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Biomarkers in cancer screening, research and detection: present and future: a review

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Abstract

Biomarkers provide a powerful and dynamic approach to understanding the spectrum of malignancies with applications in observational and analytic epidemiology, randomized clinical trials, screening, diagnosis and prognosis. Defined as alterations in the constituents of tissues or body fluids, these markers offer a means for homogeneous classification of a disease and risk factor, and they can extend one's basic information about the underlying pathogenesis of disease. The goals in cancer research include finding biomarkers that can be used for the early detection of cancers, design individual therapies, and to identify underlying processes involved in the disease. Because so many myriad processes are involved in the diseased states, the goal is similar to 'finding a needle in a haystack'. However, the development of many -omic technologies, such as genomics and proteomics, has allowed us to monitor a large number of key cellular pathways simultaneously. This has enabled the identification of biomarkers and signalling molecules associated with cell growth, cell death and cellular metabolism. These are also facilitating in monitoring the functional disturbance, molecular and cellular damage, and damage response. This brief review describes the development of biomarkers in cancer research and detection with emphasis on different proteomic tools for the identification and discovery of new biomarkers, different clinical assays to detect various biomarkers in different specimens, role of biomarkers in cancer screening and last but not the least, the challenges in this direction of cancer research.

Keywords: *Biomarkers, proteomics, screening, diagnosis*

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Introduction

The development of new throughput technologies, especially -omic technologies such as *genomics* (the study of the genome complement of the cell) and *proteomics* (the study or analysis of protein profile of the cell), has helped to unravel many complex mechanism associated with the uncontrolled division of a cell. The application of these techniques to tumour biology has generated a lot of information not only about the cellular processes that regulate proliferation, differentiation, and apoptosis in normal cells, and the disruption of these normal functions in cancer initiation and progression, but also about the identification and discovery of potential biomarkers for cancer diagnosis and prognosis based on carefully constructed mechanism behind the

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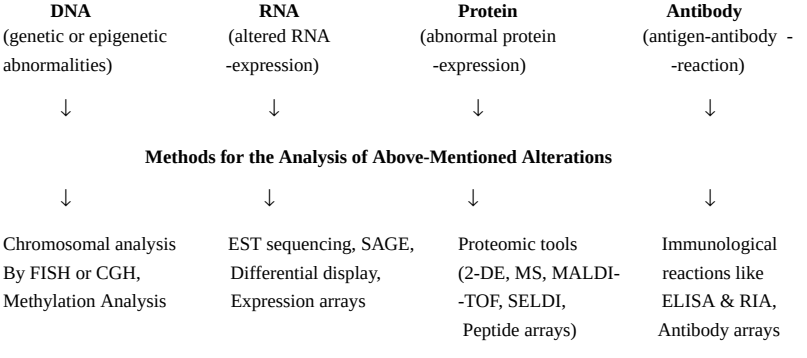
tumour initiation and progression. These biomarkers have immense potential in tumour biology to understand the mechanism behind malignancy and in cancer screening, diagnosis, prognosis and therapeutic targets.

Biomarkers are cellular, biochemical, and molecular (proteomic, genetic, and epigenetic) alterations to recognize or monitor a normal, abnormal, or simply a biological process. These biomarkers can be DNA-based, RNA-based, protein-based, or antibody-based (Figure 1). The NIH Working Group on Biomarkers and Surrogate endpoint (Biomarker Definitions Working Group 2001) has defined biomarkers as characteristics used to measure and evaluate objectively normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention. In cancer research and detection a biomarker refers to a substance or process that is indicative of the presence of cancer in the body. It might be either a molecule secreted by malignancy itself or a specific response of the body to the presence of cancer. Biomarkers can be measured in biological media such as tissues, cells, or fluids. To maximize the usefulness and minimize the cost for screening, it is advantageous for these biomarkers to be measurable in serum, urine, or sputum (Verma et al. 2003). There are many scopes for the use of valid biomarkers in clinical cancer research with new exciting opportunities emerging with new and better biomarkers. However, it is not authentic to use a biomarker in laboratory/clinical-based transnational research before going through different phases of evaluation. Pepe et al. (2001) proposed a five-step guideline for providing a systematic and comprehensive approach to develop, evaluate, and validate biomarkers and guide the results produced in biomarker discovery from the research laboratory to the clinics (Figure 2). The various phases include: (1) phase I (discovery phase), in which the expression profiles of genes or gene products are compared between cancerous tissues and normal tissues to find biomarkers that are either elevated or depressed in the cancerous tissues as compared with normal tissues; (2) phase II (validation phase) for the development of the clinical assays, usually with samples collected non-invasively; (3) phase III, a retrospective study, to determine the capacity of a biomarker to detect preclinical disease using huge



Abbreviations: FISH, fluorescent *in situ* hybridization; CGH, comparative genomic hybridization; EST, expressed sequence tags; SAGE, sequential analysis of gene expression; 2-DE, 2-dimensional gel electrophoresis; MS, mass spectroscopy; MALDI-TOF, matrix assisted laser desorption/ionization-time of flight; SELDI, surface enhanced laser desorption/ionization; ICAT, isotope-coded affinity tags; ELISA, enzyme-linked immunosorbent assay; RIA, radioimmunoassay

Figure 1. Different DNA, RNA, protein or antibody-based molecular alterations as potential cancer biomarkers along with the genetic, molecular and immunologic approaches for their detection.

