The 7th EFCC (former FESCC) Continuous Postgraduate Course in Clinical Chemistry

Under the Auspices of IFCC

NEW TRENDS IN DIAGNOSIS, MONITORING AND MANAGEMENT USING MOLECULAR DIAGNOSIS METHODS

Handbook

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GLOSSARY

Editorial

The Seventh EFCC (former FESCC) Continuous Postgraduate Course in Clinical Chemistry:
New Trends in Diagnosis, Monitoring and Management using Molecular Diagnosis Methods

The Croatian Society of Medical Biochemists and Slovenian Association for Clinical Chemistry, together with the European Federation of Clinical Chemistry and Laboratory Medicine (EFCC), former FESCC, have organized the seventh in a series of postgraduate weekend courses under the auspices of IFCC. The Course entitled "New Trends in Diagnosis, Monitoring and Management using Molecular Diagnosis Methods" promotes continuing postgraduate education of professionals in clinical chemistry and laboratory medicine, and ensures the laboratory knowledge harmonization, this time on molecular diagnosis methods in particular.

Renowned experts from European countries participating at this specialized EFCC Course have presented the state-of-the-art knowledge of molecular methods used in different clinical settings. The integrated knowledge of the authors, experts in different fields, printed in the Course Handbook, is intended to provide optimal information to the reader.

The contents of Handbook is divided into three chapters according to the Course program. The chapter Molecular Diagnosis in Inherited Disease covers topics such as Prenatal diagnosis of chromosomal disorders – molecular aspects, Postnatal molecular diagnosis of inherited diseases, Risk assessment and Global approach to biomedicine: functional genomics and proteomics. In the chapter Molecular Diagnosis in (Pharmaco) Therapy, Pharmacogenomics and personalized medicine, Dose adjustments based on pharmacogenetics of CYP450 enzymes, Pharmacogenetics of drug receptors and Application of pharmacogenetics in dose individualization in diabetes, psychiatry, cancer and cardiology are presented. The last chapter addresses, New technologies methods, Ethics, Quality assessment in molecular diagnosis where point-of-care molecular diagnosis: a near future?, Circulating nucleic acids as a diagnostic tool, European quality assessment networks in molecular diagnosis, and Ethics and legal issues of genetic testing.

We do hope that the Course program as well as this Course Handbook meets the intended goals by presenting the state-of-the-art, contributing to harmonization of new trends in the diagnosis, monitoring and management using molecular diagnosis methods.

Zagreb, October 2007 *Elizabeta Topić*

1. PRENATAL DIAGNOSIS OF CHROMOSOMAL DISORDERS - molecular aspects

Ana Stavljenić-Rukavina

The standard measures for population health outcomes is based on maternal, infant and under five mortality rates. Health care of mother and unborn child is the most important part of population health. Therefore the care for mother and child health during pregnancy and delivery, assessment of all risks during pregnancy is of utmost importance of any health care system.

It is known that perinatal mortality is caused in 20-25 percent of cases by inhaerited anomalies of fetuses and many of theese might be explained by genetic disorders. In general genetic disorder is a condition caused by abnormalities in genes or chromosomes. Chromosomes are complex bodies in cell nucleus as carriers of genes. While some diseases are due to genetic abnormalities acquired in a few cells during life, the term "genetic disease" most commonly refers to diseases present in all cells of the body and present since conception. Some genetic disorders are caused by chromosomal abnormalities due to errors in meiosis, the process which produces reproductive cells such as sperm and eggs. Examples include Down syndrome (extra chromosome 21), Turner Syndrome (45X0) and Klinefelter's syndrome (a male with 2 X chromosomes). Other genetic changes may occur during the production of germ cells by the parent. One example is the triplet expansion repeat mutations which can cause fragile X syndrome or Huntington's disease. Defective genes may also be inherited intact from the parents. In this case, the genetic disorder is known as a hereditary disease. This can often happen unexpectedly when two healthy carriers of a defective recessive gene reproduce.

Chromosomal abnormalities are disruptions in the normal chromosomal content of cell and are a major cause of genetic diseases in humans; some chromosomal abnormalities do not cause disease in carriers such as translocations or chromosomal inversions although they may lead to higher proportions of chromosomal disorder in child. Abnormal number of chromosomes or chromosome sets caled aneuploidy may cause letal condition or give rise in genetic disorders. Furthermore the gain or loss of chromosome material may lead to genetic disorder (deletion, extra copy as trisomy). Chromosomal mutations produce changes in whole chromosomes (more than one gene) or in the number of chromosomes present.

1.1 The major chromosomal abnormalities

The risk for chromosomal abnormalities increases with increasing maternal age, mainly because non-dysfunctional events in meiosis are more likely, and result in trisomies. To make it more complex the “mosaicism” must be added. A "mosaic" is a person with a combination of two cell lines with different karyotypes (normal and abnormal). When karyotyping is performed, multiple cells are analyzed to rule out this possibility. The mosaic condition is not as severe as the completely abnormal karyotype, and the features may not be as marked, and live births may be possible. Sometimes the mosaic’s is confined to the placenta ("confined placental mosaicism").

A placenta with an abnormal karyotype (confined placental mosaicism) may lead to stillbirth, even though the fetus has a normal karyotype; conversely, a placenta with a normal karyotype may allow longer survival for a fetus with a chromosomal abnormality. Rarely, a translocation of part of one chromosome to another in the parent will be passed on to the child as a partial trisomy (such as 6p+ or 16p+) which may not be as severe as a complete trisomy.

- Trisomy 21 (extra chromosome 21): Down syndrome; incidence based upon maternal age, though translocation type is familial; features can include: epicanthal folds, simian crease, brachycephaly, cardiac defects.
- Trisomy 18 (47, XY,+18): Features include micrognathia, overlapping fingers, horseshoe kidney, rocker bottom feet, cardiac defects, diapragmatic hernia, omphalocele.
- Trisomy 13 (Patau Syndrome also called D-Syndrome): Features include microcephaly, cleft lip and/or palate, polydactyly, cardiac defects, holoprosencephaly.
- Trisomy 16: Seen in abortuses from first trimester. Never liveborn.
- Monosomy X: Turner's syndrome (45,X 0); can survive to adulthood; features include short stature, cystic hygroma of neck (leading to webbing), infertility, coarctation.
- Klinefelter's syndrome (XXY, a male with 2 X chromosomes); features include elongated lower body, gynecomastia, testicular atrophy (incidence: 1/500 males)
- Triploidy: There is often a partial hydatidiform mole of placenta. Fetal features include 3-4 syndactyly, indented nasal bridge, small size.
- Idic 15 or isodicentric 15 :inverted duplication of chromosome 15 or tetrasomy 15
- Jacobsen syndrome also called the terminal 11q deletion disorder. This is a very rare disorder. Those affected have normal intelligence or mild mental retardation, with poor expressive language skills. Most have a bleeding disorder.
- XYY syndorm. XYY boys are usually taller than their siblings. Like XXY boys and XXX girls, they are somewhat more likely to have learning difficulties.
- Triple XXX syndrome. XXX girls tend to be tall and thin. They have a higher incidence of dyslexia.

A host of other chromosomal abnormalities are possible. In general, fetal loss earlier in gestation, and multiple fetal losses, more strongly suggests a possible chromosomal abnormality.

1.2 Prenatal diagnosis

Prenatal diagnosis employs a variety of techniques to determine the health and condition of an unborn fetus. Without knowledge gained by prenatal diagnosis, there could be an untoward outcome for the fetus or the mother or both.

Specifically, prenatal diagnosis is helpful for:

- Managing the remaining weeks of the pregnancy
- Determining the outcome of the pregnancy
- Planning for possible complications with the birth process
- Planning for problems that may occur in the newborn infant
- Deciding whether to continue the pregnancy
- Finding conditions that may affect future pregnancies

There are a variety of non-invasive and invasive techniques available for prenatal diagnosis. Each of them can be applied only during specific time periods during the pregnancy for greatest utility.

Indications for prenatal diagnostic testing include:age of mother, Down syndrome in previous pregnancy or family, structural aberrations in previous pregnancies or in family members, autosomal genopaties, X-linked genetic disorders, neuronal tube defects in previous pregnancies, mental retardation in family (linked to fragile X) present ultrasound suspicion, consanguinity, pathological finding in prenatal serum screening, other reasons (viral infection, radiation).

1.3 Source of samples for prenatal testing

Prenatal diagnosis of chromosomopathies as well as genetic disorders is based on invasive and non-invasive techniques.

Chorionic villi sampling (CVS)In this procedure, a catheter is passed via the vagina through the cervix and into the uterus to the developing placenta under ultrasound guidance. Alternative approaches are transvaginal and transabdominal. The introduction of the catheter allows sampling of cells from the placental chorionic villi. These cells can then be analyzed by a variety of techniques. The most common test employed on cells obtained by CVS is chromosome analysis to determine the karyotype of the fetus. The cells can also be grown in culture for biochemical or molecular biologic analysis. CVS can be safely performed between 9.5 and 12.5 weeks gestation.

CVS has the disadvantage of being an invasive procedure, and it has a small but significant rate of morbidity for the fetus; this loss rate is about 0.5 to 1% higher than for women undergoing amniocentesis. Rarely, CVS can be associated with limb defects in the fetus. The possibility of maternal Rh sensitization is present. There is also the possibility that maternal blood cells in the developing placenta will be sampled instead of fetal cells and confound chromosome analysis. The obtained material is used for fluorescent in situ hybridization (FISC), short tandem repeats (STR) , DNA and some biochemical analyses.

Amniocenthesis (transvaginal aspiration of amnionic fluid 15-20 weeks of pregnancy) is the most used method (risk below 0,5 %) for sample for all kind of analyses.

Preconception – preimplantation diagnosis is possibility applied in connection with in vitro fertilization (IVF) to make diagnosis at the gamete stage or performing the biopsy of one or two blastomeres by aspiration with micropipette. Preimplantation diagnosis is now offered as an alternative to conventional prenatal diagnosis in following cases: recessive or dominant hereditary disorders linked to chromosome X, monogenic disorders of authosomal inheritance (recessive or dominant) and the detection of translocations (couples who are carriers of chromosome abnormality of number or structure).

Maternal blood sampling for fetal blood cells is a new non-invasive technique that makes use of the phenomenon of fetal blood cells gaining access to maternal circulation through the placental villi. Ordinarily, only a very small number of fetal cells enter the maternal circulation in this fashion (not enough to produce a positive Kleihauer-Betke test for fetal-maternal hemorrhage). The fetal cells can be sorted out and analyzed by a variety of techniques to look for particular DNA sequences, but without the risks that these latter two invasive procedures inherently have. Fluorescence in-situ hybridization (FISH) is one technique that can be applied to identify particular chromosomes of the fetal cells recovered from maternal blood and diagnose aneuploid conditions such as the trisomies and monosomy X.

The problem with this technique is that it is difficult to get many fetal blood cells. There may not be enough to reliably determine anomalies of the fetal karyotype or assay for other abnormalities.

1.4 Molecular analysis

The technologies developed for the Human Genome Project, the recent surge of available DNA sequences resulting from it and the increasing pace of gene discoveries and characterization have all contributed to new technical platforms that have enhanced the spectrum of disorders that can be diagnosed prenatal. The importance of determining the disease-causing mutation or the informative ness of linked genetic markers before embarking upon a DNA-based prenatal diagnosis is, however, still emphasized.

Different fluorescence in situ hybridization (FISH) technologies provide increased resolution for the elucidation of structural chromosome abnormalities that cannot be resolved by more conventional cytogenetic analyses, including micro deletion syndromes, cryptic or subtle duplications and translocations, complex rearrangements involving many chromosomes, and marker chromosomes. Interphase FISH and the quantitative fluorescence polymerase chain reaction are efficient tools for the rapid prenatal diagnosis of selected aneuploidies, the latter being considered to be most cost-effective if analyses are performed on a large scale. There is some debate surrounding whether this approach should be employed as an adjunct to karyotyping or whether it should be used as a stand-alone test in selected groups of women.

Interphase and metaphase FISH, either as a single probe analysis, or using multiple chromosome probes, can give reliable results in different clinical situations.

It should be noted that there may be variation in probe signals both between slides (depending on age, quality, etc. of metaphase spreads) and within a slide. Where a deletion or a rearrangement is suspected, the signal on the normal chromosome is the best control of hybridisation efficiency and control probe also provides an internal control for the efficiency of the FISH procedure.

Depending on the sensitivity and specificity of the probe and on the number of cells scored, the possibility of mosaicism should be considered, and comments made where appropriate. By using locus-specific probes at least 5 cells should be scored to confirm or exclude an abnormality. Multiprobe analysis: three cells per probe should be scored to confirm a normal signal pattern. Where an abnormal pattern is detected, confirmation is advisable. In prenatal interphase screening for aneuploidy signals should be countered in at least 30 cells for each probe set. A minimum 100 cells should be scored.

When hybridisation is not optimal, the test should be repeated. When a deletion or another rearrangement is suspected, the results must be confirmed with at least one other probe.

Results should preferably be followed up by karyotype analysis. This is essential when there are discrepancies between the expected laboratory findings, and the clinical referral.

Before introducing interphase FISH as a diagnostic technique, staff need appropriate training on the type of samples to be analysed. Laboratories should set standards for classification of observations and interpretation of results.

More recently new method for fast identification of chromosomal abnormalities has been developed as high resolution array comparative genomic hybridization (aCGH) which provide genome-wide analysis of chromosome copy number and structural change .The chip technology provide investigation of genetic causes associated with dysmorphic features, mental retardation, developmental delay, multiple congenital abnormalities .The commercial chip include more tha 40 abnormalities including duplications and microdeletion regions. It is expected that evaluation of this technique will prove scientifically based evidence for named advantages.

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2. POST-NATAL MOLECULAR DIAGNOSIS OF INHERITED DISEASES

Maurizio Ferrari, Laura Cremonesi, Stefania Stenirri

2.1 Introduction

Molecular diagnostics is a discipline that combines laboratory medicine with the knowledge and technology of molecular genetics. Its aim is to provide a sensitive alternative to protein-based current methodologies by developing DNA/RNA-based analytical methods for monitoring human pathologies. This is accomplished by the identification of the disease-causing mutations which may be both known or unknown.

Completion of the human genome project have been generated, within a few years, vast information regarding molecular alterations either causing inherited disorders or predisposing to diseases (1). Many projects in current human genetics aim to dissect complex traits by making use of DNA markers, mainly by single nucleotide polymorphisms (SNPs) which are currently used for whole-genome scanning of genomes to gain first indication of interesting regions that contribute to the traits under investigation or in more focused candidate gene association studies. Once an SNP or a group of SNPs has been identified as a disease marker, it can be used for diagnostics.

In clinics some specific criteria of the DNA variations typing methods are required such as: simple protocols, which can be easily applied for medium throughput applications, short operational time because it reduces labour costs, rapid analysis which may be crucial to save the life of a diseased (e.g., infected) patient and costs which become an important issue (2).

With the advent of PCR, the battery of diagnostic tools for gene mutation screening was significantly enriched and DNA amplification is coupled to a rich repertoire of methodologies for detecting known mutations or screening for unknown sequence alterations inside the human associated disease loci (3, 4).

In clinical diagnostics, methods for SNP genotyping are applied, for example, in the diagnosis of a number of inherited diseases caused by a relatively small number of mutant alleles. In other situations, where the disease is associated with a high number of different and private mutations spread all over the gene or the mutant alleles are so rare that each family carries its own mutation, the routine clinical diagnostics must be based on mutation scanning over a complete gene (5).

Consequently, in the last years a number of rapid, robust, cost-effective and efficient methods with the capacity to detect known and unknown sequence variations on a medium-large scale have been developed (1). We will describe only the main methods that are frequently utilized in molecular diagnostic laboratories.

2.2 Detection of known mutations

Genotyping methods for the identification of known DNA variations are based on nucleic acid hybridization with short oligonucleotide probes or on the use of DNA modifying enzymes.

Allele-specific mutation detection of amplified DNA based on hybridization of PCR products to allele-specific oligonucleotide probes (ASO) can be applied in two formats. The first is the Dot-blot approach, whereby PCR products are immobilized on a membrane and hybridized to labelled ASO probes. Because of its simplicity it has become one of the most widely adopted methods in molecular diagnostics, using either radioactive or nonradioactive probes. The Dot-blot format is most useful when large numbers of samples are being screened for a small number of mutant alleles (6). The second approach is the reverse dot-blot, whereby ASO probes are immobilized on a membrane and hybridized to labelled PCR products. The latter can be considered the founding principle behind genotyping microarrays (see below). The reverse dot-blot is a widely used tool for routine screening of numerous mutant alleles in several disease associated genes (7). Automated platforms for preparing the reverse dot-blot membranes (strips) have been reported that allow printing of large numbers of strips with higher-density arraying (8) and hence commercialization of the entire process. Today there are a number of commercially available mutation detection assays for different disease mutations (4).

Enzyme-assisted genotyping, using nucleases, DNA ligase or DNA polymerase, are also employed. Restriction endonuclease analysis (RFLP) and allele-specific mutation amplification are the early and most widely used techniques to detect known gene mutations based on enzymatic reaction. In cases in which the mutation fails to create or abolish a restriction site, the latter can be artificially created by incorporating the necessary nucleotide change(s) in the amplification primer trough PSDM (PCR-mediated Site Directed Mutagenesis) (9, 10).

Allele-specific amplification using the amplification refractory mutation system (ARMS) (11) is probably one of the most popular detection methods for point mutations (10, 12), since it has the advantage of being able to detect virtually all known sequence variations. False-negative results due to amplification failure can be easily monitored using an internal control of an irrelevant genomic region, while the single-tube assay allows for the simultaneous detection of both wild-type and mutant alleles (4, 13)

The oligonucleotide ligation assay (OLA) relies on the hybridization of two allele-specific oligonucleotide probes (one specific for the wild-type allele and the other specific for the mutant allele) plus a fluorescent common probe coupled with DNA ligase reaction (14). This method have been improved to genotype a large panel of informative biallelic markers through multiplexing PCR and ligase reactions and chemically modifying oligonucleotide probes. Probes bear non-nucleotide tails which allow the mobility of each ligation product to be arbitrarily defined, regardless of oligonucleotide length or sequence (Sequence-Coded Separation) (15, 16).

A more recently developed method based on DNA polymerases is Single Nucleotide Extension (SNE) (also Single Base Extension –SBE- or minisequencing) In this case the distinction between genotypes of the SNPs is based on the high accuracy of nucleotide incorporation by the DNA polymerases (17). The primer extension reaction is robust,

allowing specific genotyping of most SNPs at similar reaction conditions. These features are advantageous for high throughput applications because the effort required for assay design and optimization are minimized.

Because the enzyme-assisted methods have proven to be more robust and to provide more specific allele distinction than allele specific oligonucleotide hybridization (18), these methods have been multiplexed, automated and adapted to various detection strategies, and they provide most of the current high-throughput SNP-genotyping platforms (19).

Finally, real-time PCR has recently emerged for rapid genotyping (20, 21) without the need for post-PCR sample manipulation. The method is based on Fluorescence Resonance Energy Transfer (FRET). FRET occurs when two fluorescent dyes are in close proximity to one another and the emission spectrum of one fluorophore overlaps the excitation spectrum of the other fluorophore (22). Commonly used FRET-based technologies include the LightCycler and TaqMan® assays and molecular beacons (16).

Whit this approach, each fluorescently labelled hybridization probes, specific for each mutation, yields a different melting curve, and genotyping is performed on the basis of a melting-curve analysis. This allows one to quickly assign hetero- or homozygosity for the wild-type and/or mutant allele, and at the same time monitor for false-positive or negative results. Although it is expensive and difficult to standardize, the assay is very fast, simple, and high-throughput, and allows the reliable detection of several mutations simultaneously (4).

There has, however, been enormous development in assay formats and labeling and detection strategies. Miniaturization and multiplexing of the mutation scanning and genotyping assays is a key element for bringing down costs and increasing throughput (5).

2.3 Scanning methods for unknown gene sequence alterations

Denaturing gradient gel electrophoresis (DGGE) and single-stranded conformation polymorphism (SSCP) analysis are two of the most commonly used methods for screening for both known and unknown mutations in human genes (23, 24). The hallmark of both techniques is their high discriminatory potential between the wild-type and different mutant alleles, since even a single base difference anywhere in the amplified DNA fragment will theoretically yield a different electrophoretic pattern (4). The DGGE when optimised displays a very high mutation detection rate (about 95%) compared with other scanning methods. The technique has been further improved by superimposing a porous gradient on the denaturing gradient [double-gradient DGGE (DG-DGGE)], which minimizes band broadening, even in prolonged runs, and permits more accurate band separation (25). Additionally, temporal temperature gel electrophoresis (TTGE), which relies on a temporal temperature gradient instead of the chemical gradient used in DGGE, has also been reported to be easier to perform and more reproducible (compared to DGGE) (4, 26).

However for all these approaches, careful adjustment of the experimental conditions is required in order to obtain reproducible results between different runs, particularly for the purposes of prenatal diagnosis.

In recent years, denaturing HPLC (DHPLC) has been gradually adopted for use in several diagnostic laboratories because it provides a semiautomated, fast, and reliable alternative to DGGE. DHPLC uses an ion-pair chromatography separation principle, combined with precise

control of the column temperature and optimized mobile phase gradient for separation of mutant DNA molecules (3). Different experimental protocols have been described for diagnostic purposes, showing about 98% sensitivity and specificity in detecting point mutations or even large deletions (27, 28). Considering the above mentioned advantages, together with the high initial investment costs for purchasing the DHPLC set up, this method seems the most appropriate for diagnostic laboratories that have large test volumes and are involved in routine carrier identification and mutation screening.

It should be noted that ideally the above-mentioned methodologies should be coupled with DNA sequencing, for either the definitive identification of unknown DNA sequence variations or the confirmation of inconclusive results, such as neutral gene variants, or ambiguous chromatograms and/or electrophoretic patterns (4).

Direct sequencing approach is the benchmark for genotyping but costs and throughput are the key limitations. Many new sequencing methods are being explored at present (sequencing by hybridisation, pyrosequencing, base-by-base sequencing by synthesis, sequencing by ligation, nanopore technology) and their integration in high throughput and automated platform may help in overcoming sequencing limits (29). An alternative to direct sequencing is the employment of mass spectrometry (MS) for the detection both identification of known and unknown DNA variations (2, 30).

2.4 Microchip

In the last few years, technology rapidly improved and new laboratory tools became available. Among them DNA chips have the potential for sample detection in integrated systems. Through miniaturization of the test platform, microchip-based nucleic acid technologies allow assay development for rapid detection of a large variety of single nucleotide polymorphisms (SNPs) and mutations in a large population sample thus reducing time and manual work.

Therefore, many important molecular biological analyses will be improved by the introduction, in both research and clinical diagnostic laboratories, of this new powerful technique that can be proved faithful for a variety of applications (1).

Two principally different approaches underlie the new miniaturized assays: development of highly parallel assays in solid phase microarray formats and homogenous assays performed in individual channels in microfluidic devices. (5).

DNA chips are referred to as high-density oligonucleotides or cDNA molecules attached to a solid support. The fundamental principle of most of them is the highly selective nature of DNA double helix hybridization. In particular, the immobilized nucleic acids are interrogated through hybridization with a fluorescent nucleic acid molecule.

The biochip technology for SNP typing mainly uses two different approaches to perform allelic discrimination: either allele specific nucleotide incorporation based on enzymatic reaction (SNE, SBE and arrayed primer extension – see above) or allele specific hybridization (1, 31).

2.5 Future trends

In practice, the requirement of a PCR amplification step to achieve sensitive and specific SNP genotyping is the principal factor that limits the throughput of assays today.

New PCR instruments that use microcapillaries instead of microtitre plate formats have been devised, and offer increased PCR throughput and reduced reagent costs as they use extremely short amplification times and small reaction volumes. Fully automated SNP analysis systems could then be designed based on homogeneous detection, or by streamlining the PCR and the subsequent genotyping procedure in microfluidic 'lab-on-chip' devices that operate with submicrolitre reaction volumes. Such microfluidic devices are now under development in several biotech companies. Additionally, recent developments of composite materials and fluorescence detection strategies offer increased detection sensitivity and specificity for SNP-genotyping assays. Hybrid gold and silver nanoparticles have been used, instead of fluorophores, as labels on allele-specific oligonucleotide probes (32). In another very promising strategy for multiplexing bioassays, multicolour optical coding is accomplished by embedding different sized "quantum dots" into polymeric microbeads at precisely controlled ratios (33), this technology has the potential for several-thousand-fold multiplexing.

Anyhow, despite the numerous technical advances in detection and multiplexing strategies, no technique clearly represents the final benchmark approach.

