Current Trends
in Science

PLATINUM JUBILEE SPECIAL

Biomarkers of diseases in medicine

MANOJ KUMAR[1] and SHIV K SARIN[1,2]

[1]*Department of Hepatology, G B Pant Hospital and Institute of Liver and Biliary Sciences, New Delhi 110 002, India.*
[2]*Department of Gastroenterology, G B Pant Hospital, New Delhi 110 002, India.*
e-mail: shivsarin@gmail.com

Biomarkers have gained immense scientific and clinical value and interest in the practise of medicine. Biomarkers are potentially useful along the whole spectrum of the disease process. Before diagnosis, markers could be used for screening and risk assessment. During diagnosis, markers can determine staging, grading, and selection of initial therapy. During treatment, they can be used to monitor therapy, select additional therapy, or monitor recurrent diseases. Advances in genomics, proteomics and molecular pathology have generated many candidate biomarkers with potential clinical value. In the future, integration of biomarkers, identified using emerging high-throughput technologies, into medical practise will be necessary to achieve 'personalization' of treatment and disease prevention.

1. Introduction

In 2001, a consensus panel at the National Institutes of Health defined the term biomarker as 'a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention or other health care intervention'. The biomarker is either produced by the diseased organ (e.g., tumour) or by the body in response to disease. Biomarkers are potentially useful along the whole spectrum of the disease process. Before diagnosis, markers could be used for screening and risk assessment. During diagnosis, markers can determine staging, grading, and selection of initial therapy. Later, they can be used to monitor therapy, select additional therapy, or monitor recurrent diseases [1]. Thus, identifying biomarkers include all diagnostic tests, imaging technologies, and any other objective measures of a person's health status. Biomarkers can also be used to reduce the time factor and cost for phase I and II of clinical trials by replacing clinical endpoints.

Biomarkers span a broad sector of human health care and have been around since the understanding of human biology and diseases began to evolve. So, why is so much attention being paid to biomarkers today? Genetics, genomics, proteomics, and modern imaging techniques and other high-throughput technologies allow us to measure more markers than before. In addition, we achieve a greater understanding of disease pathways, the targets of interventions, and the pharmacologic consequences of medicines.

2. Phases of evaluation of biomarkers

Because of diseased tissue/tumour heterogeneity and other biases that might be inherent with biomarker identification and evaluation processes, it is important that the identification of biomarkers should proceed in a systematic manner. Unlike a clinical trial design in which there are three phases (phase I, phase II and phase III), research on biomarkers has largely been guided by intuition and experience. In 2002, the National Cancer Institute's 'Early Detection Research Network' developed a five-phase approach to systematic discovery and evaluation of biomarkers. In general, biomarker development should follow an orderly

Keywords. Biomarkers; diagnosis; screening; prognosis.

process wherein one proceeds to the next phase only after meeting pre-specified criteria for the current phase [2].

Phase 1 refers to preclinical exploratory studies. Biomarkers are discovered through knowledge-based gene selection, gene expression profiling or protein profiling to distinguish cancer and normal samples. Identified markers are prioritized based on their diagnostic/prognostic/therapeutic (predictive) value that could suggest their evolution into routine clinical use. The analysis of this phase is usually characterized by ranking and selection, or finding suitable ways to combine biomarkers. Although not required, it is preferred that the specimen for this phase of discovery comes from well-characterized cohorts, tissue banks or from a trial with active follow-ups.

Phase II has two important components. Upon successful completion of phase I requirements, an assay is established with a clear intended clinical use. The clinical assay could be a protein-, RNA-, DNA- or a cell-based technique, including ELISA, protein profiles from MS, phenotypic expression profiles, gene arrays, antibody arrays or quantitative PCR. To document clinical usefulness, firstly, such assays need to be validated for reproducibility and shown to be portable among different laboratories. Secondly, the assays should be evaluated for their clinical performance in terms of 'sensitivity' and 'specificity' with thresholds determined by the intended clinical use.

During **Phase III**, an investigator evaluates the sensitivity and specificity of the test for the detection of diseases that have yet to be detected clinically. The specimens analyzed in this evaluation phase are taken from study subjects before the onset of clinical symptoms, with active follow-up to ascertain disease occurrence. It is usually time-consuming and expensive to collect these samples with high quality; therefore, phase III should consist of large cohort studies or intervention trials whenever possible. This is probably when most biomarker validation studies will end and the biomarker will be ready for clinical use.

Phase IV evaluates the sensitivity and specificity of the test on a prospective cohort. The major difference from phase III is that in phase IV a positive test triggers a definitive diagnostic procedure, often invasive and that could lead to increased economic healthcare burden. Therefore, in a phase IV study, an investigator can estimate the false referral rate based on tested biomarkers and describe the extent and characteristics of the disease detected (e.g., the stage of tumour at the time of detection). For rare diseases, phase IV requires a large cohort with long-term follow-up and might often be too expensive as a stand-alone activity. These studies are difficult to perform specifically for rare diseases.

Table 1. Performance characteristics of biomarkers.

	Disease present	Disease absent
Biomarker positive	A	B
Biomarker negative	C	D

Disease prevalence: A + C/A + B + C + D; Negative likelihood ratio (LR−): (1 − sensitivity)/(specificity); **Negative predictive value**: D/C + D; Positive likelihood ratio (LR+): sensitivity/(1 − specificity); **Positive predictive value**: A/A+B; Specificity: D/B+ D; Sensitivity: A/A + C.

Phase V evaluates the overall benefits and risks of the new diagnostic test on the screened population. The cost per life saved is one example of an endpoint for such a study. This again requires a large-scale study over a long time period and could also be prohibitively expensive.

Phases IV and V are necessary to evaluate benefits and risks of the use of a biomarker in screening and detection.

3. Characteristics of an ideal biomarker and basic statistical methods for evaluation

- An ideal biomarker should be safe and easy to measure.
- The cost of follow-up tests should be relatively low, there should be proven treatment to modify the biomarker.
- It should be consistent across genders and ethnic groups.

If the biomarker is to be used as a diagnostic test, it should be sensitive and specific and have a high predictive value table 1. A highly sensitive test will be positive in nearly all patients with the disease, but it may also be positive in many patients without the disease. To be of clinical value, a test with high sensitivity should also have high specificity; in other words, most patients without the disease should have negative test results. For predicting the likelihood of disease on the basis of the test result, rather than the converse, the appropriate measures are positive and negative predictive values. Unfortunately, the positive predictive value falls as the prevalence of the disease falls, so tests for rare conditions will have many more false positive results than true positive results.

