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Review article

The future of microarray technology: networking the genome search

In recent years microarray technology has been increasingly used in both basic and clinical research, providing substantial information for a better understanding of genome-environment interactions responsible for diseases, as well as for their diagnosis and treatment. However, in genomic research using microarray technology there are several unresolved issues, including scientific, ethical and legal issues. Networks of excellence like GA^{2}LEN may represent the best approach for teaching, cost reduction, data repositories, and functional studies implementation.

C. D'Ambrosio[1,2], L. Gatta[1], S. Bonini[1,2]

[1]IRCCS San Raffaele, Research Center, Rome; [2]Italian National Research Council, Institute of Neurobiology and Molecular Medicine, Rome, Italy

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Claudio D'Ambrosio
Allergy and Clinical Immunology Unit
Genomics Laboratory-Research Centre
IRCCS San Raffaele
Via dei Bonacolsi, 81
Rome 00163
Italy

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The development of microarray technology

From DNA discovery to microarrays

Understanding the molecular basis of normal and diseased tissues has been a major challenge for medical research since the early discovery of the DNA molecule back in the 1950s by Watson and Crick.

Edwin Southern first described the use of labeled nucleic acid molecules to interrogate, by hybridization, DNA molecules attached to a solid support, with only a one-by-one gene approach, though (1).

Techniques such as RNase protection assay, differential gene display (2) and serial analysis of gene expression (SAGE) (3) were later developed, allowing for the simultaneous study of some tens of genes.

The continuous gain of information on the sequence of entire genomes, along with the Human Genome Project (4, 5), has progressively challenged scientists with the demand to detect expression levels of multiple genes in a single experiment. The first genomic filter arrays were consequently implemented, allowing for the screening of clone libraries, based on a one-to-one correspondence between clones and hybridization signals. For this purpose, gridded cDNA libraries could be stamped onto nylon filters in fixed positions, and each cDNA could be uniquely identified by its signal intensity after hybridization. Although these filter arrays allowed to study some hundreds of genes in one single experiment, they still posed several difficulties, being made of porous materials, difficult to handle, furthermore requiring the use of radioactive labels for signal detection. But the era of genomic microarrays really began when the use of nonporous materials (i.e. glass and silicon) and fluorescence-based detection were made possible, and when a big technology boost came from automation, robotic spotters, photolithography, and bioinformatics.

The cDNA microarrays

In 1995 the first cDNA microarray was pioneered, with some 1000 cDNAs printed on a glass slide, allowing detection of gene expression patterns of two experimental conditions: RNA would be extracted from cells, reverse transcribed into cDNA incorporating distinct fluorescent labels for the two conditions (i.e. cyanine 3 and cyanine 5), and hybridized simultaneously in equal amounts onto the array. The analysis of the fluorescent signal would be performed with a laser scanner, and fluorescent signal intensity ratios would indicate differential gene expression levels (6) (Fig. 1). A key innovation has been provided by photolithography (7), the principle of Affymetrix GeneChip technology (http://www.affymetrix.com), that allows high density *in situ* oligonucleotide synthesis. Each genomic sequence is represented by a set of sense 25 mers covering precisely the sequence, and a set of missense 25 mers where a base mismatch is intentionally created in