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3. RISK ASSESSMENT

György Kosztolányi

Throughout the history of medicine, patients and their relatives at risk of a disorder have been sought information and advise on the consequences of a disorder, the probability of developing or transmitting it and of the ways in which this may be prevented or avoided. Genetic risk assessment is the basic essence of clinical genetics. Clinical geneticists focus on probability and risk estimate, and on communicating both of these to patients via genetic counselling in ways that optimize decision making about their reproductive options or prophylactic measures to decrease their disease risk. In the molecular genetics era this demand on clinical genetics has been growing and arises also as claim of symptomless healthy persons.

3.1 General recommendation for genetic risk assessment

3.1.1 Risk assessment and genetic counselling

Risk assessment is inseparably interwoven with genetic counselling. When the persons ask for counselling they want to know their individual risks, while the risk estimates are transmitted to the individual in genetic counselling settings. Risk assessment has to be provided or supervised by a health-care professional trained for genetic counselling.

The three main elements of genetic counselling are:

- *Finding diagnosis* - Without diagnosis all advice has an insecure foundation. A clear diagnosis should be made as firm as possible before risk estimates are given to those seeking advice. Collecting genetic information is the first and most important step and is best achieved by drawing up a pedigree. Recent advances in molecular genetics have begun to elucidate the genetic mutations underlying many single-gene diseases. However, it is important to emphasise that an abnormal test result in itself is not equal with a clinical diagnosis. The result of genetic tests should always be related to the associated clinical condition. It is unrelevant binding a test result to an individual, by saying simply that he/she has more/less/variant DNA without relating the aberration to a definitive clinical condition.

- *Risk assessment* - Information on genetic risks is rarely an absolute ’yes’ or ’no’. Risk figures in genetic counselling may be given either as odds or as percentages. Some people prefer to use odds and to quote risks as 1 in 10, 1 in 100, etc. Others prefer to use such figures as 10 per cent, 1 per cent. In the genomic era, complex mathematical models, algorithms and softwers are known for the multivariable logistic regression analyses of research data. Whatever method is used, there are pitfalls in interpretation which must be avoided, and this may require much practice.

Risk estimates may be based on different sorts of information and may be of greater or lesser reliability. The main categories are as follows (P.S.Harper).

Empiric risks Here the estimate is based on observed data rather than theoretical predictions; this is the form of risk estimate available for most of the more common non-mendelian or chromosomal disorders.
Mendelian risks Mendelian risk estimates can only be given when a clear basis of single gene inheritance can be recognized for disorder. They are prehaps the most satisfactory form of risk estimate because they commonly allow a clear differentiation into categories of negligable risk and high risk.
Modified genetic risks The essential feature is that a 'prior" genetic risk, based on mendelian inheritance, may be modified by 'conditional' information, usually genetic, but sometimes from other sources. Such modifying information may drastically alter the risk estimate and should always be used when available.
Composite risks Most empiric risks really fall into this mixed situation. A clinical entity may have different genetic background with different inheritance, resulting in an intermediate risk depending on the relative frequency of the various forms. Obviously this intermediate risk does not really exist at all – the family must represent one or other of the extreme positions. With improved resolution of genetic heterogeneity it may be possible to distinguish the individual components, while even within a single family additional information may resolve the situation.

- *Communication of the genetic information* - Communicative role ensuring that those seeking information actually benefit from it. Clinical geneticists and genetic counsellors should make genetic information available according to universal ethical principle which respect the individual's dignity, autonomy, religious and cultural beliefs. Free informed consent, privacy, confidentiality have to be regarded as strict conditions when persons are helped in their decision making.

Genetic counselling cannot be compulsory, no more than any other medical act, however, it should be offered and strongly recommended before and after genetic testing.

Pre-test genetic counselling has to inform the individuals what the test is for, include up-to-date, reliable description about symptoms and natural history of the disease, prospects of prevention or treatment, inheritance pattern, the risk of disease in the counsellee's situation, available reproductive choices, reliability and limitations of the test concerned, and possible psychological impact and other consequences of the test result to the counsellee and his/her family/relatives.

Post-test genetic counselling - in addition to the main points of pre-test counselling, a plan to inform relatives in relation to their risk has to be agreed with the counsellee (or, if necessary, a decision to discuss this further, after a time to reflect). Implications to the individual (including a follow-up plan, when relevant) and his/her near relatives should be discussed.

3.1.2 Some complexities in risk assessment

Penetrance
In many (but not all) single-gene, mainly autosomal dominant (AD) diseases a person with a specific genotype is virtually certain to develop the associated disease. The likelihood that a person carrying a disease-associated genotype will develop the disease is known as the penetrance of the genotype. Eg., both Huntington disease and cystic fibrosis are virtually 100% penetrant. The mutations in the BRCA1 gene are highly penetrant, but not completely so; estimates of the lifetime penetrance of these mutations vary from about 60-85%.

Variations in penetrance are caused by the modifying effects of other genes and/or by environmental factors.

Inherited or new mutations
The mutations underlying some, mainly autosomal recessive (AR) disorders (eg. cystic fibrosis) appear to have arisen many generations ago, with very few new mutations arising. There are other diseases, however, mostly AD disorders, for which new mutations appear to be more frequent, so an affected individual may carry a mutation that is not present in either parent. If this is the case, the parents usually have a low risk of having another child with the same disease.

Genetic heterogeneity
Some single-gene diseases, clinically fairly well circumscribed entities, may be caused by different mutations. Genetic heterogeneity resulting from disease-causing mutations at different genetic loci is called non-allelic or locus heterogeneity. When different mutations at the same locus cause the same disease, this is known as allelic heterogeneity. Allelic heterogeneity is very common.

Variable expressivity
Even if a disease genotype is fully penetrant, the severity and symptoms of the disease can vary in different affected individuals, presumably because they are influenced by other genetic and environmental factors. Variable expressivity can be considered the rule rather than the exception for virtually all genetic disease.

3.2 Risk assessment based on clinical data

3.2.1 Chromosomal abnormalities

The great majority of chromosomal disorders have an extremely low risk of recurrence in a family, except those of translocation type.

There is a well-known relationship between the incidence of trisomy 21, the most important chromosome disorder and maternal age. The population incidence is around 1 in 650 live births, while the risk of Down syndrome reaches 1% at the age of 40 years. Paternal age is of little significance. Other trisomies are rare as live births, while extremely common in spontaneous abortions.

Familial accumulation of chromosome rearrangements is possible when the abnormality is translocation type. The recurrence risk in such a situation depends on whether there is an abnormality in the parental chromosomes. If the chromosomes are normal, as in the great majority, the risk to further offspring is minimal. If one parent has an abnormal karyotype (balanced translocation), risks are 1-100% depending on the chromosomes involved and the parental origin.

During the past few years a new type of chromosomal anomaly was delineated by molecular cytogenetics techniques recognizing small (submicroscopic) deletions in a number of different malformation syndromes. The recurrence risk in these *microdeletion syndromes* is low, since the great majority of such cases are de novo rearrangements.

3.2.2 Single gene (or mendelian) disorders

If the clinical and genetic information for a family with a particular disease suggest single gene disorder, then it is likely that precise risk can be given regarding its occurrence in other family members. Mendelian inheritance may be established on the basis of the pedigree, by a combination of clinical diagnosis and the pedigree, or entirely on the clinical diagnosis (sporadic cases).

Identifying carriers of genetic disorders in families or populations at risk plays an important part in preventing genetic disease. In families in which there is a genetic disorders some members *must* be carriers because of the way in which the condition is inherited. These *obligate carriers* can be identified by drawing a family pedigree and do not require testing as their genetic status is not in doubt.

It should be emphasised that mendelian inheritance can not be regarded as a rigid and unvarying mechanism following a fixed set of rules. One of the most fascinating developments of recent years has been the discovery of fundamental biological mechanisms underlying non-mendelian monogenic inheritance (see below).

- *Autosomal dominant (AD) inheritance*

Although in theory AD inheritance is the simplest mode for risk assessment, in practice it provides some of the most difficult problems with special traps. The 50% risk of developing a condition for the offspring of an affected person may be modified by the age-of-onset of the disorder, the homo- or heterozygous state of the affected person (which is usually unknown), the lack of penetrance, variation in expressivity, and various factors underlying variability in mendelian disorders such as genomic imprinting, anticipation due to unstable DNA, gametic mosaicism, modifying allels, somatic mutation (see: non-mendelian monogenic inheritance).

In AD condition, obligate carrier is a person with affected parent and child. Testing for carrier state applies only to disorders that either are variable in their manifestation or have a late onset. Because of possibility of germline mosaicism (the real frequency is not known), the parental carrier state can not be excluded with certainty in families with single affected child suggesting new mutation.

- *Autosomal recessive (AR) disorders*

The principal difficulty with AR inheritance is to be sure that this is indeed the mode of inheritance in a particular family, since the great majority of cases of an AR disorder are born to healthy but heterozygous parents, whose high risk can not be detected from isolated case. Where the diagnosis makes this mode of inheritance certain, or in the minority of families where the genetic pattern is clear, risk prediction is relatively simple (see mendelian rules).

It is important to know how to estimate the chance of being a carrier for an AR disorder, both for family members and for the general population. The parents and children of a patient are obligate carriers, while second-degree relatives will have a 50% chance of being a carrier. Testing may be appropriate for the healthy siglings of an affected person (and their partner), and for consanguineous couples with a positive family history on both parental lines (and not just one!). The possibility of new mutation can be ignored, lack of penetrance is rarely encountered, and variation in expression is much less than in AD disorders.

The actual risk depends on the frequency of heterozygotes in the population, what can be estimated indirectly from the disease frequency by the Hardy-Weinberg equilibrium (direct

observation are exceptionally available). The main opportunity for preventing AR disorders would be in population screening programmes to identify individuals at risk.

One of the other problems with AR disease includes the risk assessment when both parents are affected by an AR condition (deafness, albinism). The risk to the offspring in such situation will depend on whether the parents share the same disease-causing genes (high risk), or they carry different alleles (risk is not increased).

- *X-linked disorders*

The term ’dominant’ and ’recessive’ must be used with caution, because a much greater degree of variability in the heterozygous female is seen than is the case with autosomal disorders. This is largely the result of X-chromosome inactivation (Lyon hypothesis).

Recognition of an X linked pedigree pattern is often overlooked and hence, risk assessment may be inappropriate and may be mistaken for AD inheritance. Aside from the new mutations (a mother of an affected boy is not always a carrier!), problems could be resulted from some particular conditions, eg. the disease is X-linked dominant, males with the disease do not reproduce, or the X-linked dominant disease is lethal in the male, etc.

The carrier state of the mother of an affected son may be particularly difficult to assess because of the possibility of new mutation in the child. Obligate carrier is a woman with two affected sons, or one affected son and another affected male maternal relative; the daughters of an affected man are also obligate carriers. Anyway, obligate carriers should be identified, even in the molecular era, since information from carrier testing is not always easy because of the variability of X-inactivation and gene expression in heterozygotes females.

- *Isolated cases*

Pedigrees showing only one affected person are the type most commonly encountered in clinical practice. Various causes must be considered, and risk assessment depends entirely on reaching an accurate diagnosis in the affected person. AR and X-linked recessive disorders are brought up first since, as a rule, such inheritances manifest themselves by a single case emerging in the family. Isolated cases with AD disorders may be resulted from new mutation, non-paternity, or germ-line mosaicism. In AD conditions, new mutations can usually be distinguished from transmitted cases. In X-linked recessive disorders, however, it may be extremely difficult to tell whether an isolated case represent a new mutation or whether the mother is a carrier.

- *Mitochondrial inheritance*

It is well known that any disorder following mitochondrial inheritance should be exclusively maternal in its transmission. All daughters of an affected or carrier females are themselves at risk of transmitting the disorder, as well as of becoming affected, while all sons are at risk of becoming affected (but not as transmitter).

3.2.3 Common complex disorders

Most common disorders (birth defects, chronic later-onset diseases) do not follow any of the clear patterns of mendelian inheritance. Yet, to some degree these conditions show a familial tendency, therefore, families where such disorders occur increasingly seek genetic counselling for risk assessment.

The fact that many of these disorders (alternative terms are *'multifactorial' or 'polygenic*) form the basis of activity for most medical specialists and primary care doctors, creates special challenges. Available risk information for non-geneticists is often inadequate and changing rapidly as advances in research alter specific genetic factors. This is mainly the reason why the genetic education of non-geneticists health professionals is a European primary.

The well known general rules of risk estimation in common complex disorders are based on epidemiological data available in handbooks and web sites, and such information provides the most satisfactory basis for risk assessment until the genetic basis can be resolved further. However, these empiric risk figures are not universal in their application. Eg., data on one population may not be applicable to others. Improved identification of specific causative factors may radically change risk estimates. Risks may depend also on individual factors, not only on the diagnosis.

Problems include also the involvement of more than one gene (gene-gene interaction), each of which may have only a small effect on disease susceptibility; uncertainties in disease diagnosis; different genetic polymorphisms underlying disease in different populations, and the large effects of environment and lifestyle on the development of disease (gene-environmental interactions). Although each of the underlying genes is inherited according to Mendel's rules, the disease itself is not inherited in any simple mendelian way.

Cancer is often described as a 'genetic disease' following rules of multifactorial inheritance, in the sense that it is caused by genetic alterations and influenced by environmental factors. However, the genetic alterations that lead to cancerous behaviour occur in the somatic cells of the body and are no passed on to the next generation. Therefore, risk assessment, instead of estimate the risk of disease transmission to the offspring, is focused on the outcome of cancer, and the ways in which this may be prevented or avoided by prophylactic measurements (decision-making before, eg. mastectomy in breast cancer). There are only some germ-line mutations (that is, mutations that are present in all the cells of the body including the sex cells) that predispose people who carry them developing cancer, and these are heritable.

3.3 Risk assessment based on molecular genetic testing

In growing number of diseases, genetic risk assessement is possible on the basis of genetic test results indicating whether a person is a carrier of a disease-causing mutation, or results determining whether an individual has a specific genetic susceptibility to a disease. *Clinical validity* and *clinical utility* are going to be increasingly important criteria in the professionals' offer whether or not to apply a genetic test.

3.3.1 Need for genetic counselling in various form of testing

The offer and application of molecular genetic tests should be a part of comprehensive clinical genetic service, and associated with genetic counselling which may vary according to the different form of testing.

Diagnostic testing (genetic test performed in a symptomatic individual to diagnose or rule out a genetic condition): such tests may have a status that is similar to other diagnostic tests, and pre-test counselling might be unnecessary. However, if the test result is positive, the patient

needs post-test counselling and the relatives will need risk assessment in genetic counselling setting.

Presymptomatic testing (genetic test in a healthy high-risk family member for a later-onset monogenic disorder): even if the family has already been counselled, further pre- and post-test genetic counselling has to be offered.

Susceptibility testing (simultaneous testing of several genetic markers): referred also as risk profiling for common complex disorders, it is only emerging. The clinical validity and utility of these tests needs to be proven. At present, it seems very likely, that they will be prescribed mainly by specialists other than clinical geneticists; and the need for proper risk assessment and genetic counselling by a genetic specialist will depend on the possible implications of the results of the test for the person and his/her near relatives. The same applies to pharmacogenetic testing, which tests for a genetic susceptibility for adverse drug reactions or for the efficacy of a drug treatment with a given genotype.

Carrier testing: genetic test that detects a gene mutation that will not have any consequence to the health of that individual; however, if inherited, alone (in case of X-linked inheritance, AD premutation or chromosomal translocation) or in combination with another mutation in the same gene from the other parent (in case of AR inheritance), it may confer a high risk of disease in the offspring. Pre- and post-test genetic counselling needs to be offered.

Prenatal testing (genetic test performed during a pregnancy): risk for a certain condition in the foetus should be assessed and pre- and post-test genetic counselling for the prospective parents needs to be offered.

Preimplantation genetic diagnosis means testing the presence of a mutation or chromosomal change in one or two cells of an embryo in a family with a previously known risk for a mendelian or chromosomal disorder in order to select the unaffected embryos to be implanted. Risk assessment, pre- and post-test genetic counselling for the prospective parents has to be offered.

3.3.2 The clinical application of molecular testing for risk assessment

3.3.2.1 Single gene (mendelian) disorders

Molecular analysis is potentially possible for any single-gene disorder, especially those where the gene has been isolated or mapped. However, it is necessary to point at the growing gap between what has been discovered and what is available in service.

When interpreting the finding of an apparently specific mutation, firm proof for the association between cause and effect are needed. In the early stages of research after a gene is isolated, it may be far from clear whether a particular change is a causative mutation or is a harmless normal variation (polymorphism) unrelated to the disease state (population data, testing of the healthy parents, or protein truncation test are needed to resolve this question).

Another important point about risk assessment is that genetic testing will only reveal the presence or absence of the factor(s) being tested for. Because of genetic heterogeneity, it is important wherever possible to identify the specific mutation associated with disease in an

affected member of a family. If a mutation can be found, it is then possible with great accuracy to determine whether other family members carry the same mutation.
A negative result from genetic testing, although it lowers the probability that the individual carries a disease-causing mutation in that gene, cannot eliminate it altogether. The residual risk has several components: the possibilities of a mistake in the test itself (no test can be 100% accurate!), allelic heterogeneity, and new mutations.

In mitochondrial diseases, it is not known whether there is any correlation between the proportion of abnormal mitochondria found in blood and the risk of developing or transmitting the disorder. Genetic tests are equally unhelpful in prenatal diagnosis. Thus the conclusion at present has to be that while the recognition of mitochondrial inheritance by pedigree pattern and molecular analysis is important in identifying genetic risks and in removing risk from descendents in the male line, genetic tests are of limited use in resolving the situation for those known to be at risk.

3.3.2.2 Presymptomatic genetic testing for risk assessment

Presymptomatic genetic testing is the use of genetic testing to tell whether a symptomless individual will develop a genetic disease later in life. It can only be used where the disease-associated mutation is known and is highly penetrant. Eg., in Huntington disease DNA testing at any age, even prenatally, will reveal whether the mutation is present, changing that person's individual risk from 50% to either 100% or zero. According to good clinical practice, presymptomatic tests for future severe illnesses with no options for treatment or prevention should never be performed without pre- and post-test genetic counselling, as well as psychosocial evaluation and follow-up.

3.3.2.3 Carrier testing

Carrier testing for autosomal-recessive disease is widely practiced in developed countries with ethnicities characterized by particularly high disease-causing allele frequencies, such as beta-thalassaemia in Mediterranian countries, or Tay-Sachs disease in Ashkenazi Jews. Such testing is usually carried out in the format of formal, community run programmes, with clinical validity and utility well established.

3.3.2.4 Predictive testing of genetic susceptibility for common complex diseases

The growing ability to map and isolate specific genes involved with common disorders has allowed two main categories to be defined:

- *disorders containing a significant mendelian subset*, resulting from the action of a single major gene with relatively high penetrance in the family. – The recognition of such a subset of cases gives extremely high risks for family members or very low risk if the particular gene mutation can be excluded. It is no longer meaningful to derive overall theoretical risks eg. in breast or colon cancer, since the separation of the mendelian forms will affect the recurrence risks for the remaining.
- *disorders where the genes/variants are of low penetrance*, each (and usually also the environmental factors) being of moderate or small influence. – The ability to detect different susceptibility genes or allelic variants associating with an increased risk of diseases like asthma, diabetes, psychoses, etc. will influence risk assessment and genetic counselling. However, because of incomplete penetrance of these genes or variants, they cannot be used with certainty to predict the development of disease. Furthermore, the genes (usually many)

interact in complex ways with each other and with environmental and lifestyle factors to determine whether disease will develop.
Research for most common disorders is still at an early stage in resolving the number and nature of genes involved, and risk prediction is weak. The use of genetic susceptibility tests in risk prediction is at present of minimal help and potentially of considerable harm. However, this situation may change in the future, and genetic susceptibility testing may find a place in mainstream clinical medicine.

3.3.2.5 Genetic testing/screening to predict the future health status of a healthy individual

Predictive genetic testing of seemingly healthy people can principally be applied individually or collectively. The aim is to detect individuals who are at risk of developing a particular disease or of responding badly to a particular treatment.

An *individual approach* is chosen when the person is at recognizably elevated prior risk to develop a specific disease because of a relevant family history, such as late-onset disorders, particularly familial cancer (breast, bowel, thyroid, and others) and neurodegenerative disorders, even to children, if there is a proven clinical benefit ensuing from testing before adulthood.

A *population screening* could either be applied generally (e.g. newborn screening for metabolic disorders), or to subgroups preselected on the basis of risk factors. The use of molecular genetic screening currently has some legitimacy in *certain monogenic condition*, but no established value with respect to *common complex diseases*. The risk estimates applied to individuals is generally very uncertain, with wide margin of error. The failure to replicate the majority of initial results of association studies between genetic variants and disease risk is likely to be at least partly due to inadequate sample sizes, poor or inappropriate statistical analysis, poor study design, inderect assessment of causal pathways, complexity of the phenotypes studied, and the complexity of allelic or genotypic contributions to phenotype. Further pragmatic randomised controlled trials need to be done to determine whether genetic testing has clinical benefit in management of common complex disorders

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7. www.eurogentest.org (Harmonizing Genetic Test across Europe)
8. www.eshg.org (European Society of Human Genetics)
9. www.ashg.org (American Society of Human Genetics)
10. www.nchpeg.org (National Coalition for Health Professional Education in Genetics)

4. GLOBAL APPROACH TO BIOMEDICINE: FUNCTIONAL GENOMICS AND PROTEOMICS

Krešimir Pavelić, Marijeta Kralj, Sandra Kraljević, Mirela Sedić

4.1 Abstract

Functional genomics (transcriptomics and proteomics) is a global, systematic and comprehensive approach to identification and description of the processes and pathways involved in the normal and abnormal physiological states. The most applied methods of functional genomics today are DNA microarrays and proteomics methods, primarily two-dimensional gel electrophoresis coupled with mass spectrometry. Up to date interesting research have been carried out, representing the milestones for future implementation of functional genomics/proteomics in biomedicine. Still, further systematic examination of differentially regulated genes and proteins in tissues and fluids in healthy vs. diseased subjects will be required. However, high-throughput technologies reflect biological fluctuations and methodological errors. Large amount of such different data challenges the performance and capacity of statistical tools and softwares available at the moment. Yet, further major developments in this field are pending and the intellectual investment will certainly result in clinical advances.

4.2 Introduction to functional genomics

As an emerging discipline, functional genomics has many different definitions, which depend on the research area. Functional genomics (transcriptomics and proteomics) is a global, systematic and comprehensive approach to identification and description of the processes and pathways involved in the normal and abnormal states. Why is it such an important experimental approach nowadays? It is estimated that approximately 30% of the open reading frames in a fully sequenced organism have unknown function at the biochemical level and are unrelated to any known gene. This is why recently the interest of researchers has shifted from genome mapping and sequencing to determination of genome function by using the functional genomics approach (Figure 4.1.).

Figure 4.1. *A single gene can give rise to multiple gene products. RNA can be alternatively spliced or edited to form mature mRNA. Besides, proteins are regulated by additional mechanisms such as posttranslational modifications, compartmentalization and proteolysis. Finally, biological function is determined by the complexity of these processes.*

Techniques of functional genomics include methods for gene expression profiling at the transcript level (differential display, expressed sequence tags, serial analysis of gene expression and DNA microarrays) as well as methods for proteome analysis [Celis *et al*, 2000]. Due to recent technological advances in fabrication of very precise high-throughput instruments, a complex functional genomics approach has become possible. Processing large quantity of experimental data requires powerful information systems – bioinformatics, which encompasses development of new computational methods and application of those methods to solve biological problems. Bioinformatics has also a large service component in which computational resources such as databases are operated for the benefit of the research community. It also finds its implications in other aspects of biomedical research involved in functional genomics, such as laboratory information management systems, medical record systems as well as documentation of clinical trial results for regulatory agencies [Bogusky and McIntosh, 2003].

All those high-throughput experimental procedures enabled and accelerated new and important basic discoveries, especially in the field of molecular medicine. The ultimate aim is, however, to bring these discoveries closer to clinics and therefore substantially improve daily clinical practice.

The benefits of functional (integrative) genomics approach and applications to biomedicine are likely to be: a) the understanding of physiological or pathophysiological processes, b) identification of genes/proteins which can be used for screening, diagnosis or monitoring disease severity and c) discovery of novel genes/proteins suitable for therapeutic manipulation.

The most applied methods of functional genomics today are DNA microarrays and proteomics methods, primarily two-dimensional electrophoresis (2-DE) coupled with mass spectrometry (MS).

4.3 Introduction to DNA arrays

DNA microarrays may be defined as miniaturized, systematic immobilization of nucleic acid fragments derived from individual genes on a solid support, which by specific hybridization, enables the simultaneous analysis of thousands of genes in parallel [Dudda-Subramanya *et al*, 2003; Lockhart and Winzeler, 2000]. Microarray technique is based on new technology in which DNA probes are robotically spotted on miniature slides. The length of probes varies from kilobases, for standard cDNA arrays used for RNA expression analysis, to tens of basis in oligonucleotide arrays for both RNA expression and DNA sequence analysis [Dudda-Subramanya *et al*, 2003].

