METHODOLOGY IN MOLECULAR GENETICS AND ITS APPLICATIONS TO DIABETOLOGY

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INTRODUCTION

The human haploid genome is known to contain about 3 billion base pairs of DNA and is estimated to encode between 30,000 to 1,00,000 gene products. Every individual human being inherits 2 sets of the haploid genome- one set of maternal and the other of paternal origin. The DNA of each genome is packed as 23 chromosomes. Thus each individual has a total of 46 chromosomes.

If one were to print one copy of one strand of the human genome, it would fill a text 170 times the size if Harrison's Textbook of Medicine. The analogy of the human genome to a large book can be carried further. The book can be envisioned as being bound into 46 separate volumes, each the equivalent of one chromosome. Individuals would inherit one paternal set of 23 volumes and one maternal set of 23 volumes. The defect in sickle cell anemia would be the equivalent of changing a single letter on one page of one volume from each of the sets, while deletion of the alpha-globin gene cluster in a -thalassemia might represent the equivalent of the loss of one or two pages of text in each set.

From the above examples, one can appreciate that to identify minor genetic defects or variations one would need very powerful research tools. This is what the science of molecular genetics or molecular biology is all about.

TECHNIQUES USED IN MOLECULAR GENETICS:

The major techniques used in molecular genetics are as follows (1):

i. Molecular cloning and sequencing

ii. Nucleic acid hybridisation

iii. Restriction Endonucleases

iv. Southern blotting and

v. Polymerase chain reactions (PCR) for DNA amplification

Each of this are explained in detail below:

1. Molecular cloning and sequencing

DNA recombinant technology has enhanced the feasibility for the analysis of individual traits of genes. It allows the analysis of one DNA fragment at a time and has made it possible to locate and isolate individual genes, segments of genes or nucleotide sequences from the vast DNA library. One of the most powerful of these techniques, called Cloning of DNA helps to isolate individual genes, or portions of genes so isolated. The various genes and gene products can then be utilized in diverse studies.

The actual cloning process involves the isolation of DNA fragments and insertion of a sequence into a nucleic acid from another biologic source (vector) for manipulation and propagation. The most common vector are based on bacterial plasmids or bacteriophages like phage g or m 13. Vector like yeast artificial chromosomes (YAC vectors) can also be utilized for cloning DNA fragments upto 100kb in size. Many cDNA (DNA complementary to mRNA) clones have been isolated for hundreds of human genes. Radioactive, biotinylated or otherwise modified copies of DNA can be prepared from any cloned fragment and serve as specific molecular "probes". If a gene or genomic segment has been cloned it is relatively straightforward to clone the same region from individual patients and determine the sequence of any mutation. Thus some of the fruits of molecular cloning are (a) the availability of probes for analytical procedures, (b) determination of disease mutations and(c) the availability of sequence data to allow for DNA amplification. Cloned DNA can also be used for production of proteins, detection of sequences of infective organisms and many other research activities.

2. Nucleic Acid Hybridization

Many steps in recombinant DNA technology make use of the fact that the complementary nature of nucleic acid interaction is the result of base pairing during DNA and RNA synthesis. Linear pieces of double stranded DNA can be treated with heat or

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alkali to form a single stranded (denatured) DNA. This DNA is then placed in an incubator under conditions that allow nucleic acid hybridisation i.e. the recognition of two complementary strands and re-formation of double stranded molecules by base-pairing. The nucleic acid hybridisation technique is so sensitive that a single-stranded DNA molecule can be hybridised specifically to a complementary strand of DNA or RNA present in about 1 part in 10,000. By recombinant DNA technology, it is possible to prepare a radioactive strand of nucleic acid which can be used as a probe in analysis, the specificity of nucleic acid hybridisation along with fractionation or amplification procedures allows detection of a single gene among tens of thousands of others or of a viral sequences in the midst of other nucleic acid sequences.

