case/control approach and its advantages

Population-wide direct-association analysis is a method with significantly higher statistical power than linkage analysis for detection of genes with modest phenotypic effects. The approach often used in such studies is the case-control design that compares affected individuals and unrelated unaffected controls. In association analyses, polymorphic markers are tested for differences in allele distribution between subjects and controls. Because a high density of such markers is employed, it is assumed that some of them will be in linkage disequilibrium with the disease susceptibility gene, or that the actual functional polymorphism will be tested. Linkage disequilibrium means that there is a high correlation between the state of the polymorphic marker and the allele state of the susceptibility gene, such that a specific form of the marker occurs together with the disease form of the gene more often than would be predicted by random chance. The extent of linkage disequilibrium is largely determined by the distance of the polymorphic marker from the susceptibility gene. The shorter this distance, the fewer recombination events will have occurred between the two, and the more instances in which the susceptibility allele will be associated with the marker form originally present on the chromosome where it first appeared.

The kinds of genes for which the greater statistical power of the case-control association paradigm offers the greatest advantage are genes with moderate to small genotypic relative risks. The GRR is the ratio of the risk of developing the disease among individuals with the susceptibility genotype compared to the risk for individuals with an alternative genotype. In cases where the GRR is less than 2, linkage analysis requires unrealistically large samples to provide evidence for the location of a disease gene. The higher statistical power of association studies has been theoretically calculated to allow detection even of genes with relative risks as low as 1.5 with reasonable sample sizes [4].

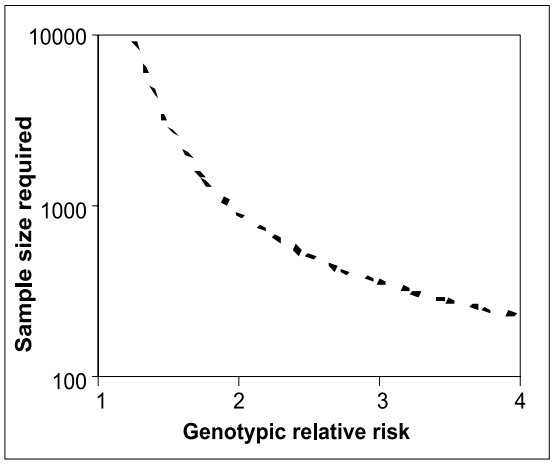

Figure 1. The theoretically required sample (cases + controls) size is reduced as GRR (genotypic relative risk) is increased. This curve corresponds to a situation where linkage disequilibrium is constant and equal to one because the tested marker coincides with the functional polymorphism.

Thus, for the study of genes with small to moderate GRRs, association studies provide the only feasible paradigm. In addition, the smaller required sample size and the greater ease of matching cases to unrelated controls without the necessity of finding and enrolling additional family members to a study contribute to making the case-control design a more economically attractive approach [Figure 1]. Finally, population-wide association analysis with high marker density has great potential for accurate mapping and identification of the actual gene in question. This is in contrast to the detection of broad chromosomal regions in family-based studies, which take advantage of the very small number of recombination events that occur over a two to three generation span to examine linkage using only a few hundred sparsely spaced markers.

Until recently, a scarcity of markers and the lack of an efficient genotyping technology have been the major limitations for applying the association strategy. These issues will be addressed later. An additional drawback of this approach that is frequently pointed out is the potential that a spurious association will be deduced, often because of inadequate ethnic matching of cases and controls and ethnic variation in allele frequencies. One way to avoid the problems of ethnic stratification in association studies is discussed below.

Advantages of a homogeneous founder population

Homogeneous or isolated populations originating from a limited number of founder individuals provide a number of advantages for the discovery of genes under the association paradigm. Firstly, they

GRR = genotype relative risk

greatly reduce the chances of confounding due to population stratification, thus virtually eliminating the potential for artefactual associations due to this factor. In addition, reduced genetic heterogeneity has the effect of increasing the GRR, since the hypothesized similarity in genetic make-up decreases background noise [5]. Finally, the extent of the chromosomal intervals on which linkage disequilibrium can be detected is enlarged because the correlation between polymorphisms is retained at larger distances. These last two factors serve to decrease the theoretically required sample size for disease gene detection in a multiplicative, rather than additive fashion [5,6]. Consequently, gene discovery is greatly facilitated.

In Israel there are a number of subpopulations that meet the criteria of an isolated population. By far the largest of these is the Israeli Ashkenazi Jewish population, which is of demonstrable genetic homogeneity.