Nylon DNA arrays, often termed macroarrays are nylon-membrane based cDNA arrays for broad-scale expression profiling. They continue to be used because of their accessibility and flexibility. This is not only due to somewhat expensive use of microarrays but also to the fact that nylon DNA arrays offer a promising alternative in a number of situations, particularly when small amounts of sample are available or when specific pathways are being studied, which is itself significant for a number of research and clinical applications.

There are several different implementations of the DNA microarray principle for expression measurement. Microarrays are the most promising in terms of throughput and should eventually allow simultaneous measurement of expression of the whole set of genes. DNA

microarrays have already had various applications: messenger RNA expression profiling for improved disease classification; genotyping of polymorphisms affecting disease susceptibility; identification of genetic lesions within malignancies; design and discovery of new therapeutic drugs and sequencing of DNA [Celis *et al*, 2000].

4.4 DNA arrays in transcriptomics studies

DNA microarrays are the preferred and wide-accepted method for transcriptomic research as most disease processes are accompanied not only by characteristic macroscopic or histological changes, but also by systematic changes in gene expression patterns. For some pathological processes such as cancer, inappropriate gene expression is a fundamental aspect of pathogenesis. For other pathological processes, the gene expression programs, both in cells directly affected by a disease and in healthy cells responding to the local and systemic effects of a disease, can provide a detailed molecular picture of the pathogenic process [Diehn *et al*, 2000].

When used for documentation of gene expression at a genome-wide scale, microarray-based transcriptional profiling allows the identification of a set of genes that defines differential biological states (Figure 4.2.). This is a crucial step in the development of novel approaches for complete diagnosis of a disease. Molecular classification of a disease combined with the ability to place a certain patient into a specific genetic subtype, holds a promise of a better comprehension of a disease and thus development of individualized health care delivery. Microarray analysis can help in prediction of disease outcomes and prognosis.

Figure 4.2. *Comparative gene expression analysis by using DNA microarray.*

Microarrays are also being applied increasingly to mutation analysis (polymorphisms analysis) by minisequencing. Single nucleotide polymorphisms (SNPs) are the commonest source of mutation in man and can be used as markers in whole genome linkage analysis of families or in association studies of individuals in a population. Single base differences between human genomes, or polymorphisms underlie differences in susceptibility to or protection from a host of diseases. Scientists believe SNP maps will help them identify the multiple genes associated with complex diseases such as cancer, diabetes, vascular disease, and some forms of mental illness. These relationships are difficult to establish with conventional gene-hunting methods because a single altered gene may make only a small contribution to the disease.

For instance, when applied to pregnancy research, the DNA array technique is extremely powerful. In that regard, interesting papers were published on newly discovered cDNAs/proteins which play a role in various stages of placentation mostly using mouse models and purified cell culture systems.

4.5 Proteomics

The term proteome was first introduced to describe the entire PROTEin complement of a given genOME, i.e. the complete set of proteins, which are expressed by the entire genome. Since the proteome is quite dynamic and it changes along with the development of an organism and with any alterations in the environment, it can be referred to as the array of proteins expressed in a biological compartment, such as cell, tissue or organ, under particular environmental circumstances at a particular time. Proteomics is a powerful tool for examining differential protein expression comparing hundreds of proteins simultaneously.

Majority of our cells contain the same genome regardless of the cell type, stage of the development or environmental conditions, unlike the proteome, that varies significantly under these diverse circumstances due to different patterns of gene expression and protein modification. There are more proteins in the proteome in comparison with the genes in a genome and it has been estimated that the human proteome is at least an order of magnitude more complex than the human genome, since it is assessed that there might be as many as a million human proteins. For example, in spite of the same genome, the same growth factor may have diverse signaling pathways in different cell types or cell states, implying that the study of the genome exclusively could not clarify the molecular mechanisms of diseases, aging and cell response to external stimuli [Godovac-Zimmermann and Brown, 2001].

It has been postulated that the average number of protein forms per gene is one or two in bacteria, three in yeast and three or more in humans. Proteomics deals with the characterization, identification and quantitative analysis of proteins in cells, tissues and body fluids. It is particularly suitable for the analysis of some body fluids, such as serum and urine that are deprived of mRNA, and thus cannot be studied by mRNA analysis.

Proteomic studies also include the analyses of the variations in the protein expression levels in cells or tissues under two different conditions and identification of the main proteins likely to have important functional roles in the cells' response to those conditions, for example, healthy versus diseased tissue or cells. A protein found only in a diseased sample may represent a useful drug target or diagnostic marker. These proteomic comparative approaches are analogous to microarray experiments, which examine whether genes are turned on or off under diverse conditions.

Furthermore, proteomics studies the numerous possible interactions among proteins as well as the molecular composition of the particular cellular structures (organelles). Knowing exactly which proteins interact with one another could help determining, for example, components of a particular enzymatic pathway.

Protein modifications not obvious from the DNA sequence, such as isoforms and post-translational modifications (e.g. phosphorylation and glycosylation) can be determined solely by the proteomic studies. It is assessed that approximately 200 diverse types of post-translational protein modifications occur.

One aspect of proteomics studies is directed towards determining the subcellular localization of proteins in order to construct an overall three-dimensional protein map of the cell, which could give an insight into the regulation of protein function.

Taken together, the scope of proteomics is quite broad and its practical application goes far beyond merely analyzing large numbers of proteins in complex mixtures [Graves and Haystead, 2002].

4.6 Short overview of proteomics methods

The method of polymerase chain reaction (PCR) amplification was probably the largest advance in genome studies, which enabled many applications for genome study to date. However, in protein-based studies it may not be possible to exploit such a tool. For that reason and because protein chemistry and enzymology differ substantially from those of the DNA, proteomics is expected to be a more complex task than genome sequencing. To meet this challenge, many new and different technologies for the whole proteome characterization must be applied synergistically.

A detailed overview of proteomics techniques could be found elsewhere [Garfin, 2003; Gevaert K, J Vandekerckhove, 2000]. In general, proteome studies include the following experimental stages: isolation and separation of proteins from a cell line, tissue or organism; characterization and identification of the particular protein species; and the information storage in databases (Figure 4.3.). Two-dimensional polyacrylamide gel electrophoresis is the most widely used method for the separation of proteins in proteomics, which allows simultaneous analysis of hundreds to thousands of gene products. The first dimension, isoelectric focusing (IEF) is an electrophoretic method that separates proteins according to their isoelectric points (pI), which are defined as the specific pH at which the protein net charge is zero. The second, perpendicular dimension, SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) resolves polypeptides according to their molecular weights. The ultimate result of two-dimensional gel electrophoresis is a gel with spots corresponding to individual proteins. The most critical step in 2-DE is the sample preparation, which depends on whether the aim of the study is to examine the complete protein profile of the cell or only proteins present in the particular cellular compartments (such as membranes, subnuclear structures, small organelles and vesicles).

Subsequent to 2-DE, the protein spots to be identified are cut out of the gel and the proteins digested into shorter peptides by a protease, most often trypsin. The peptide fragments are then analyzed by mass spectrometer for the purpose of identification. The resulting peptide masses make so called fingerprint, which is characteristic for the particular protein. This fingerprint is juxtaposed to theoretically expected peptide masses for each protein entry in the

database. Mass spectrometer measures the mass of unknown molecules by ionizing, separating and detecting ions according to their mass-to-charge ratios and consists of three main components: an ion source, a mass-selective analyzer and an ion detector. High-throughput protein identification from 2-DE gels is dominated by the use of matrix assisted laser desorption/ionization method of sample ionization and time-of-flight mass analyzer (MALDI-TOF).

Figure4.3. *Practical approach to proteome research*

If the peptide mass fingerprint fails to identify the protein, it is possible to determine a small piece of amino-acid sequence from one or more of the peptides by fragmentation of the peptide ions in the mass spectrometer that uses quadrupole orthogonal time-of-flight mass analyzer (Q-TOF). The analysis of these new masses provides some partial amino acid sequence from the peptides, which is normally sufficient to clearly identify a protein.

Together with all broadly accepted standard proteomics methods, a high-throughput, analytical tool for rapid identification of differences between large number of samples has currently being developed – protein arrays. It would be extremely powerful to analyze hundreds or thousands of protein samples using a single protein array and thus acquire large

amounts of data. However, making protein arrays is far more difficult than making DNA arrays. This is mainly due to the fact that protein function depends on its precise three-dimensional structure. Out of narrow range of environmental conditions, proteins denature. For protein arrays, it is thus critical to choose the right capture agent on the basis of its specificity and affinity for the target protein. The capture agent is immobilized on the array surface and, when exposed to samples containing complex mixture of proteins, it binds the target protein. The proteins that remain bound to the capture agents on the surface are usually detected by fluorescence and identified by mass spectrometry. Protein arrays will possibly be used as sensitive, throughput and robust technology only if they will further maturate and be constantly applied to authentic biological samples. This technology is very useful in linking gene-array expression data with protein discovery and, unlike its gene-based counterpart, can be used to examine post-translational modification of proteins.

4.7 Functional genomics in practice: is it all that perfect?

In spite of constant technological improvements that are being made in the field of functional genomics, there are still many technical and statistical obstacles hampering their usage in routine clinical practice.

When it comes to microarray technology, data analysis has been criticized for inter-, and sometimes intra- laboratory variability. Microarray experiments are quite complex and the data have proven to be noisy, susceptible to systematic errors, dependent upon sample heterogeneity (e.g. tissue) and easily affected by technical problems (e.g. sampling error, DNA/RNA isolation method, variation in protocols and handling) [Kraljevic *et al*, 2004]. When planning experiments with microarrays, some critical points should be taken into account starting from standardization of all methods and protocols, through the experimental design and ending with careful analysis and validation of the data. Before starting a microarray analysis, the reported measurements should be normalized or modified to make them comparable. The goal of normalization is to adjust for effects that are due to variations in the technology rather than the biology. Methods of interpretation of large sets of microarray biologic data are still being developed. Questions arise as to which method is considered the "right one" because of the variety of the possible outcomes. However, patterns of gene expression revealed by data analysis are just the beginning. In many cases, greater biological understanding can be attained by using expression data in conjunction with sequence data, pathway, and biomedical text sources. The limiting steps performing microarray experiments are hence not only sample handling or the analysis itself but also determination of what the obtained results actually mean which depends, as previously mentioned, on the smart use of bioinformatics tools that allow integrated analysis of multiple data types (mRNA levels coupled with proteomic analysis) resulting in the improvement of the identification of the clinical endpoints biomarkers.

However, in comparison with microarray technology, proteomics encounters some other specific problems. In particular, rather small fraction out of the total number of proteins expressed by eukaryotic cell can be routinely separated on 2-DE gel. The reason for this might be that some proteins simply fail to get into the gel due to poor solubilization (hydrophobic membrane proteins, nuclear proteins and proteins that tend to aggregate, e.g. tubulin and keratins) or molecular weight size (very large and very small proteins), others are not resolved by the pH gradient (basic and acidic proteins), or even limitations in the sensitivity of the gel staining method (disability to detect low-abundance proteins, in particular those playing an important role in the cell cycle, signal transduction and receptors).

Sample preparation is considered as the bottleneck of 2-DE in terms of quality and protein distribution, and its success lies in the efficient extraction and solubilization of proteins of interest. Unfortunately, there is no universal protocol adequate for all proteins, so that each sample (cell culture, tissue or body fluid) represents new challenge with respect to sample preparation. An important issue in this respect is the removal of non-proteinaceous particles that might interfere with 2-DE (such as salts, lipids, nucleic acids, polysaccharides) as well as of abundant proteins (e.g. albumin in human serum or fibrinogen in plasma) that might hide some low-copy proteins of biological significance.

Another matter to be considered is the artifactual modifications of proteins that might occur during 2-DE, i.e. proteolysis that occurs upon liberation of proteases subsequent to cell disruption and carbamylation, caused by the degradation of urea used in sample solutions to cyanate, which in turn reacts with the amine groups on proteins and changes their net charge thus affecting IEF separation [Garfin, 2003].

Owing to the variability from gel to gel resulting in the discrepancy in the observed amounts and number of the individual protein spots, some spots observed in one gel might not be displayed in another gel of the same sample run in parallel. Consequently, it is often difficult to come to a firm conclusion on whether the particular spot on one 2-DE gel actually matches the same protein spot on a different gel. Also, biological variations amongst samples render it difficult to establish normal protein expression profiles that can be compared with the diseased states.

Finally, 2-DE is a quite manual technique and does not seem to be easily adapted to automation for the purpose of high-throughput analyses. Nevertheless, it proves to be a method of choice for protein separation due to its excellent resolving power as well as the ability to separate different protein isoforms, and we believe it will continue to play an important role in future proteomics research.

4.8 Conclusion

The sequencing of human genome has provided the first look at all genes. The next steps however require powerful transcriptomics tools in order to verify and refine the predicted, incomplete gene models as well as define new models. Combined with complete coding sequences, protein sequences can be examined for motifs, domains, and biochemical characteristics. Proteomics is complementary to transcriptomics and it can be used to confirm the existence of an individual gene thus serving to figure out the total number of genes in a particular genome, so called “functional annotation” of a genome. Future implementation of functional genomics/proteomics in biomedicine will require a systematic examination of differentially regulated genes and proteins in tissues and fluids in healthy vs. diseased subjects. However, high-throughput technologies reflect biological fluctuations and methodological errors. Large amount of such different data challenges the performance and capacity of statistical tools and softwares available at moment. Further major developments in this field are pending and the intellectual investment will certainly result in clinical advances.

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5. PHARMACOGENOMICS AND PERSONALIZED MEDICINE

Elizabeta Topić

5.1 Introduction

Two interwoven processes, human genome sequencing and the development of new technologies using DNA as an analytical sample and as a reagent have resulted in the genetic revolution in different fields of medicine, such as the field of medical therapy leading to personalized medicine through pharmacogenetics approach.

Pharmacogenetics is a newer branch of pharmacological sciences studying the relationship between genetic predisposition of an individual and his ability to metabolize a drug. It helps understand why some individuals respond to drugs and others do not, why some individuals require higher or lower doses to achieve an optimal therapeutic response, and tries to help the physician identify those patients who will respond favorably to therapy or develop side effects.

Systematic studies involving review of published literature indicate that adverse drug reactions (ADR) are prevalent and associated with costly hospitalizations.

Individual variation in response to drug ranges from failure to respond to drug reactions and drug to drug interactions when several drugs are taken simultaneously. The clinical consequences range from patient's discomfort through serious clinical illness to the occasional fatality. Approximately 7% of patients are affected by adverse drug reactions, increasing the overall hospital costs by 1.9% and drug costs by 15%. Some 0.3% of adverse drug reactions have fatal outcomes.

Among other influences, such as physiological, pathophysiological and lifestyle factors, the intraindividual genetic variability has a major impact on drug activity.

5.2 Levels of interindividual variability in drug effects

Genetic variability is known for drug absorption, drug metabolism and drug interactions with receptors. This forms the basis for slow and rapid drug absorption, poor, efficient or ultrarapid drug metabolism, and poor or efficient receptor interaction.

Genetic polymorphism based on drug metabolism ability is associated with four phenotype classes. The phenotype of extensive (normal) drug metabolizer (EM) is characteristic of normal population. Individuals are either homozygous or heterozygous for the wild type allele. Individuals that are heterozygous for the wild type allele may have intermediate metabolizer (IM) phenotype and may require lower than average drug dose for optimal therapeutic response. The poor metabolizer (PM) phenotype is associated with the accumulation of specific drug substrates in the body due to mutation and/or deletion of both alleles responsible for phenotypic expression. Individuals with PM phenotype are either homozygotes or multiple heterozygotes for mutant alleles. The ultrarapid metabolizer (UM)

phenotype is characterized by enhanced drug metabolism due to gene amplification. Individuals having this phenotype are prone to therapeutic failure because drug concentrations at normal doses are by far too low. Five to 20% of patients can belong to one of these risk groups, depending on the population studied. It is important to mention that there are significant ethnic and racial differences in the frequency of variant alleles.

5.3 Mechanisms of genetic variations

Genetic variations are the result of multiple mechanisms such as insertion, deletion, variable tandem repeats and microsatellites but the most frequent polymorphisms are point mutations or single nucleotide polymorphisms (SNPs), accounting for over 90%. Some of the polymorphisms are without consequences, but others cause altered protein, truncated protein, unstable protein or protein due to expression level. When we talk about a polymorphism, we mean a mutation in the genetic code that occurs in more than 1% of a population.

5.4 The potential of pharmacogenetics as a discipline in laboratory medicine

Pharmacogenomics and pharmacogenetics deal with the use of information derived from analysis of gene variations with objective to guide the drugs use. The pharmacogenomics studies are focused on the contribution of multiple genes (or entire genome) to drug response variability, whereas pharmacogenetics is focused on the association between single gene and drug response variability. Single gene testing can include pharmacodynamic genes, such as serotonin transporter or dopamine receptors, but these gene tests are not ready for clinical use yet, however, pharmacokinetic genes such as CYP2D6 and CYP 2C9 have reached clinical practice. Also, testing a limited number of multiple genes is possible by several commercial laboratories, providing batteries of genotype testing such as pharmacokinetic genes CYP2D6 and CYP2C19.

Pharmacogenetics has two functional components that link pharmacology to genetics. One may predict how drugs are processed by the body (pharmacokinetics) and the other how drugs interact with receptors to cause drug response (pharmacodynamics). Pharmacokinetics is strongly linked to biotransformation of drugs by metabolic processes mostly by the liver and their subsequent elimination by kidney function. The pharmacokinetic level uncludes gene polymorphisms that modify the concentration of a drug and its metabolites at the sites of their molecular action (such as polymorphisms of drug metabolizing enzymes, drug transporters, etc.). However, pharmacodynamics deals with understanding the drug interaction with receptors and the subsequent response. In this process some biotransformation may be involved. The pharmacodynamic level includes gene polymorphisms associated with the drug effect and mechanism of action, being unrelated to the drug concentration (receptors, ion channels, etc.).

Most of the studies were done on genes encoding for CYP450 enzyme family, the most important drug metabolizing enzyme. Most drug metabolizing enzymes exhibit clinically relevant genetic polymorphisms. The potential of pharmacogenetics in differentiating responders from nonresponders in a patient population with the same diagnosis is promising for its high practical implications, especially for drugs that are substrates of highly polymorphic enzymes. However, genetic variants linked to receptors have been studied too and represent a powerful direction in predicting drugs response.

5.5 Pharmacogenetic profiles

There are two approaches to creating genetic profiles enabling optimal treatment. The first approach implies making a specific hypothesis on the genes that cause therapeutic response modification and their testing in all individuals irrespective of their therapeutic response (gene candidates).

The second approach implies the search for so-called SNP profile (SNP prints) associated with efficient or adverse events in a respective population (forensic precision). This is known as the pharmacogenetic approach, i.e. search for SNP profile. According to literature data reported by experts in the field of pharmacogenetics, for clinical use preference is given to the search for SNP profile in individuals by whole genome scanning. Examples of specific genes modifying drug response, and which could be currently used in clinical practice are the genes encoding for drug metabolizing enzymes from the families CYP450, CYP2D6, 2C19 and 2C9, then phase II enzymes NAT2 and TPMT, B2-AR receptors, and some enzymes involved in the metabolism of antitumor drugs.

5.6 Guidelines and Recommendations in Pharmacogenetics

Diversity in technology, methodology, genotyping profiles and clinical practice used in pharmacogenetics approach of therapy individualization has inspired the New York National Academy of Clinical Biochemistry Expert Group to develop the Laboratory Medicine Practice Guidelines in Pharmacogenetics (LMPG) Guidelines and Recommendations for Laboratory Analysis and Application of Pharmacogenetics to Clinical Practice. The Expert Committee led by Roland Valdes has issued a draft version of document 60806 open for comments in 2006. The draft version 0606 has been closed for comments, but can still be downloaded as pdf-file from the following web site:
http://www.aacc.org/AACC/members/nacb/LMPG/OnlineGuide/DraftGuidelines/Pharmacogenetics/
The objective of LMPG in Pharmacogenetics is to provide a systematic overview of the pharmacogenetics discipline as it applies to clinical laboratory testing and its use in clinical practice. Issues to be addressed refer to methodological (pre-analytical and analytical) consideration, standardization and quality assurance of testing; selection of appropriate pharmacogenetics testing profiles; recommended reporting of test results and interpretation; standards needed for demonstration of clinical utility and efficacy; and regulatory and other recommendations for effective use of pharmacogenetic information in clinical setting. The framework of LMPG in Pharmacogenetics offers establishing the optimal use of pharmacogenetic information obtained from clinical laboratory testing. It also defines criteria and critical pathways that should be met before testing efficacy is precisely assessed.

The whole project has been divided into nine different sections, each of them chaired by an expert in the field, member of the Committee. The approach of the Committee was to establish a series of questions in each of the sections (outlined below), listed at the beginning of each section followed by a series of respective recommendations and accompanied by a list of most relevant references. The 10th section contains the glossary devoted to pharmacogenetics.

In this paper, the material issued by the New York National Academy of Clinical Biochemistry Expert Group on Laboratory Medicine Practice Guidelines in Pharmacogenetics (LMPG) was in part taken in its integral form and in part modified for presentation to the

Course participants. The participants are encouraged to visit the original document available at the New York National Academy of Clinical Biochemistry web site: http://www.aacc.org/AACC/members/nacb/LMPG/OnlineGuide/DraftGuidelines/Pharmacogenetics/

The sections, authors, reviewers and questions are listed below.

1. Valdes R. Guidelines and Recommendations for Laboratory Analysis and Application of Pharmacogenetics to Clinical Practice (http://www.aacc.org/...)

In General introduction and scope the author describes the steps in this enormous work on the project as follow:

- Define requirements for (a) adequate and (b) optimal pharmacogenetics-testing in specific clinical settings. Examples include turn-around time requirements for test results; number of alleles needed on test reports and advisability or need for interpretative reporting,
- Define the potential links in the roles of pharmacogenetics and therapeutic drug monitoring in clinical settings,
- Discuss and formulate recommended guidelines for clinical laboratories introducing pharmacogenetics-testing services,
- Provide in vitro diagnostic companies guidance on clinical assays and their performance characteristics in pharmacogenetics-testing. Which tests are needed, with what analysis times, etc.?, and
- Provide third party payers and regulators of diagnostic laboratory testing recommendations for optimizing their reimbursement and regulatory functions.

2. Ruano G, Valdes R. Pharmacology and population genetics considerations and their applications in pharmacogenetics (Reviewers: Weber WW, DeLeon J) (http://www.aacc.org/....)

The objective of this section is to give the reader a primer in the principals of drug metabolism and population genetics with sufficient basis for understanding how the concepts of genetics are applied in the development and application of pharmacogenetics testing as a discipline. Questions for consideration in this chapter were:

- What are the essential elements of drug pharmacokinetics and pharmacodynamics necessary to understand the application of pharmacogenetics in laboratory medicine?
- What is the cytochrome P450 system and what are the relevant allele frequencies of these components (CYP2D6, CYP2C19, and CYP2C9)?
- What are key considerations and recommendations for statistical sampling of the indicated alleles in populations?

The reader can find sufficient data for understanding drug metabolism, pharmacokinetics and pharmacodynamics as well as the connection between genotyping and drug dosing requirements and adverse drug reaction. The CYP P450 system and special CYP enzymes and recommendations for their use in pharmacogenetic testing, and how the allele distribution should be evaluated in a service to a reference population are described.

3. Payne D. Methodology and quality assurance considerations in pharmacogenetics testing (Reviewer: Carr J) (http://www.aacc.org/...)

The objective of the section is quality assurance and quality control issues. Questions for consideration were:

- What is the error rate for each test and each platform?
- What potential haplotypes, single nucleotide polymorphisms, pseudogenes, epigenetic modifications, or GC ratios could produce inaccurate results for each instrument and/or assay?
- What substances or specimen types could interfere with the various instrument platforms?
- Do laboratory methods correlate closely with clinical manifestations? What roles do genotypes versus phenotype assays have? What are the roles of each of those assays? What artifacts can make the assays produce conflicting data?
- What reference/control material will be used for validation, proficiency, and lot-to-lot quality control? How much data is needed for adequate validation? How often is proficiency, analyst competency, and QC to be performed?
- If software is used in interpretation, will it automatically flag extremely rare or unlikely allele combinations?

Recommendations suggest importance of the evidence on discrepant results from various instruments or within the same instrument, corrective actions, controls of potential enzyme inhibitors in assay, assay validation, etc.

4. Linder M, Steimer W. Clinical laboratory services considerations (Reviewers: O'Kane D, Lyons E) (http://www.aacc.org/...)

