ANTIBODY BASED PROTEIN RESEARCH IN HUMAN PATHOLOGY

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Summary

The human genome is estimated to contain between 20,000-25,000 genes. The human proteome is vastly more complex due to posttranscriptional and posttranslational changes and is thought to number in the million range. Currently there are no methods for protein amplification such as polymerase chain reaction in genomic investigation which makes investigation of proteins difficult. It is therefore not surprising that only limited information is available for this very complex system.

On the other hand, proteins, not genes, are the functional units in the cell and genomic analysis may not predict protein function. Therefore, analysis of protein expression is a more direct way of assessing the complex workings at a cellular level. There are currently multiple ways to study protein expression and many of them are based on antibody-antigen interactions. They range from immunohistochemistry, routinely used in most modern histopathology laboratories to novel experimental platforms designed to quantify protein expression.

This article details antibody based routine and research techniques and discusses how such techniques will aid in mapping the extremely complex human proteome.

1. Introduction

It has been a challenge to understand how a relatively small number of genes – it is estimated that the human genome contains between 20,000-25,000 genes – can give rise to such a highly complex organism as the human body. It is now clear that this is due to a much greater total number of protein isoforms than genes. The total human proteome is estimated to number at least in the million range. Complexity is generated at transcriptional, translational and post-transcriptional levels (figure 1). At least 50% of genes are thought to have several splicing isoforms. However, most protein diversity is due to post-translational modifications. Numerous post-translational modifications take place including cleaving, cross-linking and the addition of small molecules. These may change the function of a protein but frequently alter the activity level.

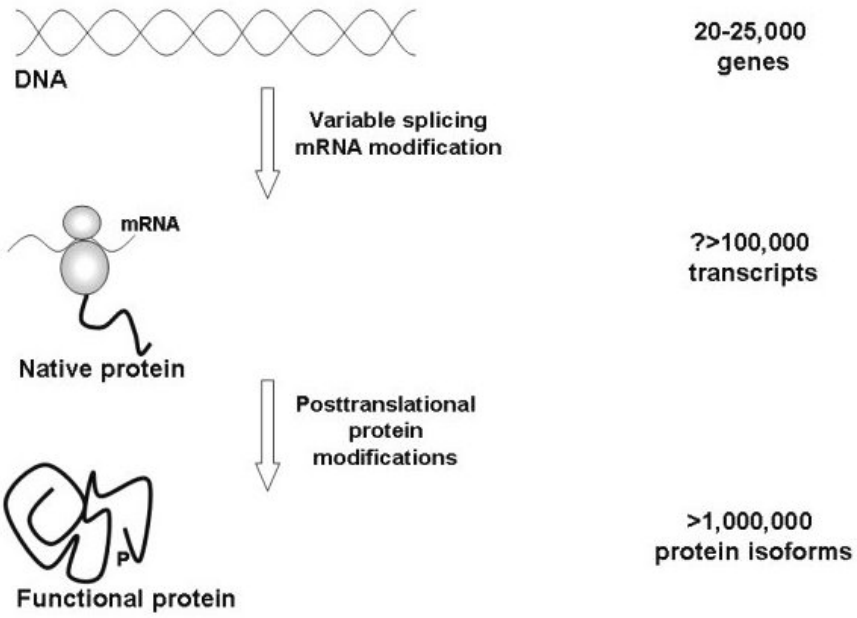

Figure 1: Schematic drawing of how the relatively limited human genome generates protein diversity.

With perhaps millions of protein isoforms and numerous interactions between different protein isoforms, it is clear that the proteome is extremely complex and so far only described rudimentarily. At the same time there two main factors that make investigation of protein expression difficult. Firstly, proteins are fragile and degrade rapidly and at unpredictable and variable rates. Secondly, there are currently no techniques available to amplify proteins such as polymerase chain reaction in genomic research.

