Identification of G-protein-coupled receptors involved in inflammatory disease by genetic association studies

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G-protein-coupled receptors are not only highly tractable drug targets but also attractive candidates for genetic association studies because they are more polymorphic than most other classes of gene and these polymorphisms frequently lead to functional changes in the levels of expression or biological activity that can predispose to common diseases. A large-scale study to identify functional variants in G-protein-coupled receptors associated with inflammatory diseases has highlighted a spectrum of novel biological insights that range from identifying the involvement of orphan receptors in certain diseases through to highlighting new therapeutic indications for existing drugs.

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Introduction

Traditionally, selection of the most appropriate drug target for the treatment of a particular disease has been the result of inspired biological insight combined with serendipity. The plethora of potential new targets emerging in recent years has increased the need for high-throughput methods to prioritise targets and to allow selection of the most appropriate disease indication. The use of disease-relevant human tissue combined with findings from animal models can often highlight fruitful avenues for exploration but, ultimately, controlled clinical trials with a drug active at a particular target are required to know whether that target is a safe and effective therapeutic intervention point. This is a painfully slow and costly process with a dismal success rate and, therefore, there is a pressing need for new approaches to target selection and validation. One attractive approach to identify clinically relevant drug targets linked to disease pathogenesis is to understand how variations in the sequence of putative drug target genes can predispose

an individual to disease. Single nucleotide polymorphisms (SNPs) or other gene sequence variants (e.g. insertions or deletions) can affect either the function of drug targets (e.g. changes in ligand binding) or the level (or location) of tissue expression. Identification of such disease-causing polymorphisms in a high-throughput mode can therefore determine which drug targets are causally linked to disease. Such drug target focused genetic research has advantages over traditional positional cloning approaches as the output is more certain to be a tractable target. Here, we discuss this approach and describe one such large-scale study in inflammatory disease conducted over the past couple of years. The drug target class selected for this study was G-protein-coupled receptors (GPCRs).

Experimental design: positional cloning or a drug target focused approach?

Positional cloning — the application of genome-wide linkage and association analysis to the pattern of co-segregation of genetic markers and phenotype in human pedigrees — has been highly successful in locating genes involved in genetic disease provided that the segregation pattern of disease itself is Mendelian (i.e. highly penetrant single gene disorders such as cystic fibrosis) [1,2]. About 1200 Mendelian genes have been mapped by this method [3]. However, success in locating genes involved in genetic disease in which inheritance does not display simple Mendelian segregation patterns — complex disease — has been much less successful; only a handful of genuine disease associations, such as *NOD2/CARD15* in Crohn's disease, have been reported [4]. As most diseases for which there is an unmet therapeutic need and, consequently, a requirement for novel drug targets are also complex in their inheritance, this renders positional cloning a long-term method of identifying novel drug targets. Moreover, even if positional cloning were more successful in identifying genes involved in complex disease, this does not necessarily provide new drug targets: it is estimated that only about one in 10 genes in the human genome is a tractable drug target [5]. Thus, a large number of positional cloning successes will be required in any therapeutic area to identify a target directly. Of course, successful identification of genes that are not targets might suggest novel lines of investigation which may themselves ultimately identify promising targets, but this will increase the time and cost of the process.

An alternative to positional cloning is to rely on direct testing of genetic variants in genes that belong to drug target classes. In this approach, the high rate of attrition of

positional cloning in identifying novel targets is avoided, and any genuine genetic association with a target can be developed with confidence. There are also several hybrid strategies: for example, testing for genetic association of drug targets that lie only in genetic linkage regions, or studying gene expression patterns of all genes within linkage regions.

The principles of association testing are simple. The frequency of a genetic variant within a gene is compared between individuals diagnosed with the disease under investigation (the cases) and individuals unaffected by that condition (the controls). If the difference in frequency is sufficiently large that it is improbable to have occurred by chance, then the gene is judged to be associated with the disease. The design and analysis of large-scale association studies, together with discussion of their strengths, weaknesses and pitfalls, have been reviewed extensively [6–8] and will not be discussed in this review.

