. . . .

Using Integrative Genomics to Elucidate Genetic Resistance to Marek's Disease in Chickens

H. Cheng[1], M. Niikura[1,2], T. Kim[1,2], W. Mao[1,2], K.S. MacLea[1], H. Hunt[1], J. Dodgson[2], J. Burnside[3], R. Morgan[3], M. Ouyang[4,†], S. Lamont[5], J. Dekkers[5], J. Fulton[6], M. Soller[7], W. Muir[8]

[1] USDA, ARS, Avian Disease and Oncology Laboratory, East Lansing, MI, USA
[2] Department of Microbiology and Molecular Genetics, Michigan State University, East Lansing, MI, USA
[3] Department of Animal and Food Sciences, University of Delaware, Newark, DE, USA
[4] Informatics Institute, University of Medicine and Dentistry of New Jersey, Piscataway, NJ, USA
[5] Department of Animal Science and Center for Integrated Animal Genomics, Iowa State University, Ames, IA, USA
[6] Hy-Line International, Dallas Center, IA, USA
[7] Department of Genetics, The Hebrew University of Jerusalem, Jerusalem 91904, Israel
[8] Department of Animal Sciences, Purdue University, West Lafayette, IN, USA

Key words: genomics, QTL, DNA microarray, protein interactions, disease, virus, chicken

Abstract: While rearing birds in confinement and at high density are very successful practices for producing poultry meat and eggs, these conditions may promote the spread of infectious diseases. Consequently, the poultry industry places great emphasis on disease control measures, primarily at the animal husbandry level. The field of genomics offers great promise to complement these current control measures by providing information on the molecular basis for disease, disease resistance, and vaccinal immunity. This briefly summarizes some of our efforts to apply several genomic and functional genomics approaches to identify genes and pathways that confer genetic resistance to Marek's disease (MD), a herpesvirus-induced T cell lymphoma of chickens. By utilizing the "top-down" approach of QTL to identify genomics regions, and integrating it with "bottom-up" approaches of transcript profiling and Marek's disease virus (MDV)-chicken protein-protein interactions, three genes that confer resistance to MD are revealed, plus a number of other positional candidate genes of high confidence. These genes can be further evaluated in poultry breeding programmes to determine if they confer genetic resistance to MD. This integrative genomics strategy can be applied to other infectious diseases. The impact of the genome sequence and other technological advancements are also discussed.

INTRODUCTION

Poultry is the third largest agricultural commodity and the primary meat consumed in the US and much of the world. Due to advanced breeding programmes and efficient animal husbandry, tremendous progress in production traits has been made to meet the growing demands of consumers. Several major issues confront the poultry industry today. Infectious diseases are certainly at or near the top of the list, e.g., avian influenza, *Salmonella*, and others, which harm the poultry industry through loss of birds, reduced public confidence, and lost market accessibility via trade restrictions. Disease outbreaks are enhanced by more concentrated chicken rearing, reduced genetic diversity from industry consolidation, and breed uniformity to meet consumer preferences. Changes in animal husbandry and new vaccines have alleviated some of the problems. However, improved or alternative control measures are still needed to address current diseases and counter emerging threats.

Genomics offers one of the more exciting avenues for addressing these issues. The ultimate goal for genomics is to relate genetic variation at the DNA sequence level to variation at the phenotypic level. By identifying quantitative trait loci (QTL) and genes that control heritable traits of agricultural importance, it is possible to select for birds with superior agricultural traits via marker-assisted selection (MAS). Other positive attributes commonly cited for MAS include greater speed and accuracy compared to traditional breeding. For infectious diseases, MAS would reduce or eliminate the expensive and environmentally hazardous pathogen-exposure trials needed to collect accurate disease-resistance phenotypes. The recent release of the chicken genome sequence [1] has increased the power of genomics. This review shows how integration of genomic and functional genomic approaches can lead to the identification of genes and pathways that confer resistance to Marek's disease (MD), the most serious chronic disease problem facing the poultry industry.

