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From Genetic Research into Clinical Practice

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Abstract. The present genome era is characterized by speedy progress and prompt transfer of results into clinical practice. This creates the need for rapid disclosure of results and renewal of laboratory's protocols. Molecular cytogenetics has provided and increased ability to identify chromosomes, correlate chromosome structure with gene location, find out cryptic aberrations, and detect specific DNA sequences. These advances have allowed the confident discovery of a number of contiguous gene syndromes. The positional cloning and positional candidate strategies have greatly expedited the search process of disease genes, and become relevant methods for genes' discovery. Understanding the molecular basis of diseases has shown an unpredicted wide genetic heterogeneity, which has splitted single disorders into many clinically similar conditions, and added complexity to the nosology of human diseases. The opposite process, allelism, where clinical diversity results from allelic mutations, has lumped together many distinct disorders, by showing that different clinical entities are not necessarily due to mutations in different genes. Dynamic mutations have provided the molecular understanding of interindividual and intrafamilial variability including anticipation, in a number of diseases. The discovery of distinct correlations between the molecular pattern and disease severity is providing a unique opportunity for using molecular results to assess the clinical outcome. Diagnostic, presymptomatic and predictive molecular testing are becoming widely used and provide enormous opportunities for improving the lot of our patients.

Key words: Spinal muscolar atrophy, Molecular cytogenetics, Contiguous gene syndromes, Williams syndrome, DiGeorge/velocardiofacial syndrome, Genetic heterogeneity, Dynamic mutations, Genetic testing

Medical research has moved into the most exciting period of its development. The large and coordinated effort elucidate the genetic architecture of the genomes of humans and, in parallel, several model organisms, providing a complete working knowledge of their DNA organization as well as an infrastructure in the form of biologic, informational and technological tools, which are markedly advancing the sophistication level of many areas

of biomedical inquiry. From a clinical viewpoint, this infrastructure is facilitating the identification and characterization of genes that directly or indirectly lead to human disease, thereby improving the ability to diagnose and treat affected individuals [7]. A number of successful methods have been developed for the diagnosis and avoidance of many disorders and the potential for a radical cure of some of these conditions is on the horizon. However, it is quite clear that for the next few years to come the main efforts of clinical genetics will be directed towards diagnosis and prevention of inherited diseases, while the benefits of improvements to large scale gene therapy are expected in a longer time period.

The present genomic era is characterised by speedy progress and prompt transfer of the results into clinical practice. This creates the need for rapid divulgation of results and renewal of laboratory's protocols.

Spinal muscular atrophies (SMAs) can be taken as an example of disorders which illustrate this point. Before 1990 we were not able to provide any molecular diagnosis for these autosomal recessive disorders, which are currently classified in three major types, I to III, according to age of onset and clinical severity. At that time, genetic counseling to the patients' parents was limited to communicating a one in four recurrence risk. Gene mapping of the SMA locus to 5q and demonstration that the three SMA types are allelic have made possible molecular characterization of affected chromosomes in these families and prenatal diagnosis, based on linkage analysis [23]. However, the SMA type I patients usually die within the first year of life, and when the relevant disease-gene was mapped, most of these babies had died without a molecular diagnosis being made. This precluded further linkage studies in their families. At that time, many laboratories, including ours, made efforts to develop original protocols to perform retrospective DNA analysis starting from Guthrie spots, stored tissue glass slides, frozen or even mummified tissues, which allowed prenatal monitoring of at risk pregnancies in families where the proband was no longer available [2]. Extensive testing of these families then showed that a minority of them is unlinked to chromosome 5q, supporting genetic heterogeneity of these disorders [24]. In addition, microsatellite analysis using markers flanking the disease gene, had shown instability of this region, with a small number of patients arising rather than from two inherited mutations, from an inherited mutation and a *de novo* parental mutation. This observation has indicated that in a fraction of these families the recurrence risk is not one in four, but negligible [3]. Cloning of the SMN and NAIP genes has made available direct molecular analysis of these diseases and proved that about 95% of the patients are deleted for either SMN or SMN and NAIP genes, while other patients have larger deletions including p44 gene. A rough correlation has been outlined between size of the deletion and disease severity [4]. However, genotype-phenotype correlations are not yet fully understood, as illustrated by the discovery of a number of SMA type II families where sibs presenting similar genotypes have discordant clinical outcome [5]. It is now established that 95% of SMA patients have no detectable SMNt, the telomeric copy of the duplicated SMN gene, which is considered the causative SMA gene. The loss of the SMNt gene occurs by two different mechanisms, deletion or conversion of SMNt to SMNc (the centromeric, non functional copy of the gene). While conversion could produce a mild SMA allele, deletion could produce a severe SMA allele. Physical evidence for these mechanisms, combined with data from assays measuring the SMNc and SMNt gene copy number have clarified the molecular