Advantages of a genetic approach

The greatest strength of the genetic approach is that any true association between a polymorphism and a disease phenotype is causative. This is not true of epidemiology in general or of experimental biology, and great care is required that any observed relationship is not a result of the disease, rather than being a cause. In genetics, there is never any doubt: the genotype results in the phenotype, never the other way around. The weakness of the genetic approach is the great difficulty in establishing associations with anything approaching certainty. However, there are additional ancillary strengths and weaknesses of a genetic approach. For example, genetics can be used not only to highlight the involvement of a drug target but also to provide information on whether it is a safe point for therapeutic intervention. Conversely, it is important to note that absence of genetic association does not rule out the possibility that the target may have therapeutic utility.

The choice of GPCRs for genetic study

GPCRs are predicted to account for ∼3–4% of genes in the human genome, of which over 50% are olfactory (or odorant) receptors. The non-olfactory receptors are divided into three major classes on the basis of their sequence homology. The largest group, class A or rhodopsin-like family, binds ligands such as biogenic amines, neuropeptides, chemokines, lipids and nucleotides. Class B (secretin-like) receptors bind peptides predominantly, whereas ligands for class C metabotropic receptors include glutamate, γ-aminobutyric acid and calcium ions.

GPCRs have been shown to be particularly amenable to pharmacological manipulation with low molecular weight 'drug-like' compounds, and the diversity and specificity of ligand interactions mean that it is possible to identify specific points of pharmacological intervention in multiple biological pathways. For this reason, the GPCR target class has been a rich source of safe and effective drugs for the treatment of a large number of diseases: 20–30% of the top-selling 500 drugs exert their therapeutic effects through an action on GPCRs. Drug discovery on receptors for the biogenic amines histamine, dopamine and serotonin have been particularly fruitful but successful drugs targeting lipid receptors (e.g. cysteinyl leukotrienes) and peptides (e.g. angiotensin II and endothelin) have also been developed. It should be emphasized that the current battery of GPCR agonists/antagonists available in the pharmacopoeia only target approximately 20 receptors (reflecting a large number of 'me too' drugs) and, therefore, there is great potential to discover novel therapies related to GPCR action. The probably impact of GPCR biology on future drug discovery is reviewed comprehensively elsewhere [9].

The cell surface location of GPCRs and consequent involvement in intercellular communication means that drugs acting on such receptors do not need to interfere with intracellular metabolism to exert their therapeutic effects. It is proposed that such a mechanism is inherently more likely to have a reduced side effect profile than is targeting an intracellular pathway which might have multiple functions vital to health. This is a particularly important consideration when the target disease is not generally life-threatening and can affect children, as is the case for asthma. Another important feature of the tractability of GPCRs is that it is possible to make both therapeutically useful agonists and antagonists of these receptors. Although antagonists are much preferred from a point of view of safety and propensity for receptor desensitisation, the ability to make an agonist does mean that disease-causing genes identified by loss-of-function polymorphisms can still be targeted. This contrasts with many other target classes, such as enzymes, which are only susceptible to inhibition, and activation is not generally considered to be a tractable option.

In addition to issues related to drug tractability, genes encoding GPCRs are also prime candidates for being disease-causing genes. It is well established that several single gene disorders are caused by both gain-of-function and loss-of-function mutations [10,11]. Some examples of where the biochemical defect is well characterised and correlated with disease are shown in Box 1. Indeed, a bioinformatics survey of OMIM (On-line Mendelian Inheritance in Man, available at the National Center for Biotechnology Information website [http://www.ncbi.nlm.nih.gov]) conducted at Oxagen indicates that GPCRs are more often found to be disease-causing genes than is predicted by chance. The human genome has been estimated to contain ∼24 000 genes, of which about 380 are predicted to encode for non-olfactory GPCRs. There are 1610 human genes listed in OMIM as being

disease-causing. Of these, 49 were identified as GPCRs. This is significantly higher than expected ($p < 0.00002$), and is not likely to be attributable to GPCR selection bias as the majority of these studies were hypothesis-free positional cloning exercises. It is also worth noting that GPCRs are reported to be more polymorphic than other genes studied [12,13••]. Given the structure of GPCRs, it is easy to imagine how SNPs in coding regions that cause minor perturbations in structure could affect receptor function. Small *et al.* [13] found that variability was prominent in the transmembrane-spanning domains (which are involved in binding for certain classes of ligand) and the intracellular loops (which could influence G protein interactions). SNPs can also affect receptor desensitisation [14] and cause constitutive activation of GPCRs [15]. The reasons for selecting GPCRs as a focus for a target class genetics approach are summarized in Box 2.