MAREK'S DISEASE

Marek's disease (MD) is a lymphoproliferative disease of poultry caused by the Marek's disease virus (MDV). The pathological manifestations of MD include immunosuppression, nerve enlargement, and lymphomas that metastasize to various visceral organs. During the 1960s as the industry converted to high-intensity rearing, MD generated tremendous economic losses. One of the great success stories was the introduction of vaccines that effectively controlled MD in the 1970s. Since then, the condemnation rate attributed to MD has decreased dramatically. However, although vaccination prevents the formation of lymphoma and other MD symptoms, it does not prevent MDV infection, replication, or horizontal spread. Moreover, increasingly frequent unpredictable and spontaneous vaccine breaks due to highly virulent strains of the MDV combined with the incomplete immunity that is elicited by vaccination [2] cause devastating damage to poultry farms. Consequently, MD remains one of the most serious chronic infectious disease threats to the poultry industry.

MDV is a herpesvirus that has lymphotropic properties similar to those of γ-herpesviruses, such as Epstein–Barr virus (EBV) in humans, where the host range is restricted and latency is often evident in lymphoid tissue. Viruses of this group are capable of transforming cells in natural hosts. MDV was re-classified, however, because its molecular structure and genomic organization more closely resembles

that of α-herpesviruses, such as herpes simplex virus (HSV) and Varicella-Zoster virus (VZV). The complete sequence of several MDV strains has been determined [2].

Genetic resistance

The best documented case for involvement of genetic resistance to MD involves the MHC or, as it is known in the chicken, the B complex. By measuring the frequency of specific blood groups or using B congenic chickens, it has been observed that certain B alleles can be associated with resistance or susceptibility. The B-haplotype also influences vaccinal immunity as some haplotypes develop better protection with vaccines of one serotype than of a different serotype. As shown by several studies, the B haplotype effect is dependent on genetic background. Other studies show that genes, other than the B complex contribute to MD resistance. In particular, in 1939, the Agricultural Research Service (ARS) laboratory in East Lansing, MI, initiated the development of inbred lines to study what was then known as the avian leukosis complex. Of the fifteen lines initially developed, lines 6 and 7 proved to be the most interesting with respect to MD. These lines share the same B^2 haplotype yet differ greatly with respect to resistance to MD. When line 6 chicks are inoculated with the JM strain of MDV at 1 day of age, typically less than 5% of the chicks will succumb to the disease. In contrast, similar inoculations into line 7 chicks will result in greater than 90% mortality. These lines are still maintained and are over 99% inbred. The level of disease resistance as measured by mortality among F1 siblings of a cross between lines 6 and 7 is intermediate to that of the parents (~60%), while levels of resistance observed in the F_2 population derived from the above cross encompass a wide, continuous spectrum indicating that there is more than one gene for resistance involved. Thus, analysis of these lines and their crosses indicate that MD resistance is a typical polygenic trait, controlled by multiple genes.

INTEGRATING GENOMICS

The presence of genetic variation in MD resistance raises the possibility of selection for genetic resistance as a complementary control strategy to augment MD vaccinal protection. Also, as noted above, MD resistant birds have the added advantage that, in general, they respond better to MD vaccines. Ideally, selection for MD resistance would be based on genetic markers within disease resistance genes or tightly linked to them. The method is known as marker-assisted selection (MAS), and is expected to accelerate genetic progress by increasing the accuracy of selection and allowing for gender-independent selection at an earlier age. Furthermore, the use of MAS for developing disease resistance avoids the necessity of challenging breeding stock with hazardous pathogens.

With this goal in mind, we have been implementing and integrating three genomic approaches to identify the genes that are involved in genetic variation in MD resistance: genome wide QTL scans, gene profiling, and virus-host protein-protein interaction screens. The rationale for using more than one approach is that the strengths of each system can be combined to yield results of higher confidence. Another justification is that given the large volume of data produced by genomics, each method provides an additional screen to limit the number of targets to verify and characterize in further experiments. The approaches are briefly described below.

Genome-wide QTL scans

With the development of a molecular genetic map of the chicken genome [3], it became possible through linkage mapping to identify QTLs (i.e., chromosomal regions containing genes or other genomic elements) controlling complex traits, including disease resistance. Our efforts have utilized resource populations based on experimental inbred lines [4,5] and commercial strains [6]. The use of the experimental lines allows for more precisely controlled environmental conditions and trait measurements while the use of commercial strains is more agriculturally relevant and permits many more birds to be produced and evaluated. In all populations, many QTLs of small to moderate effect were identified, which agrees well with earlier conclusions as to the multigenic nature of MD resistance. Some of the identified QTL locations are common across experimental and commercial populations, providing additional confidence in these QTLs and suggests that results from experimental populations can be transferred to commercial lines [6]. It should be noted, however, that all experiments to date have been implemented in White Leghorn lines, which have a known narrow genetic base and consequently, a high degree of interrelatedness. It is not known to what extent these QTLs are also responsible for genetic variation in MD resistance in broiler lines.