Diagnostic odds ratio (DOR) of a biomarker represents the comprehensive ability of the marker according to the following formula:

$$\mathrm{DOR} = \frac{\text{sensitivity}}{1 - \text{specificity}} \Big/ \frac{1 - \text{sensitivity}}{\text{specificity}}.$$

Information about the diagnostic test itself can be summarized using a measure called the likelihood ratio. The likelihood ratio combines information about the sensitivity and specificity. It tells how much a positive or negative result changes the likelihood that a patient would have the disease. The likelihood ratio of a positive test result (LR+) is sensitivity divided by 1 − specificity:

$$\mathrm{LR}^{+} = \frac{\text{sensitivity}}{1 - \text{specificity}}.$$

The likelihood ratio of a negative test result (LR−) is 1− sensitivity divided by specificity:

$$\mathrm{LR}^{-} = \frac{1 - \text{sensitivity}}{\text{specificity}}.$$

The likelihood ratio for a positive result (LR+) tells how much the odds of the disease increase when a test is positive. The likelihood ratio for a negative result (LR−) tells how much the odds of the disease decrease when a test is negative. The likelihood ratio can be combined with information about the prevalence of the disease, characteristics of your patient pool, and information about a particular patient to determine the post-test odds of disease. To quantify the effect of a diagnostic test, information about the patient is needed first. The pre-test odds, such as the likelihood that the patient would have a specific disease prior to testing should be specified. The pre-test odds are usually related to the prevalence of the disease, though it might be adjusted upwards or downwards depending on characteristics of the overall patient pool or of the individual patient. Once pre-test odds have been specified, they are multiplied by the likelihood ratio to give the post-test odds:

$$\text{odds}_{\text{post}} = \text{odds}_{\text{pre}} \times \text{likelihood ratio}.$$

The post-test odds represent the chances that a particular patient has a disease. It incorporates information about the prevalence of the disease, the patient pool, and specific patient risk factors (pre-test odds) and information about the diagnostic test itself (the likelihood ratio).

Most biological markers, however, are not simply present or absent but have wide ranges of values that overlap in persons with a disease and in those without it. The risk typically increases progressively with increasing levels; few markers have a threshold at which the risk suddenly rises, so various cut-off points must be evaluated for their ability to detect disease. Cut-off points with high sensitivity, producing few false negative results, are used when the consequences of missing a potential case are severe, whereas highly specific cut-off points, producing few false positive results, are used to avoid mislabelling a person who is actually free of the disease. Sensitivity and specificity calculated at various cut-off points generate a receiver-operating-characteristic (ROC) curve, which ideally will be highly sensitive throughout the range of specificity. The most useful clinical tests are typically those with the largest area under the ROC curve.

The use of multiple tests may also be considered for screening. When multiple tests are obtained in series and the disease is considered present when all tests are positive ('AND rule'), specificity is enhanced whereas sensitivity is diminished. When multiple tests are obtained in parallel and the disease is considered to be present when any of the tests are positive ('OR rule'), sensitivity is enhanced and specificity diminishes [3].

Even if a biomarker meets several criteria that make it 'ideal', this does not imply that the biomarker will necessarily be useful in a clinical setting. Specifically, if a novel biomarker cannot add value to tests and biomarkers are already being used in clinical settings, then it may never pass the sizeable hurdle that separates clinical practice from clinical research.

4. Specific ways to test if a biomarker adds to current risk assessment

4.1 *Model discrimination*

The C-statistic, or area under the receiver operating characteristic curve (AUC) is a popular method to test model discrimination. C-statistic for a multivariable model reflects the probability of concordance among persons who can be compared for a given outcome of interest and represents the probability that a case has a higher measure or risk score (or a shorter time to event in survival analyses) than a comparable control. The C-statistic measures the concordance of the score and disease state. The value of the C-statistic ranges from 0.5 (no discrimination) to 1.0 (perfect discrimination) and for the Framingham CHD risk score, the C-statistic is approximately 0.76 [4]. Similarly table 2 shows AUC for various markers for HCC [5].

When considering the efficacy of novel biomarkers in risk stratification, one approach is to determine to what extent entering the candidate biomarker into standard risk prediction models will actually increase the model's C-statistic. For instance, a recent investigation in the Atherosclerosis Risk in Communities Study demonstrated the extent to which several individual biomarkers increased the C-statistic for CHD prediction above and beyond age, race, sex, total

Table 2. AUC for various markers for HCC diagnosis.

Test	AUC	SE (AUC)
AFP	0.647	0.027
DCP	0.688	0.083
AFP-L3	0.695	0.166

Abbreviations: AFP, alphafetoprotein; AFP-L3, Lens culinaris agglutinin-reactive fraction of AFP; AUC, area under the curve; DCP, des-gamma-carboxyprothrombin; HCC, hepatocellular carcinoma; SE, standard error.

cholesterol level, high-density lipoprotein cholesterol level, systolic blood pressure, antihypertensive medication use, smoking status and diabetes. This study concluded that out of the panel of 19 novel biomarkers studied, lipoprotein-associated phospholipase A2, vitamin B6, IL-6 and soluble thrombomodulin added the most to the C-statistic but each only increased it marginally (C-statistic increment range 0.006–0.011) [6].

There are several limitations to using increments in the C-statistic to determine the utility of biomarkers in risk prediction [7]. First, the C-statistic depends, to a large extent, on the magnitude of the association (or odds ratio) between a dichotomous exposure and outcome. Other limitations of the C-statistic include low sensitivity for determining the relative importance of different risk factors in a multivariable model.

4.2 *Model calibration*

A complementary step when analyzing the efficacy of a biomarker is to assess the degree to which the biomarker improves model calibration. This can be thought of as the extent to which the expected risk (estimated by statistical models) agrees with the observed (or true) risk. This concept may be important when counselling patients with regards to their numeric risk or probability of developing a given condition. One statistical test that can be employed to compare these probabilities is the Hosmer–Lemeshow calibration statistic. In the Women's Health Study, investigators found that deciles of predicted and observed 10-year CVD risk for a multivariable model with Framingham risk score covariates plus C-reactive protein had a lower p-value than a multi-variable model not containing C-reactive protein ($p = 0.039$ vs. $p = 0.23$) [8]. A simple statistical test to compare model discrimination with and without the biomarker of interest would fail to provide valuable information regarding which specific groups (i.e., which deciles or quintiles and so on) of observed and expected risk are better explained by including a biomarker of interest.

4.3 *Risk reclassification*

The utility of a biomarker may also be assessed by studying how biomarker information may lead to a reclassification of individuals in low medium- and high-risk categories based on traditional risk factors. The ultimate goal of this approach is to refine risk stratification, and it has been particularly emphasized when considering biomarker information that would serve to shift individuals who are in the intermediate-risk groups (i.e., based on the Framingham risk score), upwards into the high-risk category or downwards into the low-risk category. Recent guidelines have recommended that the individuals in the intermediate-risk category be targeted to undergo screening for existing sub-clinical atherosclerosis [9].

4.4 *Model validation*

Validation, generalizability and transportability of risk scores are significant characteristics of robust risk prediction models and have important implications regarding the widespread utility of biomarkers. It is ideal if the formulation of a risk score uses separate derivation and validation samples. In the absence of an independent validation sample, the degree of over optimism in the models could be judged by using bootstrap estimations. Also, risk models that perform well in one population should be validated in other study samples. For instance, the Framingham risk score was originally developed in the Framingham heart study population, which largely consists of individuals of white European ancestry. An investigation determined that among Japanese–American and Hispanic men and Native American women, the Framingham functions systematically over-estimated the risk of 5-year CHD events and thus needed to be recalibrated (to risk factor and CHD levels within those populations) in order to maintain good performance [10].

4.5 *Considering multiple biomarkers use*

Although the ultimate aim of biomarker investigations is to develop a parsimonious set of biomarkers that will most accurately predict disease outcome, the reality is that several candidate biomarkers in a multitude of separate studies have already undergone evaluation. It is therefore difficult to extrapolate the findings of these diverse studies into one unifying conclusion which of the several potential biomarkers add substantially to risk prediction so as to be considered for measurement in routine practice. Figure 1 shows the use of single and multiple markers for HCC diagnosis [5].

