Expert Opinion

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General

The emerging importance of DNA mapping and other comprehensive screening techniques, as tools to identify new drug targets and as a means of (cancer) therapy personalisation

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Every human being is genetically unique and this individuality is not only marked by morphologic and physical characteristics but also by an individual's response to a particular drug. Single nucleotide polymorphisms (SNPs) are largely responsible for one's individuality. A drug may be ineffective in one patient, whereas the exact same drug may cure another patient. Recent advances in DNA mapping and other screening technologies have provided researchers and drug developers with crucial information needed to create drugs that are specific for a given individual. In the future, physicians will be able to prescribe individualised drugs adjusted to, for example, activities of specific enzymatic pathways that would either be targeted by these drugs, or would be responsible for drug conversion or inactivation. Furthermore, the mapping of the human genome allows broader development and application of drugs that act on the level of gene transcription rather than as simple biochemical inhibitors or activators of certain enzymes. Such new approaches will maximise desired therapeutic results and may completely eliminate severe side effects. To illustrate the potential of genetic translational research, the authors discuss available analytical methodologies such as; gene arrays, flow cytometry-based screening for SNPs, proteomics, metabolomics, real-time PCR, and other methods capable of detecting both SNPs, as well as more profound changes in cell metabolism. Finally, the authors provide several examples that focus mostly on targeting protein–DNA interactions, but also other processes.

Keywords: invader assay, matrix-assisted laser desorption ionisation (MALDI), personalised therapy, proteome, SELDI-TOF, single nucleotide polymorphism (SNP), single strand conformation polymorphism (SSCP), transcriptome

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1. Introduction

The ultimate goal of therapy in general is to correct the pathological manifestation (if possible), or at least to achieve a comfortable existence. These goals should be achieved with minimal, or no, side effects. For example, the ultimate objective of cancer therapy is to kill cancer cells while sparing normal cells. Significant research efforts have thus been focused towards discovering suitable molecular targets [1,2].

Unlike 'general drugs' that often cause severe side effects in certain patients, or that are simply completely ineffective, depending on the patient's individualised therapeutics targeting features unique to pathologically changed cells. Single nucleotide polymorphisms (SNPs) found in the genome provide an efficient way of determining how a patient will respond to certain drugs, as well as how susceptible he or she is to developing a certain disease. As these polymorphisms occur so frequently within our genome, this area of genomic research has significant potential to aid in our battle against diseases such as cancer [3-5]. Thus, drugs of the future will likely have individualised formulations with different quantities of active compounds as well as other ingredients.

Since the completion of the Human Genome Project, the effectiveness of DNA/RNA arrays has increased substantially and is helping researchers determine the expression level of genes in cancer and other diseases. 'Our' genes code for proteins and proper expression of these proteins ensures that the entire body stays in homeostatic balance. By understanding biological and cellular processes through the analysis of expression and interaction of proteins, the proteomics-based approach sheds new light on what exactly is occurring in diseased tissues and cells. Finally, in order for a gene to be expressed and eventually produce a protein, transcription factors must initiate the expression of the gene. Researchers are focusing on ways to prevent overexpression of proteins or their underexpression (depending on the disease) in order to find novel ways of treating diseases.

All of these areas in DNA mapping and genomic techniques offer a unique opportunity to eventually design drugs that are specific to each patient, and to obtain the most efficient and effective results. At the moment, high cost is preventing the use of genome mapping as a tool for diagnosis and therapy adjustment.

2. Single nucleotide polymorphisms

Variations in human DNA come in different forms such as: insertions, repetitions, deletions, duplications and, most importantly, variation at a single base. SNP are natural variations occurring within the human genome [6,7]. SNPs are the most basic form of polymorphism; nevertheless, there is a lot of information 'hidden' in these single-nucleotide changes. They can be identified when genomes from two individuals are compared. In order to be classified as SNPs, they must occur in the population with a frequency $\geq 1\%$, whereas mutations are generally defined as changes that occur with a frequency $< 1\%$ (see below). SNPs are responsible for our individuality, not only for our character or appearance, but also for metabolic activities [3,8]. They determine our susceptibility to certain diseases, as well as our response to medications and the speed by which they are metabolised. For example, low metabolism rate may cause the concentration of the drug in the blood to increase until it becomes toxic. In the US alone, > 100,000 people die annually because of

anaphylactic reactions [9]. Thus, properly applied knowledge of certain SNPs will allow for the development of 'personalised' drug or therapy protocols.

SNPs may be located anywhere in the genome. A different base pair between two individuals may be found in either the coding or non-coding regions of the genome and may, or may not have an effect on the gene product. SNPs that do not change the amino acid at a particular location are known as 'synonymous SNPs', whereas those that do change the amino acid are known as 'non-synonymous SNPs'. A 'new' protein with an amino acid substitution may have different characteristics compared to the correct form. For example, a conformational change may result in loss of function of the particular protein or a change in its activity. Therefore, each patient or group of patients with different SNPs may exhibit altered enzyme activity that can cause the body to respond to certain drugs differently than the average population.

Perhaps the best known example of a SNP is found in sickle cell anaemia. The polymorphism causes a change in the amino acid Glu to Val in position six of the beta chain of haemoglobin. This mutation in turn results in production of haemoglobin S (HbS) [10]. HbS has the tendency to aggregate more prominently under low partial oxygen pressure. The aggregated HbS forces morphologic changes in erythrocytes that can then be detected microscopically as 'sickle cells'. These changed erythrocytes are unable to perform their function as oxygen carriers, thus leading to disease manifestation.