Genome-wide scans for QTLs conferring MD resistance have been conducted on crosses between ADOL lines 6 (MD resistant) and 7 (MD susceptible). Specifically, in a 6 x 7 F2 population genotyped with 127 genetic markers, 14 QTLs (7 significant and 7 suggestive) were identified that explain one or more MD-associated trait. The QTLs were of small to moderate effect explaining 2 to 10% of the phenotypic variance, with standardized additive gene substitution effects from 0.01 to 1.05 phenotypic standard deviations. Collectively, the QTLs explained up to 75% of the genetic variance. Interestingly, 10 of the 14 QTLs displayed non-additive gene action: 3 were over-dominant and 7 were recessive. Theoretically, MAS would be useful in exploiting non-additive QTLs. Three of the QTLs were associated almost exclusively with viremia levels while the remaining QTLs could account for disease incidence, survival, tumours, nerve enlargement, and other disease associated traits. This suggests that disease resistance occurs at least at two levels: initial viral replication and cellular transformation, which occurs later. It also highlights the added value of measuring several components as this may functionally separate a complex trait into more highly heritable physiological components, as well as provide clues for identifying positional candidate genes.

More recently, with the advent of the genome sequence, millions of high-confidence SNPs [7], and high-throughput genotyping systems, it became possible to genotype large resource populations very quickly and economically. Using the Illumina platform, we were able to re-evaluate the 6 x 7 F2 MD line resource population with 578 additional genetic markers [8]. Most interestingly, partly due to the higher accuracy of calling genotypes with SNPs, a search for two-locus epistatic interactions was conducted. A large number of highly significant two-way interactions were found that could account for viremia; no highly significant interactions were revealed for MD or survival. A total of 239 highly marker-to-marker significant interactions (LRS >57.8; genome-wide P <0.001) were identified [8]. The location of loci and their interacting partners appears to be distributed throughout the genome. On the other hand, loci in specific regions on chromosomes 1 and 4 are frequently involved; a single region on chromosome 1 accounted for 166 of the 239 highly significant interactions. Interestingly, most if not all of the interacting loci are not in the QTL regions previously identified.

These results imply that (1) interactions between loci can make a substantial contribution to variation in complex traits, (2) because of the growing awareness of biological complexity, genome-wide QTL scans should attempt to incorporate resource populations for which the genotyping and analytical capabilities available today or in the near future can be used, and (3) the challenge remains to identify the underlying genes and genetic variation for QTL as the confidence intervals are fairly large and encompass many genes.

Gene profiling

The population sizes and structures that have been used for mapping QTLs affecting MD resistance can only map the causative gene or genetic element to rather broad chromosomal regions extending for tens of centimorgans and containing many hundreds of genes. For this reason, it is clear that it will be extremely difficult to identify positional candidate genes for MD resistance using approaches based on general functional annotation and positional genetics only. Gene expression profiling using microarray hybridization technology is a powerful tool for gene function studies. Our hope is that DNA microarrays will identify genes and pathways specifically involved in MD resistance, which combined with linkage mapping and functional annotation of the genome can narrow the field of positional candidate genes [9]. In other words, positional candidate genes are those that have a genetic association, are functionally relevant, and are identified as being relevant to MD resistance through gene expression analyses.

Gene profiling has been conducted to identify differentially expressed genes between lines 6 and 7 after MDV challenge [9] among B (MHC) congenic lines of chickens following inoculation with different MD vaccines, and in chicken embryo fibroblasts (CEF) infected with MDV. Analyses of these experiments have identified a number of genes and pathways that are consistently associated with either MD resistance or MDV infection. Remarkably, the results suggest that chickens with immune systems that are more stimulated by MDV infection are more susceptible. Initially, this seems counterintuitive but upon further reflection, MDV is thought to only infect activated lymphocytes. Thus, chickens with immune systems that are more responsive may present more targets for MDV to infect and later transform.