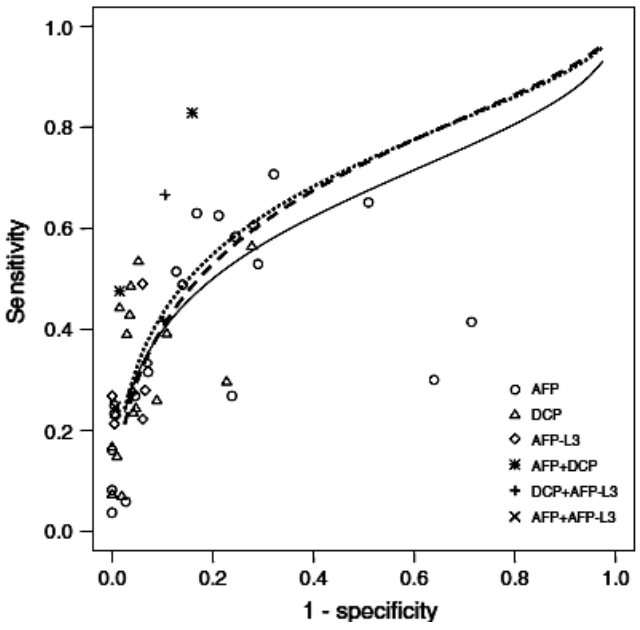

Figure 1. SROC curves for 3 diagnostic tests for HCC. Abbreviations: AFP, alphafetoprotein; AFP-L3, *Lens culinaris* agglutinin-reactive fraction of AFP; DCP, des-gamma-carboxyprothrombin; HCC, hepatocellular carcinoma; SROC, summary receiver operating characteristics.

Table 3. High-throughput technologies.

1) Genomics
- Genome sequencing
- Genome variation
- Genome annotation
2) Transcriptomics
- Microarrays
- Gene expression data
3) Proteomics
- Y2H method
- Mass spectrometry
- Protein chips
4) Metabolomics
- NMR
- Mass spectrometry

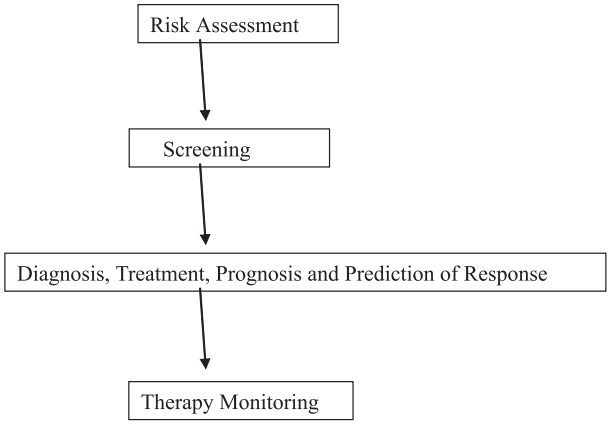

Figure 2. Schematic representation of the uses of biomarkers across the spectrum of diseases. Before diagnosis, markers might be used for risk assessment and screening. At diagnosis, markers can assist with staging, grading, and selection of initial therapy. Later, they can be used to monitor therapy, select additional therapy, or monitor for recurrent disease.

5. Biomarker discovery using high-throughput technology platforms

5.1 *High-throughput technologies – basic premises*

Historically, some screening tools (e.g., pap smears and colonoscopy) have successfully reduced mortality through early detection. Despite these successes, the field of early detection has been plagued by problems of over diagnosis (e.g. PSA), inadequate specificity of individual markers (e.g. CA125, CEA and AFP), low compliance (colonoscopy) and a lack of analytical tools for discovering new diagnostic markers. The limited number of useful markers has propelled investigators to use high-throughput platforms to identify large numbers of candidate biomarkers.

High-throughput technologies are useful to assess genomic data (which define the messages and the resulting protein sequences), transcriptomic data (which reveal the levels of messages present), proteomic data (which give the levels of each protein present), and 'fluxomic' data (which, if it existed, would provide measurements of intracellular fluxes on a complete scale) table 3.

5.2 *Genomics*

Genomics defines the genetic messages and the resulting protein sequences. Modern sequencers such as the ABI 3700 automate and multiplex the Sanger method so that it can be utilized to sequence whole genomes. The ABI 3700 has the capacity to run 12 runs a day with 96 samples of $\sim$500 nucleotides long amplified DNA fragments in parallel. This results in a nominal sequencing capacity of 576 kb a day (the whole human genome is of the order of 3 billion bases). The reported accuracy of the ABI 3700 is 98.5% indicating that there are less than 2 errors per 100 bases sequenced [11]. Once the genome is sequenced, the next important task is the study of genetic or genome variation between individuals. The types of variations that are commonly considered include single nucleotide polymorphisms (SNPs) and different types of repeats. SNPs are defined

as single base variations between individuals that occur at high enough frequency in a population to be considered to be non-random. The reason for interest in genomic variation is that these variations are a large part of what determines the difference between individuals especially when it comes to susceptibility to various diseases and responses to drug treatments. The latter aspect is known as pharmacogenomics [12].

5.3 *Transcriptomics*

After genomics, transcriptomics is probably the best developed of the different high-throughput technologies. Transcriptomics could be defined as the study of the expressed mRNA transcript complement of a cell under different conditions. The central quantity in transcriptomics is the gene (or mRNA) expression profile of the cell. While mRNAs do not play as important a role in cellular function as proteins, there are a number of reasons why one might prefer doing mRNA expression profiling as opposed to protein expression profiling. The principal reason is quite practical though – nucleic acids (such as mRNA) are much easier to separate, purify, detect and quantify than proteins. Also since protein concentrations can be considered to be integrals of mRNA concentrations, the variability at the mRNA level is usually larger than the variability at the protein level. A third reason is simply that mRNA and protein expression measurements complement each other.

The major attraction in transcriptomics is that the ability to measure mRNA concentrations of all genes under any condition allows studying regulation of gene expression at a genome-wide scale. The basic idea in transcription profiling is to measure (usually relative) mRNA expression levels of thousands of genes simultaneously in a cell or tissue sample under specific conditions. All transcription profiling techniques are based on the process of hybridization, in which a cDNA target from the sample to be studied is hybridized to its complementary single stranded DNA probe on an array. The target cDNA is created by extracting all mRNA from a sample, reverse transcribing the mRNAs to cDNAs, and simultaneously labeling the resulting cDNAs with a dye so that they can be detected and quantified. The two standard technologies for transcription profiling are cDNA microarrays (where the DNA probe on the array is a long cDNA), and Affymetrix Gene Chips (where the probe on the array is a short oligonucleotide). In addition to these major techniques there are a number of more sensitive and flexible technologies that have been developed in recent years.

Table 4. Methodologies in proteomics.

1. Protein interaction mapping
– Methods include yeast two-hybrid, co-immunoprecipitation with mass spectrometry, and protein chips
2. Protein expression profiling
– Same as gene expression profiling, but for proteins
– Methods include 2DGE or LC coupled with mass spectrometry and protein chips
3. Protein activity profiling
– Usually done using protein chips
4. Protein modification profiling
– For example, phosphorylation
– Usually done using some mass spectrometry-based approach

5.4 *Proteomics*

Proteomics could be described as a large-scale study of protein structure, expression, and function (including modifications and interactions). Some of the proteomic tasks and the methods used are given in table 4.