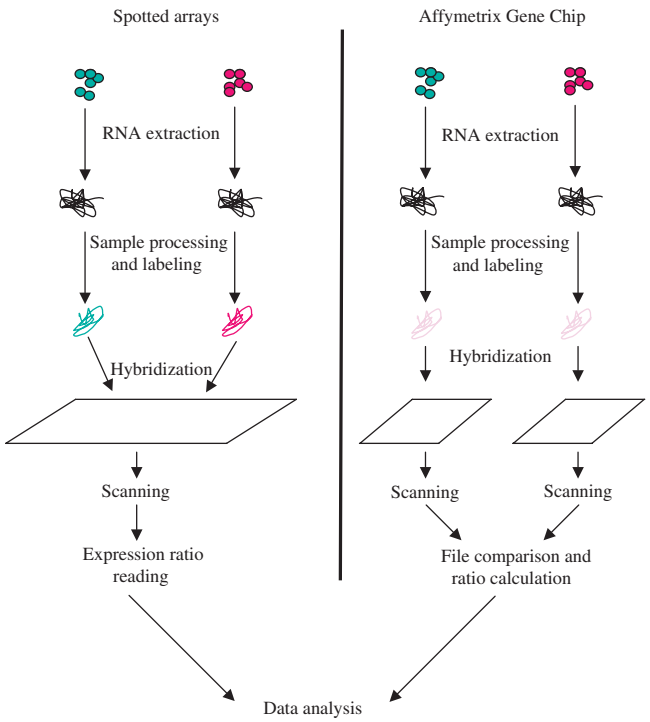

Figure 1. Flow-chart of gene expression profiling with the use of microarray technology. For spotted arrays, samples are simultaneously hybridized onto the same array after incorporation of different fluorescent-labeled nucleotides, whereas for Affymetrix GeneChips, samples are individually hybridized onto different chips, and a single fluorescent label is used.

the middle of the oligonucleotide. Using Affymetrix platforms, RNA is first transcribed into cDNA, and then reverse transcribed into biotin-labeled cRNA and hybridized individually on a single chip; streptavidin-phycoerithryn is added as a fluorescent dye both for control and test samples (Fig. 1). These features ensure sensitivity and specificity, along with best standardization and reproducibility. Photolithography has recently been applied also for genomic DNA applications, through the use of single nucleotide polymorphysm (SNP) microarrays, currently available for genotyping profiling (8–10).

Over the past decade scientists have been widely and rapidly impacted by the spread of microarray technology, that offers a whole lot of opportunities and challenges in both basic and clinical research.

Current applications of microarray technology

Gene expression profiling through genomic microarrays

At present, the main application of genomic microarrays is represented by gene expression profiling. Basically, two types of genomic microarrays are available: wide genome or focused arrays. Wide-genome arrays are designed to bear on them as many genes as possible: currently Affymetrix HU133 plus v.2 gene chips have around 47 000 genes or expressed sequence tags (ESTs) on them, and a whole human genome gene chip is expected to be released in the near future.

On the other hand, focused arrays are designed to bear few tens/hundreds of genes of interest. Furthermore, custom arrays may be specifically designed and prepared upon scientists' requirements and needs.

Genomic microarray technology has become very appealing, but it still poses some important issues. Even though genomic microarrays have nearly become a must have for any lab scientist, pros and cons should be carefully considered before planning their use in basic and clinical research (Table 1).

In the experimental design an adequate number of control and test samples should be available, purity of target cell populations should be ensured, and adequate amounts of RNA yielded from each sample. Specific guidelines for microarray experimental design are given by the Microarray Gene Expression Society (MGES) and can be easily accessed on the MGES' webpage at http://www.mged.org. While cell numbers, replicates and RNA yields might be somewhat easy to achieve when working with cell lines, it is not always the case when working with other cell sources, such as primary cultures, or with solid tissues. For blood derived cells and primary cell cultures, purity of cell populations can be obtained with the aid of antibody specific positive or negative selection via flow cytometry sorting. As for solid tissue samples, RNA and data quality are hampered by postmortem changes taking place especially in paraffin embedded tissues, and contaminating cell fractions may be confounding factors; thus, snap frozen samples should be preferentially used, and cell purity may be overcome with the aid of laser capture microdissection. Even though in early genomic

Table 1. Major pros and cons of genomic microarrays

Pros
One shot genome wide expression analysis
Rapid comparison between two states (control/diseased, untretead/treated, and wild type/knockout)
Exploration of new biological systems in a hypotheses generating rather than hypotheses testing fashion ('fishing' experiments)
Identification of markers to elucidate molecular mechanisms (signatures) underlying biological events and diseases
Rapid molecular disease classification for more accurate molecular diagnostic, prognostic and targeted treatment
Cons
Restricted access to the technology (experiments still exprehensive to perform)
Not yet approved as diagnostic tool by regulatory bodies
Not a stand alone technique (need validation/confirmation tests)
Skilled technical personnel needed (including biostatistician/bioinformatician for data analysis)
Data derived from different platforms difficult to compare
Data comparability difficult from one array version to the next
Data obtained only partially used and published
Data repositories and data sharing still not fully implemented
Ethical and legal issues when dealing with patient samples

studies at least between 3 and 15 μg of total RNA were required, at present RNA amplification protocols for much smaller samples have been established, which offer high fidelity amplification and good reproducibility (11).