Although DNA microarray experiments can be very powerful and the results enlightening, our experiences also suggest that it is critical to study the response of cells in a limited or local environment. For example, MHC class I expression was increased in MDV-infected CEF [10]. However, upon closer examination of individual cells, virus-infected cells actually expressed reduced levels of MHC on their cell surfaces but neighbouring uninfected cells had elevated levels of MHC class I. To partially circumvent the problem of heterogeneous response of individual components of samples, laser capture microdissection (LCM) and cell sorting has been used to isolate the cells of interest.

Virus-host protein-protein interaction screens

The third component of this approach to identifying the genes associated with MD resistance is to systematically examine protein interactions associated with MD resistance. Specifically, a two-hybrid screen has been used to detect MDV-chicken protein-protein interactions. Our hypothesis was that some chicken proteins that

interact with MDV proteins are involved in the immune response and genetic resistance to MD. Thus, the two-hybrid system could be used to quickly identify interacting proteins which, when combined with genetic mapping, would identify positional candidate genes for MD resistance.

The MDV *SORF2* gene was initially chosen as bait due to its potential role in the attenuation of the MDV RM1 strain [11]. Using the yeast two-hybrid system and a splenic cDNA library, growth hormone (GH) was found to specifically interact with SORF2 [11]. To corroborate the detected interaction, an *in vitro* protein binding assay using GST-fusion proteins was performed to confirm direct binding of GH to SORF2. The results show that, while SORF2 protein was not retained by GST protein alone, SORF2 can be retained by GST-GH fusion protein, presumably due to the presence of GH. This result is in agreement with the result of the yeast two-hybrid system assay and indicates that the interaction between SORF2 and GH is a direct and specific protein-protein interaction.

Based on these results, the GH gene (*GH1*) was treated as a candidate gene for MD resistance. To determine if *GH1* contributed to the genetic basis of MD resistance, an association study involving an SNP in GH1 was conducted in an MD resource population derived from commercial White Leghorn lines. Indeed, it was found that *GH1* allelic variation was significantly associated (P <0.01) with a number of MD-associated traits in MHC B^2/B^{15} chicks [11]. Furthermore, providing functional information support, DNA microarray results indicate that GH is differentially expressed between MD resistant (line 6) and susceptible (line 7) chicks after MDV challenge [9].

Thus, the combined results of a specific MDV-chicken protein interaction, differential expression of GH between MD resistant and susceptible chickens, and association of *GH1* allelic variation with MD-related traits and selected lines for MD resistance, all strongly suggest that *GH1* is a MD resistance gene. This conclusion is supported by reports demonstrating that GH modulates the immune system in many species, and frequency of *GH1* alleles differs in the expected direction in chicken strains in response to selection for MD resistance. Most importantly, it exemplifies the power of combining genetic and molecular approaches to identify positional candidate genes for QTL.

With the complete sequence of the MDV genome, it became feasible to conduct a systematic screen of the relevant MDV genes for interacting chicken partners. All the MDV genes that are considered unique to serotype I (virulent) strains were screened, and all potential MDV-host protein interactions were tested by an *in vitro* binding assay to confirm the initial two-hybrid results. As a result, 8 new MDV-chicken protein interactions were identified [12]. More importantly, genetic mapping and association analyses of the encoding chicken genes revealed that *LY6E* [lymphocyte complex 6, locus E; also known as stem cell antigen 2 (*SCA2*) and thymic shared antigen 1 (*TSA1*)] is another MD resistance gene [13] and further suggest that *BLB*, the gene for MHC class II β chain, is a strong positional candidate gene [12].

Higher order protein-protein interactions can be revealed with gentle cell lysis and immunoprecipitation of a protein complex using antibodies directed against one member of the complex. The identity of the other interacting proteins can be quickly revealed through mass spectrometry, which is now facilitated by the availability of the whole genome sequence. To extend the MDV-chicken protein interactions, we are employing tandem affinity purification (TAP) tag to several chicken-MDV protein complexes. Rather than expressing the viral gene product alone, we plan to take advantage of our infectious MDV BAC clones [14] and the ability to make defined

recombinant viruses. The use of these viruses expressing TAP-tagged MDV proteins during infection allows for the use of a system closer to normal viral infection, which will hopefully yield useful information on the biology of viral infection and the role of host-virus interactions in that context.