5.5 *Metabolomics*

In addition to genomics, trancriptomics, and proteomics data, the changes in metabolite concentration levels in the cell can be used for analysis of phenotypic behavior in the cell. Unlike genes that are encoded by 4 letters, or proteins that are made from 20 amino acids, metabolites don't have a set of codons and thus cannot be sequenced. Instead, they are characterized by their elemental composition, order of atoms, stereochemical orientation, and molecular charge.

'Target analysis' is the process of perturbing one gene and measuring the effect of this perturbation on the concentration of a target metabolite (i.e., the metabolite of interest). If more than one gene is perturbed and the changes of a target metabolite is measured following such perturbations, the analysis is referred to as 'metabolite profiling'. 'Metabolomics' is a whole-cell measurement of all the metabolites and it is considered to be equivalent to transcriptomics in mRNA expression analysis. Metabolite concentration levels can also be measured in a high-throughput and qualitative fashion. This is referred to as 'metabolic finger printing'. Primary tools for such an analysis include NMR and mass spectrometry.

The reason for using high-throughput technologies is that they provide a large number of correlative data on gene or protein expression in relation to disease. Such data are then analyzed for their association to the disease. The assumption is that multiple variables will be able to provide

information on associations more accurately than a single variable (marker). Such strong associations provide major impetus for the molecular profiling approaches to find patterns or profiles for a clinical test based on high dimensional gene or protein expression panels [13].

Comparative genomic analyses have yielded a large number of genomic expression data in relation to disease. The patterns of gene expressions that are observed represent novel signatures for the respective diseases and can be used to both develop new clinical tests based upon gene expression patterns, and identify candidate markers for diagnosis and prognosis. For example, high-throughput platforms have been developed to screen genome-wide methylation and single nucleotide polymorphism patterns (haplotypes) in tumour tissues and body fluids. Aberrant DNA methylation of CpG dinucleotides is a common epigenetic alteration that contributes to colon cancer formation [14]. Aberrant CpG island methylation results in transcriptional silencing of genes and is a mechanism for inactivating tumour suppressor genes in colon cancer. The methylated tumour DNA can be detected using methylation-specific PCR (MSP) and thus has the potential to be used as a molecular marker for cancer. In colon cancer, the tumour suppressor genes *CDKN2A*, *MGMT* and *MLH1*, as well as other genes (e.g., *TIMP-3*, *p14ARF*, *APC*, *MINT31*, *MINT2* and *THBS-1*), are commonly methylated and are thus candidate molecular markers for colon cancer. The methylation of *CDKN2A*, *MGMT*, *MLH1*, *MINT31*, *MINT2* and other genes occurs early in the adenoma-carcinoma sequence suggesting that these alterations could be used for the early detection of colon cancer.

Single nucleotide polymorphisms have also been used as genetic markers of risk, treatment response, and gene and environment interactions in both rare and common cancers. For example, SNPs within *BRCA* genes, as well as in the surrounding regions, are associated with breast and ovarian cancer risk. The HLA haplotypes have been found to correlate with the outcome of cytokine therapy for renal cell carcinoma. SNPs might also be useful for predicting outcome of 'chemoprevention' (i.e. the use of one or several natural or synthetic substances to reduce the risk of developing cancer, or to reduce the chance of cancer recurrence) [15].

Similarly, comparative analysis of serum and plasma samples by MS-based techniques, such as surface enhanced laser desorption ionization (SELDI)–MS has shown patterns of protein/peptide features indicative of a range of diseases, particularly cancer.

These high-throughput technologies have significantly increased the number of potential DNA, RNA and protein biomarkers under study. One of the major problems with high-dimensional data derived from high-throughput genomic and proteomic technologies is overfitting of the data when there are large numbers of potential predictors among a small number of outcome events. For example, a recent study of RNA micoarray analysis showed how easy it was to overfit data with a small number of samples. Simon and colleagues clearly demonstrated that expression data on 6000 genes from imaginary individuals, 10 normal and 10 cases, could be used to discover discriminatory patterns, using one common method, with 98% accuracy [16]. Many of the so-called 'omics' derived data are subjected to a similar over-fitting if the training and validation sets for analyses are small and not randomized. Most commonly used approaches to analyze 'omics' data are artificial neural networks, boosted decision tree analyses, various types of genetic algorithms and support vector machine-learning algorithms. Each approach has the potential to over fit the data. Over fitting has led to strong conclusions that are likely to be erroneous. The first step, therefore, would be to determine whether the results are reproducible and portable. For this purpose, information on samples should be blinded and samples be sent to several laboratories for running the sample sets under a fixed protocol. The data from each laboratory should be analyzed by an independent data manager to learn if each laboratory reproduced a similar result. Splitting the samples randomly between 'training sets and validation sets' should minimize the over fitting. The validation set should not contain samples used in training sets [17].

6. Types of biomarkers discovered using high-throughput technologies

6.1 *DNA biomarkers*

Increased serum DNA concentrations are associated with various types of cancers and with other diseases such as sepsis and autoimmune disease. Mutations in oncogenes, tumour-suppressor genes, and mismatch-repair genes can serve as DNA biomarkers. For instance, mutations in the oncogene *KRAS* predict metastatic spread in various tumour types, and there are mutations in the gene that encode the tumour suppressor p53 in more than half of sporadic cancers. Germline inheritance of a *TP53* mutation (Li–Fraumeni syndrome) confers a risk of developing many of the same cancers. Mutations in other cancer-related genes, such as the *RAS* oncogene or the tumour-suppressor genes *CDKN2A* (cyclin-dependent kinase inhibitor A, which encodes

p16INK4A), *APC* (the adenomatous polyposis coli gene) and *RB1* (the retinoblastoma gene), also have the potential as markers for prognosis or selection of therapy (see below) [18].

Epigenetic regulation of transcription and translation can also be important in carcinogenesis. Histone deacetylation, lysine-specific histone-H3 methylation, and promoter region CpG methylation can function through transcriptional abrogation of tumour-suppressor genes (e.g., APC or the breast cancer 1 gene, *BRCA1*) or DNA mismatch-repair genes (for example, *MLH1* or the O^6-methyl-guanine-DNA methyl transferase gene, *MGMT*). They can also function through effects on apoptosis, invasion and the cell cycle. Gene silencing by CpG methylation has received the most attention, partly because sensitive methods of measurement have become available. It has been reported, for example, that differences in methylation can distinguish prostate cancer from benign prostatic hyperplasia. Shedding of hyper methylated DNA into saliva from oral malignancies, into sputum or bronchoalveolar lavage fluid from lung cancer, and into serum from patients with lung, bladder or colorectal cancer has also been demonstrated. Pharmacogenomic effects of methylation silencing, with implications for choice of therapy, have also been shown. For example, promoter region methylation of MGMT, an enzyme that reverses 5′-guanine alkylation, predicts the response or resistance of tumours to nitrosourea alkylating agents [19].

Other potential DNA biomarkers include SNPs and mitochondrial DNA markers and oncoviral markers. Particular SNPs are associated with increased cancer risk and haplotype assessment can be predictive of several cancers like breast, prostate and lung. Similarly mutations in mitochondrial DNA occur in cancers of colon, bladder, head, neck, lung etc.

6.2 *RNA biomarkers*

Whereas most DNA markers are evaluated individually, many high-throughput technologies can assess mRNA expression comprehensively [20].