Currently, a project similar to the 'Human Genome Project' is underway that focuses on the mapping of human SNPs. Thus, in the future the 'Human Genome SNPs Map' will allow for the discovery of the linkage between, for example, patients that respond more favourably to certain therapies and certain SNPs [11,12]. Therefore, instead of having to locate genes that are associated with diseases, only specific SNPs will be searched for, and examined quickly by molecular biology techniques. Interestingly, it has been discovered that a set of SNPs can be statistically associated with certain blocks within the single chromatid [13]. With this knowledge the identification of a few alleles of a so-called 'haplotype block' unambiguously identifies all other polymorphic sites in this region. Such information is most valuable to investigate the genetics behind common diseases and is collected by the International HapMap Project [14,15]. Thus, in the near future these single base differences in a person's DNA will result in more personalised treatment and diagnosis.

Due to the large amount of sequence information available and efforts such as the Human Genome Project and due to remarkable technological progress, mapping SNPs is a cheap and effective way to identify differences in DNA among individuals. SNP markers are becoming quite popular due to the fact that these polymorphisms occur more frequently than any other in the human genome. The continuously expanding knowledge about SNPs found in the genome is developing the foundation for understanding complex disease symptoms, as well as common diseases and drug responses.

With the completion of the human genome map and SNP map, genotyping SNPs in large-scale pharmacogenetic studies will be an integrated part of the drug discovery and development process [12,16].

3. The influence of SNPs on gene expression – their effects on transcription factor activity

The transcription (copying of genetic information from DNA into an RNA transcript that is then translated into proteins) of most genes is controlled by proteins called transcription factors that have selective affinity for regulatory sequences within gene promoters. They are important control elements of differentiation, cell growth and programmed cell death (apoptosis). For these reasons interest in these proteins as possible pharmaceutical targets in many diseases, including cancer, has significantly grown. New technologies such as computer-aided modelling, proteomics and genomics, along with the huge amount of information available on these proteins to date has created excitement about the potential to develop a new generation of highly selective drugs.

Genes allow the organism to control cellular proliferation, cell cycle progression, cell death and differentiation [17]. Transcription factors play a crucial role in regulating the level of proteins that govern these processes. Thus, changes to these proteins' biochemical properties or the regulatory mechanisms that determine their activity can lead to a range of diseases within the immune and endocrine systems, not to mention cancer [18-20]. The future generation of anticancer drugs will target gene transcription. By selectively targeting specific transcription factors that malfunction in a given type of cancer, researchers will be able to stop the development and progression of the disease.

In eukaryotic cells, transcription of genes is regulated by a collection of proteins frequently referred to as the transcription initiation complex. The vital part of this complex is the enzyme RNA polymerase II (Pol II). In order for this enzyme to initiate its function, it has to be recruited to the site where transcription is initiated. Transcription factors together with other accessory proteins provide the basic requirements for the recruitment of Pol II to the site of transcription initiation. The assembly of the transcription initiation complex can further be positively or negatively influenced by other proteins known as enhancers or repressors. Mutations in the genes encoding for the proteins that facilitate the assembly of the transcription initiation complex lead to a wide range of human diseases from developmental anomalies to cancer [21]. These proteins, therefore, play a crucial role in cell physiology and changes in the expression of these proteins seriously influence the cell's fate and metabolism.

There are two major groups of known transcription factors [22]. The first group is known as basal transcription factors and they are DNA or non-DNA binding proteins that are essential for transcribing all protein coding genes [19]. The main role of these transcription factors is to bring Pol II to the

promoter region of the gene so the gene may be eventually transcribed. The region characteristically contains the usual TATA-like sequence that is located ~ 25 – 30 base pairs (bp) upstream from where transcription begins or the initiator element that overlaps the start site for transcription. The second group is known as gene-specific transcription factors (GSTF) that are essential only for the specific selection of genes that are transcribed by Pol II. They recognise specific sequence elements, typically 6 – 12 bp long, located downstream or upstream within a few hundred bp (promoter region) or several kbp (enhancer region) from the transcription start site [19]. GSTFs role may be either positive as in an activator or negative and act like a repressor. Many GSTFs are homo- or heterodimers and some GSTFs have ligand binding domains that regulate their transcription activity by the binding of other small molecules, such as hormones [19].

Disturbances in transcriptional regulation play a very important role in the development of growth-related diseases such as cancer. Mutations of transcription factors and changes in signalling pathways affect transcription factor activity and significantly contribute to a wide range of human diseases [23-25]. Thus, GSTFs are very promising targets for pharmaceutical intervention (see the next chapter). The increase in knowledge on transcriptional changes underlying malignant transformation is crucial for the development of these therapies [26]. Dramatic changes in gene transcription do indeed occur during cancer development. One third of the so far identified cellular (proto)oncogenes code for nuclear DNA binding proteins that act as GSTFs [19]. For example, the myc-family proto-oncogenes, c-myc, L-myc, and N-myc are activated in a variety of neoplasms due to chromosomal translocation or DNA amplification. Signalling pathways that are triggered by growth factor stimulation normally control the expression of myc-family genes. In tumours, myc expression is frequently uncoupled from normal mitogenic regulators leading to high levels of the Myc protein [19,27,28].

