Development of risk profiles for systemic lupus erythematosus

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Early diagnosis of autoimmune disease is a goal of ongoing research in multiple areas. Systemic lupus erythematosus (SLE) is a multisystem autoimmune disease that causes significant morbidity and premature mortality. Early diagnosis of lupus prior to the onset of major disease manifestations is currently not reliable, due in large part to the limitations of available tests. Emerging knowledge in human genetics and immunology, assisted by computer-aided bioinformatics analyses, has the potential to contribute significantly to the development of more accurate diagnostic approaches. The identification of patients in the early stages of disease, or even prior to the onset of any clinically detectable abnormalities, would permit the institution of treatments that have the potential to reverse or prevent organ damage. Potentially useful approaches to detect SLE risk include proteomic detection of autoantibodies, patterns of gene expression by cells in the peripheral blood and genetic polymorphisms detected by single nucleotide changes. While these are being investigated as individual approaches, the greatest strength may lie in the eventual combination of these nonoverlapping measures to develop a profile that can translate to a quantification of lupus risk.

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease that can cause irreversible damage to many organ systems including skin, joints, heart, lungs, kidneys and brain. Children and young adults less than 40 years of age can be affected and this demographic pattern means that even though the disease is relatively rare, affecting approximately one in 2000 persons, economic and social impacts are significant. Many studies indicate that there is a strong genetic component to lupus risk, but the genes involved are probably numerous and profiling of genetic risk remains elusive. Current approaches to diagnosis are based on a set of criteria developed over 25 years ago that include both clinical features and laboratory findings [1]. Among the latter are autoantibodies including antinuclear antibodies (ANAs) as well as antibodies to dsDNA, the Sm antigen and cardiolipin, which are associated with the disease. More than 98% of lupus patients are positive for ANA, making this an almost essential component of the diagnosis. What complicates accurate disease assessment, however, is the fact that the ANA test is very nonspecific. It can be positive in many other autoimmune disorders as well as more than 5% of normal unaffected individuals. Owing to these issues, it remains difficult to establish diagnosis in early disease stages, since the laboratory tests are not specific and the clinical criteria require the presence of abnormalities that may already be in irreversible stages at the time of the first medical evaluation.

Approaches to the early detection of autoimmune disease are being developed using data emerging from studies in genetics and immunology along with techniques for measurement of individual variations in large numbers of biomolecules [2–4]. One long-term goal of these investigations is to permit development of accurate risk profiling in persons who are in the early stages of disease, when treatments are more likely to have curative potential and perhaps even to predict disease in unaffected individuals who are in high-risk demographic categories. A model for this approach is that for defining risk in cardiovascular disease, where a combination of laboratory values (lipid profiles), genetic factors (family history), comorbid conditions (hypertension) and physical features (obesity) can predict with reasonable certainty the risk of significant life-threatening events. Three components of the potentially useful profile that may be developed for lupus risk are considered here. These are levels of autoantibodies, gene expression profiles in peripheral blood cells and genetic markers.

Autoantibodies: proteome arrays

Autoantibodies as well as other clinical criteria may precede the development of SLE and may be useful predictors of disease [5]. One of the most compelling studies demonstrating this was carried out using the Department of Defense Serum Repository, which demonstrated that 88% of stored serum samples collected from lupus patients prior

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to diagnosis had at least one SLE autoantibody [6]. Furthermore, the number of autoantibodies present in serum samples demonstrated a progressive increase up to the time of the actual diagnosis of lupus, consistent with increasing epitopic complexity of the autoantibody specificities. Similar findings have also been described in other autoimmune disorders, including rheumatoid arthritis and Type I diabetes [7–10].

However, the measurement of ANAs in an individual has low predictive value for lupus since this test can be positive in many other diseases, as well as in healthy control subjects. We have recently shown, for example, that the prevalence of ANA positivity measured by ELISA in an unselected population drawn from Dallas County, TX, was 27%, using the cutoff for positivity defined by the assay manufacturer; ANA values greater than two standard deviations over the population mean were observed in 2.5% of the subjects [11]. On the other hand, serologic markers that are highly specific for SLE and that correlate with disease activity, including complement proteins (C3 and C4) and antibodies to dsDNA, are not sensitive enough to detect preclinical disease.

