Beta-defensins and Crohn's disease: confusion from counting copies.

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Crohn's disease (CD) is a debilitating chronic inflammatory disease, which most commonly affects the terminal ileum and colon, but can affect any part of the gastrointestinal tract. In the West, the disease is frequent, with a prevalence of around 150 per 100,000 (ref 1), and patients have an increased risk of other inflammatory/autoimmune diseases such as psoriasis, ankylosing spondylitis and multiple sclerosis. It is a disease where both environmental and genetic variation make an approximately equal contribution to disease risk; and the current consensus suggests the disease is a result of an inappropriate immune response against gut microbes in a genetically susceptible host. The role in CD of a family of antimicrobial proteins and inflammatory mediators called beta-defensins is of considerable interest, yet there is much controversy on how levels of these proteins, and variation of the genes that encode them, affects susceptibility to CD. In this issue, Bentley et al. (2) present data on the genomic copy number of beta-defensin genes that not only fails to support previous work (3), but completely contradicts it. What is going on?

In recent years, there has been considerable success in dissecting the individual environmental and genetic components to CD. Smoking has been shown to markedly affect the clinical course of CD (4), and has been the most significant environmental component yet identified. Genomewide scans have been particularly successful in identifying genetic loci involved, initially by using family data and linkage analysis, followed more recently by using large case-control cohorts and association analyses. These have both confirmed susceptibility loci and identified new loci, with NOD2/CARD15 confirmed as the locus of strongest effect, with an odds ratio of 4 (refs 5, 6). This locus alone contributes over 1% of the variance in total disease risk, but all 32 loci identified to date explain only 10% of the total disease risk variance, suggesting that the variation at these loci each contribute very subtly to the etiology
of the disease. This leaves four/fifths of the genetic variation in risk unexplained, and there have been several suggestions on where this variation is hidden, including rare sequence polymorphisms and complex copy number polymorphism (where the number of copies of a gene vary between people). Case-control sample sizes of tens of thousands combined with new technologies are needed to determine whether the hidden genetic risk really is in these classes of polymorphism.

With this background, any clues from models of the etiology of the CD on where to look for genetic variation in risk are very welcome. If, as commonly accepted, CD is the result of a loss of tolerance to gut flora and a subsequent inappropriate immune response leading to tissue damage, then factors involved in mediating tolerance to gut flora may have critical roles in controlling the progression to CD. One of these factors may be the beta-defensins, which are a family of proteins sharing a conserved six-cysteine motif that forms three disulphide bridges generating a characteristic tertiary protein structure. They have multiple functions, including direct antimicrobial activity and cytokine activity in inflammatory signalling pathways. Four human beta-defensins are expressed in the gut: human beta-defensin 1 (HBD-1) is expressed constitutively and human beta-defensins 2, 3 and 4 (HBD-2, -3, -4) are induced by bacterial and inflammatory stimuli, with HBD-2 induction mediated by the NOD2/CARD15 protein (7,8). In CD, there is no induction of HBD-2 despite a severe inflammatory response. This is in contrast to the inflammatory bowel disease ulcerative colitis, where high levels of HBD-2 are characteristic of the inflammation sites; and also of the inflammatory skin disease psoriasis, where expression of HBD-2 is a characteristic event and expression level associated with clinical severity (9). Perhaps this low level of beta-defensin induction is the key characteristic of CD, and restoring beta-defensin levels would ameliorate the disease?
Analysis of the beta-defensin genes revealed that they show copy number polymorphism (CNP); that is, different individuals have different numbers of the same gene in their genomes. The genes encoding the four beta-defensins expressed in the gut (genes DEFB1, DEFB4, DEFB103 and DEFB104 encoding proteins HBD-1, -2, -3 and -4 respectively) are clustered at chromosomal region 8p23.1, and DEFB4, DEFB103 and DEFB104 (but not DEFB1) show copy number polymorphism and vary together as a group. For most genes we would expect two copies per diploid genome, but for this cluster different people commonly have between two and seven copies, with four copies being the most frequent (39% in England). The high and low extreme copy numbers are also at appreciable frequencies in the population (4% for two copies, 1.5% for 7 copies, in England) (10, 11). This prompts the question of whether the low gene copy number of beta-defensins is linked to the absence of beta-defensin induction in CD, particularly DEFB4 (HBD-2), and therefore whether low gene copy number of DEFB4 itself predisposes an individual to CD.