Most RNA-based biomarkers undergoing clinical evaluation consist of multi-gene molecular patterns or 'fingerprints'. Although such patterns can be more accurate than single-molecule markers, choosing which genes to include in the pattern adds an additional layer of statistical complexity, prompting new developments in biostatistics, bioinformatics and data visualization. Molecular markers and their patterns have been analysed by various supervised algorithms, most prominently by double hierarchical clustering methods that lead to colour-coded 'clustered image maps' (CIMs) [21]. For example, pattern-based RNA-expression analysis of clinical breast cancers has identified previously unknown molecular subtypes that are associated with differences in survival. That analysis has also provided increased prognostic capability, predicted response to neo-adjuvant therapy, predicted the likelihood of metastasis in lymph-node negative patients and correctly predicted tumour grade from laser-capture microdissected specimens. The transcript levels of enzymes important for drug metabolism have been used preclinically to predict the response to chemotherapy in lung and colon cancers. However, extensive validation studies will be required, to move those developments from clinical research to standard practice in staging [19].

6.3 *Protein biomarkers*

Most of the biomarkers in clinical use are single proteins. Just as pattern-based RNA biomarkers frequently outperform single RNA markers in tumour classification, prognosis or prediction of response to therapy, protein-based 'fingerprints' may outperform individual protein markers. Technologies such as differential in-gel electrophoresis (DIGE), two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and multidimensional protein-identification technology (Mud PIT) can be used for higher-throughput profiling with microgram quantities of protein. Other high-throughput technologies, such as the reverse-phase microarray and surface-enhanced laser desorption ionization time-of-flight (SELDI-TOF) mass spectrometry, are more sensitive (in the femtomolar range) and can cover more of the 12 orders of magnitude range of serum-protein expression levels. Emerging nanotechnologies, such as immuno-PCR, field effect transistor (FET)-based protein detection and quantum dots, promise further increases in the sensitivity of protein markers, but those techniques are currently experimental.

Protein quantity by itself might not be the salient marker parameter. Protein function is instead often dependent on phosphorylation, glycosylation, and other post-translational modifications, location in the cell and/or the location in the tissue. The important phosphorylation-dependent signalling cascades can be assessed, for example, using reverse-phase arrays. Laser-capture microdissection and similar technologies can be used to obtain DNA, mRNA or protein from precise locations within a tumour and thereby distinguish markers inherent to the malignant cells from those in other cell types within the tumour. Microdissection has enhanced expression profiling of various cancer types [22].

7. Biomarker use across the spectrum of diseases

7.1 *Risk assessment*

Risk assessment is qualitative and quantitative evaluation of the risk posed to human health by the actual or potential presence of specific risk characteristics. For example, cardiovascular risk assessment by tables and charts based on the Framingham equation are widely used [4]. Various biomarkers have been used to improve prediction by Framingham score. Lipoprotein-associated phospholipase A2, vitamin B6, IL-6, C-reactive protein and soluble thrombomodulin have been used [6].

7.2 *Screening*

Screening discriminates the healthy from the asymptomatic disease state by screening particular groups. Biomarkers are important for screening and early diagnosis. For example, the prognosis of advanced HCC is poor, whereas smaller HCC suitable for organ transplantation, surgical resection or radio frequency ablation have shown a better prognosis and longer survival. Therefore, detection of HCC at an early stage heavily affects the clinical outcome of these patients. For this reason, a surveillance program using alpha foetoprotein (AFP) and ultrasound (US) every six months has been recommended, and is widely practised. So far, AFP, the only serological marker commonly used in diagnosis has failed to be a reliable marker mainly because it shows poor sensitivity, ranging from 39% to 65% and a specificity ranging from 76% to 97%. AFP seems to be reliable at values over 400 IU/ml, but the percentage of patients with such high levels is very small; this represents one of the most important limits of this marker. Various other markers for HCC diagnosis have been evaluated including fucosylated variant of the AFP glycoprotein, having a high affinity of the sugar chain to *Lens culinaris* (AFP-L3), hepatoma-specific AFP and AFP-mRNA, Des-gamma carboxy prothrombin (DCP), Glypican-3 (GPC3), squamous cell carcinoma antigen (SCCA), immunoglobulins of the IgM class forming complexes with either AFP (AFPIC) or SCCA (SCCAIC), tissue polypeptide specific antigen, hepatoma-specific gamma-glutamyl transferase isoenzyme, transforming growth factor (TGF)-β1 and TGF-β1-mRNA, insulin-like growth factor (IGF)-II and IGF-II mRNA and genetic alterations of telomerase. However, individually used, these markers don't have good performance characteristics. The combination of SCCA, SCCAIC, AFP and AFPIC has been investigated. This combination of biomarkers allows the identification of almost 80% of tumours with normal AFP, that represent the most difficult challenge for clinicians [23].

8. Diagnosis, treatment, prognosis and prediction of response

8.1 *Classification, grading and staging*

Classification of the tissue of origin of a disease especially malignancy is the first step towards predicting survival and choosing therapy. Because a tumour's anatomical location usually indicates its tissue of origin, molecular markers are rarely required. Histological examination generally confirms the diagnosis and identifies the tumour subtype. However, new molecular markers might sometimes be helpful in the differential diagnosis. By using a combination of high-throughput RNA, protein and tissue microarray technologies, markers potentially useful for distinguishing colon and ovarian abdominal carcinomas from an unknown primary location can be identified [24].

Each anatomical site has its own histological grading system, designed to classify malignancies by degree of differentiation. Low-grade, well-differentiated tumours are usually less aggressive and more favourable in prognosis than high-grade tumours, which tend to grow faster and metastasize earlier. However, tumour grade is included in formal TNM staging only when intimately linked to prognosis, as it is for soft-tissue sarcomas, prostate cancer and primary brain malignancies. Assignment of grade is inherently subjective and dependent on the skill and experience of the reviewing pathologist, but several reports indicate that biomarker patterns can correctly score tumours according to their pathologist-assigned grades. Computer-aided diagnostic systems (CAD systems) have been used for preliminary grading of cervical smears and for assisted interpretation of radiological images such as screening mammograms, computerized tomography (CT) scans and standard X-ray films [25]. CADs are generally designed to make routine distinctions, giving the pathologist time to focus on difficult diagnostic problems. The addition of either individual or pattern-based biomarkers in the assessment of histological grade could increase the utility of grading for predicting response to therapy.

The TNM Committee of the International Union Against Cancer (UICC), has defined staging criteria for most anatomical sites. T, N and M are determined separately and then grouped, usually to classify the cancer into one of four main stages (stages I–IV) and subdivisions thereof. Clinical staging, which is primarily used to guide

initial therapy integrates information from physical examination with data such as those from standard X-ray, CT, MRI, PET, endoscopic examination, biopsy, and surgical exploration. Pathological staging on the basis of surgical specimens, if acquired, complements clinical staging with a precise determination of the extent of disease and additional histological information. Increasingly, imaging agents targeted at biomarkers are being used for anatomical localization. The most common are radioisotopes, detected by standard nuclear medicine imaging, by single-photon emission computed tomography (SPECT) or by PET. Also under study are fluorescent molecules, which are detected by optical imaging, and paramagnetic particles for enhancing MRI. The target can be any marker that delineates the cancer or its metabolism. For example, (18)F-FDG, (11)C-acetate, and dual-tracer PET/CT have recently been shown to have a relatively high sensitivity for the detection of extrahepatic metastases of HCC and may be potentially helpful in HCC staging [26].