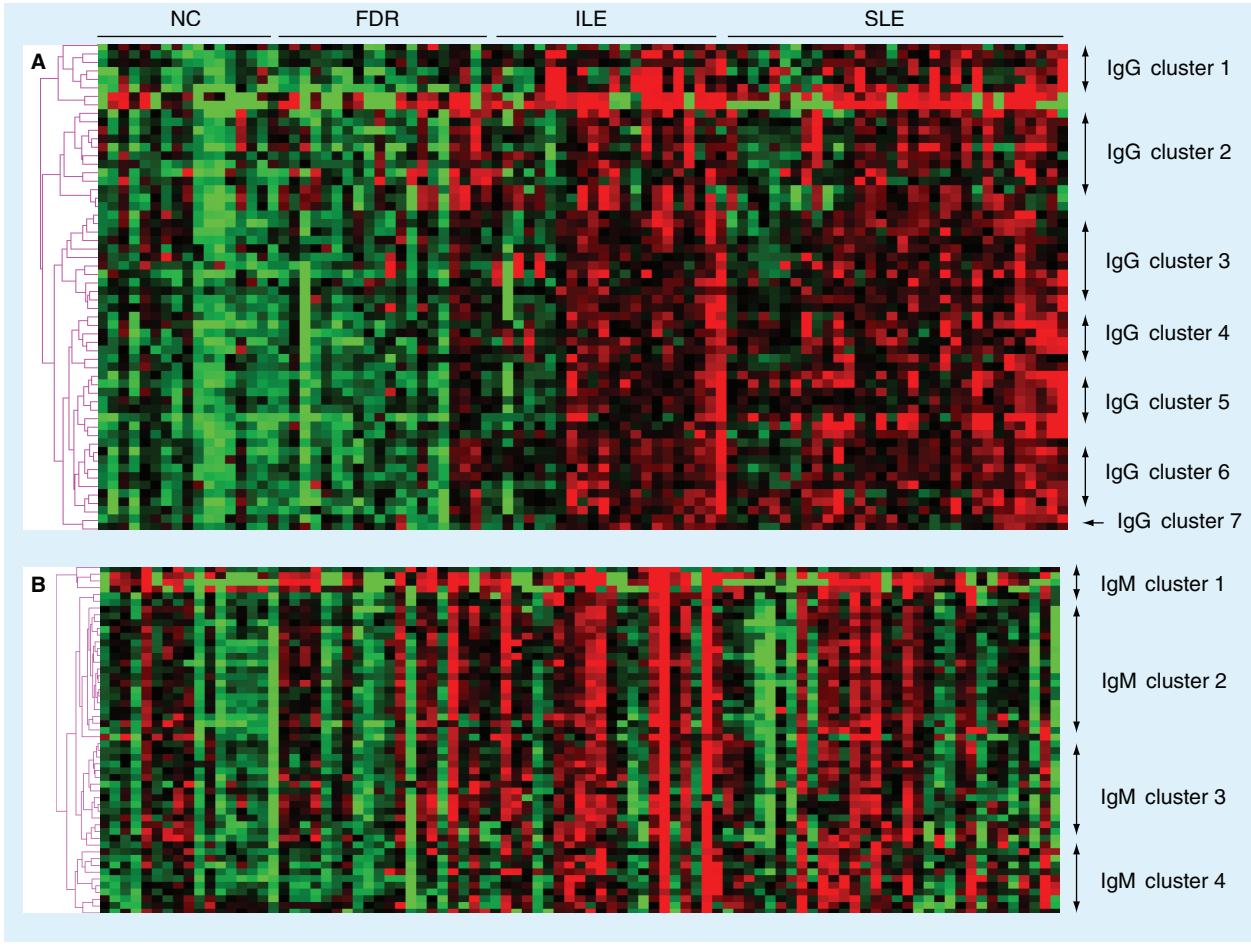
In clinical practice, panels for detection of multiple autoantibodies in a single serum sample are commonly used to assist in evaluation and classification of individuals with suspected autoimmune disease. However, even these multiplex assays, using techniques such as bead assays or solid phase strips [12] to measure 8–12 specificities, detect only a small portion of the autoantibody burden in lupus, which has been estimated to include at least 100 distinct reactivities [13]. To get a broader picture of autoreactivity, we and others have utilized arrays of autoantigens, an approach pioneered by Utz, Robinson and colleagues [14]. This technique requires only microliter volumes of serum, which can be readily collected and stored from patients and unaffected individuals. Furthermore, insights into the relatedness of seemingly unrelated specificities can be developed using the same types of computer-based clustering algorithms that have been applied to studies of gene expression. In addition, the arrays are flexible and can be easily expanded or modified to incorporate new autoantigens reported in the published literature or customized for panels to study specific disease subsets.