A slight variation in the nucleic acid hybridisation technique is the use of allele- specific oliogonucleotides (ASO). The DNA probe is a synthetic single strand oligonucleotide usually 15-20 bases in length. The oliognucleotids are synthesized to be complementary to each of 2 or more sequences that represent polymorphisms or mutations in the genome. Allele specific nucleotides are now used in combination with southern blotting but more commonly in combination with DNA amplification.

3. Restriction Endonucleases

With the discovery of micro organisms linked with restriction endonucleases, known as restriction enzymes, recombinant DNA technology was facilitated greatly. These enzymes recognize a specific oligonucleotide sequence in a double stranded DNA and cleave the DNA at this site. Many enzymes, each recognizing a particular DNA sequences are known. For example Enzyme Hae III and Mst III recognize 6 base pair sequences. The sequence specificity of restriction enzymes is a powerful tool in dissection of large genomes. When the human DNA is thus digested ,hundreds and thousands of DNA fragments (varying from few to hundred thousand base pairs in length) of great reproductibility are produced. With the use of multiple restriction enzymes to study a particular fragment of DNA, it is possible to define a detailed map of restrictions endonuclease cleavage sites in that region. Such a map can span a region from several hundred to tens of thousands of base pairs of DNA.

4. Southern Blotting

Many analysis of the human genome involve a specific application of DNA- DNA hybridization . The blotting technique developed by E. M. Southern, begins with the isolation of genomic DNA from cells like peripheral leukocytes or fetal cells. The high molecular weight genomic DNA, can be digested by restriction enzymes to yield a series of DNA fragments which are reproducible. These fragments are separated by electrophoresis in agarose gels. After this, the DNA is transferred from the gel to a membrane that binds the DNA. This membrane is next treated to denature the DNA and is then soaked in a solution containing a single-standed radioactive nucleic acid probe. This probe will form a double-stranded nucleic acid complex at sites on the membrane where homologous DNA are present. The membrane is washed to remove unbound radioactivity and the regions where homologous DNA sequences are bound are detected by using x-ray film. The sensitivity of Southern blotting is achieved by splitting the DNA into fragments, fractionating these fragment and using a sensitive method to pick out the specific fragments. This is called as Restriction Fragment Length Polymorphism or RELP The RFLP technique can detect genomic DNA fragments that represent a single gene or about 1 part in 1 million of a genome. The clinical power of the "Southern Blotting" technique is the ability to analyse a portion of the primary structure of human genomic DNA from an individual.

An analogous procedure to Southern Blotting is "Northern Blotting" which makes use of RNA. Here the presence or absence of a particular mRNA or its approximate size can be determined. Another technique called "Western Blotting" or "immuno blotting" can be used to analyse proteins antigens. Proteins are separated by electrophoresis and then transferred to a solid membrane by a blotting procedure. The membrane is analysed by incubation with antibodies followed detection of bound antibody. The Southern, Northern and Western blotting procedures provide sensitive techniques for the analysis of DNA, RNA and proteins respectively as shown in Table 1.

TABLE 1.

ANALYTICAL BLOTTING PROCEDURE

Blot Method	Material Analysed	Fractionation	Detection
Southern	DNA	Electrophoresis	Nucleic Acid Hybridisation
Northern	RNA	Electrophoresis	Nucleic Acid Hybridisation
Western of Immunoblot	Protein	Electrophoresis	Immunologic

5. Polymerase chain reaction (PCR) for DNA amplification

The technique of PCR for DNA amplification which was pioneered and patented by workers at the Cetus Corporation had a revolutionary impact on molecular diagnosis. The technique is based on knowing the nucleic acid sequence for a region which is to be analysed repeatedly.