Some GSTFs, although not (proto)-oncogenes themselves, may directly affect the action of known proto-oncogenes [19]. For example, members of the NF- κ B (Rel) family that include the proto-oncogene products c-Rel, p50/p105 (NF- κ B 1), p65 (Rel A), p52/p100 (NF- κ B 2) and Rel B [29], may affect both cell survival, as well as cancer cell sensitivity towards cytotoxic agents and ionising radiation [30]. Another promising target is the proto-oncogene C-erbB-2, a single chain receptor tyrosine kinase that is overexpressed in 25 – 30% of breast- and other solid tumours. The AP-2 transcription factor is associated with c-erbB-2 upregulation in human mammary carcinoma and it has been shown that expression of C-erbB-2 can be inhibited by molecules that interfere with the DNA-binding activity of AP-2 [31,32]. Beside transcriptional inhibition of C-erbB-2 expression, peptide-based approaches exist that inhibit the function of C-erbB-2 and other proteins important for malignant transformation or cancer therapy resistance [33,34].

Table 1. Examples of anticancer agents that interfere with transcriptional machinery of cells*

Compound	Target/mechanism of action	Literature
GW5638	Antiestrogen, induces conformational change of the estrogen receptor, alternative therapy of tamoxifen-resistant breast cancer	[35]
Thalidomide	Prevents I κ B phosphorylation (inhibitor of NF- κ B), tested in combined chemo- and radiotherapy, in multiple myeloma and colon cancer	[94,95]
MS-275	Histone deacetylase inhibitor, induces the expression of TGF- β type II receptor, suppressed in many tumours	[36]
CP-31398	Stabilises p53 by inhibition of ubiquitylation, rescues destabilised mutated p53 and promotes the activity of wild-type p53	[96]
Aminothiols (WR1065)	Antioxidant, protects normal tissues from the damage inflicted by anticancer drugs, acts on NF- κ B, AP-1 and p53	[37]
PRIMA-1	Restores proper conformation of mutated p53, increases drug sensitivity in p53-mutant-expressing cells, restores sequence-specific binding of mutated p53	[96]
Ajoene	Isolated from garlic, NF- κ B activator	[39]

*More examples can be found in reference [17], pp. 377-390.

Understanding the specific mechanisms governing the expression of genes that are deregulated in different types of cancer will allow the development of drugs that influence tumour-specific transcriptional patterns. The molecular structure of GSTFs makes them perfect candidates to be modulated by 'small molecule' drugs (Table 1). There are several ways by which designed small-molecule drugs could influence the function of GSTFs. (i) Because many transcription factors are regulated by their nuclear translocation, a potential drug could interfere with the translocation of the transcription factor into the nucleus, therefore preventing the transcription factor from binding to the DNA and expressing the gene. (ii) As most transcription factors need to form dimers/oligomers to be active, a drug could prevent this dimerisation/oligomerisation and as result leave the transcription factor inactive. (iii) The drug may prevent the transcription factor from binding to the DNA promoter region and, in turn, no transcription would occur. (iv) A potential drug may interfere selectively with the interplay of transcription factors with the basal transcriptional machinery but leaving the GSTF-DNA binding intact [19]. (v) Finally, drugs may be developed that simply trigger the proteolytic degradation of the targeted transcription factor.

To date, several anticancer agents have been reported to regulate or interfere with the transcriptional machinery in cancer cells, some of which are shown in Table 1. GW5638 (antiestrogen) causes a unique conformational change in estrogen receptors and it is used as an alternative therapy in tamoxifen-resistant breast tumours [35]. MS-275 which is derived from synthetic benzamine is a histone deacetylase inhibitor. This molecule selectively induces expression of TGF- β type II receptors which are transcriptionally repressed in many tumours [36]. Aminothiols (WR-1065) protects normal tissue from the hazardous effects of anticancer drugs while not altering desired effects in diseased tissue. This effect is accomplished by changes in the activity (binding) of NF- κ B, AP-1,

and p53 to their cognate DNA elements [37]. Daunomycin originates from anthracycline and causes the induction of p53, partially by binding NF- κ B to the NF- κ B response element of the p53 promoter [38]. Finally, ajoene is a compound of garlic which activates NF- κ B [39]. By using these molecules and various strategies transcriptional targets can be successfully manipulated to obtain desired results. Because most transcription factors do not recognise a single 'perfect' sequence, but rather they allow a certain degree of target sequence variation (degeneration), a new class of drugs that interfere with GSTF/DNA-interaction may specifically target this phenomenon by positively or negatively modulating the affinity of the GSTF to certain 'imperfect' recognition sequences.

Beside the promoter sequences that directly respond to certain transcription factors, gene regulation also depends on epigenetic information that is not directly encoded in the sequence of A, C, G, and T nucleotides. Examples of 'epigenetic coding' involve DNA methylation and changes in chromatin proteins most frequently at histone tails. These changes directly influence, for example, chromatin compactness, and thus also the accessibility of transcription factors to their regulatory elements. These widespread 'epigenomic' features can be mapped and characterised by alternative applications of similar technologies that have been used for conventional transcriptional profiling. The rapid progress that is being made in developing and applying epigenomic profiling methods means that epigenomic profiling is likely to become a standard research tool for understanding chromatin structure and gene expression [40].