Investigators in our group have shown that this approach can be used to detect the autoantibody clusters that best predict lupus activity [15]. In these studies, glomerular protein arrays revealed distinct clusters of IgG autoantibodies in the sera of lupus patients. Two of these clusters demonstrated association with disease activity. One was characterized by antibodies to DNA-related proteins, such as chromatin, and the other included structural or basement membrane proteins, such as myosin, laminin and vimentin. This approach thus revealed some specificities in the DNA-related group that have been known to be present with active disease, which validated the approach, while at the same time detecting novel autoantibodies that may be important in the pathogenesis of organ damaging lupus.

We have more recently reported the application of this approach to characterize patients with SLE and incomplete lupus (ILE), defined as individuals with less than four diagnostic criteria [16]. Advantages of studying ILE patients include the possibility that a subset is in the early stages of SLE and the lower prevalence of immunosuppressive medication use. Results in these two groups of patients were compared with unaffected first-degree relatives (FDR) and unrelated nonautoimmune control (NC) individuals. The 93 serum samples were applied to a 70-antigen protein array and detection for IgG and IgM specificities was carried out. After eliminating those specificities that did not react in any of the samples, 58 IgG and 50 IgM autoantibodies were detected and subjected to clustering analysis (**Figure 1**). While the overall degree of autoreactivity was similar in the ILE and SLE groups, further analysis demonstrated that IgG antibodies predominated in SLE while in ILE the reactivity was primarily in the IgM class. These results are consistent with the hypothesis that IgM autoantibodies are less pathogenic than IgG autoantibodies, and may even be protective [17]. Whether these results indicate that ILE patients are in transition to SLE or that ILE tends be a more stable form of lupus with less organ damage will require further longitudinal studies.

Correlation analyses were carried out in the ILE and SLE groups to compare the IgG autoantibody reactivity level within each cluster with the number of ACR diagnostic criteria fulfilled. IgG clusters four (largely containing DNA-related antigens) and five (U1-snRNP-related cluster) demonstrated positive correlations with the number of lupus criteria (R values of 0.50 and 0.52, respectively; $p < 0.001$), while cluster three (collagens) was weakly correlated with these criteria ($R = 0.28$; $p = 0.03$). Other analyses demonstrated that samples from SLE patients with renal disease had

Figure 1. Autoantibody reactivities in serum samples.



(A) Heat map of 58 IgG autoantibody reactivities in serum samples.Normalization was to average intensity of total IgG as detected by six replicates within the array. For each antigen, reactivity intensities are colored red for those above the mean of all samples, green for those below and black for those that are close to the mean. Reactivities clustering together are shown by the left margin dendrogram. **(B)** Heat map for 50 IgM autoantibodies in the serum samples. Most of the array antigens were proteins obtained from commercial sources; additional information is available in the previous publication as well as directly from the authors.
FDR: First-degree relative; ILE: Incomplete lupus; NC: Nonautoimmune control; SLE: Systemic lupus erythematosus.
Adapted with permission from [9].

significantly higher IgG reactivities than those without renal involvement for three of the autoantibody clusters, namely those related to collagen (including collagens I, II and III; $p = 0.0016$), DNA (including ssDNA, dsDNA and chromatin; $p = 0.0014$) and histones (including histones three and four and total histones; $p = 0.0092$).

These data illustrate how an autoantigen or protein array can be used to develop a profile that greatly enhances potential insights into the nature of individual autoreactivity including both qualitative and quantitative features. The findings demonstrate clinical correlations, point to novel specificities and suggest designs for longitudinal studies to assess development of risk for individuals and for groups.