Figure 2. Jewish communities in the seventeenth century when 10,000–30,000 Jews lived in 60 communities in the Pale of Settlement (Map reproduced from Beyond the Pale: The History of Jews in Russia: http://www.friends-partners.org/partners/beyond-the-pale/index.html)

Ashkenazi Jews – a unique population resource for genetic studies

The majority of the founders of the Ashkenazi community made their way from the region of present-day Israel, to Spain, France and Italy and then in the tenth century to the Rhineland valley in Germany. The Crusades in the eleventh to the thirteenth centuries, and the charters of protection granted to the Jews in 1264 in order to rebuild the Polish cities, encouraged the migration of many Jews to Poland and Lithuania. The early Jewish settlements in these areas often consisted of only a few families that were isolated genetically, not only from their non-Jewish neighbors, but also from other Jewish communities. Until the end of the Middle Ages the Jewish population of Eastern Europe was very small. It is estimated that in the year 1300 there were only about 5,000 Jews in all of Poland and Lithuania.

The Eastern Europe Jewish population subsequently experienced cyclic periods of flourishing punctuated by persecution and decimation. By 1490 the number of Jews had increased to between 10,000 and 30,000 [Figure 2], but in the mid-1600s the Cossack uprising led to the massacre of many of the Jews of Poland and the Ukraine. In the eighteenth and nineteenth centuries there was a large growth in the number of Ashkenazi Jews, which reached 750,000 in a 1765 census and 6 million at the end of the following century, shortly before the Holocaust. Subsequent to persecutions and economic difficulties in the nineteenth century, and in the aftermath of the Holocaust, many Ashkenazi Jews emigrated to the United States and to Israel. The present-day Ashkenazi Jewish population is estimated at more than 10 million, of whom 2.8 million live in Israel.

There are several rare genetic diseases that occur with a particularly high incidence among Ashkenazi Jews. These include: Tay-Sachs, Gaucher disease, Bloom syndrome, idiopathic torsion dystonia, familial dysautonomia, factor XI deficiency and more. For many of these disorders, a causative gene has been identified and a specific mutation demonstrated to be involved in most cases of the disease among Ashkenazi Jews. The frequencies of the common mutations of most of these diseases are between 1/16 and 1/110 in this population, and each is found mainly in the context of a single haplotype (a linear, ordered arrangement of alleles on a chromosome), a finding consistent with a single founder for each mutation. For example, the gene causing familial dysautonomia was mapped to a segment of chromosome 9 using Ashkenazi Jewish families. The candidate region was confirmed by haplotype analysis; haplotype sharing among 435 of 441 familial dysautonomia chromosomes revealed a strong founder effect [7,8].

Age estimations have been calculated assuming a genetic clock for most of the high frequency diseases listed above. The origin of the investigated mutations can all be dated to between the ninth and fourteenth centuries, and is consistent with the early migration of Jews to Europe and the founding of the Jewish community in Eastern Europe. The high frequency of some genetic disorders among Ashkenazi Jews (less than 1/100) indicates that the founder chromosome carrying the disease allele was introduced into a very small population, probably in the order of about 100 unrelated individuals. The demographic expansion that followed the early migration maintained the high frequency of these alleles and was probably also affected by genetic drift [9,10].

Thus, the Ashkenazi Jewish population is a unique population in terms of its demographic history and genetic architecture. The

factors that make it so include emergence from a limited number of founders, exceptional expansion and contraction of the population size, and a long history of marriage within the faith (endogamy) and often within the family. These factors have made the Ashkenazi Jews an invaluable population source for the study of a number of monogenic diseases, and have also begun to show their benefits in the study of common, complex diseases. Indeed, this population is instrumental in the deciphering of the contributing roles of mutations in the *BRCA1* and *BRCA2* genes in the tendency to develop breast and other cancers [11–13]. In addition, this population is easily accessible since it actively seeks medical treatment. However, as recently pointed out on the pages of this journal [14], the Ashkenazi Jewish population is a resource that must be harvested quickly – before the "melting pot" effects of intergroup marriages in Israel result in the mixing of different ethnic subpopulations.