Technical protocols are by now well standardized and reproducible, even though they might vary slightly across different platforms.

Data analysis

Since in gene expression studies data might be collected from up to hundreds of experiments, data sets can vary greatly in size. The data heterogeneity is where the big challenge lies for the scientist, and a crucial role is thus played by bioinformatics and biostatistics. The use of dedicated software packages best suited to handle such massive amount of data is recommended. Several packages are commercially available, such as Microarray Suite (Affymetrix, Santa Clara, CA, USA), GeneSpring (Silicon Genetics, Redwood City, CA, USA), Partek Pro (Partek Inc., St Charles, MO, USA), etc. All software packages are equipped with visualization tools, such as self-organizing maps, hierarchical clustering, principal component analysis, relevance networks, etc. In self-organizing maps genes are plotted on a two-dimensional graph based on their expression level across all samples. Thus, groups of genes with a similar expression pattern will have a similar trend within the graph (12, 13).

In hierarchical clustering analyses (Fig. 2) genes are plotted against samples with a dendrogram, which is sort of a mock philogenetic tree, whose branches connect genes related by a similar expression pattern: the shorter the branch, the stronger the correlation. The dendrogram is connected with its branches to the clustering map, where genes are represented by squares colored in green/red or blue/yellow, based on their differential expression level (up- or down-regulation), which define specific molecular fingerprints (14, 15).

Principal component analysis represents gene expression data in a multidimensional space according to their level of concordance to several set parameters, such as sample features and array lots, and may be used both as a visualization tool and as a mean to predict data quality based on the parameters chosen (16, 17).

Most interestingly, relevance networks are used to build networks of several characteristics of the experimental setting including not only gene expression data, but also phenotypical features and clinical measurements (18).

Even though visualization tools can give a comprehensive overview of the whole study performed, they are usually derived from the application of filters resulting in lists of at least hundreds of genes that might be difficult to interpret and make a sense of, without additional statistical analyses. Furthermore, once a specific set of genes is identified as the significant one, validation studies are required in order to confirm gene expression profile

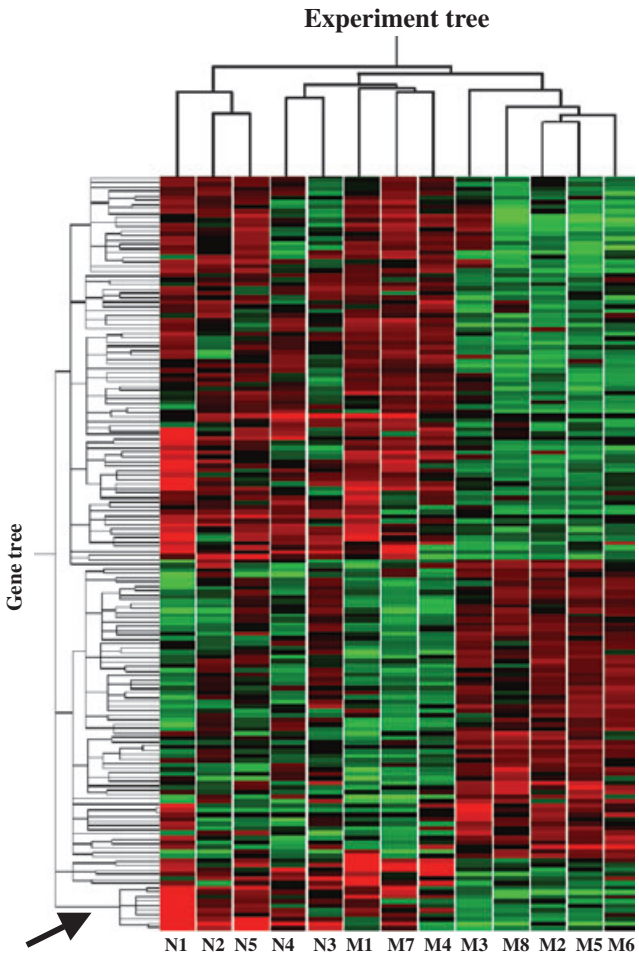

Figure 2. Hierarchical clustering analysis. In this study (39), gene expression analysis of bone marrow mononuclear cells from mastocytosis patients (M1-8), as compared with normal volunteers (N1-5), defined a highly consistent profile (arrow) with candidate molecular markers (modified from JACI 2003;112:1162–1170).

observations. Such validation should be preferentially done by RT-PCR, quantitative PCR (Taqman), Northern and Western blotting, RNA protection assay, flow cytometry, mice knockout models, all targeted to reconfirm previous gene expression profiling data.