4. SNP identification methods

Several techniques are available to identify mutations/polymorphisms and the following chapters will highlight some of the most common ones. Perhaps the two most popular methods used to specifically identify SNPs are conformation-based

mutation scanning, also known as single strand conformation polymorphism (SSCP) and direct DNA sequencing. A commonly used method to identify SNPs is to select and amplify a gene of interest by PCR followed by scanning the products for the presence of DNA variants by confirmation-based mutation scanning methods [16,41]. With access to 'world wide web' databases, it is becoming faster and easier to compare DNA sequences obtained by PCR amplification to the same fragments of the human genome previously sequenced from other individuals. With a vast amount of human expressed sequence tags and genomic clones in the public domain, computer-based sequence alignment and clustering also provide a rich resource for SNP identification [16].

Conformation-based mutation screening (SSCP) is a cost effective and easy way to detect novel polymorphisms. Single strand conformation analysis is a very popular method used for detecting mutations. Regions of DNA where polymorphisms are believed to occur need to be amplified first by PCR. The products from the PCR amplification are then denatured so that single stranded DNA molecules can be separated on a polyacrylamide gel. A strand of DNA that contains even one base modification generally forms a different conformer and migrates differently when compared with wild-type DNA.

Other alternative conformation-based mutation screening methods include: chemical or enzymatic mismatch cleavage, denaturing gradient gel electrophoresis, matrix-assisted laser desorption ionisation (MALDI)-TOF (see below) [42], and denaturing high performance liquid chromatography (HPLC). Once it has been confirmed that a region of DNA contains potential polymorphisms, these regions can eventually be sequenced to locate the final polymorphic sites. DNA sequencing has become fully automated due to substantial advances in computer software and detection systems.

Detecting known polymorphisms can be done by either gel-based genotyping techniques or non-gel-based genotyping techniques. Gel-based genotyping techniques include PCR restriction fragment length polymorphism analysis and oligonucleotide ligation assay genotyping. The first technique includes gel electrophoresis coupled with PCR and restriction fragment length polymorphism analysis. The desired region of DNA is amplified by PCR and then restriction enzymes cut the DNA into fragments that are eventually visualised after electrophoresis is complete. If a polymorphism is present, different lengths of DNA fragments will be observed. A major drawback of the PCR-restriction fragment length polymorphism method is the requirement that the polymorphism changes the site where the restriction enzyme would cut the DNA.

A second method that involves gel electrophoresis is the oligonucleotide ligation assay. The oligonucleotide ligation assay (OLA) relies on hybridisation with specific oligonucleotide probes that can effectively discriminate between wild type and 'changed' variant sequences. A total of three oligonucleotides are used in this assay. Two of the oligonucleotide probes are created specifically for the desired alleles. They are customised

for the wild-type allele and for the mutant allele. The third probe is a fluorescent common probe that recognises where on the DNA the polymorphism is located. The piece of DNA where there is believed to be a polymorphic site is amplified by PCR and then incubated with the three probes. In the presence of a thermally stable DNA ligase, ligation of the fluorescently labelled probe to the allele-specific probe(s) occurs only when there is a perfect match between the variant or the wild-type probe and the PCR product [16]. The products from these ligations are then separated by gel electrophoresis and wild-type genotypes, variants, heterozygotes and non-ligated probes can be recognised.

Among the non-gel-based techniques, TaqMan[®] genotyping and Invader[®] assays are frequently applied. The TaqMan-based method uses a modified real-time PCR protocol in order to analyse polymorphisms. Two probes are used in a biallelic system, each specific to an allelic variant and labelled with a different reporter fluorophore [either 6-carboxy-fluorescein or 6-carboxy-4,7,2',7'-tetrachloro-fluorescein [43]. The probe is an oligonucleotide that is complementary to the sequence with the desired SNP and is labelled at the 3' end with a quencher dye tetramethylrhodamine and at the 5' end with a reporter dye. If the probe is not disturbed and left intact, the closeness of the reporter dye to the quencher dye reduces the fluorescence that is observed. The specifically designed target probe is degraded by the 5'–3' exonuclease activity of the DNA Taq polymerase as the forward primer extends during PCR. With each round of amplification, there is an increase in the intensity of fluorescence related to the accumulation of PCR product which is measured directly in the reaction well by an ABI PRISM[™] 7700 Sequence Detector (Applied Biosystems, Warrington, UK) [43]. In addition to the sample being analysed several controls are used. One control contains no DNA template and the others are DNA samples homozygous for their respective alleles. Once PCR is complete, a laser collects and develops a fluorescence spectrum and algorithms. The software determines how much each component dye contributed to the observed spectrum. A scatter diagram is eventually produced which plots the allele-specific components of each reaction [43].

The Invader assay, on the other hand, uses two oligonucleotide probes that hybridise to DNA containing an SNP or polymorphic site. At the SNP location the two oligonucleotides hybridise to the target and form an overlapping invader structure. The Invader oligonucleotide is complementary to the target sequence 3' end of the polymorphic site and ends with a non-matching base overlapping the SNP nucleotide [44]. The second oligonucleotide, the allele-specific probe, contains the complementary base of the SNP allele and extends to the 5' end of the polymorphic site [44]. Thus, a three dimensional invader structure is formed at the SNP site when the two oligonucleotides anneal to the target DNA. This site is then recognised by a cleavase that is a FEN enzyme. Cleavage is detected by several different methods. The most common method of detection involves the cleavage product triggering a

secondary cleavage reaction on a fluorescence resonance energy transfer cassette which releases a fluorescent signal that can then be detected and measured. Cleavage may also be detected by using fluorescence polarisation probes or by mass spectrometry [44].