CONCLUSION

It has been over 30 years since MDV was identified as the causative agent of MD, and effective vaccines produced. In spite of our ability to detect and protect against MD, there is a surprising lack of information on the components of the chicken immune system that confer disease resistance and contribute to vaccinal immunity. With the advent of new technologies, we believe that our ability to tangibly improve upon MD control mechanisms is at hand. The key to making rapid gains will be the ability to integrate various methods and information. If this occurs, then we should be able to accelerate the transition from serendipity to rational, mechanism-based control of disease and food production in the poultry industry.

ACKNOWLEDGEMENTS

REFERENCES

1   Hillier LW, Miller W, Birney E, Warren W, Hardison RC, Ponting CP, et al: Sequence and comparative analysis of the chicken genome provide unique perspectives on vertebrate evolution. Nature 2004;432:695-716.

2   Osterrieder N, Kamil JP, Schumacher D, Tischer BK, Trapp S: Marek's disease virus: from miasma to model. Nature Rev Micro 2006;4:283-294.

3   Groenen MAM, Cheng HH, Bumstead N, Benkel BF, Briles WE, Burke T, et al: A consensus linkage map of the chicken genome. Genome Res 2000;10:137-147.

4   Vallejo RL, Bacon LD, Liu HC, Witter RL, Groenen MAM, Hillel J, Cheng HH: Genetic mapping of quantitative trait loci affecting susceptibility to Marek's disease virus induced tumors in F2 intercross chickens. Genetics 1998;148:349-360.

5   Yonash N, Bacon LD, Witter RL, Cheng HH: High resolution mapping and identification of new quantitative trait loci (QTL) affecting susceptibility to Marek's disease. Anim Genet 1999;30:126-135.

6   Heifetz EM, Fulton JE, O'Sullivan NP, Arthur JA, Wang J, Dekkers JCM Soller M: Mapping QTL affecting susceptibility to Marek's disease in a backcross population of layer chickens. Genetics (in press).

7   Wong GK, Liu B, Wang J, Zhang Y, Yang X, Zhang Z, et al: A genetic variation map for chicken with 2.8 million single-nucleotide polymorphisms. Nature 2004; 432:717-722.

8   Cheng HH, Zhang Y, Muir WM: Evidence for widespread epistatic interactions influencing Marek's disease virus viremia levels in chicken. Cytogen Genome Res 2007;117:313-318.

9   Liu HC, Cheng HH, Sofer L, Burnside J: A strategy to identify positional candidate genes conferring Marek's disease resistant by integrating DNA microarrays and genetic mapping. Anim Genet 2001;32:351-359.

10   Morgan RW, Sofer L, Anderson AS, Bernberg EL, Cui J, Burnside J: Induction of host gene expression following infection of chicken embryo fibroblasts with oncogenic Marek's disease virus. J Virol 2001;75:533-539.

11 Liu HC, Kung HJ, Fulton JE, Morgan RW, Cheng HH: Growth hormone interacts with the Marek's disease virus SORF2 protein and is associated with disease resistance in chicken. Proc Natl Acad Sci USA 2001;98:9203-9208.

12 Niikura M, Liu HC, Dodgson JB, Cheng HH: A comprehensive screen for chicken proteins that interact with proteins unique to virulent strains of Marek's disease virus. Poultry Sci 2004;83:1117-1123.

13 Liu HC, Niikura M, Fulton J, Cheng HH: Identification of chicken stem lymphocyte antigen 6 complex, locus E (*LY6E*, alias *SCA2*) as a putative Marek's disease resistance gene via a virus-host protein interaction screen. Cytogen Genome Res 2003;102:304-308.

14 Niikura M, Dodgson JB, Cheng HH: Direct evidence of host genome acquisition by the alphaherpesvirus Marek's disease virus. Arch Virol 2006;151:537-549.

Dr. Hans H. Cheng, USDA, ARS, Avian Disease and Oncology Laboratory, East Lansing, MI 48823, USA.
E-mail: Hans.Cheng@ars.usda.gov