Despite the seemingly insurmountable complexity of the proteome and technical difficulties, there are significant reasons to concentrate on protein expression: It is the

most direct way of assessing cellular processes. Genetic analyses can only suggest the level of protein production but cannot truly determine predict protein levels as they are also affected by the rate of protein breakdown. More importantly posttranslational protein modifications cannot be predicted by genetic analyses and may be much more important than the total protein level as such. Phosphorylation of an enzyme can for example increase its function by orders of magnitude.

Another reason to concentrate on protein expression in human pathology is that many novel drugs modulate (usually inhibit) specific protein function.

Protein kinase overactivity is implicated in many cancers which is not surprising. Phosphorylation usually activates enzymes and many cancers are driven by abnormal activity of proliferative pathways. Several protein kinase inhibitors are already used in oncological practice today. They are low molecular weight molecules and often bind to protein kinases competing with ATP for the ATP-binding site. The best example is imatinib mesylate (Gleevec) which inhibits abnormal kinase activity in chronic myeloid leukaemia, gastrointestinal stromal tumours and other malignancies. Targeting protein kinases represents a promising approach for effective *targeted* anti-cancer therapy with minimal side effects.

Protein expression can be studied in various ways but many of the platforms are based on visualisation of antigen-antibody interactions.

1.1 Antigen-Antibody Interactions

Antibodies are glycoprotein immunoglobulins produced by plasma cells in response to an antigen stimulus. They bind to antigen target molecules. In a body, the introduction of an antigen will lead to a polyclonal antibody response.

An antibody binds through weak non-covalent forces with the antigen epitope at several sites and affinity is proportional to the number of interactions. All antigen-antibody binding is reversible and the number of antigen-antibody complexes formed is a function of the total number of antibody and antigen molecules present and the affinity constant. The affinity constant is affected by temperature, pH and the medium in which the reaction takes place. The above highlights an important feature of antibody-antigen reactions: That of 'non-specific' binding. Antibodies are never *completely* specific and will bind to a number of different epitopes with varying strength. A 'specific' antibody merely has much greater affinity to 'its' antigen. With enough antibody or antigen and/or variation in pH, temperature and medium, an antibody can always bind 'non-specifically' with an irrelevant antigen.

Protein antigens well suited for antibody detection usually are stable with immunogenic regions accessible for antibody. The amino acid composition and charge is also important and extremely small peptides may not be very suitable for antigenic detection.

Many antibodies used in protein research are purified polyclonal antibodies produced by immunising animals and harvesting the antibody from their serum. They usually recognize multiple epitopes, making them more tolerant of changes in the antigen and

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thus are often the choice for detection of denatured proteins. Monoclonal antibodies are produced by hybridoma techniques. They have a number of advantages over polyclonals: Since they are more specific, monoclonal antibodies will often give significantly less background staining than polyclonal antibodies. Also, since they are very homogenous, they are usually more reproducible, between experiments. Their main drawback is lower sensitivity than polyclonal antibodies.

2. Routine Antibody Based Methods in Pathology

2.1 Immunohistochemistry in Histological Material

2.1.1 Principles and History

Immunohistochemistry (IHC) is the method for localising specific antigens in tissues or cells based on antigen-antibody reactions. This can be visualised at a light microscopic level. IHC has been used since 1940 when Coons developed an immunoflouresence technique to detect antigens in frozen tissue sections. The use of antibodies labelled with the enzyme horseradish peroxidase in the presence of suitable colourgenic substrates allowed visualisation of antigen-antibody reactions by light microscopy. Greater sensitivity was achieved by developing the detection systems from one step direct conjugation to multiple step techniques. The development of hybridoma techniques facilitated the manufacture of abundant highly specific monoclonal antibodies. Finally, with the application of antigen retrieval IHC is now widely used in wax embedded tissue sections.