5. DNA- and RNA-arrays

The most versatile technique to probe global as well as specific changes in transcriptional activity are DNA- or RNA-arrays. The expression 'DNA-arrays' and 'RNA-arrays' sometimes also called 'DNA-chip', indicates which type of nucleic acid is being hybridised to the array (chip). Since the human genome was mapped, the DNA-chip has become the prominent gene expression profiling method because they are easy to use, do not require large-scale sequencing and let the experimenter to quantify thousands of genes from many samples. DNA arrays work by having probes (i.e., genes of interest) attached to a solid surface and then targets from a biological sample hybridise to the probes. The hybridisation image is then retrieved by scanning and the signals are automatically quantified. The mRNA expression level corresponds to the signal generated on each probe on the array. After detection, quantification and integration of signals with specialised software, intensities are normalised for technical deviations and provide a gene expression profile. A 'transcriptome' is generated for each sample and is compared to profiles from other samples [45].

5.1 Preparation and application of DNA arrays

There are two main ways that DNA arrays have been successfully applied. The first array consists of cDNA clones robotically spotted on a solid surface in the form of PCR products [45]. There are several different ways this method can be executed. The solid surface may vary from nylon or glass and the targets may be labelled with a radioactive element, colour or fluorescence [46,47]. This method is very flexible giving researchers a wide range of possibilities and even enabling them to use experimental gene samples. The second implementation involves arrays of oligonucleotides directly synthesised on the array's matrix [45]. In order to design the probes for this method, the gene sequence needs to be known. After the completion of the Human Genome Project this is hardly a limitation; however, in the past this lack of knowledge limited the use of this method. The high cost of this technique, and the requirement for timely sample processing, still prevents it from being common in most laboratories and in clinical settings.

DNA and RNA arrays allow researchers to conduct large-scale experiments that were previously impossible or just too time consuming. With this technology, expression levels of thousands of genes can be measured simultaneously. Significant advances in human genome research along with advancing technologies such as DNA arrays now allow researchers to get a better idea of the molecular complexity of cancer cells and predict the cellular responses to many drugs. As gene expression can now be measured in particular tissues or cells

at different disease stages, a clearer picture is developing as to how diseases, and specifically cancer, develop and spread. Furthermore, due to DNA array technology that scores changes in mRNA expression levels, the role of genes with previously unknown function (so called 'unannotated genes') are now becoming associated with certain signalling pathways or other cellular functions. When applied to the genome sequence itself, microarrays have been used to identify novel genes, binding sites of transcription factors, changes in DNA copy number, and variations from a baseline sequence such as in emerging strains of pathogens or complex mutations in disease-causing human genes [48]. Thus, DNA arrays are a powerful tool in the lab that have serious potential in changing the way diseases are diagnosed and treated.

The most common use for DNA arrays is mRNA expression profiling, but several other techniques also contribute to our understanding of gene functions and the functional relationship between genes and gene groups. For example, pathogens can be detected and characterised by the detection and analysis of their genomic DNA. This technique is executed with random primed PCR or PCR with selected primer pairs for the desired, usually unique, target DNA-region [48]. As genetic maps of more and more pathogen organisms are becoming available, this approach is gaining feasibility and in the near future it may allow for fast multiple diagnoses of pathogens in a single biological sample.

DNA arrays have the ability of greatly enhancing molecular diagnosis, but more specific classification needs to be established that reflects the diversity and dynamic nature of cancer. Because cancer specifically is such a complex disease, several markers as opposed to one are likely to be more accurate in correctly identifying the cancer. For example, tissue sample profiles of acute myeloid leukaemia and acute lymphoblastic leukaemia can now be successfully distinguished [49]. Expression profiling done on groups of cancer cell lines matched with the organ from which the cells were taken [5,50]. For example, in lung cancer, a group headed by Gordan recently identified eight genes that when expression of these genes was measured, distinguishing between malignant pleural mesothelioma and adenocarcinoma of the lung was possible [51].

Transcriptional targets can also be used as 'markers' in identifying cancer in individuals. Most cancer targets or signatures are identified at specific times, which only reflect gene expression at that particular time point. From this information it is very difficult to recognise and distinguish 'cause and effect' of gene expression [52]. For example, does one gene activate another or a group of genes all together? If a detailed transcription network can be established then interpreting cancer signatures will be significantly easier. When all the targets of transcription factors are known, one could then easily determine which transcription factor is activated and eventually leads to observable cancer signatures [52]. New technologies, such as chromatin immunoprecipitation coupled with promoter microarrays (chip-chip) allow for genome-wide identification of transcription factor binding sites [53-55], but only a

few human transcription factors have been profiled to date [52]. With the discovery of hundreds of 'consensus binding sequences' it is possible to execute large-scale integrative analysis of potential cancer signatures. In humans, with respect to various phases of the cell cycle, known transcription factor binding sites among promoters of expressed genes have been identified [56]. In a similar type of experiment, gene expression was compared to sequence-derived transcription factor binding site profiles and in a significant number of cases a specific transcription factor profile was found within the cancer 'signature' [52]. Binding sites (recognition sequences) for transcription factors like E2F, Myc, Ets-1, Rel and ATF have frequently been found within these signatures [52]. Detailed knowledge of such signatures would allow for the development of 'signature-specific', individualised therapy.