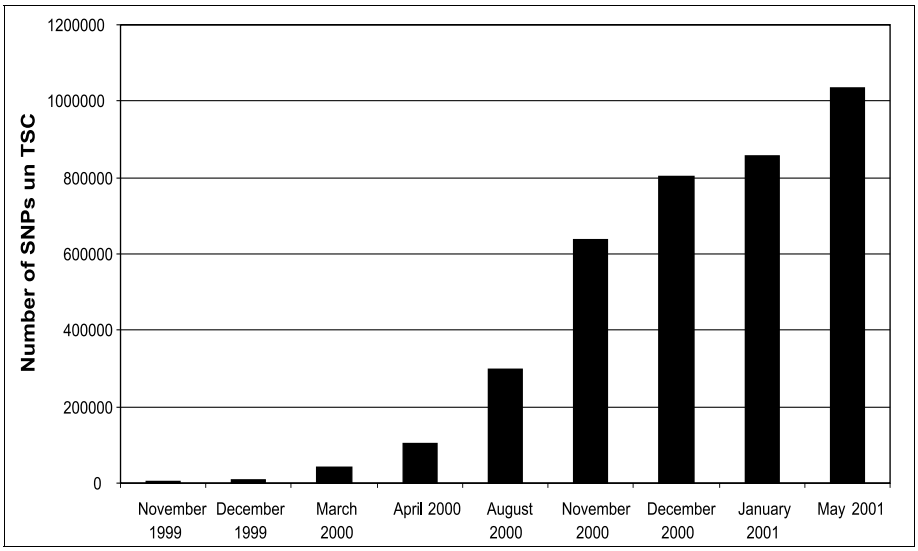

Figure 3. The increase in the number of SNPs reported in The SNP Consortium (TSC) (http://snp.cshl.org) from November 1999 to May 2001.

SNPs as tools in genetic analyses

An impressive by-product of the sequencing endeavor by the International Human Genome Sequencing Consortium and Celera Genomics is the ongoing identification and mapping of a large number of single nucleotide polymorphisms. These single-point substitutions dispersed along the genome cause any two random human genomes to be 0.1% different from one another and account for most of the heritable variation among individuals, including susceptibility to disease or particular drugs.

About 1.4 million SNPs have been found thus far by The SNP Mapping Consortium, mainly through comparison of a small number of random reads against draft sequences from the Human Genome Project [Figure 3]. Given a genome size of 3.2 billion base pairs, this implies the presence of a SNP approximately every 2,300 bp. In fact, in a 1.5 million bp subset of the genome for which high quality sequence was available, the SNP discovery rate was 1 per 1,331 bp based on two haploid genomes. Even this SNP density is likely to be greatly under-estimated since SNPs with a relatively low allele frequency can only be revealed by comparison of a larger number of genomes. (Polymorphisms that occur at a frequency of 1% or more qualify as SNPs. In order to detect a SNP with a 1% allele frequency with 95% certainty, 96 genomes, i.e., 48 individuals, would have to be compared. In order to increase the certainty of discovery to 99%, 192 genomes would have to be compared.) Taking into consideration that the present method of SNP scoring allows description primarily of common variants, but not all of them, 11 million SNP sites have been inferred in the human genome. If this turns out to be the case, the average SNP frequency would be 1 per 290 bp [15].

Because SNPs can be expected to be present in a large fraction of patients and controls, they are the focus of two kinds of association studies – direct association studies of SNPs with potential functional significance, and indirect association studies to track disease loci through linkage disequilibrium.

Direct association studies make use of SNPs in the coding or regulatory regions of genes that may directly influence the trait under study. cSNPs, or those in gene coding regions, are of interest if they change an amino acid. Such non-synonymous cSNPs comprise about 40% of all cSNPs [15]. Variants, such as those in promoter regions that impact on gene expression, are also clearly of functional importance. It was recently shown that SNPs at exon-intron boundaries affect gene function by altering mRNA splicing [16]. Interestingly, the number of SNPs in these categories is under-represented as compared with, for example, synonymous SNPs, which is consistent with the notion that these variants have phenotypic significance and are subject to selection. Theoretically, in order to study all SNPs with potential phenotypic consequences in the currently estimated 30,000 genes of the human genome, each of which has an average of 4 cSNPs, one would have to examine about 50,000 non-synonymous cSNPs and an unknown number of SNPs in regulatory regions. Presently, the number of cSNPs in the SNP Consortium is approximated at 15–30% of the total, which would already bring the number to more than 200,000 [15,17].

Indirect association studies are based on linkage disequilibrium, as described above, and the current debate lies in the number of random SNPs that will have to be assayed in a whole-genome study in order to detect association with a complex disease. The debate surrounding the number of SNPs required for this strategy stems from the fact that the actual extent of linkage disequilibrium in the human genome in general, and particularly in specific populations, is not known. While some maximalist theoreticists insist on the need to assay more than 500,000 evenly spaced polymorphisms in

bp = base pair

order to detect linkage disequilibrium of sufficient magnitude for mapping purposes, it is more likely that the appropriate number is in the range of 50,000 to 100,000, and the smaller numbers may specifically apply to founder populations. In either case, assuming a relatively random distribution along the genome, the present catalog of 1.4 million SNPs already provides the required tools to carry out whole-genome association studies.

The technological bottleneck

The rate of SNP discovery has clearly outpaced the capacity of the scientific community to genotype these variants. In order for the tremendous potential of SNPs in large-scale correlation studies to be realized, technologies permitting rapid and accurate genotyping of multiple individuals with numerous markers and at low cost must become available. While a number of approaches for various degrees of high-throughput SNP profiling has been developed, none has thus far emerged as the leading "golden standard" technology offering the necessary scoring capacity to carry out several million genotypes at a truly economically feasible cost per genotype [Table 1].