This section is focused on the importance of how clinical laboratory is expected to provide services of pharmacogenetic testing consistent with the needs of healthcare providers. Questions for consideration were:

- What level of certification should be required for clinical laboratories and personnel performing pharmacogenetics testing?
- What are the recommended specimens for testing?
- What should be the primary test-result output?
- What test result turn-around times are optimal for pharmacogenetics testing?
- What criteria should be used to establish which genetic variants of a locus should be included for diagnostic purposes?
- Is it necessary to have evidence to demonstrate cost effectiveness before recommending clinical use of pharmacogenetic tests?

In this section, the personnel performing pharmacogenetics testing rank first in the recommendation, followed by robust and optimized diagnostic methods for DNA analysis from fresh whole blood, dried whole blood spots, isolated nucleated blood cells, and oral epithelial cells obtained from either buccal scraping or saliva. Laboratories should report a description of all physical characteristics of the genetic locus that are being determined by the assay. Laboratories should provide turn-around times that are consistent with the clinical

application of pharmacogenetic test results. In general, the goal is for the physician to be advised of the patient's genotype in due time to avoid any risk for the patient.

Before the pharmacogenetics testing be considered for purposes of cost-effectiveness as applied to general screening, a series of important questions should be addressed. Some examples are:

- What is the frequency of the genetic polymorphism?
- How closely is the polymorphism linked to a consistent phenotypic drug response?
- Are there metabolic, environmental or other significant influences on drug response?
- What are the sensitivity and specificity of the genomic test?
- What alternative tests are available to predict drug response?
- How prevalent is the genotype of interest?
- Is the genotype or haplotype important – does the test detect genotype or haplotype?
- What are the characteristic outcomes associated with the genotype with and without respective knowledge?
- How does the pharmacogenomic strategy alter these outcomes?
- What is the therapeutic range of the drug involved?
- What alternative therapeutic options are available?
- How effective are current monitoring strategies for preventing severe ADRs and predicting drug response?

The recommended factors that should be assessed are presented in table below.

The use of pharmacogenetics will probably prove most cost efficient in case of drugs with a narrow therapeutic index, high inter-individual variability, problems in monitoring ADR or treatment response, and few alternative therapeutic options.

5. Morello JP, Valdes R. Reporting and interpretation of pharmacogenetic test results (Reviewers: Kirchheiner J, Reynolds D) (http://www.aacc.org/...)

When reporting genotype information from pharmacogenetic tests, the clinical laboratories must keep in mind that the end user of this information will most likely be the physician or other healthcare provider. It is usually the physician who will report the findings to the patient and take action using the test result. For the physician to correctly interpret the genotype information, it is beneficial that he or she have the complete diagnostic setting of the patient including present and past drug regimens, medical history and lifestyle. Questions for consideration are:

- What information should accompany the reported result?
- Should the result be linked to a specific drug usage (as indicator)? Should drug "dosing and usage" information accompany the test result?
- Should laboratories reporting pharmacogenetic test results have a genetic counseling component or service available or by referral?
- Should manufacturers of IVD used for providing pharmacogenetic tests be required to supply evidence of specific use for every drug, or by class?
- Are there unique or specific limitations to be considered regarding confidential reporting of pharmacogenetic test results?

In recommendation, the authors state that laboratories reporting pharmacogenetic genotype test results should be prepared to provide an educational resource to recipients of the test results to explain the complexity of the metabolic pathways involved and also be prepared to provide guidance as to which genes should be tested for a given clinical situation when that is known. Many other recommendations are counted in consideration of the section.

6. Ruano G, Linder MW. Clinical practice considerations (Reviewers: DeLeon J, Flockhart D) (http://www.aacc.org/...)

This section describes one of the most challenging aspects of transitioning the science of pharmacogenetics to the bedside while establishing criteria for its clinical application. The approach for these present practice guidelines is to take several key examples and use them as a basis on which to set criteria for documenting future development of these services to medical practice. Three models are presented in which pharmacogenetic test results have proved useful to establish criteria for clinical applications: warfarin (anticoagulation), atomoxetine (psychiatry), and irinotecan (oncology). These three models combined demonstrate several strategies and concepts for future development of pharmacogenetics applications. Questions for consideration were:

- Which are the most current variant alleles for CYP2D6, 2C9, and 2C19 recommended for clinical use?
- What set of criteria (characteristics) should be required of a pharmacogenetic test to make it clinically useful?
- What examples are available that can presently be used as models for application of pharmacogenetic testing in clinical settings? warfarin (2C9 and VKOR), atomoxetine (2D6), irinotecan (UGT1A1)?
- What models of pharmacogenetics-genotyping are available which can serve to establish dosing adjustment rules based on pharmacogenetic information?

- What role should the clinical laboratory play in developing the use of pharmacogenetic testing?

In recommendation, the most current variant alleles are recommended when performing pharmacogenetics-genotyping for CYP2D6, 2C9, and 2C19, as well as its clinical use. As examples, warfarin, atomoxetine and irinotecan are presented.

7. Shaw L, Burckart G. TDM and pharmacogenetics interface considerations (Reviewer: Linder M) (http://www.aacc.org/...)

This section addresses medications which require monitoring of their concentrations in blood, i.e. those where narrow therapeutic concentration ranges in blood are required for efficacy, and where toxicity is a persistent problem. As the discipline of pharmacogenetics finds its way into clinical practice, the combination of traditional TDM and pharmacogenetics must be explored to achieve optimum utilization of the combined information they provide. In essence, pharmacogenetics provides information that allows the clinician to make a determination of appropriateness and risk of drug therapy prior to the initiation of therapy. Pharmacogenetics may then have an additional place in selecting drug or dosage alterations during the treatment for a disease process. Questions for consideration are:

- Will use of pharmacogenetics information preclude or require TDM in future? and, How should standard TDM practices be modified to account for pharmacogenetic variation?
- Are there specific clinical situations demonstrative of both TDM and pharmacogenetics information having complementary value?
- How can TDM be best utilized in establishing the predictive value of pharmacogenetic tests, as end point, etc.?

It is recommended that pharmacogenetics testing information be used for the initial selection of drugs or doses for some agents, with a note that the clinician should be aware of the possibility of a significant variability using pharmacogenetics information to design a drug regimen, and that TDM is still essential to monitor therapeutic response and toxicity.

8. Jortani S, McLeod H, Wong S. Ancillary applications (drug prescription /dispensing, forensics) (Reviewer: Wu A) (http://www.aacc.org/...)

This section is focused on other applications of pharmacogenetics including its use in: production of targeted drugs by pharmaceutical companies; forensics; safer distribution of medications by pharmacist; environmental toxicology (toxicogenomics); predicting addiction to substances; etc. The information is still rather green, however, evolving rapidly in many of these areas. Yet, in the areas of forensics and drug dispensing some recommendations are worthy of consideration.

A) Applications in dispensing of medications

Questions for consideration are:

- Should information related to pharmacogenetic test availability be made part of the information provided to patients as part of the drug dispensing mechanism? If so, by whom or how?
- Should pharmacogenetic test information be considered an integral part of the drug-dispensing safety awareness practice?
- Should information-related relationships be fostered between drug dispensing providers and clinical laboratories providing pharmacogenetics testing services?

According to the recommendation, after appropriate consent from the patient, pharmacogenetic genotype information should be made available to drug-dispensing organizations to be used as part of their drug-dispensing safety verification procedures.

Hospital-based drug dispensing departments and clinical laboratories should work in close collaboration and establish policies to make available timely genotyping information useful for guiding the dispensing of medication for hospitalized patients and for recommendations after discharge.

B) Applications in forensics

Questions for consideration are:

- In forensic applications of pharmacogenetics testing, what is (are) the preferred specimen(s), and what diligence should be established for purposes of evidence acquisition?
- What type of information and correlations should be used to optimize the application of pharmacogenetics data in forensic cases?
- What qualifications by way of training and experience should be required for individuals reporting and interpreting pharmacogenetics information when applied to forensics?
- What type of information should accompany a pharmacogenetic test report as it applies to applications in forensics?
- Are there any particular or specific ethical considerations that may apply to the use of pharmacogenetics data with regard to applications in forensics?

According to the recommendation, in forensics blood is considered to be the preferred specimen of choice and should be used whenever available. Chain of custody should be maintained for forensic samples according to the established protocols by each laboratory. Whenever possible, in cases in which polymorphic enzymes are suspected as factors in drug toxicity, other relevant issues such as polymorphisms in receptors, transport proteins, genes that affect pharmacodynamics, etc. should also be considered. Interpretation of pharmacogenetic testing results in forensic toxicology should be done by toxicologists with adequate training in pharmacogenetic testing and familiarity with metabolic pathways.

9. Frueh F, Rahman A. Regulatory considerations (Reviewers: Collins J, Rudman A) (http://www.aacc.org/...)

Considering regulatory considerations in this section, the evaluation and approval of pharmacogenomic tests can be categorized into two broad aspects: analytical validation and clinical usefulness of the test. Questions for consideration are:

- When will a test be "required", when is a test "recommended"?
- When should the label state that the test is "available"?

According to the recommendation, a test may be required for therapy when the drug or the biological is co-developed with a test. Patients are eligible to receive a treatment only if a test result is obtained prior to treatment initiation. The test may be recommended prior to the selection of a therapy and/or the selection of a dose for a particular population deficient in activity of a polymorphic enzyme involved in the inactivation of the drug/biological.

10. Farkas D. Glossary - definition useful in understanding pharmacogenetics. The Glossary is printed as a whole text at the end of this Handbook.

5.7 In Conclusion

A hundred years ago, clinicians prescribed a drug only on the basis of physical examination. At the end of the 20th century, therapeutic decision was greatly facilitated by laboratory support and the process of therapeutic drug monitoring. Now we have entered a new era with pharmacogenetics and pharmacogenomics, which appear highly promising in enhancing the support to therapeutic decision making, predicting patients who are most likely to respond best to a particular drug, or in whom the drug will yield optimal effects.

The development and release of these LMPG in Pharmacogenetics in terms of methodology, genotyping profiles, interpretation of pharmacogenetic results, quality control and standardization, thereby upgrading the overall healthcare level and service cost effectiveness while reducing the morbidity and mortality rates due to ADR, will ensure appropriate and systematic assessment of pharmacogenetic testing and its optimal application in therapy individualization.

Recommended literature:

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6. DOSE ADJUSTMENTS BASED ON PHARMACOGENETICS OF CYP450 ENZYMES

Ron H.N. van Schaik

6.1 Introduction

In today's medicine, drug therapy represents an important tool to treat or control disease. Dosing drugs is based on the assumption that a particular dose will yield a predefined concentration in the circulation and thereby establish the desired therapeutic effect. The fact that liver and kidney function may affect this relationship is appreciated, as is the fact that the use of co-medication potentially may interfere with the relationship between dose and blood concentration. Also environmental factors, like diet, alcohol use, smoking behaviour and caffeine intake may alter the rate at which particular drugs are metabolized, and will thus affect exposure of individuals to these drugs. A factor that has been neglected for a long time is genetics: differences in genetic make up may affect the relation between drug dose and blood concentration. Variant alleles encoding drug transporters or drug metabolizing enzymes with altered activity, present in the population, will give rise to unexpectedly high or low blood concentrations in response to a particular dose of a drug in certain individuals. The variation in drug metabolizing capacity between individuals is known for quite some time, but only recently this is becoming available as a routine diagnostic to provide better therapy. An important group of enzymes involved in drug metabolism is the cytochrome P450 enzyme system, which is mainly (but not only) active in the liver and the intestine. It consists of a family of isoenzymes, each with their own substrate specificity, and catalyzes oxidative reactions in order make potentially toxic and harmful substances more soluble. Purpose is to protect the body against toxic substances. The most important cytochromes with respect to drug metabolism are CYP3A4, CYP2C9, CYP2C19 and CYP2D6. For these enzymes, however, several variant alleles encoding enzymes with decreased activity exist (see www.cypalleles.ki.se), giving rise to interindividual differences in capacity to metabolize drugs. The best known example in this family is the enzyme CYP2D6, for which even 10% of the Caucasian population is a poor metabolizer, due to inheritance of two inactive variant alleles.

The documented genetic variability in cytochrome P450 enzymes has increased considerably the last years, and it has become clear that inherited capabilities of drug metabolism by this important detoxifying system may be of importance for predicting outcome of drug treatment. Especially the fact that this concerns inherited information makes it possible to assess prospectively the drug metabolizing capacity of patients. Whereas detection of variant alleles is an important first step, the real clinical benefit should come from adjusting drug therapy based on this genetic information. It is at this stage where the field of pharmacogenetics nowadays finds itself.

6.2 CYP450 isoenzymes and variant alleles

6.2.1 CYP2D6

CYP2D6 is the best characterized polymorphic cytochrome P450 enzyme to date. Whereas it represents only 2% of total CYP450 protein in the liver, it is involved in the metabolism of approximately 20-25% of commonly prescribed drugs. Based on debrisoquine and sparteine phenotyping, the Caucasian population was shown to be divided in poor (5-10%), extensive (90%) and ultra extensive (or ultra rapid) (1-2%) metabolizers. This trimodal distribution proved to be caused by genetic factors. Already in 1977, the most common CYP2D6 null (deficient) allele, CYP2D6*4 (1846G>A, splicing defect), was discovered. This variant has an allelic frequency of 20%, and is thus not a rare allele. Also a CYP2D6 gene deletion (CYP2D6*5 variant allele) is not uncommon: it has an allele frequency of 3.8%. Together with the simultaneous analysis for CYP2D6*3 (2549delA, frameshift; allele frequency 1-2%) and CYP2D6*6 (1707delT, frameshift; allele frequency 1%), over 98% of CYP2D6 poor metabolizers can be identified in the Caucasian population with these tests. At this moment, many more variant alleles for CYP2D6 have been described (up to CYP2D6*63 at this moment (www.cypalleles.ki.se)). These variant alleles can be divided in functional alleles (like *1, *2, *35, encoding normal activity), null alleles (like *3, *4, *5, encoding no activity at all) and decreased activity alleles (like *9, *10, *41), encoding enzymes with residual metabolic capacity. Based on genotyping, we can predict CYP2D6 extensive metabolizers (EMs; normal metabolism: 2 active alleles)), intermediate metabolizers (IMs; only 1 active allele), poor metabolizers (PMs; 2 inactive alleles) and ultra rapid metabolizers (URs or UEMs; gene duplication). However, the overlap in enzyme activity between these groups can be substantial, and also depends on the drug. In addition, CYP2D6 variant alleles show a large difference in distribution between ethnicities: the CYP2D6*10 decreased activity allele is present in a large proportion of Asians, and causes a shift towards a lower mean CYP2D6 activity for this population compared to Caucasians. The CYP2D6 gene duplication occurs in approximately 1-2% of Swedes, 3.6% of Germans, 7-10% of Spaniards, 10% of Italians, 20% of Saudi Arabians and 29% of black Ethiopians, thus revealing a strong north-south gradient, probably caused by evolutionary pressure with respect to diet. Based on the occurrence of alleles, a semi-quantitative gene dosage (SGD) score was introduced by Steimer *et al*, to better predict CYP2D6 activity. In this scoring system, active alleles were assigned a score 1, decreased activity alleles a score 0.5 and null alleles a score 0. This leads to SGD groups of 0, 0.5, 1, 1.5, 2 and >2.

6.2.2 The CYP2C subfamily

The CYP2C subfamily consists of four genes, located on chromosome 10q24. CYP2C9 is the main enzyme of the CYP2C subfamily in the human liver, and is involved in the metabolism of 10-20% of commonly prescribed drugs. Approximately 1-2% of the Caucasian population is a poor metabolizer for CYP2C9, while for Africans and Asians this percentage is thought to be much lower. The CYP2C9*2 (430C>T, Arg144Cys) and CYP2C9*3 (1075A>C, Ile359Leu) variant alleles encode both significant altered CYP2C9 activities and do show high population frequencies: 11% (CYP2C9*2) and 7% (CYP2C9*3) in Caucasians, 4% (CYP2C9*2) and 2% (CYP2C9*3) in Africans and 0% (CYP2C9*2) and 3% (CYP2C9*3) in Asians. The decrease in activity is most profound for the CYP2C9*3 allele: mean clearances in homozygote CYP2C9*3/*3 individuals are 25% of that of wild type for a number of substrates, while heterozygosity for this variant corresponded to clearance of 29% compared to wild type. For the CYP2C9*2 allele, the Vmax displays a 50% reduction compared to the

CYP2C9*1 allele and residual clearance of tolbutamide for CYP2C9*1/*2 heterozygotes was 70% compared to CYP2C9*1/*1 individuals. In total, approximately 30 CYP2C9 variant alleles have now been described.

The second important enzyme of the CYP2C subfamily is CYP2C19, for which the first genetic polymorphism was identified based on aberrant metabolism of the anticonvulsant drug mephenytoin: 3-5% of Caucasians and 12-23% of Asians were shown to be CYP2C19 poor metabolizers. In African populations, 4% CYP2C19 poor metabolizers can be found. The two predominant variant alleles, encoding CYP2C19 protein lacking enzymatic activity, are CYP2C19*2 (681G>A, splicing defect) and CYP2C19*3 (636G>A, W212X). At present, nomenclature has reached the CYP2C19*21 allele, and does include several null alleles with relative low allelic frequencies. An interesting allele, CYP2C19*17 (991A>G, Ile331Val), was recently characterized as encoding an increased activity on proton pump inhibitor metabolism, due to increased transcription compared to the CYP2C19*1 allele, predicting 35-40% higher activity in homozygote individuals. Also part of the CYP2C family is the CYP2C8 enzyme, which plays a role in paclitaxel metabolism. CYP2C8 has variant alleles CYP2C8*2, *3, *5, *7 and *8 allele that were shown to have decreased activity. From these alleles, the CYP2C8*2 allele is found predominantly in African Americans (allele frequency of 18%) and has a low frequency in Caucasians (allele frequency 0-0.7%). In contrast, the CYP2C8*3 allele was found in 2% of African Americans and in 8-13% of Caucasians.

6.2.3 The CYP3A subfamily

The CYP3A subfamily gene cluster is located on chromosome 7; this family is responsible for the metabolism of approximately 50% of all drugs. It is the largest subfamily of CYP450 enzymes in the liver, and comprises the isoenzymes CYP3A4, CYP3A5, CYP3A7 and CYP3A43. For CYP3A4, the most important member of this subfamily, over 20 genetic polymorphisms have been described, but almost all have low (<1%) allelic frequencies making them unlikely targets for pharmacogenetic screening. The only exception on this might be the promoter variant CYP3A4*1B (-392A>G), originally referred to as CYP3A4-V, which has an allelic frequency of 2-9% in Caucasians, 35-67% in African Americans and which is rare in Asians. This polymorphism is associated with a moderate (1.2-1.9 fold) increase in transcription rate and thus enzymatic activity, although this effect was questioned by other authors. Because CYP3A expression is highly susceptible to induction and inhibition of transcription, it is not clear whether this modest difference in induction of the CYP3A4*1B allele will contribute significantly to the observed 40-fold variation in CYP3A4 activity in vivo. From the other genetic polymorphisms, the CYP3A4*2 (664T>C, Ser222Pro) and CYP3A4*17 (566T>C, Phe189Ser) were shown to have decreased enzymatic activity, while this is also suggestive for CYP3A4*4 (352A>G, Ile118Val) and CYP3A4*5 (653C>G, Pro218Arg). In addition, two null alleles have been identified: CYP3A4*6 (830-831insA; frameshift) and CYP3A4*20 (1461-1462insA; frameshift), but these alleles are extremely rare. In addition, the CYP3A4*18 (878T>C, Leu293Pro) variant allele surprisingly was shown to encode increased enzymatic activity. This allele is not found in Caucasians, but has an allele frequency of 10% in Chinese.

A second member of the CYP3A family, CYP3A5, is not expressed an all Caucasians. In 10-40% of this population, homozygosity for the inactive CYP3A5*3 allele causes absence of protein and thus CYP3A5 activity. The allele frequency of the CYP3A5*3 allele varies from 89-94% in Caucasians, 71-75% in East Asians, 60-65% in Hispanics to 29-35% in Blacks. At this moment, 13 genetic polymorphisms have been described fro CYP3A5, but CYP3A5*3

seems to be by far the major variant allele. The relevance of CYP3A5 genotyping will depend on the contribution of this enzyme to total CYP3A mediated metabolism of a specific drug. For many drugs, this contribution is not (yet) exactly known.

The CYP3A cluster contains also two minor CYP3A genes: CYP3A7 and CYP3A43. CYP3A7 is expressed mainly during fetal life and is down regulated after birth. However, 3% of Caucasians and 6% of African Americans still express CYP3A7 during adult life due to inheritance of the CYP3A7*1C allele, which contains 60 bp of the CYP3A4 promoter. Up to now, no correlations with altered pharmacokinetics with specific drugs have been described, but a correlation with blood levels of the CYP3A7 substrate DHEAS apparently exists, demonstrating an impact of this polymorphisms in adults. The role of CYP3A43 with respect to drug metabolism is unclear at the moment This enzyme is expressed in very low levels in human liver, and no information is available as to which drug metabolism it might contribute.

6.3 Dose adjustments based upon CYP450 genetic polymorphisms

6.3.1 Pain

CYP2D6 plays an important role in the metabolism of codeine, used to treat pain. Codeine needs to be activated by conversion to morphine by CYP2D6 in order to achieve pain relieve. Because of this activation, CYP2D6 poor metabolizers will not benefit from codeine as pain medication. Dose adjustments are thus not appropriate, and another analgesic should be considered. Knowledge about drug metabolism is important here, because also the analgesic tramadol requires activation by CYP2D6. Another important problem may arise for CYP2D6 ultra rapid metabolizers: because of the increased conversion of codeine to morphine, severe morphine toxicity may occur. Several case reports have been published on this, with a tragic story in 2006 in the Lancet of a new born child dying on day 13 because his mother was on codeine. Because the mother was a CYP2D6 ultra rapid metabolizer, the normal codeine dose translated in fatal morphine concentrations in the mothermilk. Whereas dose adjustments could be appropriate here, it is much easier to prescribe another analgesic lacking these dangerous side effects.

6.3.2 Psychiatry

Important, and widely used substrates of CYP2D6 are antidepressants (tricyclic antidepressants and SSRIs) and antipsychotics. CYP2D6 genotype was shown to correlate strongly with drug metabolism. Based on available literature, Kirchheiner *et al* extrapolated dose recommendations based on genotype for several antidepressants and antipsychotics. From this work, it is very clear that although multiple drugs are a substrate of CYP2D6, the clinical consequence of being a poor metabolizer very much depends on the drug. As example: whereas CYP2D6 poor metabolizers would need a 72% reduction in dose compared to standard dosing to reach target concentrations, citalopram needs to be adjusted only 2%, whereas both drugs are CYP2D6 substrates. In our own hospital, we showed that the obtained steady state dosages for imipramine could be well predicted by CYP2D6 semi quantitative gene dosage, and indeed got a 70% dose reduction for imipramine in CYP2D6 PMs. For IMs, slightly higher doses were found than predicted by Kirchheiner *et al*, indicating that clinical validation of extrapolated data is important. Also apparent was that considerable overlap exists between different genotyping groups. Pharmacogenetic based dosing is thus certainly possible, but it cannot replace therapeutic drug monitoring.

6.3.3 Anticoagulation

A major topic in today's medicine is the use of CYP2C9 genotyping to improve anticoagulant therapy: warfarin and acenocoumarol are both depending on CYP2C9 activity for metabolism. Based on studies on warfarin, special websites (like www.warfarindosing.com) with algorithms are now available which will enable physicians to target the INR of patients more quickly, based on CYP2C9 genotyping information. In addition, the genetic information on the polymorphic VKORC1 gene, encoding the target molecule for warfarin and acenocoumarol, needs to be provided to be able to retrieve the optimal dosing. This system seems to be now the most advanced with respect to CYP450 genetic information in relation to dose prescription.

6.3.4 Immunosuppressants

One of the first drugs in which CYP3A5 proved of importance, is the immunosuppressant tacrolimus, used in kidney transplantation. CYP3A5 expressers (20% of Caucasians) require 1.5 – 2.0 fold higher tacrolimus dosages to obtain target concentrations. Although there is a difference in drug metabolism rate between CYP3A5 expressers and non-expressers, this did not translate into differences in kidney transplantation rejection, making the clinical utility yet unproven. Currently, a prospective trial is performed in which tacrolimus dosing is based on CYP3A5 genotype, which should demonstrate the clinical relevance of CYP3A5 genotyping.