6. Flow cytometry-based detection of SNPs

Although flow cytometry-based detection of SNPs is inferior in terms of the quantity of SNPs that can be screened for simultaneously, the method allows for a limited number of SNPs to be scanned quickly. Flow cytometry is available in most hospital and research centres. SNPs are analysed by a bead-based assay in which DNA structures are attached to beads. In one configuration of the assay, biotinylated primers that stop one base shy of the SNP site are attached to avidin-coated beads. DNA from test subjects, either direct genomic DNA or PCR amplified segments that span the SNP site, are hybridised to the primers. At this point, the beads with numerous copies of the primer/template duplexes attached are divided into four aliquots to which dideoxy nucleotides (that terminate the DNA-strand extension) are added along with DNA polymerase. In each of the aliquots, only one of the terminating nucleotides is fluorescently labelled. Thus, upon analysis of each of the four bead sets by flow cytometry for a homozygous SNP only one bead type will be fluorescent.

This assay has been multiplexed by the addition of unique DNA sequence tags to the primers and sequences complementary to the tags attached to beads that are different for each bead type in the multiplex set [57]. After thermal cycling, the single base primer extension reaction is performed at multiple sites on the target DNA, the tagged DNA primers are then melted from the template DNA and the tagged beads pick up their extended primers. Analysis by a flow cytometer of the bead-associated fluorescence determines the nucleotide at the SNP site. Sequence tags or 'ZipCodes' have also been used for SNP analysis by OLA [58] and single base extension [59] assays. A previous method involves a fluoresceinated oligonucleotide reporter sequence added to a 'capture' probe by OLA. Capture probes are designed to hybridise both to genomic 'targets' amplified by polymerase chain reaction and to separate complementary DNA sequences that have been coupled to a microsphere. These sequences on the capture probe are called 'ZipCodes'. The OLA-modified capture probes are hybridised to ZipCode complement-coupled

microspheres. The use of microspheres with different ratios of red and orange fluorescence makes a multiplexed format possible where many SNPs may be analysed in a single tube. Flow cytometric analysis of the microspheres simultaneously identifies both the microsphere type and the fluorescent green signal associated with the SNPs genotype [58]. In the single base extension assay, ZipCode at the 5' end of the capture fluorescent oligonucleotide probe allows the DNA polymerase reaction product to be captured by its complementary sequence (cZipCode) which has been coupled to a specific fluorescent microsphere [59]. Both assays can be used with standard flow cytometers (e.g., B-D FACSCalibur) with individual loading, or with the much less expensive bead-designed Luminex system.

7. Proteomics

Ever since the Human Genome Project was completed, focus has now shifted towards understanding what our ~ 30,000 genes are programmed to do. Thus, proteomics, the technology that globally studies the structure, function and expression of proteins is gaining importance. It involves the characterisation of biological and cellular processes by analysing the expression and interaction of proteins. Several techniques are used in proteomics to identify proteins present in a cell, tissue or organism. The most common proteomic technique for separating proteins based on size and charge is 2D polyacrylamide gel electrophoresis. Initially, proteins are separated in the '1st dimension' on a thin gel, or within a gel-filled capillary according to their iso-electric charge. In the '2nd dimension' the proteins are separated based on their size when the first gel is placed across a larger gel. 2D polyacrylamide gel electrophoresis is too labour intensive to be effective for clinical use, but it is extremely valuable for research purposes. This technique can separate > 1000 proteins on a single gel. Individual proteins can then be isolated from the 'spot' and identified using mass spectrometry.

In comparison to genomic mapping approaches, the proteomics approach typically requires much larger quantities of biological material. Furthermore, unlike DNA-proteins, peptides cannot be easily amplified. As this still young but powerful technology evolves, it may eventually gain broader clinical application because it directly examines proteins that are the active components of the cell. Once target proteins are identified, treatment and diagnosis of a particular disease has the potential to become much easier and efficient and eventually, proteomics will lead to the development of individualised patient treatment. Being able to identify specific genetic mutations and protein profiles linked to diseases will give way for the creation of proteomic based assays that could be used for diagnosing diseases much earlier than presently possible. Due to the application of proteomic techniques specifically relating to cancer, several research groups from around the world have identified proteome patterns associated with prostate-, colorectal-, ovarian- and other cancers [60]. There is no

doubt that significant breakthroughs have been made to date; however, there still are problems with reproducibility, standardisation, and 'coherence of experimental results' between leading researchers on the results being produced.

8. Protein microarrays as a cost effective approach to study a limited cluster of proteins or to screen for protein-protein interactions

Proteomic experiments are currently laborious, expensive and require significant quantities of biological material. Some more focussed scientific hypotheses can be efficiently investigated by using protein microarrays instead. Protein microarrays detect specific interactions between proteins or proteins and other molecules. At least one of the interaction partners must be known. Protein microarrays may be composed of various molecules such as antibodies, phage displayed antigens, other types of recombinant proteins or drugs permanently attached to a slide in order to 'capture' desired proteins. These arrays then undergo image analysis and the signals detected from each 'catch' are compared and interpreted. Recently, this method was successfully used to measure the humoral immune response to α -methylacyl-coenzyme A racemase (AMACR) which is a protein marker of prostate cancer [61,62]. The mentioned studies revealed that the immunoreactivity against AMACR was discovered to be significantly higher in sera from prostate cancer patients compared with control samples by using protein microarrays [61,63].