Most of the known examples of diseases caused by inherited defects in GPCRs are rare disorders. However, the CCR5del32 mutation is quite common in Europeans and shows a cline or gradient from southern to northern Europe, reaching an allele frequency of about 14% in

Scandinavians [16]. This mutation leads to the production of a truncated protein that is degraded before it reaches the cell surface. CCR5 is the receptor for chemokines such as macrophage inflammatory protein (MIP) 1α, MIP1β and RANTES (regulated on activation, normal T cell expressed and secreted), which cause activation and migration of monocyte/macrophages, T cells and certain other leukocyte populations [17]. In the mid 1990s, it was discovered that CCR5 also served as a co-receptor for HIV entry into leukocytes [18,19], explaining the ability of MIP1α, MIP1β and RANTES to inhibit HIV infection *in vitro* [20]. The discovery that CCR5 could serve as a co-receptor for HIV was rapidly followed by reports that carriers of the CCR5del32 mutation were resistant to HIV infection [21,22]. Homozygotes are almost completely resistant to HIV infection, whereas heterozygous individuals show slower disease progression. It is now appreciated that during the early stages of asymptomatic infection HIV-1 strains (termed R5) use CCR5 as co-receptor whereas, during the later stages of infection when full blown symptoms are apparent, other HIV-1 strains emerge that bind to CXCR4 (termed R5X4 strains) [23]. This suggests that blocking CCR5 should prevent infection and slow progression during the early stage of AIDS, which usually lasts 6–9 years. In addition to providing direct clinically relevant evidence that CCR5 antagonists are likely to be efficacious, the genetic information provides confidence that blocking this receptor is likely to be safe, as there are no apparent health problems in individuals lacking CCR5. Preliminary reports on clinical trials support this: the Pfizer CCR5 antagonist UK-427 857 reduced viral titres without causing serious adverse events when dosed at 100 mg twice daily [24]. Given the high mutability of HIV, there are worries that in the presence of CCR5 antagonists strains could emerge that utilize other chemokine receptors, such as CXCR4, for cell entry [25]. However, although *in vitro* studies have found emergence of escape mutants, these strains did not utilize CXCR4 [26]. Rather, there is evidence that blocking CCR5 *in vitro* may result in the generation of strains that have stronger binding at CCR5 or can even utilize drug-bound receptors for cell entry [26].

Output from a GPCR programme

Several significant associations have emerged from Oxagen's GPCR programme that have highlighted a spectrum of therapeutic opportunities, from identifying potential new uses for marketed drugs through to discovering the involvement of orphan receptors in inflammatory disease. The genetic association study focused on GPCRs that had tissue expression profiles consistent with a role in inflammatory disease. The target cell types and tissues studied are shown in Table 1. The overall objective of studying the expression profiles was to define a GPCR present in diseased tissue that is critical in regulating a cell function relevant to disease but that is not expressed elsewhere in the body. On average, each cell

Table 1

Examples of cells and tissues used to define the inflammatory disease profile of GPCRs.

Disease	Cell types	Tissue types
General Immune	CD4$^+$ T cells CD8$^+$ T cells B cells Activated macrophages Dendritic cells Granulocytes	Spleen Thymus
Asthma	Th2 lymphocytes Eosinophils Mast cells Bronchial epithelial cells	
Colitis	α4β7 gut-homing T cells CD4$^+$ T cells from ulcerative colitis patients Colonic epithelial cells	Ulcerative colitis colon
Psoriasis	CD4$^+$ T cells Keratinocytes	Psoriatic skin
Rheumatoid arthritis	CD4$^+$ T cells Activated macrophages Synoviocytes Chondrocytes Osteoclasts	Rheumatoid synovial tissue Rheumatoid synovial fluid cells

expressed approximately 50 GPCRs (including several orphan receptors), of which about five were novel (see Table 2). GPCRs with attractive expression profiles were then prioritised for genetic association studies and the following are examples of genetically associated targets identified by such an approach.