6.3.5 Cancer

The most challenging field for applying molecular genetics to predict drug response, is cancer. Also here, CYP2D6 has become important the last years, since it catalyzes the activation of tamoxifen, which is used in the treatment of breast cancer. CYP2D6 PMs do benefit less well from standard tamoxifen therapy. Therefore, the FDA is considering to indicate CYP2D6 genotyping in the label for tamoxifen. However, it is currently not clear whether increased tamoxifen dosages should be used, or that alternative drugs (like aromatase inhibitors) should be considered. Other interesting findings in this field are correlations of CYP2C8 genotype with paclitaxel metabolism, and CYP2B6, CYP2C19, CYP3A4 and CYP3A5 genotype with cyclophosphamide and ifosfamide effectivity. Also etoposide and vincristine metabolism were correlated with CYP3A5 genotype, and several studies have investigated the role of CYP450s in the metabolism of IRESSA, Gleevec, irinotecan and docetaxel. Most of these studies, however, do not yield consistent results to favour CYP450 genotyping.

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7. PHARMACOGENETICS OF DRUG RECEPTORS

Janja Marc

7.1 Introduction

Drug treatment is in many cases ineffective. Besides patients who do not respond to the treatment can develop so called adverse drug reactions (ADRs) as a consequence of the treatment (cause over 100 000 deaths per year in USA). Pharmacogenetics is the discipline, which takes the patient's genetic information of drug transporters, drug metabolising enzymes and drug receptors into account leading to optimal choice and dose of the drugs in question. It represents a kind of patient-oriented medicine or personalized medicine. It is believed that a lot of costs for the society can be saved by so called individualized drug therapy mainly based on pharmacogenetic data.

Genetic variations (mostly single nucleotide polymorphisms, SNPs and micro satellites) can be present in drug transporters, in majority of phase I and phase II drug metabolizing enzymes or in drug receptors. SNPs in drug transporters could alter the absorption, distribution and the elimination of the drug from the body. The polymorphisms in drug metabolising enzymes can cause abolished, quantitatively or qualitatively altered or enhanced drug metabolism. Several examples exist where subjects carrying certain alleles suffer from a lack of drug efficacy because of ultrarapid metabolism caused by multiple genes or by induction of gene expression. On opposite site, adverse effects can develop in slow metabolisers as a result of the accumulation of drug in presence of defective alleles. Finally, certain polymorph genes can be used as markers for optimisation of the drug therapy. It is likely that predictive genotyping is of benefit in 10–20% of drug treatment and thereby allows for prevention the appearance of ADRs and thus improves the health for this part of the patients.

7.2 Drug receptors

Receptor is a macromolecule in the membrane or inside the cell that specifically (chemically) bind a ligand (drug). The binding of a drug to receptor depends on types of chemical bounds that can be established between drug and receptor. The strength of this chemical bonds (covalent, ionic, hydrogen, hydrophobic) determine the degree of affinity of ligand to receptor. Ligands (drugs) that attracted the receptors may be classified as agonists or antagonists. Agonists produce the biological response as a results of receptor –ligand interactions therefore agonists posses efficacy. On the contrary antagonists did not provoke any biological activity after binding to its receptor.

There are different types of receptors (1):

1. Transmembrane ion-channels receptors
2. Transmembrane G-protein-coupled receptors
3. Transmembrane receptors with cytosolic domain
4. Intracellular (cytoplasm or nucleus) receptors

7.2.1 Transmembrane ion-channels receptors

The most rapid cellular responses to receptor activation are mediated via ligand-gated ion channels. These kind of transmembrane receptors composed of multiple peptide subunits and each of it contains four membrane-spanning domains (Figure 7.1.).

Figure 7.1. *Ion-channel receptors (1)*

The ligand binding causes the conformational changes of receptor and ion channel forming. The binding of Ach to each of four subunits of AchR induces change in receptor and opening the sodium selective channel through the centre of the receptor protein. It causes the depolarisation of surrounding membrane. In this type of receptors belong nicotinic acetylcholine receptors and receptors for GABA, serotonine and some other neurotransmitters.

7.2.2 Transmembrane G-protein-coupled receptors

The most abundant type of drug receptors are G-protein coupled receptors (GPCR). This are family of (over 100 different) transmembrane receptors which share a well conserved structure and transduce their signals via activation of intracellular guanidine nucleotide binding protein (G-protein) (Figure 7.2.). A variety of ligands for this receptors include biogenic amines (Ach, noradrenalin, serotonin), amino acid neurotransmitters (glutamat, glycine) and peptide hormones (angiotensinII, somatostatin). There are multiple GPCR types for a single ligand. The result is the possibility that single ligand can activate a variety of transduction pathways. Thus receptor is defined not only just by which ligands binds to it but also by second messenger systems (cAMP, PLC, Na/H exchange) and signal transduction pathway, which is activated by receptor activation.

Figure 7.2. *G-protein coupled receptors (1)*

7.2.3 Transmembrane receptors with cytosolic domain

The intracellular domain of this transmembrane receptor is either enzymatic active (catalytic receptors) or is bound to specific enzyme(s) in cytosol (enzyme coupled receptors). The catalytic receptors are activated predominantly by peptide hormones (insulin, growth factors, etc). Catalytic part of receptors has the protein kinase activity. Mostly dimerisation of catalytic as well as enzyme-coupled receptors is necessary for kinase activity. Phosphorilation of intracellular proteins by these receptors results in effects such as opening the ion channels, initiation of gene expression or as in the case of enzyme coupled receptors activation of signal transducers and activators like JAKs and STATs.

7.2.4 Intracellular (cytoplasm or nucleus) receptors

Those receptors are not associated with cell membrane. In general their protein molecule consists from three main domains: Hsp-90 and DNA and ligand binding domains. Ligands are mostly lipid soluble and passively pass cell memmbrane. Agonists include nitric oxide, steroid hormones and vitamin D. Ligand binding activates receptor and initiates the dissociation from Hsp-90. The complex then translocates to nucleus and bind to specific DNA sequences mostly located in gene promoter region (Figure 7.3.). This kind of signal tranduction is slow, but duration of response can last long.

Figure 7.3. *Schemes of nuclear receptors function (1)*

7.3 Pharmacogenetics of human beta adrenergic receptors

Beta adrenergic receptors (ADRBs) are transmembrane G-protein-coupled receptors that bind adrenalin or noradrenaline in sympathetic nervous system. There are three types of ADRBs: ADRB1, ADRB2 and ADRB3. ADRB3 has been least studied to date and the role of ADRB3 in cardiovascular disease is not known. ADRB1 are the predominant type expressed in the hearth. ADRB2 are abundantly expressed in bronchial smooth cells and activation of them results in bronchodilatation.

ADRB1 and ADRB2 are intronless genes encoding 477 and 413 amino-acid proteins, respectively. They share a common structure with an extra cellular amino terminus domain, seven transmembrane spanning domains and a cytoplasmic carboxyl terminus. Binding of ligand (adrenalin or noradrenalin or other agonists) to these receptors coupled to G- protein lead to conversion of ATP to cAMP. Increased cAMP stimulates a chain of events that culminates with removal of calcium from contractile protein and increase the activation of contraction through greater calcium cycling. The global effect is improved systolic and diastolic function (2).

7.3.1 ADRB1 polymorphisms

23 polymorphisms have been described and 13 of these change amino-acid sequence. As ADRB1 has not a recognised role in asthma, the majority of association and pharmacogenetic studies were done by cardiovascular phenotypes. Ser49Gly and Arg389Gly have been widely studied owing to studies supporting the functional effects on ADRB1 activity and phenotypes.

The Gly49 allel showed altered glycosilation and a more pronounced agonist-induced receptor down regulation in a fibroblast model what could explain the resistance to chronic beta-adrenergic stimulation through diseases or medication. The Arg 489 alleles showing higher basal levels of cAMP. Thus, the Arg389 may have inherently coupling to Gs and increase the signal transduction. Population association studies show Ser49 homozygotes have higher mean heart rates and are associated with reduced 5-year survival. However different studies gave divergent results. The Arg389 variant is associated with elevated diastolic blood pressure and higher resting heart rate. This finding was strengthened by similar conclusions of different studies. Furthermore Arg389Gly polymorphism appears to interact with 4-amino-acid deletion in the alpha-2-adrenergic receptor gene. Homozygotes with both polymorphisms have increased risk for heart failure (odd was 10,1). However it is approved in black population, the number of double homozygotes in Caucasians was too small (2).

7.3.2 ADRB2 polymorphisms

From 12 SNP identified only 5 of these predict the change of amino-acid sequence. The most studied SNPs are Arg16Gly, Gln27Glu and Thr164Ile. Functional analyses showed that the first two SNPs down regulate ADRB2 in fibroblasts and enhanced isoproterenol-mediated desensitisation in humans carrying the Arg16. The Ile164 allele has increased binding affinity for endogenous ligands. SNPs in ADRB2 have been studied in patients with asthma, obesity and diabetes. Recent studies concluded that Gly16 alleles predisposes to nocturnal asthma and asthma severity as well as to response to beta-agonist therapy (albuterol) in asthmatics. Association studies of ADRB2 gene variations with hypertensive phenotype yielded mixed results. In healthy individuals ADRB1 is far more abundant than ADRB2. However in the heart failure diseases proportion of ADRB2 is increased (to 40%). This data could be interpreted to mean that ADRB2 SNPs might have great effects on heart failure.

7.3.3 Pharmacogenetic studies of ADRBs

As the ADRBs are the major target for pharmacological therapy (beta receptor agonists and antagonists) some of pharmacogenetis studies were published. Studies showed that Arg389 homozygotes have increased effects of ADRB1 agonist dobutamine and ADRB1 antagonist metoprolol as compared to Gly 389 homozygotes. SNPs in ADRB2 influence the response to methacholine and albuterol therapy of asthma. In conclusion for ADRB1 the Gly49 and

Arg389 alleles seem to be variants that perform more favourably in heart failure patients given beta-blockers, in case of ADRB2 the data suggest a negative effect of Arg 16 alleles on short term beta agonist therapy (2).

7.4 Pharmacogenetics of estrogen receptors

7.4.1 Type and function of estrogen receptors

Estrogen receptors (ERs) are intracellular nuclear receptors that belong to steroid hormone receptor family. There are two types of ER; ER alpha (ER 1) and ER beta (ER 2) encoded by two different genes ESR1 and ESR2, respectively. ESR1 and ESR2 are expressed mostly bones, breasts, ovaries, cardiovascular system and central-neural system, but ESR2 mRNA was also found in kidney, lung, colon and testis tissues. ERs function as transcription factors activated by a ligand. Ligands bind to ERs are endogenous estrogen hormones (estradiol) or estrogens administered at hormone replacement therapy (HRT). A new group of drugs, "selective estrogen receptor modulators" (SERMs) act as ER-agonist in a specific tissue (like raloxifen in bone) but as an ER-antagonist in other tissue (like tamoxifen in breast). Irrespective of ligand, ligand binding to ER results in activation and translocation of ERs to nucleus, where the complexes bind to specific DNA sequences (estrogen responsive element). In association with other coactivators and represors alters the expression of target genes. For example binding of ER1-ligand complex to fos/jun complex facilitate the binding of fos/jun heterodimer to AP-1 site and activation of many genes including IGF-1. On the contrary, ESRs inhibit binding of NF-kB to IL-6 promoter.

7.4.2 Pharmacogenetics of ERs

Pharmacogenetics of ERs was mostly studied regarding the role of ERs genes polymorphisms in determine the response to estrogen and SERM therapy and the risk of cardiovascular events. Clinical data of hormone replacement therapy (HRT) for maintain the health of postmenopausal women showed an increase in cardiovascular complications as the adverse drug reactions. However our data at raloxifen (SERM) treatment showed opposite effect with lowering of cholesterol level. Both, estrogens and SERMs activity could be modified with genetic polymorphisms of ER alpha gene. In this regard 10 different SNPs or microsatelite polymorphisms over regulatory, coding and uncoding regions were analysed. Cluster of four SNPs located at the 3' end of intron 1 were significantly associated with higher increase of HDL level at HRT treatment. Among these SNPs the IVS1-401T>C was most strongly related to HDL increase (3). Our data approve positive effects of exonic but not intronic SNPs on total cholesterol level at postmenopausal osteoporotic women treated with raloxifen (4).

Several other studies have also examined the effects of ER1 IVS1-401T>C polymorphism on response to HRT or bisphosphonates treatment of osteoporosis. IVS1-401C allele was associated with greater effects of estrogen on bone mineral density. Our data on nearly 60 postmenopausal women treated with raloxifen didn't show any influence of ER1 SNPs on SERM effect.

7.5 Conclusion

The information about the role of polymorph drug receptors for efficiency of drug therapy are more scarce, although promising examples are seen in drug treatment of asthma where the efficiency can be severely enhanced by predictive genotyping of the drug targets. To move the

field to clinical practice, future studies should be larger and have to consider the complexity of drug response (receptor protein with proteins in signal transduction). Finally, for those drugs with multiple pharmacological effects or effects in different organs, the genetic contributions have to be considered separately (5). Discovering highly predictive genotype-phenotype associations during drug development and demonstrating their clinical validity and utility in well-designed prospective clinical trials will no doubt better define the role of pharmacogenetics in future clinical practice.

Recommended literature:

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2. Taylor MRG. Pharmacogegenetics of human beta-adrenergic receptors. Pharmacogenomics J 2007;7:29-37.
3. Herrington DM. Role of estrogen receptor-a in pharmacogenetics of estrogen action. Curr Opin Lipidol 2003;14:145-50.
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8. APPLICATION OF PHARMACOGENETICS IN DOSE INDIVIDUALIZATION IN DIABETES, PSYCHIATRY, CANCER AND CARDIOLOGY

Dorota Tomalik-Scharte

8.1 Introduction

Following administration of any medication, it is not always possible to predict its effects in the individual patient. Due to the major inter-individual variability in response to pharmacotherapy, in some patients, adverse drug reactions or therapeutic failure instead of therapeutic success are observed. The list of possible factors contributing to the individual drug response involves e.g. age, sex, body weight, liver of kidney function, co-medication or smoking status. Moreover, inter-individual differences in the efficacy and toxicity of many drugs could also be affected by polymorphisms (sequence variants) in genes encoding drug-metabolizing enzymes, transporters, receptors and molecules of signal transduction cascades. Such polymorphisms may contribute to pronounced variability in pharmacokinetic processes (absorption, distribution, metabolism and elimination) and pharmacodynamic effects which finally results in differing drug response. Pharmacogenetics/pharmacogenomics tries to define the influence of genetic variations on drug efficacy and adverse drug reactions. Although both terms are often used interchangeably, pharmacogenetics concentrates on individual drug effects having regard to one or a few gene polymorphisms only, whereas pharmacogenomics assumes application of modern genomic technologies for drug assessment and discovery taking into account the entire genome.

The importance of genetic variations in drug response was recognized about 50 years ago, when in some individuals, live threatening adverse drug reactions following application of the muscle relaxant succinylcholine were observed and in patients treated with the tuberculostatic drug isoniazid, pronounced differences in pharmacokinetic parameters (bimodal distribution) were measured. Later, it was determined that these prime examples of variable drug disposition were caused by inherited differences in genes coding respective drug metabolizing enzymes. Since that time, contribution of genetic polymorphisms in drug metabolizing enzymes, transporters and targets (e.g. receptors) to drug disposition and/or drug effects has been investigated in numerous in vitro and clinical studies. Although more prospective studies with clinical endpoints are required to establish a definite role of molecular genetic diagnostics in individually tailored pharmacotherapy, in many situations pharmacogenetics/pharmacogenomics allows for an improved drug response, yet. Possibilities of individual dose adjustment in some important medical fields are briefly discussed below.

8.2 Diabetes

Type 2 diabetes is one of the most important public health problems and its complications like angio- and neuropathy are associated with pronounced morbidity and mortality. In addition to lifestyle modification programs, an appropriate therapy with oral antidiabetic drugs plays a key role in blood glucose control. Several classes of antidiabetics such as sulfonylureas, meglitinides, biguanides, α-glucosidase inhibitors, thiazolidinediones or insulins belong to the

approved drugs for patients with type 2 diabetes. The action of oral antidiabetic drugs and their adverse drug reactions such as hypoglycemia are subject to wide inter-individual variability. Most oral antidiabetic drugs are metabolized with participation of cytochrome P450 enzymes of the class 2C, which is genetically polymorphic. Whereas sulfonylureas are mostly CYP2C9 substrates, CYP2C8 is the main enzyme responsible for the biotransformation of thiazolidinediones (rosiglitazone and pioglitazone) and repaglinide. For tolbutamide, an oral sulfonylurea hypoglycemic agent used in the treatment of type 2 diabetes for many years, the contribution of CYP2C9 genetic polymorphisms to pharmacokinetics and blood glucose lowering effects was very well documented. Consequently, a careful monitoring of the hypoglycemic effects upon tolbutamide administration in patients heterozygous and especially those homozygous for CYP2C9*3, which is an allele with decreased enzymatic activity, was recommended. Moreover, dose adjustments for carriers of CYP2C9*3 polymorphism were suggested i.e. half and 20% of tolbutamide standard dose, respectively, for heterozygous and homozygous carriers of CYP2C9*3. The impact of CYP2C9 polymorphism on pharmacokinetics of the second generation sulfonylurea drugs like glibenclamide (glyburide), glimepiride and glipizide have also been studied. Similarly, it could have been shown that total clearance of these oral antidiabetics in carriers of CYP2C9*3/*3 genotype was only about 20% of that in wild types (CYP2C9*1/*1), whereas in heterozygotes, this parameter was reduced to 50-80%. Interestingly, the resulting magnitude of differences in drug effects (insulin concentrations) seems to be much less pronounced than for the pharmacokinetic parameters. Nevertheless, it has been considered that respective CYP2C9 genotype-based dose adjustments may reduce the incidence of possible adverse reactions. At the same time, the presence of another common CYP2C9 variant allele i.e. CYP2C9*2 seems to be without clinical relevance for the therapy with sulfonylureas since it has been considered to reduce the CYP2C9 enzymatic activity to a minor extent only.

Both nateglinide and repaglinide are meglitinides, which, like sulfonylureas, act by stimulating insulin release from beta cells of the pancreas via ATP-sensitive K+ channels and on voltage-sensitive Ca^{2+} channels. For nateglinide, predominantly metabolized via CYP2C9, it could be shown that CYP2C9*3 polymorphism, but not CYP2C9*2, has a moderate impact on pharmacokinetics and pharmacodynamic effects of the drug in healthy volunteers. Furthermore, following administration of repaglinide, which is metabolized via CYP2C8, reduced plasma concentrations have been determined in carriers of CYP2C8*3 variant allele. The possible role of CYP2C8*3 polymorphism in pharmacokinetics of thiazolidinediones rosiglitazon and pioglitazone should be assessed in further clinical studies.

Biguanide metformin belongs to oral antidiabetics widely used in overweight patients with type 2 diabetes. It could be shown that organic cation transporter 1 (OCT1) is mainly responsible for metformin entry into enterocytes and hepatocytes. To date, several genetic polymorphisms in OCT1, some of them leading to reduced transporter activity, have been identified. In one clinical study, carriers of at least one OCT1 variant allele, determining reduced function of the transporter, showed higher glucose levels following administration of metformin. However, before OCT1 genotyping could be established as a reliable method for prediction of clinical response to metformin, prospective clinical studies in large numbers of patients must be performed.

It appears that personalized medicine could promise an optimization of treatment choices in patients with type 2 diabetes, however, due to pronounced complexity of the disease and

individual drug response, further research is needed to establish the role of pharmacogenetics in therapy of diabetes.

8.3 Psychiatry

Major psychiatric disorders, endogenous depression and schizophrenia, often require a life-long medication with drugs characterized by a narrow therapeutic index and wide inter-individual variability in therapeutic response. Moreover, it is estimated that about 30-50% of patients treated with antidepressants and antipsychotics do not respond sufficiently to the first treatment given to them, which imposes significant costs on public health services. It is expected that identification of genetic factors determining individual drug response in psychiatric disorders could notably improve therapeutic outcomes.

Most antidepressants from the group of tricyclic antidepressants are metabolized with participation of CYP2D6, which is characterized by a high inter-individual variability in catalytic activity mainly determined by the number of functional CYP2D6 alleles. Carriers of two, one or none functional copies of the gene are phenotypically extensive (rapid), intermediate or poor metabolizers, respectively. Furthermore, inheritance of three or more functional alleles by gene duplication or gene amplification determines the ultrafast metabolizer phenotype characterized by higher-than-average enzymatic activity. Tricyclic antidepressants undergo similar biotransformation reactions in the liver, whereas hydroxylation reactions are catalyzed by CYP2D6. For a number of common tricyclics like amitriptyline, clomipramine, desipramine, imipramine, nortriptyline, doxepin and trimipramine, large differences in the pharmacokinetic data depending on CYP2D6 genotype have been documented, so that in poor metabolizers of CYP2D6, reduced (50% or more) clearance values have been observed. On the other hand, following the administration of nortriptyline and desipramine, extremely high clearance was measured in ultrarapid metabolizers of CYP2D6. In addition, CYP2C19, another genetically polymorphic enzyme, can also contribute to metabolism (demethylation) of some tricyclics like imipramine, amitriptyline and clomipramine, however, a possible impact of CYP2C19 polymorphism on the pharmacokinetics of the drugs is not so well documented as that of CYP2D6. Furthermore, CYP2D6 also plays a role in metabolism of another class of antidepressants, i.e. selective serotonine re-uptake inhibitors (SSRIs) and some of them like fluoxetine, fluvoxamine and paroxetine were shown to be strong inhibitors of CYP2D6 activity. For that reason, conversion from extensive to slow and from ultrafast to extensive metabolizer phenotype in course of the therapy with the drugs has been observed. Therefore, for SSRIs, the problem of CYP2D6 inhibition appears to be more relevant than CYP2D6 genetic polymorphisms.

Unfortunately, the data considering potential clinical implications of CYP2D6 genotype in patients treated with antidepressants is very limited, but it seems that poor metabolizers of CYP2D6 tend to be more affected by relevant adverse effects, whereas the role of CYP2D6 in response to antidepressants is rather controversial.

CYP2D6 polymorphisms can also affect the pharmacokinetic parameters of commonly prescribed conventional as well as atypical neuroleptics like haloperidol, levomepromazine, perazine, thioridazine, clozapine, olanzpaine or risperidone. Moreover, CYP2D6 genotype has been associated with an increased risk of antipsychotic-induced extrapyramidal symptoms, which frequently accompany the therapy with conventional antipsychotics. For haloperidol, pseudoparkinsonic adverse events were significantly more frequent in poor metabolizers of

CYP2D6, whereas with a higher number of active CYP2D6 gene copies, a tendency toward a lower therapeutic efficacy was observed.

For some antidepressants and neuroleptics, possible dose adjustments have been calculated on the base of CYP2D6 and CYP2C19 genotypes. In carriers of CYP2D6-related poor metabolizer genotype, dose reductions to about one third of the standard dose have been suggested for drugs like tricyclics impiramine, trimipramine, doxepin or antipsychotic drug perphenazine, to name a few examples. At the same time, dose enhancements by about one third of the standard treatment for extensive metabolizers were calculated for these drugs. Likewise, dose extrapolations resulting from CYP2C19-mediated quantitative influences on pharmacokinetics of some antidepressant drugs are possible. Notably, assessment of both genes CYP2D6 and CYP2C19 has found the way into clinical practice by means of the recent approval of the respective pharmacogenetic tests by the Food and Drug Administration.

As genetic polymorphisms in genes coding for drug metabolizing enzymes can explain only a part of the large inter-individual variability in therapeutic response in psychiatric disorders, other candidate genes which code for target molecules should also be considered. However, data on the possible medical impact of the particular polymorphisms affecting targets like neuronal serotonin transporter, serotonin and dopamine receptors as well as several molecules of signal transduction are not so well documented or partially controversial, so that conclusive clinical evidence is missing in many cases and no respective treatment recommendations are possible at present.

In summary, there is a strong evidence first of all for CYP2D6 genotype affecting pharmacokinetics of numerous antidepressants and antipsychotic drugs and respective dose extrapolations for carriers of genetic polymorphisms have been calculated. However, before dose individualization based on genotype could be routineously implemented in clinical practice, it should firstly be validated in prospective and controlled clinical studies.

8.4 Oncology

Application of pharmacogenetics to individualization of therapy with antineoplastic drugs, most of them characterized by a narrow therapeutic index and life-threatening adverse reactions, seems to promise improvement of drug effects in some cases.