Microarrays in Allergy and Clinical Immunology

Gene expression profiling has been widely applied in the field of Allergy and Clinical Immunology and it has been well reviewed in some interesting review articles (19–22) (Table 2).

Genomic microarrays have brought significant insights into a better understanding on the Th1/Th2 paradigm. Studies performed on both human and murine Th1 and Th2 type cells have shown differential expression profiles and signature genes both for cytokine and for transcription factor genes, consistently with previous reports on

Table 2. Selected microarray papers published in the field of allergy and clinical immunology

Granucci 2001 (19)	Review article on gene expression profiling in immune cells
Ono 2003 [20]	Review article on gene expression profiling in the study and management of allergic diseases
Pawliczak 2003 [21]	Review article on application of functional genomics in allergy and clinical immunology
Benson 2004 [22]	Review article on pros and cons of microarray technology in allergy research
Sayers 2005 [43]	Review article on pharmacogenomics of bronchial asthma
Rogge 2000 [23]	Transcript imaging of the development of human T helper cells
Chtanova 2001 [24]	Differential gene expression in both CD4(+) and CD8(+) types 1 and 2 T cells
Hamaleinen 2001 [25]	Gene expression profiles of human types 1 and 2 T helper cells
Nomura 2003 [29]	Gene expression patterns in skin lesions from atopic dermatitis and psoriasis patients
Matsumoto 2004 [30]	Analysis of gene expression in T cells from patients with atopic dermatitis
Nagata 2003 [31]	Analysis of gene expression in peripheral blood monocytes from atopic dermatitis patients
Hashida 2003 [32]	Analysis of gene expression in peripheral blood eosinophils from atopic dermatitis patients
Benson 2002 [33]	TGF-β and related transcripts in nasal biopsies from patients with allergic rhinitis
Fritz 2003 [34]	Gene expression profiling of nasal mucosa in allergic rhinitis patients with and without nasal polyps
Liu 2004 [35]	Characterization of gene expression profiles in human nasal polyp tissues
D'Ambrosio 2003 [36]	Identification of a highly consistent gene expression profile in mastocytosis
Chehimi 2001 [37]	Chemokine and cytokine gene expression profiling in Hyper-IgE syndrome
Rus 2004 [38]	Analyses of peripheral blood mononuclear cells from lupus patients with active and inactive disease
Olsen 2004 [39]	Identification of a gene expression signature for recent onset rheumatoid arthritis
Laprise 2004 [40]	Functional classes of bronchial mucosa genes expressed in asthma
Yuyama 2002 [41]	Identificaiton of novel disease-related genes in bronchial asthma
Zimmermann 2003 [42]	Chracterization of arginase role in asthma pathogenesis
Drysdale 2000 [45]	Pharmacogenomics of β2-adrenergic receptor
Tantisira 2004 [48]	Pharmacogenomics of corticosteroid drugs

individual genes (23–25). A recent study on cord blood derived mast cells has shown that Th2 cytokines increase mast cell growth and differentiation (26), consistently with previous reports (27, 28) and strengthening the hypothesis that tissue resident mast cells exert their role primarily in innate immunity, whereas bone marrow mast cell progenitors, under the influence of IL-4, can still be directed towards helminth infection or allergic inflammation.

Atopic dermatitis has been extensively studied with gene expression profiling applied not only to skin lesion biopsies (29) but also to peripheral blood effector cells such as T cells (30), monocytes (31) and eosinophils (32).

The DNA microarray analysis proved also to be an effective way to study cytokine effects *in vivo* in allergic rhinitis patients (33), as well as a useful tool for identification of expression patterns associated with nasal polyposis (34, 35).

Genomic microarrays have shed also light into the understanding of some rare diseases such as mastocytosis and hyper-IgE syndrome (36, 37), as well as for other immune-mediated diseases, such as SLE (38) and rheumatoid arthritis (39).

With the aid of microarray analysis, novel candidate genes have been identified for bronchial asthma in both bronchial biopsy (40) and epithelial cell culture (41) models, and the role of arginase in asthma pathogenesis has been better defined (42).