Autoantibodies: peptoid arrays

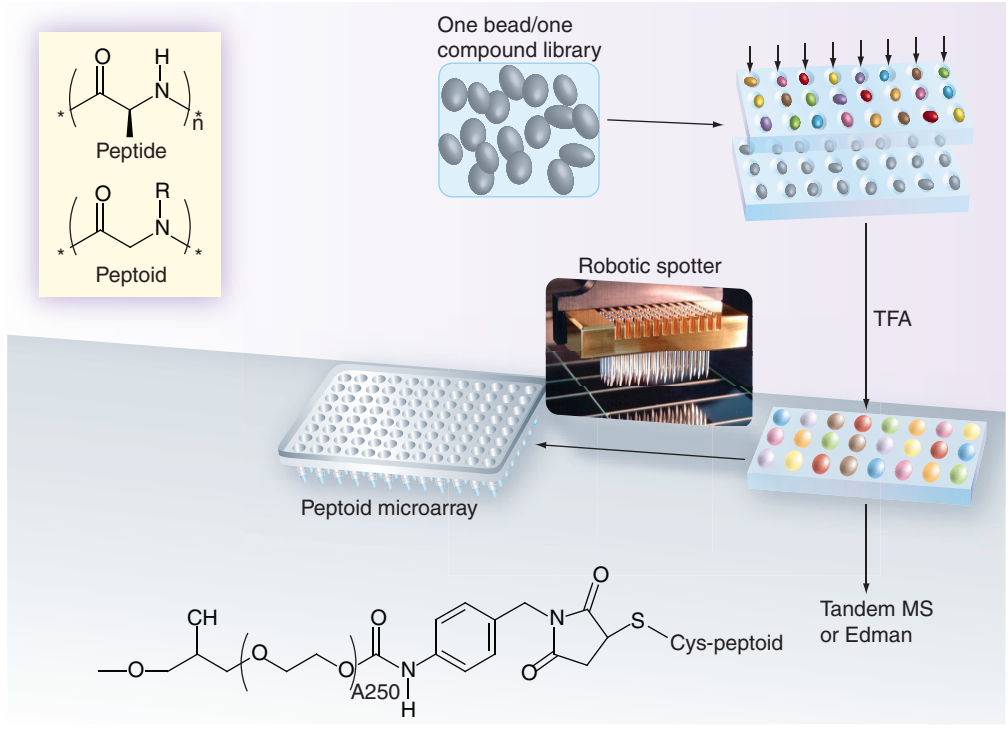
One of the limitations of the proteome array is that the number of antigens, while large, is still relatively small compared with the probable number of potential autoantigens. Furthermore, these studies provide a somewhat biased view of the immune response because the autoantigens on the slides are chosen based on things that are already known. An alternative approach is to use large numbers of randomly synthesized peptides as antigens. In theory, any linear antibody epitope can be represented on an array of sufficient complexity. A variation of this technique has been developed by Thomas Kodadek, in the Division of Translational Research of the Department of Internal Medicine at UT Southwestern Medical

Center, TX, USA [18]. This method uses 'peptoids' as targets for protein binding. Instead of using naturally occurring α amino acids to create short oligomers, *N*-substituted glycines are used. These peptoids are similar enough to natural peptides to be recognized as ligands for protein binding while exhibiting resistance to proteases. In addition to being unbiased, the total number of epitopes that can be detected in a 1 µl serum sample is greatly expanded. A schematic of the peptoid microarrary methodology is shown in **Figure 2**. Preliminary studies with human serum samples suggest that patients with SLE have a large number of binding specificities compared with rheumatoid arthritis sufferers, a finding that is consistent with the known prevalence of autoreactivity in these two conditions. As this approach is refined, it is likely that sets of peptoids will be identified that can reliably discriminate clinical subgroups of individuals. Further characterization of the nature of these autoantibodies might then be carried out by using the peptoids for affinity purification of the targeted autoantibodies from serum. Whether the peptoid approach turns out to be useful in SLE remains to be determined. Other candidate systems, such as those based on aptamers, also show promise for identifying novel elements of the human proteome in health and disease [19].

Gene expression

Identification of patterns of gene expression in peripheral blood is another profiling approach for detecting early lupus changes. In previous studies, we have shown that a signature present in patients with autoimmune diseases including SLE is also found in their unaffected FDRs, suggesting that the liability for development of autoimmune disease is inherited as a genetic trait [20]. These data are compatible with long-standing observations in families suggesting that autoimmune disease is transmitted as a dominant trait with a variable penetrance that probably depends on other genes or on nongenetic factors such as pathogen exposure or hormonal milieu [21]. Other signatures characteristic of SLE have been described, including upregulation of a set of genes that are responsive to stimulation by

Figure 2. Schematic of peptoid microarrary.