Thiopurines, like 6-mercaptopurine and thioguanine, largely used in the treatment of acute leukemia, are one of the earliest examples of importance of pharmacogenetics in individualized drug therapy. Following the activation to thioguanine nucleotides via the purine salvage pathway and incorporation into DNA as false purine bases, they are metabolized by the enzyme thiopurine-S-methyltransferase (TPMT) to inactive compounds. The individual enzymatic capacity is a subject to large inter-individual variability which is determined by genetic polymorphisms, with three variant alleles *2, *3A and *3C explaining about 80-95% of enzymatic deficiency. In the Caucasian population, about 89% of people exhibit a high TPMT activity, whereas in 11 and 0.3% of individuals, respectively, intermediate and low activity, is observed. Following a treatment with conventional doses of thiopurines, patients showing diminished catalytic TPMT activity are at increased risk of bone marrow suppression, which may result in fatal outcomes and require discontinuation of therapy. Hepatic TPMT activity can be reliably determined by genotyping or measurement of the catalytic activity of cytosolic TPMT in erythrocytes using established radiochemical or HPLC methods (i.e. phenotyping). Measurement of TPMT activity should routinely precede

onset of therapy with thiopurine-derived drugs in order to minimize myelotoxic adverse events. For patients being carriers of two non-functional TPMT, thiopurine dose reduction to 5-10% of standard dose was recommended to allow for an efficacious therapy. In heterozygous patients, the therapy begins with a full dose, but a subsequent dose reduction may be required. Although only a small percentage of patients could be affected by inherited differences in TPMT activity, the clinical consequences may be crucial. For that reason the Food and Drug Administration has already implemented respective pharmacogenetic data into the product label of 6-mercaptopurine, widely used for childhood leukemia.

Another antineoplastic drug for which pharmacogenetic diagnostics prior to therapy onset would promise selection of potentially toxic patients is 5-Fluorouracil (5-FU). Dihydropyrimidine dehydrogenase (DPD) is a key enzyme in the hepatic metabolism of 5-FU and its derivatives such as capecitabine, so that the enzyme activity affects pharmacokinetics, efficacy, and toxicity of the drugs. Diminished enzymatic activity has been observed in about 3-5% of Caucasians and can potentially result in severe adverse drug reactions like mucositis or granulocytopenia in cancer patients treated with 5-FU. DPD is genetically polymorphic and allelic variants in the gene coding the enzyme have been associated with reduced catalytic activity. One of the best described mutations is the the so-called exon 14-skipping mutation at the 5'-splice donor site of exon 14. Although this polymorphism is present in only about 1% of Caucasians, it has been detected in 24% of patients developing severe toxicity (WHO grade IV) following treatment with 5-FU. Nevertheless, further research is needed to evaluate possible benefits of pharmacogenetic strategies upon therapy with 5-FU.

At the same time, pharmacogenetics of irinotecan, a potent antineoplastic agent used in the treatment of colorectal cancer and small-cell lung cancer, seems to be one of few promising examples of the implementation of pharmacogenetics to individualized drug therapy. Following its application, irinotecan is metabolized to the active compound SN-38, which is a topoisomerase I inhibitor. In the next step, SN-38 is glucuronidated to its inactive form by various isoenzymes of uridine diphosphate glucuronosyltransferase (UGT), first of all UGT1A1, which is also responsible for glucuronidation of bilirubin. Reduced glucuronidation activity of the UGT1A1 enzyme has been connected to elevated levels of SN-38 and toxic effects like severe diarrhea and neutropenia in patients treated with irinotecan. To date, several genetic polymorphisms leading to impaired UGT1A1 activity have been determined in the gene coding for the enzyme. In the Caucasian population, the UGT1A1*28 polymorphism (TA repeat in the promoter region) is the most frequent variant contributing to reduced glucuronidation activity. It could be shown that even in heterozygous carriers of the variant allele, pronounced changes in irinotecan disposition and severe toxicity occur. For that reason, genotyping for UGT1A1 polymorphisms before the onset of ironotecan therapy has been recommended. Interestingly, the measurement of total bilirubin level seems to be an easy surrogate parameter, if genotyping is not possible. Patients with diminished glucuronidation capacity should be administered a reduced initial dose of irinotecan to avoid the above mentioned severe toxicities.

Possible implications of polymorphisms in genes coding for other drug metabolizing enzymes like CYP2D6 and CYP3A, drug transporters like ATP-binding cassette transporter ABCB1 (P-glycoprotein) and drug targets like thymidylate synthase in patients treated with common prescribed antineoplastic drugs have also been considered in numerous studies, but their potential impact on clinical outcomes is still controversial.

In summary, oncology is the clinical area where achievements of modern pharmacogenomic diagnostics have already been used to tailor individual therapy with some antineoplastic drugs, but for a wide implementation of genotyping in cancer patients, more clinical data and a precise cost effectiveness analysis of this approach are required.

8.5 Cardiology

Cardiovascular diseases like coronary heart disease, hypertension or heart failure are still a leading health problem in developed countries and respective pharmacotherapy is an established approach in affected patients. It appears that pharmacogenetics throws some new light on the question of treatment amendment with respect to cardiovascular diseases.

For several beta-blockers, which belong to the most often prescribed drugs in patients with cardiovascular diseases, possible effects of genetic polymorphisms in drug metabolizing enzymes like CYP2D6 were assessed. CYP2D6 is the key enzyme in metabolism of metoprolol and pronounced differences between CYP2D6 extensive and rapid metabolizers with respect to the phramacokinetics of the drug have been observed. Moreover, *CYP2D6* polymorphism has been shown to contribute to pharmacodynamic response following the administration of metoprolol, since reduction of exercise induced heart rate by the drug in the group of ultra rapid metabolizers (carrying a duplication of the *CYP2D6* gene) was only circa half of that observed in extensive metabolizers. Also for carvedilol, the role of the CYP2D6 polymorphism was studied. However, respective pharmacokinetic differences resulted from the genetic polymorphism seem to be without any effects on heart rate and blood pressure so that they will have no clinical significance.

Another class of drugs, AT_1 (angiotensin II type 1) receptor antagonists (sartans), used to treat hypertension or heart failure, could be potential candidate for consideration of pharmacogenetic data in therapy optimization. Most sartans are metabolized with participation of genetically polymorphic CYP2C9. Losartan is a pro-drug which is transformed to its active form, i.e. E-3174, via CYP2C9 and CYP3A4. Unfortunately the role of the *CYP2C9* polymorphism for therapy with losartan is quite controversial. Whereas in one study, presence of *CYP2C9*3* was shown to be associated with decreased formation of E-3174, in another study, no differences with respect to the pharmacokinetics of the parent drug and its active metabolite between the wild types and carriers of the best investigated *CYP2C9* variant alleles related to impaired intrinsic enzymatic activity *CYP2C9*3 and CYP2C9*2* were determined. There is also some clinical data suggesting the role of *CYP2C9* polymorphism in the pharmacokinetics and/or -dynamics of other AT_1 receptor antagonists like irbesartan or candesartan. However, if potential dose adjustment of sartans according to the *CYP2C9* genotype might be beneficial is furthermore doubtful.

Recently, importance of pharmacogenetic implications has also been discussed for statins (HMG-CoA reductase inhibitors), administered to lower cholesterol level in numerous patients with or at risk for cardiovascular problems. Statins are the most prescribed and most effective drugs in lipid lowering therapy but large variability in response is observed and in nearly one of three patients treatment goals could not be met. It has been reported that in patients treated with pravastatin, cholesterol lowering effects are poorer in carriers of two common and tightly linked single nucleotide polymorphisms localized in the gene coding for HMG-CoA reductase, which is the target enzyme for statin therapy. However, no data is available, if possible genotyping approach with a following dose adjustment, in terms of

application of a higher dose of pravastatin in patients carrying the variant haplotype, could be advantageous in clinical practice.

Last but not least, the meaning of pharmacogenetic approaches for therapy with oral anticoagulants (coumarin anticoagulants) should be briefly discussed. These vitamin K antagonists, used widely in patients at risk of thromboembolic disorders, are characterized by a narrow therapeutic index, so that the therapy with them is often complicated by dangerous bleeding episodes or lack of efficacy, in case of under- or overcoagulation, respectively. Two polymorphic genes, *CYP2C9* and vitamin K epoxide reductase complex subunit 1 (*VKORC1*), can contribute significantly to the known inter-individual variability in the effectiveness of oral anticoagulants. The role of the enzyme CYP2C9 in metabolism of the warfarin and its analogues acenocoumarol and phenprocoumon is well documented. The variant alleles with decreased enzymatic activity *CYP2C9**2 and *CYP2C9**3 have been demonstrated to impact considerably the pharmacokinetics of S-warfarin (which is 3 to 5 times more potent than the R-isomer) and so to influence the antithrombotic activity of the drug. Patients carrying at least one variant allele, show a longer induction period to achieve a stable warfarin dosing and tend to have increased values of international normalized ratio (INR). They are also at increased risk of life threatening bleedings. Similarly, there is a good evidence for the role of *CYP2C9* polymorphism in the anticoagulation effects of acenocoumarol and phenprocoumon in the literature data. For that reason, *CYP2C9* genotyping was suggested as a useful approach to select a population of patients who are potentially at risk of complications associated with oral anticoagulants and who may require a reduced dose of the drugs.

VKORC1 is the target molecule of vitamin K antagonists and polymorphisms in *VKORC1* gene, in addition to *CYP2C9* and demographic factors, seem to explain a significant part of the inter-individual variability in pharmacokinetics and –dynamics of the drugs and consequently could be essential for determination of the individual dose. For warfarin, an algorithm for individual dosing adjustment on the base of *CYP2C9* and *VKORC1* genotype, age and height has been proposed, but prior to introduction into clinical practice it should be proved in prospective clinical studies.

In summary, in the light of current knowledge, it seems that with respect to cardiovascular diseases, only for vitamin K antagonists, there is a place for pharmacogenetic approaches to optimize the therapy and avoid adverse events.

8.6 Conclusion

Looking back at more than 50 years of pharmacogenetic experience, we have learnt that an important part of the inter-individual variability in drug response is caused by polymorphisms in drug metabolizing enzymes, transporters or target molecules. For some treatments, it was shown that efficacy and safety profile of pharmacotherapy could be improved if respective allelic variations are taken into account. Although it seems that the first genotype-specific dose recommendations have already reached clinical practice in some medical fields, unquestionably more prospective clinical studies validating pharmacogenetic approaches as well as cost-effectiveness evaluations are needed before pharmacogenetics makes a great jump form bench to bedside.

Recommended literature:

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9. POINT-OF-CARE MOLECULAR DIAGNOSIS: A NEAR FUTURE?

Ana-Maria Šimundić

9.1 Introduction

Is point-of-care molecular diagnosis a near future or not – it may not be too difficult to perceive and answer the question. The future of medicine is a logical consequence of the sequence of events, and some achievements and possibilities probably are currently closer than we can imagine. Some questions unavoidably emerge when considering the future of medicine and the role of molecular diagnosis in it, e.g.:

- What influences the development of medicine and medical biochemistry as tightly related professions (market economy?; science?; technology?)?
- What is the impact of market economy on the course of development of diagnostic methodology?
- What is the course of technological development? What are the predominant fields of development?
- What is the course of scientific development?

As many as 80% of medical decisions are made on the basis of laboratory findings. Therefore, it can well be stated that the results of *in vitro* laboratory tests are a vital aspect of medical decision making. However, the costs of *in vitro* laboratory diagnosis account for a negligible percentage of overall health care expenditures in the context of global market. Although still insignificant, this share has shown some increase in the last few years.

Investment in new reagents and technologies in general results in advanced methods used to detect various diseases and states. According to reports and estimates of renowned economy experts, in medicine these developments are currently most pronounced in the fields of infectious diseases, diabetes mellitus, cardiovascular diseases, and malignant diseases.

Considering technologies, in the last decade the most intensive development has been recorded in the methods of molecular diagnosis, e.g., various variants of the polymerase chain reaction (PCR) method, microarray and nanoarray technologies. This trend is anticipated to continue in the future, with ever increasing investments in this segment of market economy.

Hospital laboratories remain the most extensive consumer of diagnostic equipment and reagents. In the future, some non-institutional health care segments such as private practice and polyclinics will probably take a growing share. These segments will be subject to strict control of the tests and methodologies licensed to perform, and thus of the technologies available.

9.2 The future of medicine

Technological development definitely plays the key role in the development of medicine. These exponential technological advancements make achievements once inconceivable to human mind now really accomplished. Novel technologies entail profound changes in medical profession and mode of approach to patient.

What trends are perceived in the development of modern medicine? Generally, the main idea is to make medicine a proactive rather than reactive in nature. What does it mean? According to Professor R.M. Satava from the University of Washington, USA, one of the pioneers in the field, the future of medicine will follow the 5P principle, according to which modern medicine is:

- predictive
- preventive
- point-of-care (POC)
- parametric
- personalized

Our understanding of factors that significantly influence the risk of particular disease development is expanded in parallel with the advent of the genetic screening methods and results acquired in large epidemiological studies. Predictive medicine tends to identify individuals or populations carrying some features in common and requiring some specific preventive measures, and diagnostic or therapeutic approach.

Preventive medicine integrates new concepts and is focused on the implementation of preventive measures in a population at risk, thus preventing the onset and development of the disease in the population. Such an approach is by far more efficient than the treatment of disease and is from the economic point perceived as a significant step forward in rationalization of overall health care costs.

There is no need to explain the POC aspect of modern medicine to medical biochemists. Modern medicine tends to the conditions that will enable continuous monitoring of all relevant parameters in a minimally invasive or completely noninvasive way, and in a setting most suitable for the patient, i.e. at home, at bedside, at work place, etc.

The current information technology enables observation and collection, analysis and understanding of the very complex systems of inter-related data. As such, it has created conditions for the development of so-called parametric medicine. Parametric medicine implies collection of many parameters, along with determination and monitoring of their changes over time, with due consideration of individual characteristics such as age, sex, race, etc.

Personalized medicine tends to adjust every diagnostic and therapeutic approach to the individual patient's needs. The constitution of each individual is absolutely unique, with a specific genetic code and phenotype, while living in inimitable environment. Thus, the hypothesis that every human being is distinguished by some aberration from all other individuals appears to be quite justified. Although the field has lately been the subject of in-depth research, it should by no means be perceived as a novelty in medicine. The concept of variability is very old indeed, dating from the times of Hippocrates, Galen and their disciples.

Sir William Osler (1849-1919), a Canadian physician, father of modern medicine, wrote: "It is more important what patient category is affected with a disease than what type of disease develops in a particular patient".

Accordingly, current medicine is based on care for patient. The medicine of tomorrow will make a step forward towards population screening and identification of individuals with a particular predisposition, their follow up over time, and implementation of preventive measures.

The development of molecular diagnosis is keeping up with the above mentioned changes in medicine. The molecular diagnosis of tomorrow will be (or already is?) a predictive, preventive, POC, parametric and personalized discipline.

9.3 Molecular diagnosis

Scientific advancements and knowledge of the sequence of genome, transcriptome, proteome and metabolome on the one hand, and technological development of novel sequencing platforms, microarray devices and other systems on the other hand make the basis of further improvements towards medicine of a new future era to come soon. What is the role of laboratory in such a new medicine? How will such a modern laboratory and new technologies meet the needs of emergency diagnosis?

Not more than some ten years ago, the idea of molecular diagnosis in emergency states seemed inconceivable and unfeasible. Today it is not so anymore. Since lately, POC systems for molecular diagnosis have been released to the market. These systems are intended for various fields, e.g., infectious diseases, pharmacogenetics and genetic testing.

One of these systems is the GeneXpert® released by Cepheid (Sunnyvale, CA, USA), proclaimed product of the year by the Small Times Magazine in 2005. It is the first system that allows for the process of isolation, purification and concentration, followed by DNA amplification and detection to be performed in a fully integrated and automated manner. While the current DNA technology involves several completely separate steps, and requires specific equipment and properly trained personnel for considerable manual manipulation over 1-3 days, the GeneXpert® real-time PCR system enables the desired sequence to identify within 30 minutes. These systems were initially employed for rapid analysis and detection of the potential biological war and terrorist agents at the national level within the United States Postal Service. Thousands of these systems were mounted across the United States, and the GeneXpert® system has since been certified and validated as an efficient anti-terrorist technology. GeneXpert® uses PCR technology for rapid analysis of air samples and for detection of DNA in traces originating from the bacterium *Bacillus anthracis* spores.

Cepheid Co. has also developed other methods for detection of various pathogens such as group B streptococci, some enteroviruses, Epstein-Barr virus, cytomegalovirus and methicillin-resistant *Staphylococcus aureus* (MRSA). In addition to this microbiological panel, some other genetic tests, e.g., rapid method for detection of BCR/ABL translocation, have recently been launched.

DNA isolation by the GeneXpert® system is based on the principle of ultrasonographic destruction of cell membrane or bacterial spore wall. The complete procedure proceeds in

disposable cartridges that contain mixing needles and physically separated chambers for different process phases. Upon membrane destruction, DNA is extracted into a microfluid column coated by DNA probes that bind the released DNA, while cellular debris passes and flows out through the column. The bound DNA is then released from the binding site and washed into the system segment for amplification. Various sample types and amounts (up to 5 mL of sample!) can be applied onto the device. Blood, tissue and various swabs can be used as a sample. Results can be obtained in no more than 30 minutes, whereas RT-PCR analysis takes about 2 hours.

Besides Cepheid, some other manufacturers also offer equipment for rapid molecular diagnosis testing (Table 9.1.). These instruments have not yet been routinely used; however, there are numerous ongoing clinical studies the results of which will make the basis to decide on the potential justifiability of introducing these tests in routine practice.

Manufacturer	Seat	URL address
Cepheid	Sunnyvale, CA	www.cepheid.com
Enigma Diagnostics	Witshire, England	www.enigmadiagnostics.com
LGC	Middlesex, England	www.lgc.co.uk
IQ^{uum}	Allston, MA	www.iquum.com
Nanosphere, Inc.	Northbrook, IL	www.nanosphere-inc.com
Lumora Ltd.	Cambridge, England	www.lumora.co.uk
Nanogen, Inc.	San Diego, CA	www.nanogen.com
HandyLab, Inc.	Ann Arbor, MI	www.handylab.com
IMM	Mainz, Germany	www.inn-mainz.de

Table 9.1. *List of manufacturers of point-of-care equipment in the field of molecular diagnosis (from Holland CA , Kiechle FL. Curr Opin Microbiol 2005;8:504-9.)*

9.3.1 Rapid detection of the cause of sepsis

Sepsis is a syndrome characterized by systemic response to infection which, if not appropriately treated, may lead to multiorgan dysfunction and death in a very short period of time. Sepsis is the third leading cause of death in the world. Eighteen million people are affected with sepsis *per* year, and every day 1400 patients die from sepsis worldwide.

The costs of treatment for severe sepsis account for almost 40% of overall intensive care unit (ICU) expenditures, amounting to 7.6 billion EUR in Europe and 16.7 billion USD in the USA. Thus, sepsis is one of the major challenges for ICU physicians. Therefore, the Surviving Sepsis Campaign was launched at the international level in 2002, with the objective to reach the fastest possible detection and diagnosis of sepsis at all hospital wards. As in sepsis the probability of survival correlates with the time of antibiotic therapy initiation, it is of utmost importance to introduce intravenous antibiotic therapy as early as possible, i.e. within the first few hours of the diagnosis of sepsis.

One of the major problems in the approach to sepsis patient is related to the choice of appropriate antibiotic, which should be based on proper identification of the causative agent in order to be efficient. According to literature data, more than 25% of patients receive inappropriate therapy. Microbiological methods are too time-consuming for this purpose. Traditional methods for detection of the causative agent take several days and have inadequate

inadequate diagnostic accuracy. Fungal infections are especially difficult to identify as the cause of sepsis (Figure 9.1.).

Figure 9.1. *Candida albicans as viewed by electron microscope (with permission from Dr. John Bennett).*

At the beginning of 2006, Roche released LightCycler SeptiFast, a new diagnostic kit for rapid detection of the cause of sepsis. It is a very rapid and reliable procedure to detect and identify as many as 25 most common causes of sepsis, which account for some 90% of sepsis cases. This method of pathogen type analysis is based on the principle of real-time PCR, as a method of amplification and melting curve analysis. The turn around time (TAT) from the initiation of sample processing to the result reporting is somewhat more than 5 hours. The first step of sample preparation takes 2 hours, followed by sample analysis that takes some more than another 2 hours. The last step is agent identification by use of computer program and finding report.

The example of the SeptiFast test points to the current achievements in the molecular diagnosis methodology. In formal terms, this method could not be included in emergency tests because it does not meet the professional criteria (TAT <1 h). Yet, the method definitely means a breakthrough as compared with the traditional microbiology methods, reducing TAT from several days to only a few hours. At present it appears irrelevant whether or not it is short enough to proclaim the method an emergency test. Sepsis is a condition that requires emergency intervention from the physician and as such imposes the need of the earliest possible diagnosis. If we assume that emergency means as early as possible, then in sepsis the earliest possible result is currently available in 5-6 hours, and from this point of view this test could be considered an emergency test.

It certainly is not the end of technological development. It can be anticipated that quite soon it will be possible to identify the cause of sepsis within a shorter period of time. The shortcoming of the current method lies in the procedure of sample preparation, which is too time consuming due to the still existing technological limitations. Some other manufacturers have already significantly reduced this step by use of the above mentioned methods of ultrasound destruction of cell membrane.

9.4 Pharmacogenetics

Among other fields where molecular diagnosis is employed for emergency diagnosis, mention should be made of pharmacogenetics. A rapid method to identify genotype which determines the patient's therapeutic response may occasionally be of crucial importance indeed. In this way, the incidence of unwanted and toxic drug effects that occur due to inappropriate therapeutic drug dosage and may pose vital threat for the patient would be significantly reduced. Target pharmacogenetic analysis prior to therapy introduction will certainly be routinely performed in the near future. These studies belong to the aspect of personalized

medicine, described in the Introduction section. The main obstacles to these developments are not technology related. There are no technological limitations to halt or hamper the implementation of emergency pharmacogenetic analysis in clinical practice. The adoption and introduction of novelties in clinical routine need to be founded on strong arguments according to the principles of evidence based medicine. Large randomized controlled clinical trials have to be conducted on many of the known pharmacogenetic polymorphisms to demonstrate the justifiability and efficiency of this approach in clinically relevant situations.

9.5 Conclusion

The systems enabling the use of molecular diagnosis methods in emergency are yet to take hold in daily routine; however, it is just a matter of time for them to become a generally accepted standard. There also are some obstacles to overcome, which is not impossible but we have to be aware of them in order to approach and master them successfully. If molecular diagnosis is to be performed beyond central clinical laboratory and after hours, it will require proper training of the personnel working on these instruments. Also, the economic aspect of such changes needs to be taken in consideration, i.e. which systems from the ample offer to choose? Who will bear the cost of these tests? Are these tests cost-effective considering overall health care cost *per* patient? Medical biochemists should not only take the role of silent witnesses but also of active participants and visionaries of this technological and information revolution. We have to keep abreast with the fast progress and acquire due education and training in line with the high professional standards to be able to offer the latest and most sophisticated diagnostic methods to our patients when necessary.

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10. CIRCULATING NUCLEIC ACIDS AS DIAGNOSTIC TOOL

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10.1 Introduction

Circulating nucleic acids are present in small amounts in the plasma of healthy individuals. However the discovery of circulating nucleic acids has long been explored for the non-invasive diagnosis of a variety of clinical conditions. The first studies concerning the detection of circulating DNA were investigated for finding various forms of cancer. Metastasis and recurrence in certain tumour types have been associated with the presence of high levels of cancer-derived DNA in circulation. The detection of fetal DNA in maternal plasma in pregnant women is useful in detecting and monitoring fetal diseases and pregnancy-associated complications. Similarly, levels of circulating DNA in acute medical emergencies including trauma and stroke, have been reported to be increased and have been explored as indicators of clinical severity. In the last few years, other than circulating DNA, much attention and effort has been put into the study of circulating RNA, starting from the detection of tumor-derived RNA in the plasma of cancer patients. Soon after that, detection of circulating fetal RNA in maternal plasma was described. Plasma fetal RNA detection looks to be a promising approach for the development of gender- and polymorphism-independent fetal markers for prenatal diagnosis and monitoring complications during pregnancy. This development also opens up the possibility of non-invasive prenatal gene expression profiling by maternal blood analysis.

10.2 Noninvasive prenatal diagnosis

The discovery of cell-free fetal DNA in maternal plasma by Dennis Lo in 1997, outlined new scenarios for non invasive prenatal diagnosis. The quantitative analysis of the free fetal DNA showed that this can be made up of as much as 6.2% of the total DNA present in the maternal plasma (1). A deep and extensive search for non-invasive techniques of fetal DNA sampling has been carried out to substitute invasive prenatal diagnosis that carry a significant risk of miscarriage.

Fetal DNA release into maternal plasma has been shown to be a very early physiological phenomenon increasing progressively throughout pregnancy (2-3). Circulating fetal DNA molecules (SRY gene) have been detected in maternal plasma in the first trimester (starting from the 5th gestational week) onwards with an accuracy approximately of the 100% (2-3). This approach has been used for the prenatal investigation of sex-linked diseases and fetal RhD status condition in which the fetus presented a gene absent in the mother .