TSHR

The gene encoding thyroid stimulating hormone receptor (TSHR) is an obvious candidate gene for autoimmune thyroid disease. Several small association studies, examining amino acid changing polymorphisms, have generally yielded negative or weak results [27]. Initial genotyping of 86 individuals for 38 SNPs with minor allele frequencies of >1% identified three blocks of linkage disequilibrium covering the gene. From these 38, 20 tagging SNPs were then selected and genotyped in a two stage process over 1059 cases and 971 controls. A haplotype, running over blocks one and two, showed association (p < 0.000001) to Graves' disease. No association to autoimmune hypothyroidism was found. The most associated single SNP from this study was replicated in an independent study of 1366 cases and 1061 controls (p = 0.000002). Although the causal polymorphism and mechanism for increased risk have not been identified, the strength of the original association, its replication and the well-established role for this receptor in the aetiology of autoimmune thyroid disease leave little doubt that this is a genuine genetic association. Moreover, the presence of association for Graves' disease but absence for autoimmune hypothyroidism is informative and could indicate new approaches in research for these diseases.

EDG6

EDG6 encodes for S1P4, one of five sphingosine-1-phosphate (S1P) receptors. A search for SNPs within the exon of *EDG6* established that it carries many rare polymorphisms. Sequencing of 406 ulcerative colitis cases and 531 controls showed that amino acid changing mutations were present in higher numbers in the disease cases. Moreover, for the subgroup of amino acid changing mutations predicted to have the greatest effect on protein function, the difference between cases and controls was enhanced further: cases carried 0.067 mutations per individual, controls 0.032. Evidence for the association is not strong (p = 0.016) and requires replication. Nevertheless, this result increases the rationale for use of S1P modulators such as FTY720 in the treatment of inflammatory bowel disease. FTY720 is a natural product which is metabolised to a S1P analogue that interacts with several SIP receptors, including SIP4, and, consequently, exerts immune

Table 2

Repertoire of GPCR expression in several cells and tissues relevant to inflammatory disease.

Cells and tissues	Non-olfactory GPCRs	
	Known	Novel
CD4$^+$ T cells	29	3
CD8$^+$ T cells	41	7
Activated macrophages	50	6
Dendritic cells	44	6
α4β7 gut-homing T cells	49	3
Th2 cells	36	2
Granulocytes	49	3
Ulcerative colitis colon	62	3
Psoriatic skin	66	8

suppressant activity by preventing lymphocyte egress from lymph nodes [28,29].

CRTH2

The *CRTH2* gene is located within an asthma linkage region on chromosome 11q13 and was found to be significantly associated with the disease in a Caucasian asthma cohort. It was recently reported that this gene has shown particularly strong association with asthma in both an African-American and a Chinese population [30]. The SNPs associated were located in the 3′ untranslated region and were shown to increase mRNA stability in transcriptional pulsing experiments. This is likely to lead to an increase in receptor expression and, potentially, enhanced receptor function.

CRTH2 (chemoattractant receptor expressed on Th2 cells) was originally identified as a marker for Th2 lymphocytes [31] and has subsequently been shown to function as a GPCR. CRTH2 is selectively expressed by Th2 lymphocytes, eosinophils and basophils, and mediates chemotaxis of these cells in response to its ligand prostaglandin D2 (PGD2) [32]. PGD2 is the major prostanoid produced by mast cells and it has been detected in high concentrations in the airways of asthmatics after antigen challenge [33]. The discovery of CRTH2 is important as it explains how PGD2 mediates allergic responses *in vivo*, an effect that could not be accounted for by the other known receptor for PGD2 (i.e. DP), which had been identified many years earlier. Through a combination of clinical genetic and biological studies, it has become evident that antagonism of CRTH2 is an attractive approach to suppress airway inflammation and, consequently, improve lung function in allergic asthma.

Conclusions

Large-scale genetic studies show great promise for the identification and validation of therapeutic targets for the treatment of human disease, and have highlighted several novel therapeutic opportunities. It will, however, be some time before sufficient clinical trial data have been generated to determine if drugs directed at genetically validated targets are more successful than those identified by other means.

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