Some tumours (for example, carcinoid, phaeochromocytoma, and cancers of the prostate, thyroid and colon) can be targeted by specific radiolabelled ligands. Carcinoid tumours, for example, are often localized using a radiolabelled analogue of octreotide (111-indium pentetreotide), which avidly binds to the somatostatin receptor, a protein commonly overexpressed in those tumours. Nuclear medicine-based imaging modalities are also clinically useful for evaluating tumour-related phenomena including angiogenesis, apoptosis, proliferation, metabolism, hypoxia and drug resistance (such as P-glycoprotein function). Molecularly targeted functional imaging has enormous potential for staging, as it does for other aspects of diagnosis and management [19].

Staging could also be useful in non-malignant diseases. For example, from a clinical management viewpoint, accurately assessing the extent and progression of liver fibrosis in cases of chronic liver disease is important. Liver biopsy is the current gold standard but is poorly suited for active monitoring because of its expense and morbidity. Thus, development of alternatives that are safe, inexpensive, and reliable is a priority. There have been tremendous advances in biomarkers for non-invasive assessment and staging of liver fibrosis. Table 5 shows the various blood biomarkers evaluated for staging of liver fibrosis. Routine laboratory tests [aspartate aminotransferase (AST) to alanine aminotransferase (ALT) ratio; gamma glutamyl transferase (GGT); cholesterol; platelet count; AST to platelet ratio and insulin resistance], various proprietary test panels ['PGA index,' which combines prothrombin time, GGT, and apolipoprotein A1, which was later modified to include alpha-2-macroglobulin ('PGAA index'); 'Fibrotest,' which combines α-2-macroglobulin, haptoglobin, GGT, apolipoprotein A1, and total bilirubin;], specialized tests of liver function [indocyanine green; sorbitol; galactose clearance tests; 13C-galactose breath test; 13C-aminopyrine breath test and MEGX test], serum ECM markers of fibrosis ['Fibrospect panel comprising hyaluronic acid, TIMP1, and −2-macroglobulin; collagen IV; collagen VI; amino terminal propeptide of type III collagen (PIIINP); apolipoprotein A-IV; complement C-4; serum retinol binding proteins; serum N-glycans etc.] have been assessed and are being developed for staging liver fibrosis [27].

8.2 *Prognosis and treatment selection*

Tumour classification, stage and sometimes grade are used to assess prognosis. Biomarker expression often supplants or complements tumour classification, stage and grade when biologically targeted therapeutics are under consideration. Prominent examples include CD20 positivity for treatment of lymphomas with rituximab, HER2/NEU positivity for treatment of breast cancer with trastuzumab, BCR-ABL translocation for treatment of chronic myelogenous leukaemia (CML) with imatinib, and KIT or platelet-derived growth factor receptor-α (PDGFRA) positivity for treatment of gastrointestinal stromal tumours (GIST) with imatinib [19].

Both prognosis and prediction of response are necessary for the selection of neoadjuvant or adjuvant chemotherapy. Tissue classification, TNM staging, molecular biomarkers, grade and other factors might be used in combination for that purpose. The combinations of variables might not be easy to analyse manually, but computer decision support systems (DSS) can make the assessments automatically [28]. Biomarkers can also be used to avoid idiosyncratic drug toxicity such as the sustained, life-threatening leukocyte suppression seen when mercaptopurine is given to leukaemia patients with homozygous mutations of the thiopurine methyltransferase (*TPMT*) gene [29].

8.3 *Therapy monitoring*

With advances in understanding of tumour biology, interest in molecular biomarkers of carcinogenesis has grown, both in terms of their prognostic significance and also their potential as therapeutic targets. For example, surgery, including transplantation, remains the only potentially curative modality for HCC, yet recurrence rates are high and long-term survival poor. The ability to predict individual recurrence risk and subsequently prognosis would help guide surgical and

Table 5. Blood markers used to detect and stage liver fibrosis.

Name	Components	Sensitivity/ Specificity for advanced fibrosis	PPV/NPV for advanced fibrosis
AST/ALT ratio	AST/ALT	53%/100%	100%/81%
'Forns' test	platelets, GGT, cholesterol	94%/51%	40%/96%
APRI	AST, platelets	41%/95%	88%/64%
PGA index	platelets, GGT, apolipoprotein A	91%/81%	85%/89%
Fibrotest	GGT, haptoglobin bilirubin, apolipoprotein A, alpha-2-macroglobulin	87%/59%	63%/85%
Fibrospect	hyaluronic acid, TIMP-1, alpha-2-macroglobulin	83%/66%	72%/78%
FPI	AST, cholesterol, HOMA-IR	85%/48%	70%/69%
ELF	collagen IV, collagen VI, amino terminal propeptide of type III collagen (PIIINP), matrix metalloproteinase 2 (MMP-2), matrix metalloproteinase 9 (MMP-9), tissue inhibitor of matrix metalloproteinase 1 (TIMP-1), tenascin, laminin, and hyaluronic acid (HA).	90%/41%	35%/92%

Abbreviations: AST, aspartate aminotransferase; GGT, gamma glutamyl transpeptidase; APRI, AST to platelet ratio index; TIMP-1, tissue inhibitor of metalloproteinase 1; ECM, extracellular matrix; HOMA-IR – homeostasis model assessment (for insulin resistance).

Table 6. Molecular markers of prognostic significance in hepatocellular carcinoma.

Hepatocarcinogenic process	Potential prognostic marker
Proliferation, self-sufficiency in growth signals, insensitivity to antigrowth signals	p53*, nm-23, Rb, PTEN*, c-met*, c-myc*, cyclin A, cyclin D, cyclin E, p15, p16, p18, p19, p21, p27, p57, TGF-b, EGFR family, growth factors proliferation indices*
Avoidance of apoptosis	p53*, Bcl-2, Bcl-xL, Bax, Bak, Bcl-xS, survivin
Limitless replicative potential	Telomerase (including TERT)*
Sustained angiogenesis	MVD, VEGF*, HIF-1a*, NOS, bFGF, PD-EGF, tissue factor, endostatin/collagen XVIII, interleukin-8, angiopoietin
Tissue invasion and metastasis	MMPs*, uPA, cadherin/catenin complex
Genomic instability	Chromosomal instability, aneuploidy*, microsatellite instability

Abbreviations: nm-23, non-metastatic protein-23; Rb, retinoblastoma gene; PTEN, phosphatase and tensin homolog; TGF-b, transforming growth factor beta; EGFR family, epidermal growth factor receptor family; TGF-a, transforming growth factor alpha; HB-EGF, heparin-binding epidermal growth factor; TERT, telomerase reverse transcriptase; MVD, microvessel density; VEGF, vascular endothelial growth factor; HIF-1a, hypoxia-inducible factor-1 alpha; NOS, nitric oxide synthase; bFGF, basic fibroblast growth factor; PD-EGF, platelet-derived endothelial growth factor; MMP, matrix metalloproteases; uPA, urokinase plasminogen activator.

chemotherapeutic treatment. As understanding of hepatocarcinogenesis has increased, the myriad of genetic and molecular events that drive the hepatocarcinogenic disease process, including angiogenesis, invasion and metastasis, have been identified. A number of molecular biomarkers with prognostic significance have been identified in hepatocellular carcinoma (table 6) [30].