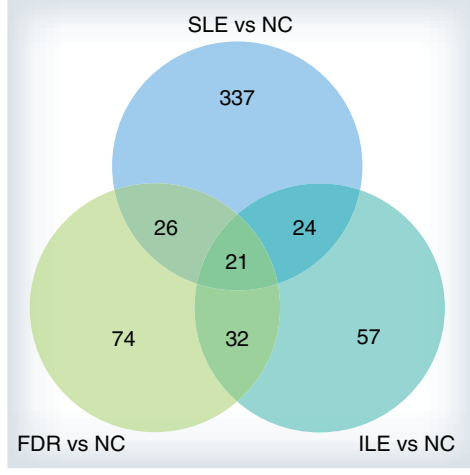


Random peptoid octamers are created by combinatorial chemistry on beads which are then distributed into 384 well plates. The peptoids are cleaved from the beads with TFA and can be identified with tandem mass spectrometry. Replicate slides are then generated, samples are added and processing is carried out for data collection and analysis. **Inset**: Comparison of peptides to peptoids. R = a variety of side groups.
TFA: Trifluoroacetic acid.

interferon [22]. Correlations between the type I interferon signature and disease activity in lupus have been described by our group and others [23–25]. However, these patterns that are associated with active disease might not be sensitive to detecting early changes, when immune and inflammatory alterations are likely to be at lower levels.

We have carried out preliminary studies to look at gene expression correlations with ILE syndromes as a window into early stages of disease. These studies have become feasible for investigations in clinical settings due to the development of blood collection tubes that stabilize RNA for later isolation and analysis. Our initial examinations have used the Illumina® 48K gene platform. Application of computer-based algorithms to the data permits detection of gene profiles for the various subsets of subjects. When each disease-related group is compared with NCs, a set of dysregulated genes can be readily identified. The size of this set is greater for the comparison of SLE to NC than for the ILE or FDR groups (**Figure 3**). The data suggest that a relatively small number of genes might serve to identify each of the subsets and that other insights could be developed. For example, examination of genes that are dysregulated in FDRs as well as in SLE patients might point to pathways involved in liability for autoimmune disease. Genes that are differentially expressed in both ILE and SLE may be components of early disease changes. Other preliminary analyses of these data suggest that at least a portion of the IFN-related signature, including some genes that are involved in host defense against viruses, are upregulated in a subset of the ILE patients. These patients tend to have greater numbers of SLE-related autoantibodies as well, suggesting a greater disease burden. Longitudinal studies have not been carried out and will be needed to determine the clinical consequences of these gene differences, and to identify those that have value for prediction of disease risk.

Figure 3. Genes with differential expression of at least twofold ($p < 0.01$) in three subject groups.



FDR: First-degree relative (n = 20); ILE: Incomplete lupus (n = 30); NC: Nonautoimmune controls (n = 13); SLE: Systemic lupus (n = 21).

Once the gene expression data can be reduced to a handful of over- or under-expressed specificities, it becomes feasible to sort disease-related subsets using real-time PCR techniques. In patients with another autoimmune disease, multiple sclerosis, this type of analysis has been carried out with a mathematical approach to identify four different genes with the highest discriminatory capacity. In comparing multiple sclerosis patients with unaffected controls and other autoimmune diseases, the derived equation with these four genes had a sensitivity of at least 90% and a specificity of 95% or greater [26]. Similar approaches to designing gene equations for SLE and ILE are being developed.

Genetics

SLE has a strong genetic component and epidemiologic data indicate that FDRs of lupus patients are at significantly elevated risk of disease [27]. This means that family history itself will contribute to assessment of risk stratification, just as it does in cardiovascular disease. Nevertheless, the exact genes that contribute to this risk remain elusive due to the polygenic nature of the disease. Unlike Type 1 diabetes or multiple sclerosis, SLE genetics is not dominated by the powerful effect of a single locus. Instead, there are at least 17 confirmed and published linkages [28]. These regions most likely contain genes that are associated with SLE, and 12 such candidate susceptibility genes outside the HLA locus have been identified (**Table 1**). Current approaches to look for genetic variability related to disease involve the search for single nucleotide polymorphisms (SNPs), which represent the most common genetic variation in the human genome. A single SNP itself is not highly polymorphic because only one of two choices is available at any individual loci. Power in the analysis therefore comes not from the SNP itself, but from the frequent occurrence of SNPs throughout the genome. These

Table 1. Confirmed genetic associations in human systemic lupus erythematosus outside of the HLA locus.