basis of phenotype in SMAs [1]. This information explosion around SMAs has created the need for prompt circulation of information concerning advances in the disease molecular biology. For this purpose, we have opened a window on Internet, SMANET, to provide these informations to the patients, their families and specialists involved with this disease [25].

The consistent changes in the diagnostic management of a single disorder in a six-year period exemplifies a general rule which applies to most genetic diseases amenable of molecular testing. In this respect the advances in human cytogenetics, particularly molecular cytogenetics, are quite impressive. Chromosome abnormalities are a leading cause of genetic diseases, including congenital disorders and acquired diseases, such as cancer. Chromosome analysis using conventional banding techniques, although highly precise, requires skilled personnel and it is labor intensive, time consuming and expensive. Automated karyotyping is useful for some diagnostic applications, but the available computer algorithms are not sufficiently robust and effective in analyzing small-sized or complex rearrangements. This has led investigators to seek alternative methods for identifying chromosome abnormalities. The development and implementation of a variety of non-isotopic *in situ* hybridization techniques allows detection and, in some instances, quantification of specific DNA sequences and their location to specific chromosomal sites. Fluorescent *in situ* hybridization (FISH) techniques use different fluorochromes which allow the microscopic visualization on a single chromosome segment or DNA/chromatin fiber of different and differently tagged probes, and the relative position of their binding sites [18, 19]. These techniques have provided the cytogeneticist with an increased ability to identify chromosomes, chromosome segments, to correlate chromosome structures with gene locations, to find out cryptic abnormalities, to analyze and describe complex rearrangements [10], to detect specific DNA sequences, including single gene sequences onto chromosomes [27] and interphase nuclei [6]. The late development of the multiplex FISH, which hybridizes to metaphase chromosomes pool of human chromosome painting probes, each detected with a different fluorochrome combination, has reached the goal of visualizing the 22 autosomes and the 2 sex chromosomes in 24 different colors [31].

The revolutionary impact of FISH techniques onto the diagnosis of human diseases, is illustrated by their ability to detect thin chromosome rearrangements, mainly microdeletions. Disorders like Prader-Willi syndrome, Angelman syndrome, Williams syndrome, DiGeorge syndrome and other mendelian or contiguous gene disorders are diagnosed confidently using this molecular cytogenetic approach. In addition, subtelomeric specific probes are disclosing the molecular defect underlying a number of non-syndromic mentally retarded subjects, by showing in 2-3% of them a microdeletion at the distal chromosomal ends [12].

These techniques have also contributed to discover and understand some genetic patterns not explained by traditional Mendelian inheritance. An example is genomic imprinting, which refers to modifications in the genetic material occurring depending on whether genetic information is maternally or paternally derived. Prader-Willi syndrome is caused by paternal deletion or, less frequently, maternal disomy in an area of chromosome 15 q, which contains genes expressed only onto the paternal chromosome [20].

Another concept is contiguous gene syndrome, which refers to a phenotype arising from involvement of genes that are adjacent on a chromosome. A number of these condi-

tions are now known, which are usually due to deletions of variable degree. Williams syndrome is an example. This disorder is characterised by growth and mental retardation, with a friendly, outgoing personality, dysmorphic facial features, hypercalcemia in early infancy and congenital cardiovascular malformations, in particular supravalvular aortic stenosis. The syndrome occurs almost always sporadically with a birth incidence of about one in 20,000. Hemizygosity of the elastin gene is responsible for most of the clinical features, including cardiovascular defects [11, 21], while mental retardation has been tentatively related to defect of other genes, including LIMK1 [13] and syntaxin [26]. It is likely that other flanking genes are deleted in a number of these patients, accounting for phenotypic diversity. Thus, WS is indeed a true contiguous gene syndrome, where the phenotype results from the combined dosage effect of genes located within the deleted region. Most important to clinical practice, discovery of the disease-genes has made possible the molecular diagnosis of this syndrome, contributed to set up a number of diagnostic probes, and in the near future is expected to improve the understanding of clinical variability between patients.