9. Metabolomics

Metabolic profiling, or metabolomics, is able to detect the unique chemical fingerprints that are generated by cellular machinery. It focuses on small-molecule metabolite profiles and proteins and RNAs in a broader sense. In order to avoid overlap with previous chapters, the authors stick to the narrow definition of metabolomics. The term was first coined by Oliver and co-workers in 1998 with respect to yeast metabolome [64], although some authors also credit Linus Pauling for the concept of metabolomics. Genomics and proteomic analysis do not provide all the information about the metabolic events occurring within a cell. Metabolic profiling gives a true picture of the physiology of that cell. The full potential of metabolomics, proteomics, and genomics will be realised when these systems are integrated to provide a complete picture of living organisms. For now, however, it is rather difficult to contemplate all the metabolites within the cell at once, thus the majority of recent papers focus only on groups of metabolites rather than the whole picture. Such 'focused metabolomics' is increasingly popular among pharmaceutical industry researchers because it can produce data that is easier to analyse. The most developed focused metabolomics area is lipid profiling [65].

Regardless of the focus and broadness, assayed metabolites must be separated and then detected and quantitated so that

their significance in a cell's metabolism can be determined. The methods used for that purpose often overlap with peptide detection methods used by proteomics, thus they are broadly discussed in the next paragraph, which is dedicated to peptide (protein) identification. Popular separation methods for precisely identifying metabolites include: gas chromatography alone or combined with mass spectrometry. Identification of metabolites is done by determining retention times or index comparisons with pure compounds [66]. Compared with gas chromatography, HPLC has a lower resolution; however, it allows for a wider range of compounds to be measured. The HPLC-mass spectrometry combination is the standard analytical tool in pharmaceutical qualitative and quantitative analysis of potential drug candidates, and their related metabolites [66]. Finally, capillary electrophoresis is emerging as a more and more popular metabolite separation method. This technique has higher theoretical separation efficiency than HPLC, and it can be used with a wider range of metabolites than gas chromatography, however as all electrophoretic techniques it can only be applied for the separation of charged analytes. For all these methods, the individually separated metabolites are usually identified by mass spectrometry. Another method sometimes used for discriminating between metabolites is nuclear magnetic resonance. This method does not require separation of the sample and as a result the sample can be used for further experiments. Nuclear magnetic resonance, however, generates a large amount of data points that for complicated mixtures may be difficult to analyse.

With the recent completion of the human genome, the demand for determining the role of 'unknown' genes has greatly risen. Metabolomics provides the key to unlocking the door between genotype to expressed phenotype. Genomics still plays an important role in determining the function of genes and their products, but for diseases such as cancer there is a great need to understand how the genetic and protein networks contribute to the particular metabolic and particular phenotypes found in tumours. Metabolomics largely complements current studies of genomics and proteomics by providing a link between biochemistry and functional genomics that relates the expression of genes and gene products to cellular biochemical and physiological events. Understanding the complexity of the metabolic network in transformed cells will create new opportunities in fighting cancer by metabolism-targeted therapies. Metabolomics, however, comparably to other '-omics', demands even higher-throughput and higher-information content analyses. Moreover, interpretation is currently a major obstacle, which is still needs to be perfected. There are currently very few metabolomic studies focussed on the development of new cancer therapeutics despite the great need and technological potential of metabolomics [67].

10. Protein identification

The typical outcome of proteomic 2D gel analysis is a series of spots with each spot ideally containing a single protein.

The identity of these proteins are then confirmed, either based on their already known localisation within the gel or by sequencing the protein from pieces of gel believed to contain the protein. In the 'old days', this was done by the 'Edman Degradation' procedure that required a significant amount of protein. Recent advances in mass spectrometry techniques allow for the sequencing of minimal quantities of protein, typically within the 1 pM (10^{-12} M) range, whereas peptide profiling (identification of peptide by comparison to known peptide standard spectra) is even 10x more sensitive (100 fM, 10^{-13} M range). Higher sensitivity can be achieved upon sample-specific optimisation of the technique.

Mass spectrometry measures the mass-to-charge ratio (m/z) of ions in the gas phase along with the number of ions in each m/z value [68]. Before mass spectrometry can be used to identify proteins, the protein or its fragments (peptides) have to be ionised without being destroyed. Currently, there are two methods that are the most popular; the electrospray ionisation and MALDI, as well as their variations (see below) [60,69]. Mass spectrometry involves first breaking down the protein with enzymes and the resulting amino acid fragments are then energised and separated according to their mass-to-charge ratio. A detector then measures the signal intensity of each fragment. The most common combinations of ionisation sources, analysers and fragmentation devices used in proteomics are: MALDI-TOF (time of flight), ESI (electrospray ionisation), ESI-Q-TOF, ESI-ion trap and FT-ICR [70]. Unfortunately, all have certain limitations and cannot reliably identify proteins in a wide range of conditions. Presently, the most widespread method in proteomics seems to be based on surface enhanced laser desorption ionisation (SELDI) profiling [71]. SELDI technology arose from the MALDI technique and is based on the process of affinity capture on special chemical surfaces which is then followed by laser desorption/ionisation based detection for mass analysis [68]. In the meantime, several variations of SELDI have emerged and have since successfully been used for functional genomic studies [72].