Oligonucleotide primers are prepared which are complementary to opposite strands of DNA and are separated by up to a few hundred base pairs. These primers are then incubated with the target DNA to be amplified and with a DNA polymerase which synthesizes a complementary strand. The reaction is usually carried out using heat resistant "Tag" (from Thermus aquaticus) polymerase such that the polymerase remains active during the temperature cycles which usually range from 50° to 95° C. After a number of such cycles ,typically 20 to 30 or more, hundreds of thousands of copies of the original target sequence are synthesized. The bulk of the product is a double stranded DNA fragment of specific length. Molecular diagnosis with PCR depends on determining the presence or absence of an amplified product, digesting the amplified product with a restriction enzyme, hybridising the product with allelespecific oligonucleotides and direct sequencing of the PCR product. But there are many variations and modifications which take advantage of the PCR concept. The PCR technique offers rapid analysis, ease of automation, relative economy and extraordinary specificity and hence has become one of the most powerful tools in the filed of molecular genetics.

USE OF MOLECULAR GENETICS IN DIABETES

Let us now see how molecular genetics can be applied to the filed of diabetology. During the last 2 decades, our understanding of the etiology, pathogenesis and classification of diabetes has undergone radical changes as a result of explosion of research in the filed of molecular genetics. Some of the applications of molecular genetics in diabetes are listed below.

1. Southern Blotting (RFLP) and PCR techniques are extensively used to correctly classify diabetes. eg. HLA typing in type diabetes.

2. Detection of newer types of diabetes based on certain genetic defects has been possible.
   eg. MODY 3, chromosome 12, HNF – 1 a MODY 2, Chromosome 7, Glukokinase MODY 1a, Chromosome 20, HNF – 4a Mitochondrial inherited Diabetes with Deafness (MIDD).

3. Demonstration of genetic defects in insulin action e.g. type A insulin resistance

4. Study of animal models in diabetes, e.g. knock-out mice. Using "anti sense" technology, it is now possible to produce different animal models with "no insulin resistance" "marked insulin resistance" etc. for various research studies

5. Recombinant DNA technology has been used to produce human insulin and recently various insulin analogues.

Need for Molecular Genetic Studies in Indian Diabetics

As practising diabetologists and physicians, it is probably unnecessary to do HLA typing or make an accurate genetic diagnosis of MODY to treat a given diabetic patient. One could very well rely on clinical methods to do this. However , if one were to undertake research , then an accurate classification of diabetes would certainly be of interest. For example in India, diabetes in the young presents several fascinating types. While the classical Insulin Dependent Diabetes Mellitus (IDDM) or type 1 is also seen, it is probably less common. Type 2 diabetes (NIDDM) appears to occur at a much younger age. Additionally , special types of diabetes such as Fibrocalculous Pancreatic Diabetes (FCPD) and Protein Deficient Diabetes Mellitus (PDDM) now known as Malnutrition Modulated Diabetes Mellitus (MMDM) are also seen. Studies of molecular genetics have thrown light on the genetics of FCPD (2) and PDDM (3). It has also helped to differentiate IDDM from PDDM.

Similarly, recent advances in the genetics of diabetes has helped to separate out the different forms of MODY. It is well known that MODY is very common among Indians (4). Further studies of genetic markets of MODY may throw more light on the frequency of the subtypes of this entity among Indian diabetics.

Finally studies on genetic markets may help to throw light on diabetic complications. Recent studies from our group have shown that there may be a genetic susceptibility to diabetic retinopathy

(5,6,7). This may explain why some diabetics are more prone to diabetic retinopathy than others.

Setting up a genetic laboratory

To set up a genetic laboratory is extremely difficult for the following reasons:

i. Initial cost of equipment

ii. Non availability of probes in India

iii. Import restriction

iv. Rapidly changing filed leading to rapid obsolescence

v. High degree of technical expertise is needed.

In this background , multicentric collaborative projects are perhaps the best answer. With relatively little training the local labs can prepare their own blood samples or even separate out DNA. This can be then sent to central laboratories which have specialized genetic techniques and thus collaborative studies can be set up. In this way , multicentric studies in different parts of India can be done to study the genetics of diabetes. Such collaborative effort has produced several research publications in the part from different parts of the country. [8-10].

CONCLUSION

The field of molecular genetics is ever widening and the methodology is being upgraded continuously. In the future, one can except that our understanding of diabetes will grow rapidly, thanks to the rapid advances being made in the field of molecular genetics.

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