Nevertheless, not all the so-called contiguous gene syndromes are explained by the additive effect of physically linked genes [8]. DiGeorge syndrome and Velo-cardio-facial syndrome are associated with deletion of 22q11.2, which in general affects a region of about 2-3 Mb. However, some patients have only a small deletion or even result from a translocation which disrupts the critical region. Molecular analysis has shown that hemizygosity at this region can result in other phenotypes additional to DGS and VCFS, including conotruncal-anomaly-face syndrome, Cayler syndrome, Opitz GBBB syndrome, absent pulmonary valve syndrome, and occasionally, isolated or familial conotruncal defects [30]. Thus, the overlap of clinical features in these patients led to the proposal that they represent part of a clinical spectrum caused by hemizygosity of a genomic segment containing dosage sensitive genes. However, no genotype-phenotype correlation can be established at present. This suggests that deletion 22q11 does not fit with the classical additive contiguous gene syndrome model. One possibility is that this region contains a number of functionally related genes, the architecture of which is either abolished or disrupted by microdeletions or translocations. Progress in understanding the molecular defect of this region has been important in many respects, since has improved the definition of clinical spectrum related to the deletions of 22q11; has allowed to set up a diagnostic protocol to identify those one in 3,500 newborns affected by this condition; has improved genetic counseling by showing that in not less than one tenth of the patients the deletion is transmitted by a mildly affected parent [9].

Although FISH analysis is becoming a major tool in diagnosis of genetic disorders its impact on clinical diagnostics is less than predicted some time ago. The reasons for this are manifold. In fact, FISH is costly and does not circumvent the need for a complete cytogenetic analysis. In addition, sensitivity and specificity have not been established and the FISH probes approval for clinical use by regulatory agencies is quite slow. Finally, the efficacy of FISH analysis is directly related to the ability of the clinician to suspect the diagnosis. In this respect a larger impact of molecular cytogenetic techniques on diagnosis of diseases awaits a parallel education of practitioners to the proper use of these new devices.

The positional cloning strategy, which locates disease-genes solely on the basis of map position, and the positional candidate approach, where the knowledge of map posi-

tion is combined with the increasingly dense human transcript map, have greatly expedited the search process of disease genes and become relevant methods for genes' discovery [7]. This has resulted in the rapid crowding of the human gene map. The ability to apply this information to diagnostics has had a profound impact upon medicine and in particular promises to transform medicine into a more preventive discipline, based on analysis of disease genes, DNA testing for carrier status, and presymptomatic diagnosis.

However, the unfolding of human biological complexity may at the same time assist or become an obstacle to the laboratory diagnosis. This point is raised by discovery of genetic heterogeneity in an unpredictably high number of disorders. In fact, many if not most of these diseases are clinically or phenotypically similar but genetically or fundamentally distinct, originating from different gene mutations. Retinitis Pigmentosa (RP) illustrates a disorder affecting 1 in 3500 persons where pigmentary deposits to the retina result in the progressive narrowing of visual fields and blindness. Not less than 35 different disorders are known in which RP is a feature. Isolated RP can be due to mutation in not less than 15 genes which have now been either mapped or cloned [33]. Thus, genetic heterogeneity can be an overwhelming obstacle to molecular diagnosis, mainly in sporadic patients, where identification of the affected chromosome can be precluded; in each case it represents a work overload and an extremely high cost to the diagnostic laboratory.