Fellerman et al (3) tackled this question by analysing two CD case-control cohorts from Germany and the United States. They found that CD patients had slightly lower mean DEFB4 copy number than unaffected controls from the same population, suggesting that low DEFB4 copy number was a susceptibility factor for CD. In addition, there was a correlation between DEFB4 copy number and expression levels of DEFB4 mRNA in mucosal biopsies from patients, supporting a direct causative link between low gene copy number, low expression and development of CD. The genetic association of low copy number and disease was significant only in those with colonic CD rather than ileal CD, and the authors suggested that this reflects a difference in the levels of HBD-2 between ileum and colon. However, in this issue of the AJG, Bentley and colleagues present data showing a completely opposite effect
of *DEFB4* gene copy number, suggesting that high copy number is associated with both ileal and colonic CD (4). Another report published recently adds more data into this maelstrom of mixed messages: by measuring HBD-2 protein levels in colonic biopsies in ex vivo culture, they find no association of *DEFB4* copy number with expression level in patients or controls (12).

What are we to make of these contradicting results? Findings from early genetic association studies on single nucleotide polymorphisms (SNPs) were often not reproduced in subsequent studies. An example is in Alzheimer's disease, where an association study examining the threonine-alanine polymorphism at amino acid position 13 of the *SERPINA3* gene (encoding alpha-1-antichymotrypsin) produced an increased risk associated with the threonine allele (13). A subsequent study, also using samples from the Japanese population, showed a significantly decreased risk due to that same threonine allele (14). A meta-analysis, where these two studies and many others were analysed together, produced strong evidence that this SNP had no effect at all on susceptibility to Alzheimer's disease (15). In this and other cases, these conflicting results are usually due to small sample size limiting the power to detect small effects and enhancing the possibility of detecting false-positive results. When one genetic locus is analysed, samples sizes of thousands needed for genomewide association studies (GWAS) are not necessarily required. This is because GWAS tests many thousands of loci for association with the disease of interest, and because of the power lost due to multiple testing, very large sample sizes are required to detect weaker effects. Nevertheless, sample sizes of several hundred, at least, are needed to minimise false-positive rates, and independent replication in another cohort of the same, or larger, sample size allows a high degree of confidence in the result. Bentley has a respectable sample size (466 patients and 329 controls) but no replication (2), and Fellermann has replication, but small sample sizes (85 patients, 20
controls and 149 patients, 169 controls, ref 3). With this cohort size, any observations are at
best only suggestive, at worst merely anecdotal.

Accurately typing gene copy number is difficult using current technologies, certainly much
more difficult than the robust and reproducible single nucleotide polymorphism (SNP)
typing that is now commonplace. This is for two reasons, firstly the ubiquity and importance
of SNPs has been realised for some time, allowing sufficient time for new technologies to be
developed. Secondly, the inherent physical nature of SNPs allows more discrimination: there
is a qualitative chemical difference between a cytosine and an adenine at a particular
nucleotide position, whilst for CNPs there is only a qualitative difference - less or more of the
same DNA sequence. Quantitative real-time PCR assays, such as the assay used by both
Bentley and Fellermann, have been used extensively to robustly detect differences in mRNA
expression levels, levels that typically differ by several orders of magnitude. Yet for gene
copy number analysis using genomic DNA, these assays are required to distinguish four from
five copies, a relative increase of 0.25, which is a considerable challenge. Bentley and
colleagues produce lots of data using this approach, and tackle the issue of experimental error
rate, but even in their hands repeating the assay five times still produces a 5% error rate (2).

These two aspects of copy number association studies can confound a third source of error
which may lead to false positive associations. In an association study, we interpret a
difference in allele frequency (for a SNP) or copy number distribution (for a CNP) between
cases and controls as evidence of an influence of that particular polymorphism on disease.
But there may be other reasons for that difference: population stratification or a difference in
the physico-chemical properties of the DNA between cases and controls. An example of the
latter was given in a case-control SNP association study where case and controls produced
biased genotype data, despite their being tested blind to disease status, and randomly assorting cases and controls among experiments (16).

The contribution of Bentley et al. is valuable because it shows that the case for low $DEFB4$ gene copy number association with CD is doubtful. But we should be wary of concluding that the opposite is true; as the authors state: "A consensus view of the relationship of beta-defensin genomic copy number with CD will only be obtained through further association and expression studies in large phenotypically well-defined cohorts." (2) The cohorts should be as large as possible, and the methods used as reliable as possible in order to get the right answer, and to prevent time and money being spent on research with shaky foundations. Until the publication of the paper in this issue of AJG, the consensus was that the jury was out on the case of association of low copy number $DEFB4$ with CD. Now, the jury is back in the courtroom, and the verdict is "not proven".

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