Research into the molecular biology of hepatocarcinogenesis has identified a multitude of molecular biomarkers with potential prognostic significance. Markers of particular interest include p53-mutation, PTEN, c-met, c-myc, p18, p27, p57, serum VEGF, HIF-1a, MMP-2, -7, and -12, as well as proliferation indices, telomerase activity and aneuploidy. Combining panels of molecular

biomarkers with more traditional histopathological characteristics may enable more accurate prediction of those at high risk of disease progression and more appropriate targeting of resources. In addition to biomarker expression in resected specimens or biopsy samples, further emphasis should be placed on the role of circulating serum biomarkers. Assessment of molecular biomarkers in serum (for example pre-operative serum VEGF), as well as other body fluids including urine, may allow formulation of pre-operative prognostic criteria to identify patients most likely to benefit from particular therapies, such as hepatic resection and transplantation, as well as predict those most likely to respond to different chemotherapeutic agents. It may be that high-risk patients achieve no advantage in undergoing hepatic resection compared to a less invasive treatment modality, such as tumour ablation, with its reduced morbidity, mortality, and cost. In addition, the ability to stratify patients' prognoses pre-operatively would improve provision of patient information when obtaining informed consent, allow assessment of the need for adjuvant therapies, and facilitate comparative studies and clinical trials. Serum and urinary biomarkers may also have a potential role in screening for recurrent disease following treatment. Ho and colleagues [31] used microarray to identify 14 genes that could discriminate between those patients with vascular invasion from those without. They subsequently tested the prognostic value of this finding on a separate group, finding a significantly poorer disease-free survival in those patients predicted to have vascular invasion, and therefore to be at higher risk of recurrence. Work by Iizuka and colleagues based on microarray analysis identified a group of genes that could predict intrahepatic recurrence with a positive predictive value of 88% and a negative predictive value of 95% [32].

9. Drug development based on molecular biomarkers and targetted personalized therapies

In the treatment of diseases especially cancer, there is a shift from the traditional clinical practices to novel approaches. Traditionally, cancer patients were treated with drugs of low toxicity or of high tolerance regardless of their efficacy in a given patient if the benefits of that drug are proven in both experimental and clinical conditions. However, recent advances in basic and clinical research have provided opportunities to develop 'personalized' treatment strategies. These novel approaches are intended to identify individualized patient benefits of therapies, minimize the risk of toxicity and reduce the cost of treatment. The biggest challenge for researchers and clinicians today is, to decide on which type of biomarker to use across the wide spectrum of disease processes. In cancer, genomic studies are valuable because every cancer cell shows some degree of genetic damage, which might not be present in normal cells of the body. Contrary to genomic DNA markers, phenotypic expression markers (RNA/protein) will vary among cell types and change over time and show different posttranscriptional or posttranslational modifications. However, proteins, peptides or metabolites are abundant, easily accessible in body fluids, such as blood, urine, cerebrospinal fluid and secretions, and show promise for measuring outcomes and studying changes in disease state. Another challenge in characterizing biomarkers is the complexity of the expression profile of potential markers in benign conditions close to the disease phenotypes. The evolving trend is the usage of patterns of markers instead of a single marker. This approach could, to some extent, reduce the error rate in predicting the outcome or severity of side effects during the targeted therapies.

With the increasing knowledge of the molecular pathways underlying the development of various diseases, the selection of patients and their efficacy in future will be based on molecular profiling or phenotypic expression of their target molecules in malignant tissues. These targeted drugs shut down their specific pathway or sets of pathways. The predictability of the response to targeted drugs rules out their use in all patients, which helps to avoid unnecessary drug-associated side effects.

For example, HCC is a tumour with several genomic alterations. There is evidence of aberrant activation of several signaling cascades such as epidermal growth factor receptor (EGFR), Ras/extracellular signal-regulated kinase, phosphoinositol-3-kinase/mammalian target of rapamycin (mTOR), hepatocyte growth factor/mesenchymal-epithelial transition factor, Wnt, Hedgehog, and apoptotic signaling. Recently a multikinase inhibitor, sorafenib, has shown survival benefits in patients with advanced HCC. This advancement represents a breakthrough in the treatment of this complex disease and proves that molecular therapies can be effective in HCC. It is becoming apparent, however, that to overcome the complexity of genomic aberrations in HCC, combination therapies will be critical. Phase II studies have tested drugs blocking EGFR, vascular endothelial growth factor/platelet-derived growth factor receptor, and mTOR signaling. Future research is expected to identify new compounds to block important undruggable pathways, such as Wnt signaling, and to identify new oncogenes as targets for therapies through novel high-throughput

Table 7. Molecular targeted agents in clinical development in cancer.

Cancer cell function	Agent (type)
Signal transduction	
Growth factor receptors	
EGFR	Gefitinib (TKI), Erlotinib (TKI), Cetuximab (mAb), Panitumumab (mAb)
HER2	Trastuzumab (mAb), Lapatinib (TKI)
PDGFR	Imatinib (TKI), Sunitinb (TKI), Sorafenib (TKI)
FLT3	Lestaurtinib (TKI), PKC 412 (TKI), Sunitinib
IGFR1	IMC-A12 (mAb),
c-MET	SU11274, JNJ-38877605, ARQ197
c-KIT	Imatinib, Dasatinib
Intracellular signaling	
RAS	Farnesyl transferase inhibitor Tipifarnib
RAF	Sorafenib
MEK	Vandetanib, AZD6244
mTOR	Temsirolimus, Everolimus, Rapamycin
Angiogenesis	
Growth factors	
VEGF	Bevacizumab (mAb)
Growth factor receptors	
VEGFR	Sorafenib, Sunitinib, Brivanib, Cediranib, Valatanib, IMC1121B (mAb)
PDGFR	Sorafenib, Imatinib, Sunitinib
Apoptosis	
Intrinsic pathway	
BCL2	GX15-070, Oblimersen
Extrinsic pathway	
Apo2L/TRAIL	Mapatumumab, Apomab, AMG-655, rhApo/TRAIL
Protein turnover	
Proteasome	Bortezomib
Chromatin remodeling	
HDAC	SAHA
DNAmethyltransferase	Decitabine
Cell cycle	
CDKs	Flavopiridol (CDKI)
Migration and invasion	
SRC	Dasatinib, XL228

technologies. Biomarkers and molecular imaging should be part of the trials, in order to optimize the enrichment of study populations and identify drug responders. Ultimately, a molecular classification of HCC based on genomewide investigations and identification of patient subclasses according to drug responsiveness will lead to a more personalized medicine [33] (table 7).

10. Future directions

A large concerted effort is required to advance the field of biomarker discovery. Most current biomarkers do not satisfy the required characteristics for use among the spectrum of diseases. Validation of new biomarkers is necessary. Generation of prospective data will be necessary for validation and demonstration of clinical utility. High-throughput technologies have begun to define disease processes and other biological processes with molecular biology detail and thus offer the potential to identify and characterize novel biomarkers. Molecular biology is now seen as encouraging more 'personalized medicine' – the closer alignment of biological information (derived from molecular diagnostics) and therapy selection. Well-designed efforts will be needed to develop general knowledge about the molecular history of diseases, to keep up with the progress with biomarkers development. The evolution of molecular medicine, coupled with the discovery and clinical application of new biomarkers, will play a significant role in reshaping medicine as a science.