A majority of research groups use sequences of chromosome Y in male embryos as a marker of fetal DNA and standardization of the assays, due to the fact that a woman (46,XX) does not possess this chromosome in her genome. Sexing analysis is also important, mainly for diseases with a recessive X-linked pattern of inheritance, with female being normal or being carriers of the mutation, but healthy, while male are normal or affected by the disease. An application of fetal sexing is Congenital Adrenal Hyperplasia (CAH) an autosomal recessive genetic disease which carries a defect in 21- hydroxylase deficiency. Homozygous girls for

this pathology are born with masculinization of the external genitalia and often require surgical operations as opposed to affected boys whom present normal external genitalia. Prenatal treatment of CAH with dexamethasone to prevent genital ambiguity has been successfully used (4). However, to minimize the side effects, the interruption of therapy has been indicated in the case of affected or normal male embryos and normal female embryos. For this reason, fetal sexing is necessary during pregnancy and is usually carried out by invasive methods. Noninvasive fetal sexing based on free fetal DNA in maternal plasma would bring the additional advantage of early discontinuation of medication in the case of male embryos (5).

Moreover, many genetic diseases are caused by mutations that result in subtle differences between the sequences of maternal and fetal DNA, such as achondroplasia (6), â and ß thalassemia (7- 9).

Rh alloimmunization is a crucial problem in medical and obstetrical clinical practice, potentially leading to hemolytic disease in the newborn. For pregnant negative Rh women (15% of the population), a positive Rh embryo involves a 16% risk of sensitization to the Rh antigen. Diagnostic procedures and invasive therapy may be necessary to reduce perinatal mortality of positive Rh embryos (10). Thus, the early detection of fetal RhD status through fetal DNA in the plasma of negative Rh mothers is of great importance in defining the need for interventions, with known risks of gestational loss, or of gestational immunoprophylaxis.

10.3 Preeclampsia/Intrauterine Growth Restriction (IUGR)

Preeclampsia is a hypertensive disorder affecting approximately 5% of pregnancies and is still one of the main causes of mortality of both the fetus and the mother. Typical symptoms are maternal hypertension and proteinuria, which usually develop in the late second or third trimester of pregnancy (11).

Increased fetal DNA release can be a marker of pathological conditions affecting both the fetus and the placenta (12-19). Abnormal placentation has often been found to be involved in the pathogenesis of intrauterine growth restriction (IUGR) and preeclampsia, which can occur either isolated or in combination. IUGR is defined as the presence of an ultrasonographically estimated fetal weight below the fifth percentile confirmed post-natally, in the absence of chromosomal and structural abnormalities. Preeclampsia and IUGR have been linked to abnormalities in trophoblast invasion into the placental bed. During normal pregnancy, trophoblastic invasion of uterine spiral arteries takes place reducing the vascular resistance and allowing adequate fetoplacental blood supply. In IUGR and preeclampsia this adaptive phenomenon is often insufficient, resulting in a diminished infiltration and modification of the spiral arteries, which lead to the maintenance of a high-resistance uterine circulation (20-21). Several studies have addressed the issue of quantifying fetal DNA in maternal plasma in pregnancies complicated by preeclampsia, and there is a general agreement of up to fivefold increased fetal DNA levels in the presence of this pathology (19, 22).

Since IUGR is mostly caused by impaired placental perfusion, similar to what is found in preeclampsia, it might be also associated with high levels of fetal DNA in maternal circulation.

The increase in the rates of circulating fetal and maternal DNA would correspond to the degree of severity of the illness and, therefore, the level of fetal DNA may serve as a marker of the prognosis and severity of the clinical picture (23-24).

Although most researchers use the Y-chromosome in this specific application, other non-gender markers have been studied, including epigenetic markers, to improve the number of pregnant women that could be submitted to quantitative investigation (25).

Recently, fetal RNA has also been found in maternal plasma. Such fetal RNA has been shown to originate from the placenta and to be remarkably stable. The use of microarray-based approaches has made it feasible to rapidly generate new circulating RNA markers. It is hoped that further developments in this field will make the routine and widespread practice of noninvasive nucleic acid–based prenatal diagnosis for common pregnancy-associated disorders feasible in the near future.

10.4 Cancer

Cancer is a common malignant disease in industrialised countries. Early diagnosis of tumours and accurate identification of haematogenic metastases can improve the success of treatment (26–29). Therefore, the detection of single tumour cell released in the blood in early stages could help physicians choose the most advantageous therapy for patients. The presence of small amounts of cell free tumor DNA (cfDNA) circulating in the plasma or serum of cancer patients was first demonstrated 30 years ago and provides another possibility of examining tumour derived genetic material in the circulation and to detect haematogenic spread of tumour cell DNA (30). Qualitative alterations in circulating DNA, such as microsatellite alterations (31), oncogene mutations (32), mitochondrial DNA, tumour-specific methylated DNA (33) and viral DNA (34), have been found in patients with different types of cancer. Quantitative alterations of circulating cfDNA have also been observed in several tumours, such as prostate cancer (35), lung cancer (36), pancreatic cancer (37), leukaemia and lymphoma (38). High levels of circulating cfDNA were correlated with tumour metastasis, response to therapy and recurrence (36-39). Therefore, tumour-derived circulating nucleic acids in the plasma or serum of cancer patients were introduced as a tool for detection and surveillance of cancers (40).The proportion of patients with altered cfDNA varies with the pathology and the nature of the marker. However, several studies have reported the presence of altered cfDNA in over 50% of cancer patients (41), suggesting that this marker may be common and amenable for a variety of clinical and epidemiological studies. Because the mechanisms and timing of cfDNA release in the blood stream are poorly understood, only few studies have addressed the use of cfDNA for early cancer detection or as a biomarker for mutagenesis and tumourigenesis in molecular epidemiology. (41). In some circumstances, cfDNA alterations are detectable ahead of cancer diagnosis, raising the possibility of exploiting them as biomarkers for monitoring cancer occurrence.

10.5 Trauma

The mechanisms by which cell-free DNA is freed into the circulation of human subjects are unknown; one possibility is that DNA is released following cell death (42-43).

It is also possible that direct damage or hemodynamic compromise of the organ systems responsible for circulating DNA clearance may also lead to increased plasma DNA. Candidate

organ systems include the liver, spleen, and kidneys, which may have a role in both liberating and clearing circulating DNA.

Along this line of reasoning, Lo’s group hypothesized that DNA may be liberated from body tissues into the plasma after trauma and that plasma DNA may be a potentially useful prognostic tool (44).

In this study Lo shows that circulating plasma DNA in the peripheral blood of trauma patients increases early after injury and that these increases are related to the development of posttraumatic complications, suggesting that plasma DNA may be a potentially useful marker for monitoring patients after trauma.

10.6 Stroke

Stroke ranked as the second leading cause of all deaths worldwide in 1990, accounting for 4.4 million victims (45), and is also currently the leading cause of brain injury in adults (46). Preventive strategies have led to a decrease in the rate of stroke attacks and deaths. Increased concentrations of several neurobiochemical protein markers have been detected in the peripheral blood of patients with stroke, but at present there is no simple and accurate blood test that may be used to determine the severity of a stroke or to predict mortality and morbidity in stroke patients on arrival in emergency wards in clinical practice.

In Lo’s paper, the authors concluded that plasma DNA concentrations correlate with stroke severity and may be used to predict mortality and morbidity in the emergency room (47).

As both hemorrhagic and ischemic strokes (48) involve cell death and disruption of the blood–brain barrier, they hypothesized that DNA would be liberated into the plasma early after the onset of stroke and that it might be useful for assessing disease severity and for predicting mortality.

They have also shown that plasma DNA measurements may be useful for early risk stratification and for predicting in-hospital and 6-month disability and mortality. The greatest differences in plasma DNA concentrations between patients with good and poor outcomes occurred within 3 h of the onset of symptoms.

The mechanisms by which circulating cell-free DNA increases after stroke require further study but are likely to be a result of increased liberation from damaged cells. Strokes involve a complicated cascade of events involving cerebral ischemia, altered cerebral blood flow, inflammation, the production of reactive oxygen radicals, neuronal necrosis and apoptosis, and neurologic dysfunction (49-53). DNA may be liberated from cells undergoing apoptosis or necrosis.

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11. EUROPEAN QUALITY ASSESSMENT NETWORKS IN MOLECULAR DIAGNOSTICS

Michael Neumaier

Molecular Diagnostics has been growing steadily over the last decade and has now conquered a firm place in diagnostic testing in the surrounding of health care and scientific biomedical applications. Aside from testing for xenogenic nucleic acids in infectious diseases, molecular tests are mostly being carried out on human genetic material for the characterization of underlying defects in illness, a presymptomatic diagnosis or a predisposition testing. With the exception of somatic genetic defects e.g. observed in malignant diseases that characterize the phenotype of cancer cells and thus may be helpful for prognosis and therapy, many molecular tests describe the germline genetic make-up of a patient. It must be clear that the increase of potential genetic biomarkers will be enormous within very few years, Specifically, while the complete sequence of the human genome as determined by the Human Genome Project [www.sanger.ac.uk] forms the basis of our knowledge of the human genome, several large-scale international studies have started to improve our knowledge of the extent of genetic variation and its importance as a marker for disease and disease risk. Such projects include the international SNP-consortium [www.snp.cshl.org], the HapMap-Project [www.hapmap.org] and recently started Medical Sequencing Program [www.genome.gov/17516031]. This situation poses important questions pertinent to the issue discussed below in more detail.

Molecular genetic testing results may have important consequences for health and the well-being of an individual and his relatives. Indeed, constituting a permanent individual feature, they possess a potential to stigmatize a carrier. This has led to the perception that genetic test results are exceptional and should be considered separately from other medical information, a perspective termed “genetic exceptionalism”. Similarly, the notion to restrict genetic testing to the medical specialist in Human Genetics has been explained by the necessity for genetic counselling before and after the performance of the test. While such restrictions certainly apply to the classical Human Genetics approach to rare monogenic disorders, they will become less and less feasible in the clinical routine molecular testing in the context of multifactorial or polygenic diseases, e.g. diabetes, obesity, psychiatric conditions, cardiovascular diseases or cancer. In these highly prevalent diseases, a multitude of genetic markers has been associated with low or moderate increases of risks of morbidity and additional ones are being added to such panels as our understanding of metabolic networks increases. In contrast, the contributions of single such markers for the clinical presentation of disease conditions with multifactorial background are unclear at best due to the low diagnostic power of the single marker. It is therefore safe to expect the extended usage of multiparametric testing strategies most probably by technologies like DNA microarrays or high-throughput DNA sequencing in the near future. Indeed, first DNA arrays have already been introduced into clinical research and clinical diagnostics. Examples are the ROCHE AMPLICHIP for pharmacogenetic testing, the ROCHE SEPTIFAST system for microbe characterization. Also, small and medium enterprises in the biotechnology/biomedical field offer highly sophisticated tests like the extensive array for the multiplex NAT testing of 34 bacterial and 6 fungi species together with typing of 5 resistance genes as introduced by SIRSLAB, a company situated in Jena, Germany. Moreover, developmental studies propose the complete sequencing of genomes of infectious agents.

Taken together, the clinical laboratories face a rapid increase in different nucleic acids tests for monogenic and multifactorial diseases and predisposition conditions not only from regional senders. Indeed, it has been pointed out by the OECD that a high increase of cross-border traffic of human genetic material is currently being observed, thus providing a challenging issue for new international regulations in biolegal and bioethical fields. It should be sufficiently clear from the above introductory remarks that quality management in molecular testing must be a highly prioritized activity. Indeed, the importance of quality assurance in molecular diagnostics is now increasingly being emphasized. While there are few active legislative regulations at present, some recommendations and guidelines have recently been published.

Currently, few European countries i.e. Norway, Austria and Switzerland have active national legislations on molecular genetic diagnostics. In others like in Germany, specialist committees have been installed to draft bills that will also specifically address the quality issues that need to be followed when performing molecular diagnostics in a health care setting. For example, in Austria the compliance of molecular genetics laboratories with quality standards may be controlled directly through the State Department for Health. The “molecular diagnostics portfolios” offered by genetic testing laboratories and the respective external quality programs, in which they participate are to be communicated to the department, which will support the availability and access to additional schemes to the labs, as appropriate. In this respect, the Austrian legislation closely follows guidelines issued by the OECD in 2007 (see below).

As for recommendations and guidelines, a number of initiatives have addressed different aspects of molecular diagnostics on national levels. In 2005, a working group for the EU commission has laid down very concise recommendations on genetic testing entitled “25 Recommendations on the ethical, legal and social Implications of genetic Testing”. These recommendations put genetic test results - together with other medical (and for that matter confidential) information - into the general realms of medical laboratory diagnostics and are specifically opposed to the concept of genetic exceptionalism.
[http://ec.europa.eu/research/conferences/2004/genetic/recommendations_en.htm]!
Somewhat in contrast, the 2007 OECD “Guidelines for Quality Assurance in Molecular Genetic Testing” address the special nature of genetic test results (possibly due to the different national composition of the OECD consortium). Both documents give broad room to the various issues related to quality assessment. The OECD paper is highly recommended for their detailing aspects. For example, all molecular genetic testing should be provided and practised under a quality assurance framework, which is subject to adaptation and interpretation by regulatory and professional bodies. Accreditation or equivalent recognition has been recognized as effective procedure to assure the analytical and diagnostic quality. The instalment of monitoring systems is proposed to address instances where laboratories do not meet the standards. It is clearly emphasized in the OECD guidelines that proficiency testing should be implemented to monitor the quality of laboratory performances. Accreditation or equivalent measures should be the basis for the international recognition of providers of external quality assessment programs. Proficiency testing providers need to develop their schemes to keep up with technological advances. Importantly, where proficiency testing is not available for a molecular test performed, the lab should strive to use alternative methods to assure the quality of the test result. Best practise includes the identification of persistent poor performance in each and all single steps including the result reporting. Quality of reporting includes the definition of requirements for adequate reporting by medical care professionals.

Finally, the OECD guidelines address procedures to ensure proper education and training of laboratory staff and medical professionals involved in molecular genetic testing.

While internal quality procedures can be expected to be implemented in every laboratory, External Quality Assessment Programs (EQAP) are important tools to increase the analytical or diagnostic proficiency of the diagnostic lab. Moreover, EQAP allow comparing the performance between laboratories with agreed independent standards. Although there is a general recognition of the necessity of EQAPs, they are usually not mandatory in EU countries so far. We speculate that the minority of labs actually participate in EQAPs on a regular basis. While legislation aims to define genetic testing or the protection of individual personality rights, or regulates the legitimation of professionals to perform genetic analyses, EQAPs are less strictly fixed. Indeed, it may be argued that a proficiency framework will contribute to this patient protection in a number of ways. In this context it is interesting to note that the 25 EU recommendations have defined quality management and assurance in molecular testing as an ethical task in its own right.

In Europe there exist a number of EQAPs for molecular genetic testing that operate on national and in few cases on supranational levels (they can be addressed via the directory of the Eurogentest network, see below). Molecular Diagnostics EQAPs have been initiated in the 1990s in different fields of Laboratory Diagnostics. For example, the European Molecular Genetics Quality Network (EMQN) started in October 1998. EMQN has been funded by the EU during the Standards Measurement and Testing Programme until 2002. It has since then supported itself by subscriptions from it users. EMQN concentrates on monogenic disorders and offers a range of services including methodological EQAPs and proficiency assessments for reporting of finding of inherited diseases. Currently, EMQN currently offers 18 schemes mostly for rare monogenic disorders in the context of Human Genetics (http://www.emqn.org/emqn/EQA/mainColumnParagraphs/04/document/EMQN_scheme_catalogue_2007v2.pdf). Similarly, the European Union Quality Control Concerted Action (EU-QCCA) in diagnostic virology has been established in 1998 and has been replaced in 2001 by QCMD [http://www.qcmd.org/Index2.htm], a not-profit organisation dedicated to molecular diagnostics EQAPs in microbiology and virology.

In Clinical Chemistry, an EQAP for molecular genetic testing has been established in Germany by the German Society for Clinical Chemistry as one area within their extensive quality assessment program that is covering 29 different schemes from all areas of Clinical Chemistry. These first EQAPs were run in 1997 and 1998 (1) and have been offered twice a year since. The program has been extended ever since (2) today encompassing 19 different genetics parameters including metabolic diseases, thrombophilia and pharmacogenetics [http://www.dgkl-rfb.de/index_E.shtml]. Currently, more than 250 laboratories participate regularly, thereby allowing detailed analysis of the results. Additionally, EQAPs on molecular methodology like DNA-sequencing are performed. Finally, this scheme provides so-called “case-based EQAPs” that present clinical cases e.g. in context with biochemical or haematological data, asks the participants to perform molecular diagnostics and communicate back qualified reports.

There is an increasing awareness to improve the quality of molecular genetic testing as recognized by the EU commission ever since the first funding schemes in the 1990s. Indeed, the FP6 has supported a specific support action called EQUAL between 2004 and 2006. In EQUAL, methodological quality assessment schemes have been devised that concentrated on genotyping (EQUALqual), quantitative rt PCR (EQUALquant) and DNA sequencing

(EQUALseq). EQUAL was carried out by a group of laboratory scientists under coordination from researchers of the University of Florence. This program has proved immensely successful and featured offers of training courses for participants with below-the-average performances in all 3 subprograms, also showing demand in this area. A number of reports on the results of EQUAL have been published recently (3-6). Finally and most importantly, the FP6 has funded a Network of Excellence (NoE) called Eurogentest coordinated by Jean-Jaques Cassiman from the University of Leuven. The necessity for such network to support collaboration and the exchange of expertise had been felt after previous reports on high percentages of incorrect test results from Human Genetics testing in rare genetic disorders like cystic fibrosis (7). Since then, Eurogentest has rapidly grown into an extensive network that covers a multitude of information on numerous aspects of molecular diagnostics, proficiency and education, ethics, patient rights etc. It is also a website for active members of the molecular diagnostics community. An updated directory of active EQAPs in genetic testing can be found there [http://www.eurogentest.org/web/info/unit1/molecular.xhtml.

The new Framework 7 (FP7) commissioned by the European Commission in 2007 has again encouraged projects that cover areas of quality assessments and control in molecular genetic testing in different programs, and calls have been issued. While this continued EU activities concede that even with today´s advances in molecular techniques many areas still need to be optimized, it also is reassuring that the importance of quality on both the national and the international level is now increasingly being acknowledged. This emphasis is certainly well deserved considering the rapid further development and throughput of genetic data in the life sciences and medicine.

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12. ETHICS AND LEGAL ISSUE OF GENETIC TESTING

Jana Lukač Bajalo

12.1 Abstract

The Expert Group, invited by European Commission's Research Directorate-General, prepared 25 recommendations related to the use of genetic testing in health care systems and genetic testing as a method of research. This paper presents in detail the recommendations crucial for laboratory diagnostics and research. Genetic testing and genetic data, are a part of the spectrum of all health-care information, equally available to individuals and with identical high standards of quality and confidentiality. It is the individual's right to specify the scope of privacy and confidentiality with regard to all health-care information, including data originating from genetic testing, as well as with regard to biological samples as data carriers. The national Ethics Commission gives the opinion on ethics of any testing, including genetic testing for research purposes, in line with the recommendations of competent bodies of the EU, and the Code of Ethics applicable in the Member State. Test subjects must give informed consent for current or future tests. The recommendations for using the existing and forming new "biological databanks", cross-border exchange of samples, and samples of the deceased are also presented in the paper. The general public should be impartially informed in all forms possible, and public dialogue must take place within the general public on clinical applicability of genetic testing, on genetic screening and pharmacogenetics. Screen testing in the EU may differ between Member States, and it has to be verified and evaluated in regular intervals in each Member State within the framework of the public health-care system. Genetic counselling requires specifically trained experts and should be non-directed counselling with the objective of helping individuals or families to understand a genetic disease or to cope with it. Curricula at all levels of study programmes should include subjects regarding the progress and possibilities of genetic testing in medicine.

12.2 Introduction

Every person working in bioscience, whether it is related to humans (including patients and their biological samples), animals (including test animals), agricultural products (including those genetically modified), or the environment (in the narrow and broad sense of the word), must adhere to the principles of bioethics, meaning that (s)he is responsible for making ethical decisions the whole time. Ethical attitude towards particular phenomena, new discoveries, and new technologies is a process carried forward with varying speed in different environments. The biggest shift regarding ethical issues in biomedicine stems from sequencing of the human genome, and from new biotechnologies enabling new diagnostic approaches and new possibilities for treatment and prevention of diseases. Genetic testing is gaining recognition for the many advantages. Among their many uses, genetic tests most commonly present an opportunity for individuals to become informed about their genetic predisposition to disease and disability, important decisions about their future and for couples to be aware of the possible genetic characteristics of their unborn children., therefore supporting their right to make a informed choice. Genetic information can have important implications also for their relatives. Respecting a patient's confidentiality by not disclosing the results of a genetic test to third parties can therefore conflict with the well-being of family members, who could

benefit from this knowledge. Finding the right balance between the patient's privacy and confidentiality of genetic information, and what is in interests of family members, is an ongoing ethical and social challenge.

This predictive power makes genetic testing particularly liable for misuse. Employers and insurance companies have been known to deny individuals essential health care or employment based on knowledge of genetic disposition. This type of discrimination can be socially debilitating and have severe socio-economic consequences. Stemming from the informative potential of genetic testing some critical ethical, legal and social issues come to the forefront It is important, therefore, to ensure the confidentiality of test results, and to establish legislation permitting only selective access to this information (1).

The Brussels-based European Commission's Research Directorate-General, was established in the EU in 2004. The Expert Group of experts from various backgrounds (representatives from the industry that produces or uses genetic tests, from patient organisations with clear interests in the subject, scientists and representatives from academic institutions with different backgrounds specialised in the field (law, philosophy, ethics, and medicine), prepared 25 recommendations related to the use of genetic testing in health care systems and genetic testing as a method of research. The participants came from various national backgrounds within Europe and numbers were well balanced between men and women. Eryl McNally, Member of the European Parliament, acted as Chair of the Group and ensured that the experts achieved the necessary and agreed recommendations which should be useful for decision-makers in Europe (2).

12.3 The 25 recommendations

The following 25 recommendations are organised into three chapters addressing:

- General framework;
- Implementation of genetic testing in healthcare systems; and
- Genetic testing as a research tool.

12.3.1 General framework

1. Need for universal standard definitions

There are various definitions of genetic testing and genetic data. Clarification of the issues at stake is an important prerequisite to any debate or official position.

Recommendation 1

That:

a) any official statement or position should refer precisely to an explicit definition of the terms used or topic addressed;
b) a consensus definition of genetic testing should be developed globally by all respective public and private bodies involved (including the World Health Organisation, the Organisation for Economic Co-operation and Development, the European Commission, the International Federation of Genetic Societies, and the International Conference on Harmonisation);
c) the European Commission should consider taking the initiative on this topic.

A broad definition was used for genetic testing, i.e. "any test that yields genetic data". Genetic data or information relate to inherited or acquired properties that are transmitted during cell division and that affect subsequent generations of offspring ("germinal genetic data") or cells and tissues ("somatic genetic data"). The Group focused mainly on genetic

data transmissible at the germinal level, pertaining to heritable diseases or traits, and not on somatic genetic data which are subject to increasing interest as tools for identification of disease mechanisms and pathways, disease classification and identification of targets for new medicines.

2. Germinal and somatic genetic testing

Further issues are at stake with respect to non-germinal (somatic) testing that require more indepth reflection and investigation.

Recommendation 2

That:

a) a specific working group be set up to discuss further issues relevant to genetic testing for acquired genetic properties.

3. "Genetic exceptionalism"

The sentiment that genetic data are different from other medical information ("genetic exceptionalism") is inappropriate. Genetic information is part of the entire spectrum of all health information and does not represent a separate category as such. All medical data, including genetic data, must be afforded equally high standards of quality and confidentiality at all times. However, the current public perception that genetic information is somehow different is acknowledged by the Group. This perception is due to a number of factors. These include historical reasons (eugenics), the current predominance of predictive genetic tests for rare monogenic diseases which may give rise to particularly sensitive information affecting patients' relatives, the fact that no treatment is available yet for most monogenic diseases, potential loss ofbcontrol over samples, plus a number of other reasons. Current efforts to establish guidelines, recommendations, rules, regulatory texts and laws that apply specifically to genetic testing and data handling should be viewed as an understandable response to specific public concerns. They are, however, only acceptable as a stepping stone to more considered and inclusive legal and regulatory frameworks that encompass all medical data and testing, and which reflect advancements made in healthcare provision.