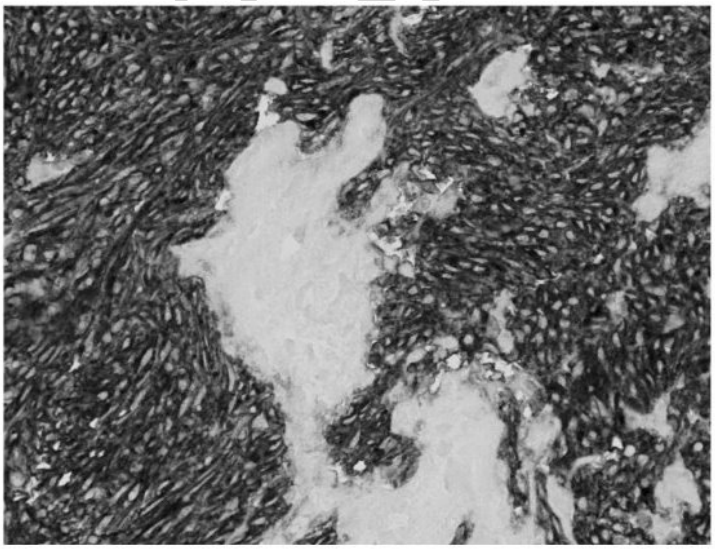

Figure 2: Example of IHC. Light micrograph of a histological section from a gastrointestinal stromal tumour stained with antibody for CD117 (c-kit). This signal is predominantly from the cell membrane whereas nuclei are negative. Also, areas of dense stroma lack signal. Magnification X400.

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The application of modern IHC to routinely processed formalin fixed paraffin wax embedded tissue sections allows specific identification of cell subtypes and even of subcellular component thereby supporting a particular histological interpretation. [see figure 2]. The basic critical principle of IHC is a sharp localisation of target components in the cell and tissue, based on a high signal to noise ratio. Amplifying the signal while reducing non-specific staining (noise) is the strategy used to achieve a satisfactory result.

2.1.2 Antibodies

Antibodies in IHC are evaluated based on their sensitivity and specificity. Sensitive antibodies can detect low levels of target antigen. Specific antibodies will detect the correct antigen without cross-reacting with non-specific antigens. The best antibodies are both sensitive and specific. In general monoclonal antibodies are specific but less sensitive, while antibodies polyclonal antibodies are sensitive and less specific. A new technique for producing rabbit monoclonal antibodies has recently been introduced and seems to combine high sensitivity with specificity.

The application of optimised dilution factors, sensitive detection systems and appropriate positive and negative controls allows the specific and sensitive use of monoclonal and polyclonal antibodies.

2.1.3 Blocking Non-Specific Background

Background staining can in IHC be due to non-specific antibody binding or the presence of endogenous enzymes or molecules.

Non-specific antibody binding is mainly a problem of polyclonal antibodies. The greater the optimal working dilution the smaller the problem. Pre-incubation of tissue sections with normal serum from the same species of animal as the one in which the polyclonal antibody was raised will block/occupy unwanted binding sites. The incubation must occur before primary antibody application and will reduce non-specific binding.

IHC detection systems utilise specific enzymes and molecules to produce coloured end products (see below, 3.1.4). If the tissue section under analysis contains these enzymes and molecules endogenously false positive reactions may occur. Endogenous peroxidase enzyme and biotin molecules can for example cause false positive reactions. Supplying the enzyme with its substrate (hydrogen peroxide) blocks endogenous peroxidase activity.

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Biographical Sketches

Colm Buckley received his primary degree in laboratory science in 1987. He currently works as a senior medical scientist in Dublin, Ireland. He has special interest in immunohistochemistry and is the lead scientist in routine immunohistochemistry in a major teaching hospital. He gives regular presentations on quality assurance in immunohistochemistry.

Robert Cummins received his primary degree in 1994. He currently works as a senior scientist in a research laboratory in Dublin, Ireland. During his career, he has had a particular interest in research and has significant experience with molecular techniques as well as proteomics and tissue microarrays. He has authored several papers.

Christian Gulmann received his primary degree in medicine in 1997 from Aarhus University, Denmark. Much of his research has involved electron microscopy, proteomic methods and tissue microarray techniques. After completing specialist training in histopathology, he worked as a research fellow at the National Institutes of Health, MD, USA, under the proteomics programme. He has authored more than 25

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research papers as well as presented at multiple scientific meetings. He is a reviewer for several pathology and oncology journals. He currently works as a Consultant Histopathologist in Dublin, Ireland.

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