11. Examples of recent discoveries made by proteomics-based studies

Proteomic research can be applied to many different areas within medicine such as: cardiovascular and neuromuscular diseases, prostate and ovarian cancer, organ transplantation and infertility [68]. Proteomics has the potential to discover cancers in their early stages and as a result significantly improve remission rates. The most important factor in successfully dealing with the majority of cancers is the clinical stage at the time of diagnosis. A significant number of diagnosed cancers are often in too advanced stages and curative treatment is no longer possible. For example, earlier detection and differential diagnosis would be significant for differentiation between advancing ovarian- and colorectal cancer in women. Frequently, at the time of diagnosis the disease is

already at the advanced stage, and thus the 5-year survival rate is only 35% [73]. Moreover, fast and correct differentiation between ovarian and colon cancer in such cases is critical for the proper choice of treatment [60]. Recently, by using genomic and proteomic techniques, researchers were able to identify villin in colon cancer and moesin in ovarian cancer as reliable novel tumour markers [74].

Pancreatic cancer has one of the lowest survival rates, which is partly due to the late development of clear symptoms [75]. Using the proteomic approach with the subsequent SELDI-based protein identification, Goggins and co-workers have recently identified hepatocarcinoma-intestine-pancreas/pancreatitis-associated protein I (HIP/PAP-1). This protein is released from pancreatic acini during acute pancreatitis, is also overexpressed in hepatocellular carcinoma, as well as pancreatic cancer [76].

Breast cancer is the second leading cause of cancer-related death in American women [77]. Like with many cancers, early detection is the key factor in survival. Using SELDI-TOF mass spectrometry, Li and co-workers have identified three protein peaks that discriminate between stage 0 – 1 cancer patients and non-cancer controls. Other recently identified breast cancer biomarkers using SELDI include Hsp27, 14-3-3 sigma, and mammaglobin/lipophilin B complex [77-80].

12. Expert opinion: the future of transcriptome and proteome-based medicine, and other developments towards individualised therapy

Some trends are beginning to develop with respect to microarray technology. This technology is only starting to make advances in substantially decreasing the amount of a biological sample required to perform the procedure [48]. In the future the microarray technology will become more sophisticated and sensitive enough to be executed efficiently in 96 well plates [81]. By knowing the genetic makeup and enzymatic activity of tumour tissues, this information will eventually lead to significantly more accurate diagnosis and treatment [45]. In the near future, proteomic and metabolic profiling will reach the same level of sophistication as DNA microarrays and these types of profiling will be able to shed more light on biological pathways. This information will no doubt make an impact in understanding what exactly is taking place in the cell. It will be easier to identify cancers and other diseases as well as eventually being able to produce treatments that are safer and more effective. Ideally, if all three measurements could be done on the same tissue or cell sample, a clearer picture of all the interactions taking place would emerge.

Many barriers still remain to be crossed before clinical medicine starts fully benefiting from the amazing progress made recently in genome research. Although the SNPs databases are rapidly expanding with a number of new entries on weekly bases, little is known about the clinical significance that these single nucleotide changes cause in given clinical settings. As indicated earlier, proteome analyses of various cancers led to

discoveries of new, more specific tumour markers. At present however, proteomic studies require rather large quantities of biologic material, they are laborious, and attempts to automatise these processes have only been fully successful at certain steps. Furthermore, low abundance proteins with high biological activity, such as, for example, transcription factors and some cell-signalling proteins may frequently be missed by these techniques due to high 'background signal' from more abundant proteins. Protein fractionation and enrichment techniques, such as immunodepletion combined with increasingly sensitive instruments are beginning to overcome this problem [60]. A single gene may also produce on average up to three protein splice variants, not to mention the potential post-translational modifications that may take place. Thus, splice variants and post-translational modifications significantly complex the 'gene to protein' identification process. Probably the biggest challenge of all is integrating proteomic data gathered with related biochemical and genetic information in order to better understand how the detected quantitative or qualitative (with altered activity) changes in the expressed proteins contribute to the patho-mechanism of a disease, and what are the central factors that perpetuate a particular disease. Nevertheless, the combined genomic and proteomic approach towards clinical pathologies has already significantly developed and it will only continue to improve our understanding of the relationship between the genome and disease. In the foreseeable future personalised clinical care will be the norm.

Yet, besides straightforward genomic approaches, other ways to combat some diseases and to individualise therapy should also be taken into account. (i) Because our immune system can recognise and remove 'changed proteins', immunostimulation is becoming a promising, individualised and selective approach towards curing cancer and other diseases [82-84]. (ii) Some viral proteins, for example apoptin and E4orf4, show

significant tumour-selective toxicity [85-88]. Thus, development of therapies based on these proteins, or on their principles of action may allow for the selective targeting of 'changed cells'. Every human being is genetically unique and this individuality plays a role in how well an individual responds to a particular drug. A drug administered to one patient may show no improvement compared to another patient where the particular drug completely cures them. (iii) Modulation of programmed cell death, for example induction of apoptosis in cancerous cells or prevention of apoptosis in tissues affected by degenerative diseases holds great promise for the development of new treatments against medical problems such as: myocardial infarction, neuro-degenerative diseases, or pathologies within the alimentary tract [89-91]. (iv) Precise, *in vivo* monitoring of cancer therapy would allow drug dose individualisation and quick identification of emerging resistance towards an administered drug [92,93].

Recent advances in DNA mapping and technologies will provide researchers and drug developers with crucial information needed to create drugs that are specific for each individual (i.e., different drug doses, or amounts of certain components within the drug). Novel tests will allow rapid and precise *in vivo* therapy assessment. Physicians will then in turn be able to prescribe drugs in more precise doses that will maximise positive results in the patient and almost eliminate severe side effects.

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