Recommendation 3

That:

a) "genetic exceptionalism" should be avoided, internationally, in the context of the EU and at the level of its Member States. However, the public perception that genetic testing is different needs to be acknowledged and addressed;
b) all medical data, including genetic data, must satisfy equally high standards of quality and confidentiality;
c) in order to track the evolution of public perception of genetic testing and to identify issues for future debate:

- further research on ethical and social perceptions of genetic testing is necessary and should be promoted by the European Commission and national bodies; and
- questions relevant to genetic testing should be included in pan-European surveys such as the Eurobarometer.

4. Public information and education

The new knowledge has to be disseminated quickly. As the scientific background is complex and not always easy to understand, it is imperative that opportunities for education are organised for both the public and the media. It is important to increase public awareness, education and understanding of genetic concepts. Genetic research and its clinical

applications should be represented in an impartial way, and realistic expectations as to what they can achieve should be set.

Recommendation 4

That:

a) materials and resources be developed and made available at the EU,national, and local level to provide information about genetic testing, genetic screening, and pharmacogenetics through a variety of media;
b) science curricula at all levels (from primary to university level and vocational training) include reference to progress and potential in the field of medical genetics;
c) national education systems ensure an adequate supply of appropriately trained scientists and teachers, including technicians and clinicians, to ensure that benefits arising from genetic research and genetic testing can be made real and delivered to all EU citizens;
d) concerted efforts to promote dialogue, education, information and debate be encouraged;
e) the 'Science and Society' component of the EC research and development framework be strengthened further.

5. Public dialogue

Dialogue should have a defined scope, be multidisciplinary, organised transparently and carefully designed to involve all relevant and interested stakeholders in an egalitarian fashion. Participants in the dialogue should be encouraged to be open-minded, willing to listen, respectful of local cultural values, and should treat this dialogue as an exchange of opinions rather than as an opportunity for proselytising. An effective dialogue will require discussion leaders to ensure that all participants in the debate are given equal opportunities to voice their respective positions, and that provision is made for questions and answers.

Recommendation 5

That:

a) opportunities for public dialogue between different stakeholders be organised, offering participants equal opportunities for expression;
b) different formats of dialogue and debate be organised as no single format will fit all purposes and all publics.

12.3.2 Implementation of genetic testing in healthcare systems

6. Medical genetic testing and its context

Provision of genetic testing in the context of human healthcare requires appropriate procedures for obtaining informed consent, equitable access to testing as well as to appropriate counselling, and protection of confidentiality and privacy. These requirements will ensure that genetic testing offers new possibilities for individual choice rather than generating further constraints.

The Group acknowledges the present activity of the Council of Europe (3) on such matters. Predictive genetic testing has real potential to provide options for personal choice. However, it is imperative to recognise both the right to know and the right not to know as important individual rights. Equitable access to information and to quality genetic services must be assured. In addition, measures must be taken to prevent undesirable societal consequences as a result of genetic testing. The application of genetic testing for non-medical reasons requires careful consideration with regard to its potential consequences for society.

Recommendation 6

That:

a) medically relevant genetic testing be considered an integral part of health service provision;
b) medically relevant genetic testing should never be imposed and should always be a matter of free personal choice;
c) comprehensive information about the availability of genetic tests be freely available from a range of reputable sources including public authorities, physicians, and patient groups;
d) national healthcare systems ensure that genetic testing will be accessible equitably to all who need it.

7. Quality assurance

Genetic-testing services in Europe, while based on high-quality scientific know-how, tend to suffer from an intolerably high level of technical error and poor reporting. This is caused by a lack of both structuring and complementarity at European level and by the absence of a common European objective to provide quality services to all its citizens now and in the future. Diverse and heterogeneous quality schemes, a lack of reference systems, and differing Member State regulations, have added to the overall fragmentation of services. Nevertheless, genetic services face an ever-increasing number of requests for testing, while widespread susceptibility testing and pharmacogenetic tests are lurking on the horizon. In the context of human healthcare, a test should only be offered when it has been shown to perform reliably and when there is a sound medical reason to consider it. A system for the validation of tests should be established.

Recommendation 7

That:

a) the European Union institutes a consistent regulatory framework to assure specific standards of quality for all genetic-testing services and their providers, including a system of accreditation for genetic-testing laboratories;
b) test providers ensure that the information provided is accurate, by conforming with internationally agreed quality standards;
c) national healthcare systems establish consistent quality requirements for genetic testing.

8. Population screening programmes

Genetic screening for increased disease risk will progressively become possible for common disorders. Offering screening to specific subgroups of the population for certain disorders may be beneficial, but may also carry risks. There must be agreement between medical professionals, patients, and the community that the benefits outweigh the risks. There is a need for meaningful information and regulation with respect to the provision of genetic screening on a population-wide level as well as the testing of certain subgroups within that population.

Recommendation 8

That:

a) measures be put in place to ensure that tests are meaningful: the condition screened for must be serious, the test highly predictive, and follow-up actions must be available in terms of healthcare interventions (including reproductive choices);
b) the relevance of the genetic condition being screened for be validated and regularly evaluated within the framework of the public health context (this may differ from country to country in the EU);

c) the appropriate medical environment for providing information prior to testing and relevant post-test counselling be in place prior to offering such screening;
d) pilot programmes be performed prior to general introduction of the screening;
e) the economic dimension of envisaged screening programmes should be considered carefully.

9. Genetic counselling

The provision of specialised genetic counselling is considered an essential requirement for certain genetic tests, particularly for highly predictive tests for serious disorders. Provision of such counselling requires professionals who have received specific training. In other instances, the physician or other non-specialised members of the healthcare team may provide the patient with the relevant information. The provision of simple, printed information that can be consulted by the individual after counselling has been shown to be extremely valuable, so such materials should always be available. Non-directive counselling must be the rule. The main goal of genetic counselling is to help individuals or families understand or cope with genetic disease, not to decrease the incidence of genetic disease. Appropriate explanations and sufficient time should be allocated to people in need of support. General information about genetic testing and counselling is part of regular medical practice and relevant education must be provided to physicians. Specialised counselling should be devoted to individual situations where it is of particular relevance.

The Group acknowledges, however, that while there should be a common basis of shared principles regarding standards of genetic counselling, practices will vary depending on cultural settings and values and therefore a certain heterogeneity of practices will continue to exist throughout the EU.

Recommendation 9

That:

a) in the context of healthcare, genetic testing be accompanied by the provision of key information and, where appropriate, by the offer of individualised counselling and medical advice (in the case of highly predictive genetic tests for serious disorders, the offer of specific counselling should be mandatory, and patients should be strongly encouraged to take advantage of it);
b) specific educational programmes on counselling and exchange of experience in the field be organised at the European level;
c) specific qualifications and quality standards for those engaged in the provision of specific genetic counselling, whether clinicians or non-clinicians, be established and made mandatory;
d) appropriate financial means for such training and the subsequent accreditation be made available;
e) Europe-wide general standards for fundamental principles of genetic counselling be developed by relevant medical professional groups, with due consideration given to patients' views.

10. Data protection: confidentiality, privacy and autonomy

Public concern regarding genetic testing is grounded to some extent in the fear of misuse of genetic data and of inappropriate access to such data by third parties. Confidentiality and privacy with regard to all personal medical data, including those derived from genetic testing, are basic rights and must be respected. An individual's right to determine the extent of privacy and confidentiality that he or she will be afforded encompasses both access to personal information and data and to tissue samples as carriers of information. The individual

has both the right to know and not to know. Affording legal protection in this field is of paramount importance and the existing EU Data Protection Directive (4) provides an appropriate framework for the need to protect personal data.

Recommendation 10

That:

a) genetic data of importance in a clinical and/or family context should receive the same level of protection as other comparably sensitive medical data;
b) the relevance for other family members has to be addressed;
c) the importance of a patient's right to know or not to know be recognised and mechanisms incorporated into professional practice that respect this. In the context of genetic testing, encompassing information provision, counselling, informed consent procedures, and communication of test results, practices should be established to meet this need;
d) these issues are of particular relevance to vulnerable populations, whether in the EU or elsewhere in the world.

11. Protection from discrimination

Irrespective of confidentiality issues, personal medical data including genetic data must not be used to discriminate unfairly against individuals. The Group acknowledges the risk of discrimination as a result of genetic testing but notes that the field of discrimination extends far beyond genetics. Such discrimination may affect individuals, families or larger groups. Regarding prevention of discrimination in insurance and employment, the Group refers to the work of other bodies such as the European Group on Ethics in Science and New Technologies (5), the Council of Europe (3), and the European Society of Human Genetics (6).

Recommendation 11

That:

a) data derived from genetic sources should not be used in ways that disadvantage or discriminate unfairly against individuals, families or groups in either clinical or non-clinical contexts, including employment, insurance, access to social integration, and opportunities for general well-being;
b) EU-level regulations addressing these issues should be promoted;
c) timely access to genetic testing should be based on need and appropriately resourced with no discrimination based on gender, ethnic origin, social or economic status.

12. Ethnicity and genetics

There may be differentiation of patient populations, based on ethnic groupings, with respect to genetic testing. Some genetic variants are more common in certain populations or groups in the EU and elsewhere. Specific attention should be paid to such groups in the context of test development and in the conditions set up for the use of these tests, both to ensure fair access and to avoid stigmatisation or stereotyping. Specifically, genetic tests are inappropriate to determine ethnicity and must never be used for this purpose.

Recommendation 12

That:

a) genetic tests be clinically evaluated in the populations in which they are to be used;
b) those who are involved in genetic research, the provision of genetic testing and healthcare policy-making be sensitive to the risks of stereotyping and stigmatisation based on ethnic origin, and recognise and respect ethnic and cultural sensitivities;

c) minority ethnic groups should not be excluded from access to those genetic tests appropriate for them.

13. Gender issues and genetics

There are well-established differences between men and women regarding the risk of certain disorders, including genetic diseases. The Group considers that specific gender issues may arise in the context of genetic testing. However, little evidence for this exists so far.

Recommendation 13

That:

a) further studies at EU level address the impact of genetic testing, in particular in societies where women and men are given different rights or privileges;
b) governments and society be aware of the possible consequences of the application of genetic testing to aid reproductive choice for prospective sex selection;
c) criteria be established at EU level to ensure that no gender discrimination occurs in the course of, or as a result of, EU-funded research projects.

14. Social, cultural and economic consequences

Relatively little information is presently available at EU level on the impact of genetic testing on health systems and on health economics.

Recommendation 14

That:

a) the European Commission funds more research relating to the impact of genetic testing on the social, cultural and economic aspects of healthcare provision.

15. Professional development

The responsibilities of primary and specialised care providers will expand as genetic testing, including pharmacogenetics, evolves. This will require appropriate changes in medical school curricula. Other healthcare professionals will also require enhanced initial professional education and continuing professional development in the area of genetics.

Recommendation 15

That:

a) initial educational and professional requirements be coordinated in all countries of the European Union;
b) continued professional training be offered for healthcare professionals.

16. Partnerships and collaborations

The Group considers that all stakeholders, including governmental authorities, scientists, healthcare providers, industry and patient organisations should work together in a partnership approach to optimise future advances in healthcare that may become possible as a result of genetic testing, such as new treatment options and disease prevention. For effective development of new genetic tests and diagnostics, a productive interchange between academic scientists and those working in the healthcare industry will continue to be essential.

Recommendation 16

That:

a) the European Union stimulates and supports partnerships between stakeholders;

b) a framework for transparent collaboration between industry and academic scientists be established.

17. Regulatory framework and criteria for test development and use

The Group considers that a more explicit regulatory framework for test development needs to be set up throughout the EU.

Recommendation 17

That:

a) the regulatory framework for genetic testing be further developed by the EU and other international organisations in a way that recognises both the need for new tests and the importance of safety, clinical validity and reliability;
b) all newly developed tests must conform to the standards established before introduction into clinical use, based on a review process by an organisation or body independent of the test developer to ensure that the patient will benefit from the test;
c) priority-setting for the development of accurate genetic tests be guided by the degree of unmet medical need, independently of disease prevalence;
d) the EC takes measures to facilitate the availability of genetic testing for rare diseases as well as for more common diseases;
e) the EC actively promotes the regulatory framework on these topics.

18. Rare diseases

The Group acknowledges that few countries have instituted screening programmes for serious rare diseases.

Recommendation 18

That:

a) an EU-wide network for diagnostic testing of rare genetic diseases be created and financially supported as a matter of urgency;
b) an EU-level incentive system for the systematic development of genetic tests for rare diseases be created and financially supported;
c) for rare but serious diseases for which treatment is available, Member States introduce universal neonatal screening as a priority.

19. Pharmacogenetics

The term 'pharmacogenetics' describes the study of differences between individuals in drug response (efficacy or adverse reactions) which result directly from variations in DNA sequences and in differences in protein sequence, structure, or gene expression that are the direct consequence of such DNA variations. The main aim of pharmacogenetics is to help deliver medicines to patients who are most likely to benefit and least likely to experience adverse reactions. Although pharmacogenetics is currently still in a mainly exploratory phase, an increase in its application in healthcare is expected and appropriate measures should be prepared in time for this evolution.

Recommendation 19

That:

a) national health authorities play a more active part in encouraging development of the field of pharmacogenetics:

- by providing particular incentives to enable the development of pharmacogenetic tests and associated therapeutics which are clinically desirable but which may not be economically viable; and
- by enhancing the possibilities of co-operation between industry, patients, and academia in this field;

b) an appropriate harmonised legal, regulatory, and healthcare policy framework for pharmacogenetics be developed at EU level, taking into account research, therapy development, and clinical practice.

12.3.3 Genetic testing as a research tool

A large part of research relevant to genetic testing relies on the use and exchange of human tissue samples and associated data because all biological samples contain DNA. These may allow, to a variable degree, identification of the individuals who are the source of the samples and data. Genetic studies also rely on genealogical, population, clinical, and personal data. The Group has concentrated on research aspects in terms of sample and data resources of human origin as indispensable means for the identification, validation, and development of genetic testing for potential clinical use, including pharmacogenetics, and on the relevant ethical, legal, and societal issues.

20. Existing and new ‘biobanks’

The term ‘biobank’ is used variably to designate several types of collections of biological samples. The Group has considered biobank collections to mean the biological samples themselves, plus the related databases, allowing a certain level of accessibility, availability and exchange for scientific studies. Public trust in research surrounding genetic testing is largely dependent on how the use of samples and data in and from such biobanks is undertaken and communicated. This applies in particular to the areas of informed consent, storage, data protection and the degree of anonymity of samples, the communication of study results and, where appropriate, of individual test results. A harmonised approach to these issues at EU levelis considered desirable. The Group is aware that the Council of Europe is developing an activity regarding storage and research use of biological material and related data. Depending on their scope and context, official identification and registration of such biobanking activities, as well as assurance of their long-term financial sustainability, are desirable and may be required. There is a need for education about biobanking and for guidelines to assure quality control of both the collections and the ethical management of such repositories. For the study and validation of the association of genes with specific diseases, including the development of diagnostic products, devices and other tools, access to well-characterised human tissue samples for research and development use is essential.

Recommendation 20

That:

a) guidelines be developed and coordinated across the EU to ensure that the use of samples, including those from archival collections, is not unduly delayed or impeded, particularly if proper consideration of their level ofidentification has been taken into account;
b) action be taken by Member States to ensure that approval by a competent review committee is obtained before research is undertaken;
c) an inventory of existing biobanks across the EU be created, indicating standards and rules of access, to identify which of their contents may or may not be used for genetic studies;
d) a system be implemented to evaluate and monitor the current usage of existing biobanks throughout the EU;

e) the task force on ‘biological resource centres’ set up by the OECD be followed closely by the European Commission regarding development of standards;
f) the European Commission closely follows this activity.

21. Collections of human biological material and associated data and their uses
It should be noted that biobanking issues extend far beyond genetic testing. Few Member States have a well-developed legal framework in this domain. There is currently no EU directive regulating the use of tissue and cell conservation for research purposes.

Recommendation 21
That:
a) the European Commission follows closely relevant activities and developments of the Member States in this field and in the global context;
b) action be taken at the EU level, in coordination with other initiatives, to follow and address regulatory issues related to collections of human biological material and associated data and their uses.

22. Cross-border exchange of samples
Cross-border exchange of samples and data is important to improve European co-operation in this field. Ethical questions, such as validity of informed consent, play an important role in this transfer.

Recommendation 22
That:
a) the European Commission evaluates the need for, and the feasibility of, developing harmonised standards for the research use of human samples and associated data (including informed consent issues), taking into account relevant international conventions on cross-border exchange of samples.

23. Informed consent
Consent issues for samples from individuals and groups entering new collections may differ from those applicable to existing collections, where contact may not have been maintained with the source individuals over the years. Explicit written consent is the rule but may not always be feasible for future uses of the samples and/or data not envisaged at the time of collection. The issues at stake include respect for individual or community autonomy regarding their control over the use of samples, the distribution and flow of data and samples across national borders, and methods that are both compatible with the nature of the research yet respect human rights. Various existing and practices exist in the EU. Professionals working in human genetics have developed recommendations and several ethical bodies have taken positions. Genetic data represent personal information which may or may not have individual medical relevance, as well as medical or cultural relevance at the community level. Biological samples and associated or derived genetic or medical information of any kind and of any origin must not be collected, stored or used without obtaining informed consent based on adequate process and procedure, including appropriate approval by relevant review bodies, and consideration of community-related aspects where appropriate, regardless of the purpose of the collection and the level of anonymity. Samples for genetic diversity studies should only be obtained in compliance with local or national ‘traditions’ and regulations or laws. The sources of samples and individual data and the relevant agreement regarding their use should be documented in all protocols and publications. The informed consent process and procedures used should be fully transparent with regard to the planned research, including

policies on provision of test results to individuals and to the population, as well as to the handling of samples and to the rights of sample donors. The Group considers that views of both patients and patient organisations, and public debate about biobanks, in particular with regard to consent issues, should be explored further.

Recommendation 23
That:
a) the European Commission promotes opportunities for dialogue between stakeholders to support exchange of experience throughout Europe on issues of sample and data use for research, at the individual, family and population level;
b) the European Commission funds multidisciplinary research into the ethical, legal and social issues related to informed consent procedures for human genetic research and other relevant areas essential for any evolving research in genetics.

24. Samples from the deceased
As regards samples and genetic data from the deceased, the Group considers that in the case of the overriding interest of one or more blood relatives, even in the absence of consent given before death, their use could be legitimate: absence of consent should not be considered as equivalent to non-consent. Furthermore, their use should be permitted, when made anonymous, for research, development of genetic tests, and for teaching purposes.

Recommendation 24
That:
a) Member States take actions to promote the right of access to samples and data from a deceased person, in the case of overriding interest for blood relatives;
b) Member States take actions to allow the use of anonymous samples from the deceased for the purposes of genetic research, development of new genetic tests, as well as for teaching purposes.

25. Consent procedures for children and vulnerable individuals in human genetic research
As regards the participation of children or vulnerable people in genetic studies, the principle of acting in their best interest applies and specific protection is the rule, as in any research on humans. An important issue raised by long-term conservation and use of data and samples obtained from children relates to the question of when assent or consent, respectively, need to be obtained. The Group recognises that few data are available on "what children think" and how information should best be presented to them.

Recommendation 25
That:
a) the use of tissue and accompanying data from minors or vulnerable individuals in research be permitted if, in so doing, their interests are served;
b) specific consideration be given to children's views, the information provided to them, and issues of children's assent and/or consent.

12.4 Conclusion

Ethical attitude towards particular phenomena, new discoveries, and new technologies is a process carried forward with varying speed in different countries. Expectations are, that ethical attitude towards genetic testing in health care systems and genetic testing as a method of research in EU Member States will carry forward with a proper speed!

Recommended literature:

1. Mefhan B. Bioethics, an introduction for the biosciences, Oxford University Press 2005;3-227, ISBN 0-19-926715-4 978-0-19-926715-6
2. European Commission: EUR 21120 – 25 recommendations on the ethical, legal and social implications of genetic testing, Luxembourg: Office for Official Publications of the European Communities 2004 – 25 pp, ISBN 92-894-7308-8
3. http://conventions.coe.int
4. Directive 95/46/EC
5. http://europa.eu.int/
6. http://www.eshg.org/

Glossary

Definitions useful in understanding pharmacogenetics; Author: D. Farkas
http://www.aacc.org/AACC/members/nacb/LMPG/OnlineGuide/DraftGuidelines/Pharmacogenetics/

allele - version of a gene at a given locus

amplicon - copy of a target DNA created by PCR or other amplification methods

central dogma (of molecular biology) - fundamental tenet of molecular biology stating that DNA is copied via replication, RNA is derived from DNA via transcription, and protein is derived from RNA via translation. The discovery of reverse transcription disrupted the central dogma of molecular biology by showing that genetic information could also flow from RNA to DNA, not just DNA to RNA.

complementary DNA (cDNA) - DNA produced using an RNA template via the enzyme reverse transcriptase

compound heterozygote - individual with two abnormal alleles at a given locus, each with a difference polymorphism or mutation.

deletion - mutation resulting from the removal of base(s)

DNase - enzyme that degrades DNA

DNA ligase - enzyme that joins two pieces of DNA

DNA polymerase - enzyme that uses DNA as a template to produce a complementary strand of DNA; cDNA is made with a type of DNA polymerase called reverse transcriptase (an RNA-dependent, DNA polymerase)

DNA sequencing - base-by-base determination of the exact sequence of target DNA

epigenetic - referring to heritable changes to the genome that do not alter the coding sequence

frame-shift mutation - insertion or deletion of base(s) that alters the reading frame of a coding sequence, thereby changing the amino acids encoded downstream and/or producing a stop codon

gel electrophoresis - separation of DNA by size via migration in an electric field in an agarose or polyacrylamide matrix

gene - segment of DNA transcribed into RNA that (i) is translated into a protein or (ii) forms structures such as ribosomes

genetic variant - alternative forms of a gene which may or may not lead to altered phenotype

genome - all the genetic material of an organism

genotype - the alleles at a given locus in an individual; see also phenotype

haplotype - analogous to genotype, haplotype is the set of alleles (or SNPs) on one chromosome or part of a chromosome that are linked and usually or often inherited together

heterogeneous - regarding PCR, requiring separate amplification and detection steps

homogeneous - regarding PCR, having combined amplification and detection steps

hybridization - process of forming a double-stranded molecule from a single-stranded probe and a single-stranded nucleic acid target

hybridization probe - type of fluorescently-labeled probe used in real-time PCR that produces signal following hybridization to a target DNA

missense mutation -base change resulting in coding of a different amino acid

molecular diagnostics - diagnosis of disease using nucleic acids as analytes, often used synonymously with molecular pathology

mRNA - messenger RNA, translated into protein

mutation - generally harmful DNA sequence change; compare to polymorphism

nucleic acids - deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), the molecules containing the genetic code

nucleoside - nucleotide lacking a phosphate group

nucleotide - building block of nucleic acids composed of phosphate group(s), a five-sided sugar molecule, and a nitrogenous base

oligonucleotide - short sequence of nucleotides, often used as primers for PCR or DNA sequencing

penetrance - percent expression in a population of the phenotype of a given mutant genotype

pharmacogenetics - the hereditary basis for inter-individual differences in drug response

pharmacogenomics - the convergence of pharmacogenetics and genomics used to mean the influence of DNA sequence variation on the effect of a drug on an individual

phenotype - traits resulting from a given genotype

point mutation - mutation that changes a single base

polymerase chain reaction (PCR) - enzymatic in vitro nucleic acid amplification method using temperature cycling to produce repeated cycles of DNA replication

polymorphism - variant DNA sequence change, typically benign, found in 1% or more of individuals; compare to mutation

primer - oligonucleotide used in PCR or DNA sequencing to target an area of interest

primer-dimers - nonspecific products formed during PCR by the interaction of primers

probe - relatively small piece of DNA or RNA used to find or detect a specific piece of target nucleic acid

proteomics - study of the entire complement of proteins in organisms

quantification standard - synthetic nucleic acid standard spiked into samples before processing to serve as a reference in quantitative PCR

real-time PCR - PCR in which detection of product is simultaneous with amplification

replication - process of duplicating DNA with DNA polymerase

restriction endonuclease (RE) - enzyme purified from bacteria that recognizes and cleaves unique sequences

restriction fragment length polymorphism (RFLP) - polymorphism that changes the electrophoretic banding pattern of DNA fragments generated by digestion with a restriction endonuclease

single nucleotide polymorphism (SNP) - polymorphism that is a single base change

Southern blot hybridization - DNA detection method where digested sample is separated by electrophoresis, transferred to a membrane, and probed

transcription - process of producing mRNA from a DNA template

translation - process of converting the information contained in mRNA into protein

uracil-N-glycosylase - enzyme used to prevent amplicon carryover contamination that degrades any DNA containing uracil (uracil-containing DNA is not natural and is produced in vitro during some PCR protocols)

variant allele - specific alternative forms of a gene, generally causing a known alternative phenotype

wild-type - normal allele; compare to mutant or variant

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