While genetic heterogeneity is the biological mechanism which leads to the splitting of a disease in many clinically similar disorders, adding complexity to the nosology of human disorders, affinity or allelism is the opposite process, where clinical diversity results from allelic mutations [28, 37]. Thus, affinity is also a mechanism for lumping together clinically distinct diseases. In some instances allelic phenotypic variations refer to differences in disease severity, as in the Duchenne and Becker forms of muscular dystrophy. In other instances allelic mutations result in non-overlapping phenotypes affecting the same apparatus. This is illustrated by mutations of the FGFR3 gene, in which the X-rays skeletal changes unambiguously distinguish between achondroplasia, hypochondroplasia, thanatophoric dwarfism (types A and B), Crouzon syndrome with acanthosis nigricans, Pfeiffer syndrome, and isolated craniosynostosis phenotypes [36]. Finally, some allelic mutations cause widely separate phenotypes, as shown by mutations of the CFTR gene, which either result in cystic fibrosis or congenital bilateral absence of the vas deferens [32]. Most relevant to clinical management, genetic affinity provides the possibility of predicting clinical outcome, as experienced by genotype-phenotype correlation analysis [35].

Another turning-point of molecular genetics is mosaicism, which refers to the coexistence of genetically different populations in the same individual. Indeed all human beings are mosaics, at least in the mitochondrial genome, although in general the proportion of mutated cells is below the clinical threshold level. A very peculiar situation is the mosaicism resulting from dynamic mutations. This definition applies to those mutations that, rather than following the general rule of being stable on transmission, are unstable both on meiotic and mitotic transmission. This results in mosaic phenotypes, where expansion of the mutation beyond a given threshold causes the pathological phenotype. A correlation can be established between the size of expansion and age of onset and disease severity. This provides the biological basis to interindividual and intrafamilial clinical variability and tendency towards progressive expansion of the mutation through gen-

erations and disease anticipation [14]. Common disorders such as FRAXA-mental retardation syndrome, Huntington chorea, a number of spinocerebellar ataxias, myotonic dystrophy and other neurological disorders are due to a triplet expansion within the disease gene. The correlation between expansion and disease severity provides a unique possibility of using molecular results to assess the clinical outcome. This predictive testing proves to work in some of these disorders, as in myotonic dystrophy, where CTG triplet expansion classify the patients into three major phenotypic classes. Prenatal monitoring or presymptomatic testing of an at risk subject provide informations both of inhertance of the disease and age at onset and disease severity. In spite of mosaicism, this prediction is quite accurate and improves genetic counseling in at risk families [15]. However, predictive testing can at time be problematic. For example the CAG expansion in the range of 36 to 39 in the Huntington chorea gene can be associated either with an unaffected or an affected phenotype [29]. This underlines the need for caution while delivering predictive testing results.

All together these results recommend a prudent use and interpretation of genetic testing, as properly stated by Hubbard and Lewontin [17]: "our increased knowledge about DNA sequences that constitute genes is transforming the concepts of wild-type or normal genes and their mutations. The relations between such sequences of nucleotides and their clinical manifestations can be complex and unpredictable, even in conditions with mendelian inheritance". In spite of these recommendations molecular testing has become widely used and provides a fundamental support to practical medicine, where is considered a major tool to the diagnosis. Genetic tests also provide prognostic informations by foreseeing onset of diseases; they are predictive by identifying factors related to the risk of developing one disease; they assess the presence of genetic susceptibility to environmental factors or to develop a disease with an higher probability compared to that in the general population; they may disclose empirical associations between a given polymorphism and an increased frequency or resistance to diseases. In this respect genetic testing include "diagnostic" test, which is done on people who either have or are suspected of having a particular disorder and where the question to be answered is whether the patient has the particular disorder; "presymptomatic" test which applies to those situations where an abnormal result will always inevitably lead to the diseases later in life; "predictive" test which applies to situations in which the risk of a disorder occurring is increased or reduced, with a lesser degree of certainty [16]. In some circumstances genetic tests clearly are not medical investigations, as shown by paternity testing or forensic analysis. However, it is possible that analysis of molecular polymorphisms prove important disease associations.

As molecular genetics develops, the overall opportunities for improving the lot of our patients and the future human generations are enormous, provided that it is never forgotten that medicine, rather than a mere diagnostic laboratory, will always remain an art. In this respect, while progress is offering a powerful approach to the avoidance and management of genetic disease, we, as medical geneticists, must remind that "clinical genetics must remain a medical discipline even in the era of molecular genetics" [22], and "molecular diagnosis is only one part of a battery of tests in which clinical suspicion and our own clinical expertise are the basis of most diagnoses" [34].

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