Bronchial asthma has also provided a disease model suitable for large pharmacogenomic studies, aimed at a better phenotype classification and prediction of treatment response, for an effective disease management based on genetic variation. Clinical studies have been indeed conducted for the three major classes of therapeutic agents used for asthma control, namely β-agonists, anti-leukotriene agents and corticosteroids, to all of which asthmatic patients show a whole pattern of response variability (43).

A least 19 polymorphisms and 12 haplotypes have been identified for the β2-adrenergic receptor gene and their effect on the β2-agonist bronchodilator response has been described (44, 45). As for anti-leukotriene therapeutic agents, two polymorphisms and several mutations in the 5-lipoxygenase gene have been identified that might account for variability in treatment response to leukotriene synthesis inhibitors (46). Furthermore, overexpression of leukotriene C4 synthetase has been described in aspirin induced asthma, which explains the greater benefit of leukotriene receptor antagonist therapy in this subclass of patients (47). The glucocorticoid receptor has also been shown to have several possible polymorphisms resulting in different affinity to glucocorticoid agents (48).

Even though these findings may explain many features of clinical phenotypes of asthmatic patients encountered in clinical practice, some important aspects are still to address. It should be in fact considered that the mechanism of action of any drug is multifactorial and involves also genes that act downstream from the receptor-ligand interaction. Furthermore, it should be noted that these studies on asthma pharmacogenomics have been focused on individual polymorphisms and haplotypes, rather than integrating them into a comprehensive picture. Genomic microarrays, with both gene expression and genotyping (SNPs) applications, are certainly the most suitable and powerful tool to attain such needed integration. It is thus understandable that the power of microarray technology will soon have a profound impact not only on basic biomedical research, but also on common clinical practice. As a matter of fact, in the near future, genomic microarrays are expected to better and better clarify the

molecular basis of disease and identify novel therapeutic targets. On the other hand, they are also expected to become a routinely and widely tool used for disease diagnosis and classification – probably even by incorporation into disease management guidelines – providing orientation for effective and targeted patient treatment.

Noteworthy and with particular relevance to the field of allergy and clinical immunology, microarray technology has been recently applied for allergen-specific IgE detection in an immunofluorescence microassay, with the use of recombinant allergens bound to a glass slide. This particular allergen microarray will enable multiple IgE measurements in a single miniaturized assay and might easily become a tool to design and monitor patient-tailored specific immunotherapy in the near future, as an example of what is referred to as component-resolved diagnosis (49).

Thus, allergists and clinical immunologists need to become more and more familiar with microarray technology and with the management of information derived in both basic and clinical research, in order to better capture its chances and challenges.

The future: networking the genome search

The potential of genomic microarray technology will be fully exploited only when it will be available to the widest number of scientists and when data will fully turn into translational studies (50).

Technology accessibility is still limited; experiments are still rather expensive to perform, due to high costs of both instrumentation and consumables, and complaints are raised for restricted access to the technology (51).

Microarray core facilities and service providers have been developed, challenged with the need to achieve maximal technical consistency and standardization, ensuring data reproducibility and comparability across different research centers. Microarray user groups have also been set up by both industrial and academic initiatives, aimed at addressing specific technical issues.

Nearly 15 years after its conception, the difficulty of microarray technology no longer lies in the technical aspects, but rather in the handling of the massive amount of data derived from genomic studies, and in the subsequent use to make of the data. But the need of networking centers using microarray technology mainly derives from another consideration. Multiple gene analysis generates a massive amount of data, which are only partially utilized and functionally tested by individual laboratories. This affects significantly the potential of genomic microarrays, since there should be no advantage to investigate multiple genes if only some of the data have an experimental follow up.