There is a large number of SNP typing chemistries and detection systems available. Most of these are polymerase chain reaction-based, requiring the prior amplification of a small DNA fragment, usually a few hundred bases long, around the SNP to be analyzed. The two most common SNP typing chemistries currently employ hybridization, or primer extension followed by different detection systems to then resolve the allelic state of the fragment at the polymorphic point (usually a two-base alternative).

In allele-specific oligonucleotide hybridization [18], the basis of the "chip technology," the amplified fragments are hybridized over an array of oligonucleotides, using high-stringency hybridization conditions that allow single-base mismatches to be detected [19]. Hybridization is then monitored by fluorescent-based systems. In primer extension technologies, an oligonucleotide primer is annealed immediately upstream to the polymorphism in the PCR-amplified fragment. After the primer is extended by one or more bases, the alleles are distinguished. This can be on the basis of the size or mass of the extended product by, for example, mass spectrometry (such as MALDI-TOF) or denaturing high performance liquid chromatography, or by the use of distinct fluorescent labels for the alternative dideoxynucleotides at the SNP site [20–22]. In the Pyrosequencing™ strategy [23], pyrophosphate molecules are detected as they are released during incorporation of nucleotides in the primer extension reaction.

Many additional SNP discrimination approaches exist, some of which have managed to eliminate the prerequisite PCR reaction. The latter make use of more exotic strategies, such as the ability of a cleavase enzyme to cut a matched (and not mismatched) three-strand hybridization structure [24], or allele-specific rolling-circle amplification of universal probes [25]. While it is not clear which will ultimately be the technology of choice, what is certain is that, given the overwhelming potential benefits to humanity of the study of genetic variants, the technological obstacles to highly parallelized, high-throughput, low cost SNP genotyping will be overcome in the near future.

Table 1. Features of SNP genotyping systems

Technique	Features	Throughput*	Approximate cost per genotype
Allele-specific amplification	Low capital cost Low assay set-up costs Highly adaptable system	250–400	0.5–1.0 $
Pyrosequencing™	Medium capital costs Medium throughput Automation enhances throughput Multiplexing** in some cases	600–1,000 (manual) 2,300–10,000 (automat)	3.0 $
Minisequencing with dye terminators	Low capital costs (based on a multi-application device) Medium throughput Multiplexing reduces cost	4,000–7,500 (24 hours) 23,000 (multiplex)	5.0 $ 0.5 $ (multiplex)
MALDI-TOF	High capital cost Always automatic Very high throughput Multiplexing possible	5,000–20,000	0.5 –2.4 $***

* Throughput relates to allele discrimination, assuming PCR products are already available, in one working day unless otherwise specified.

** Multiplexing is the simultaneous processing of independent SNPs on the same DNA sample and in the same test tube.

*** A few companies have developed slightly different techniques based on MALDI-TOF. These variations are reflected in the assay costs.

Population genetics: Israel and the world

The great challenge in identifying the genetic basis of common diseases in the window of opportunity that has presented itself today has attracted the interest of several academic and commercial institutions. One of the best known projects in this direction is being carried out by deCODE Genetics, which is trying to recruit virtually the entire Icelandic population to its studies and has gained access to the extensive medical and genealogic records of all the country's citizens. deCODE's genealogic approach links the family trees of living Icelanders suffering from certain diseases. Patients with particular diseases are then clustered into large extended families, sometimes containing hundreds of individuals. Several companies have also arisen in the U.S., including Myriad Genetics, DNA Sciences Inc. and others, which employ the case-control strategy without regard to ethnic origin. Each of these companies is addressing the issue of predisposing genes in a large number of common complex diseases.

If there are so many players in the arena, can Israel also play a part in the post-genomic era? A similar debate that surrounded Israeli involvement in the

PCR = polymerase chain reaction

Human Genome Project was recently described on these pages [26]. At the time that this issue had to be resolved, it was realized (correctly, in retrospect) that even a minimal amount of involvement by Israel would better position the scientific community for the "post-genome" era. In fact, Israel's actual contribution to the creation of sequence data and, certainly to the development of data search and analysis tools (as exemplified by GeneCards and the Unified Database, two internationally employed databases that were developed at the Weizmann Institute, and the sequence analysis programs of Compugen), has been far-reaching beyond initial expectations. It is with this optimistic lesson behind us that we should view the entry into the arena of IDgene, an Israeli-based company that intends to make use of population genetics and the country's statistical, biological and computational scientific capabilities, in the hunt for a greater understanding of the role of common molecular variation in phenotypic diversity, and, in particular, disease susceptibility.

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