Gene	Location	Odds ratio	Populations
*C1*Q0*	1p36	10	European, Asian, Spanish
CTLA4, +49G	2q33.2	1.3	European, Asian
FcγRIIA, R131	1q23	1.3	African–American, European
FcγRIIIA, F176	1q23	1.6	European, Korean
FCRL3	1q23	1.3	Asian, Spanish
IL-10	1q32	1.4	European, Asian
IRF5	7q32	1.6	Nordic, European, Hispanic
MBL	10q22	1.4	European, Asian, African–American
Osteopontin	4q22	1.6–2.3	European
PDCD1	2q37	1.38	Meta-analysis
PTPN22	1p13	1.6	European
TYK2	19p13	1.6	Nordic

Taken from [19].

analyses require platforms, such as those that have been developed by Affymetrix and Illumina, which allow hundreds of thousands of SNPs to be assayed at once. Many of the SNPs that are sought in candidate genes are probably markers of non-functional polymorphisms without major consequence in the expression of the gene or its function. These types of SNPS may be in linkage disequilibrium with the functional variation that represents the 'true mutation'. But a SNP itself can cause differences in gene expression, splicing variations and/or coding variation, whether it leads to an amino acid change or not. The current status of genotyping for SLE is at the level of candidate genes, and identification and validation of genetic associations will be needed before this information can be added to a risk profile.

An example of a candidate gene identified through this approach is interferon regulatory factor (IRF)5 [29]. An association between IRF alleles coding for different isoforms of the associated protein and SLE has been described in several cohorts, including patients from northern Europe and Latin America. This gene is involved in the type I interferon signaling pathway and it has been postulated that altered isoforms with changes in protein stability or binding could alter signaling effects on interferon genes. These genetics studies require large datasets of cases and appropriate controls, in part due to the need to control for ethnic and racial factors that demonstrate correlations with genetic differences. A consequence of the need for large sample sizes has been the development of consortiums of investigators who are willing to pool samples, and the IRF5 study is an example of this approach.

New data are accumulating at accelerating pace, using technologies that are now within the reach of a larger number of investigators. Hopefully this means that identified loci can be entered into risk calculations in the not-too-distant future.

Conclusion

The most useful risk profiles for SLE are likely to be assembled from different kinds of measured parameters. One potential application will be to examine FDRs who are already known to be at increased risk for development of SLE. The FDRs who are found to have high-risk genotypes could be further examined for protein and gene expression patterns that demonstrate activation of SLE-related pathways. Careful longitudinal evaluation of these individuals would be advisable. It is plausible to anticipate that treatment of these high-risk subjects with drugs such as hydroxychloroquine would ameliorate, cure or even prevent the disease [30].

Future perspective

It is likely that the next 5–10 years will see an increase in the attention paid to early SLE, similar to that which has been given to early RA. This might include early SLE clinics in which risk can be assessed. The profiling applied will most likely incorporate both patient-derived information, including family history and environmental exposures, as well as laboratory data

Executive summary

Currently available tests do not reliably detect lupus in early stages

- Early detection is an important goal that is likely to improve treatment outcomes.
- Antinuclear antibody testing is sensitive but lacks specificity for disease.

Protein arrays offer many advantages for developing insights into lupus-related autoantibodies

- A total of 100 or more specificities can be detected using microliter volumes of serum.
- Clustering algorithms can suggest relatedness of seemingly different autoantibodies.
- Identification of novel epitopes is feasible.
- The related technique of peptoid arrays expands further the scope of detected serologic markers.

Peripheral blood gene expression is a complementary approach with potential for detecting early lupus

- Dysregulated genes are present in first-degree relatives, suggesting autoimmune liability independent of disease expression.
- A relatively small number of genes may define disease-specific pathways.

Lupus has a strong genetic component

- Multiple gene loci are probably involved in susceptibility.
- Over 17 loci have been identified but the responsible genes remain largely unknown.
- Technologies for identification of large numbers of single nucleotide polymorphisms are increasingly available.
- Large databases pooled from collaborating investigators are being assembled to find the significant genetic associations.

from genotyping and gene expression analyses. Proteome analysis techniques are progressing rapidly and it is likely that this as well as gene expression analysis will become available to greatly expand clinical laboratory testing. Identification of SLE genes and pathways will permit development of new and more specific therapies. If these are of low risk, then interventions in preclinical patients to achieve disease prevention has the potential to become a reality. All of these advances are likely to give SLE patients a more complete understanding of why they have this disease and give their doctors tools to offer them a highly functional and normal life.

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