Since one of the basic principles of medical research is reproducibility and comparability, it is claimed that expression and genotyping data should be entirely published within scientific publications (20) or in public repositories, such as those maintained by the DDBJ, EBI, and NCBI. In fact most scientific journals require authors to do so, when submitting manuscripts on studies done with the use of genomic microarrays. If on one hand this is perfectly consistent with the principle of scientific honesty, on the other hand intellectual property issues arise and scientists need to find means to protect their work and their data. Industry can effectively protect inventions for commercial delivery by patents, but no such protection exists, though, for scientific data often difficult to reproduce in print, and thus published as supplemental material on the online version of a journal, or in public repositories. Therefore, they can become rapidly accessible by any user in any part of the world in a matter of seconds. From a merely scientific perspective, once a study is being published, access to these data cannot be restricted. Thus, efforts should be driven in gathering together basic and clinical researchers with common scientific interests, for the development of networks of excellence, devoted to speeding up translational research, which is ultimately the most effective way to protect and direct data for follow-up studies. Even though genomic microarrays are not yet usable as a routine diagnostic tool, there are many legal, ethical, social and regulatory issues, which have been poorly addressed so far (Table 3).

Because of the huge amount of information brought into knowledge and all the potential diagnostic, prognostic, therapeutic and preventive implications, modern genomic biomedical research is faced by many issues for which no specific and comprehensive regulatory framework has been prepared yet (52).

Good clinical and ethical practice in biomedical research certainly falls as such under the Declaration

Table 3. Unresolved issues in genomic research

Scientific issues
Experimental design
Experimental comparability
Data analysis procedures
Data sharing
Effective networking
User groups
Consensus working groups
Ethical issues
Informed consent
Biobanks
Communication of relevant information to subjects and families
Legal issues
Intellectual property
Patents
Patient's confidentiality
Health insurances
Protection of patient in the workplace
Worldwide regulatory frameworks
EU regulatory frameworks
National and local regulatory frameworks

of Helsinki (1964) of the World Medical Association, and following amendments and additions. The issue of research dealing with genomic material has been particularly addressed by the Universal Declaration of the Human Genome and the Human Rights (1998) of the United Nations, where the right of a person is declared to decide whether or not information should be given to the person on data related to studies on his/her own genome.

The issue, though, becomes rather complex, considering that information derived by genomic examinations may impact not only the person primarily involved in the study, but also his/her own family. As an example, should any disease gene or disease susceptibility gene profile be uncovered, should the subject notified anyway? Should the family be informed as well? Would the latter be a case of confidentiality breach? Ultimately, since, according to the Declaration of Helsinki, anyone should be able to withdraw his/her own consent from the study at any time during the study, in the case of biobanks, should it be ensured that the patient have at any time the possibility to ask for destruction of the sample of his/her own? Should this apply also to any related data? Most large genomic studies may be run on biobanks, for which samples may have been transferred to third parties and there might be a considerable lap of time between sample collection and gene profiling. What if a sample donor died in the meantime? Should relatives be contacted anyway for relevant information? Biobanks should certainly have the least possible conflict of interest, protecting above all patients' interest, and ensuring at the same time public interest in biomedical research (53).

In this context, informed consent is particularly crucial. All these individual issues should be clarified, giving all possible details on the use of experimental samples and data, and it should also be discussed and agreed on how to handle possible genomic findings which might affect the person's or his/her family's health.

The existing regulatory framework on genomic research is rather heterogeneous and varies greatly geographically, mainly driven by the different socio-politic conditions of individual countries. In recent years, the use of genetic testing and genome-wide analysis has been addressed in the USA, where many initiatives have been taken at the legislative level by States and by the Congress, in order to prevent discrimination by employers and health insurance companies, based on genetic testing and genome-wide analysis. In Europe issues related to genomic research have been sporadically addressed by member States and the regulatory framework is very heterogeneous.

GA^2LEN: a network for genomic search

The Global Allergy and Asthma European Network (GA^2LEN) is a project implemented within the Sixth EU Framework Program, specifically aimed at creating networks of excellence in the field of allergy and asthma across EU countries.

In the genomic era, networks of excellence, like those that GA^2LEN is intended to create, have a crucial role in: (i) establishing collaborative networks among centers conducting genomic research in the areas of allergy, asthma and immunology, including core facilities, and service provider centers; (ii) identifying valid research targets, with carefully monitored basic and clinical research protocols; (iii) integrating data centers, for a quick information exchange and easy turn into translational research; (iv) addressing legal, ethical and regulatory issues related to genomics, establishing consensus papers; (v) addressing proposals for a more integrated regulatory framework at a transnational EU level.

Only working on these issues, encompassing all aspects related to the unrevealing of the molecular basis of disease, will result in a real networking of genomic research, and through integration with newer technologies like proteomics, will allow smooth and effective translational research.

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