Science in India could make a significant impact on the global scene if scientists and policy makers could agree to dedicate sufficient time and resources to the field of biomarkers. This should be much beyond task-force and excellence initiatives, and should be output-driven in a defined time line.

References

[1] Atkinson A J *et al* 2001 NCI-FDA Biomarkers Definitions Working Group; Biomarkers and surrogate endpoints: preferred definitions and conceptual framework; *Clin. Pharmaco. Ther.* **69** 89–95.

[2] Sullivan Pepe M 2001 Phases of biomarker development for early detection of cancer; *J. Natl. Cancer Inst.* **93** 1054–1061.

[3] Sackett D L, Hayens R B, Guyatt G H and Tugwell P 1991 The interpretation of diagnostic data; In: *Clinical epidemiology. A basic science for clinical medicine* (2^{nd} edition) (Boston: Little Brown) 69–152.

[4] Wilson P W, D'Agostino R B, Levy D, Belanger A M, Silbershatz H and Kannel W B 1998 Prediction of coronary heart disease using risk factor categories; *Circulation* 97 **18** 1837–1847.

[5] Tateishi R, Yoshida H, Matsuyama Y, Mine N, Kondo Y and Omata M 2008 Diagnostic accuracy of tumour markers for hepatocellular carcinoma: A systematic review; *Hepatol. Int.* **2** 17–30.

[6] Folsom A R, Chambless L E, Ballantyne C M *et al* 2006 An assessment of incremental coronary risk prediction using C-reactive protein and other novel risk markers: The atherosclerosis risk in communities study; *Arch. Intern. Med.* 166 **13** 1368–1373.

[7] Cook N R 2007 Use and misuse of the receiver operating characteristic curve in risk prediction; *Circulation* 115 **7** 928–935.

[8] Cook N R, Buring J E and Ridker P M 2006 The effect of including C-reactive protein in cardiovascular risk prediction models for women; *Ann. Intern. Med.* 145 **1** 21–29.

[9] Greenland P, Bonow R O, Brundage B H *et al* 2007 ACCF/AHA 2007 clinical expert consensus document on coronary artery calcium scoring by computed tomography in global cardiovascular risk assessment and in evaluation of patients with chest pain: A report of the American College of Cardiology Foundation Clinical Expert Consensus Task Force (ACCF/AHA Writing Committee to Update the 2000 Expert Consensus Document on Electron Beam Computed Tomography) developed in collaboration with the Society of Atherosclerosis Imaging and Prevention and the Society of Cardiovascular Computed Tomography; *J. Am. Coll. Cardiol.* 49 **3** 378–402.

[10] D'Agostino R B Sr, Grundy S, Sullivan L M and Wilson P 2001 Validation of the Framingham coronary heart disease prediction scores results of a multiple ethnic groups investigation; *JAMA* 286 **2** 180–187.

[11] Venter J C, Adams M D, Myers E W, Li P W, Mural R J *et al* 2001 The sequence of the human genome; *Science* 291 **5507** 1304–1351.

[12] Syvanen A C 2001 Accessing genetic variation: genotyping single nucleotide polymorphisms; *Nat. Rev. Genet.* 2 **12** 930–942.

[13] Verma M and Srivastava S 2003 New cancer biomarkers deriving from NCI early detection research; *Recent Results Cancer Res.* **163** 72–84.

[14] Tycko B 2000 Epigenetic gene silencing in cancer; *J. Clin. Invest.* **105** 401–407.

[15] Ellerhorst J A *et al* 2003 Heterozygosity or homozygosity for 2 HLA class II haplotypes predict favorable outcomes for renal cell carcinoma treated with cytokine therapy; *J. Urol.* **169** 2084–2088.

[16] Feng Z *et al* 2004 Research issues and strategies for genomic and proteomic biomarker discovery and validation: A statistical perspective; *Pharmacogenomics* **5** 709–719.

[17] Ransohoff D F 2004 Rules of evidence for cancer molecular-marker discovery and validation; *Nat. Rev. Cancer* **4** 309–314.

[18] Pantel K and Brakenhoff R H 2004 Dissecting the metastatic cascade; *Nature Rev. Cancer* **4** 448–456.

[19] Ludwig J A and Weinstein J N 2005 Biomarkers in cancer staging, prognosis and treatment selection; *Nat. Rev. Cancer* 5 **11** 845–856.

[20] Gray J W and Collins C 2000 Genome changes and gene expression in human solid tumours; *Carcinogenesis* **21** 443–452.

[21] Petricoin E F, Zoon K C, Kohn E C, Barrett J C and Liotta L A 2002 Clinical proteomics: Translating benchside promise into bedside reality; *Nature Rev. Drug Discov.* **1** 683–695.

[22] Verma M, Wright G L Jr, Hanash S M, Gopal-Srivastava R and Srivastava S 2001 Proteomic approaches within the NCI early detection research network for the discovery and identification of cancer biomarkers; *Ann. NY Acad. Sci.* **945** 103–115.

[23] Giannelli G, Fransvea E, Trerotoli P, Beaugrand M, Marinosci F, Lupo L, Nkontchou G, Dentico P and Antonaci S 2007 Clinical validation of combined serological biomarkers for improved hepatocellular carcinoma diagnosis in 961 patients; *Clin. Chim. Acta.* 383 **(1–2)** 147–152.

[24] Nishizuka S *et al* 2003 Diagnostic markers that distinguish colon and ovarian adenocarcinomas: Identification by genomic, proteomic, and tissue array profiling; *Cancer Res.* **63** 5243–5250.

[25] Erickson B J and Bartholmai B 2002 Computer-aided detection and diagnosis at the start of the third millennium; *J. Digit Imaging* **15** 59–68.

[26] Park J W, Kim J H, Kim S K, Kang K W, Park K W, Choi J I, Lee W J, Kim C M and Nam B H 2008 A prospective evaluation of 18F-FDG and 11C-acetate PET/CT for detection of primary and metastatic hepatocellular carcinoma; *J. Nucl. Med.* 49 **12** 1912–1921.

[27] Rockey D C and Bissell D M 2006 Noninvasive measures of liver fibrosis; *Hepatology* **43** S113–S120.

[28] Ravdin P M *et al* 2001 Computer program to assist in making decisions about adjuvant therapy for women with early breast cancer; *J. Clin. Oncol.* **19** 980–991.

[29] Relling M and Dervieux T 2001 Pharmacogenetics and cancer therapy; *Nature Rev. Cancer* **1** 99–108.

[30] Mann C D, Neal C P, Garcea C, Manson M M, Dennison A R and Berry D P 2007 Prognostic molecular markers in hepatocellular carcinoma: A systematic review; *European J. Cancer* 4 **3** 979–992.

[31] Ho M C, Lin J J, Chen C N *et al* 2006 A gene expression profile for vascular invasion can predict the recurrence after resection of hepatocellular carcinoma: A microarray approach; *Ann. Surg. Oncol.* 13 **11** 1474–1484.

[32] Iizuka N, Oka M, Yamada-Okabe H *et al* 2003 Oligonucleotide microarray for prediction of early intrahepatic recurrence of hepatocellular carcinoma after curative resection; *Lancet* 361 **9361** 923–929.

[33] Llovet J M and Bruix J 2008 Molecular targeted therapies in hepatocellular carcinoma; *Hepatology* **48** 1312–1327.