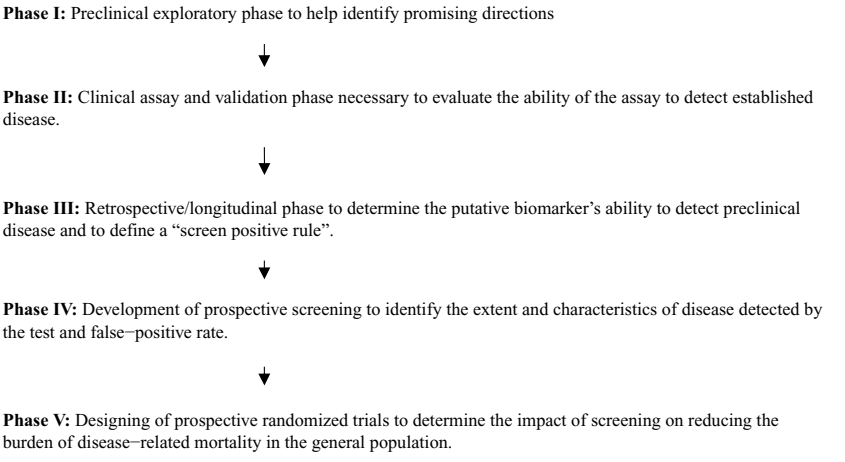


Figure 2. Flow chart of various phases in the biomarker discovery as proposed by Pepe et al. (2001).

sample size collected from apparently healthy individual who are monitored for the development of cancer; (4) phase IV, in which usually asymptomatic or individuals with a high risk of developing cancer are screened for a selected biomarker, and those who test positive are generally followed-up to determine if they have cancer and, hence, assessing the use of selected biomarker in early detection programs; and (5) phase V studies are designed to check whether the screening of a large population results in decreasing disease-related morbidity and mortality.

Biomarker discovery

The completion of initial draft of human genome (Venter et al. 2001) and development of new technologies have set the pace for biomarker discovery and provided stimuli for the next level of molecular inquiry that have the potential to characterize simultaneously alterations in the expression and structure of thousands of genes and their downstream products. Recently these methodologies have been used to identify novel biomarkers for the early detection of premalignancy and cancer and some of these biomarkers are currently being validated on assays of tumour sets. For example, in non-small cell lung cancer (NSCLC), the α-catalytic subunit of phosphatidylinositol 3-kinase was found to be frequently amplified as detected by comparative genomic hybridization (Massion et al. 2002). Measurement of kinase activity revealed a tumour-specific increase most prominently in squamous cell carcinoma (Massion et al. 2002). Analysis of NSCLC by cDNA microarrays also revealed the overexpression of a group of protease inhibitors (e.g. maspin) as compared with matching normal lungs. Reverse transcription-polymerase chain reaction and immunohistochemical studies also confirmed elevated and nearly ubiquitous expression of maspin gene products in SQC (Heighway et al. 2002, Smith et al. 2003). Maspin was also expressed rarely in normal or hyperplasic bronchial tissue but was expressed in half of bronchial dysplasias (Smith et al. 2003). Overexpression of cytoskeletal proteins, collagens, and metalloproteinase were found in stage 1A NSCLC patients as compared with normal lung by cDNA microarray

analysis (Nakamura et al. 2003). As compared with the higher stage lung cancer, 30 genes were found to be differentially expressed in stage 1A NSCLC. cDNA microarrays were used to identify the overexpression of cancer testis antigens in SCLC and NSCLC (Sugita et al. 2003) to identify genes differentially expressed between normal and tumour cell as well as among different tumour cell types (Wang et al. 2000, Chen et al. 2001, Feng et al. 2001), for molecular classification of human malignancies (Ramaswamy et al. 2001, Pomeroy et al. 2002) and have shown promising results so far. High-density oligonucleotide microarrays (OMAs) have been used recently to profile gene expression in lung carcinoma tissue homogenates (Bhattacharjee et al. 2001). Laser-capture microdissection followed by cDNA microarray analyses has been used in lung cancer to delineate genes associated with both lymph node metastasis and sensitivity to a panel of anti-cancer drugs (Kikuchi et al. 2003). By using molecular profiling in cell culture and a time-course analysis, genes associated with the development of cisplatin resistance have been determined (Whiteside et al. 2004). In addition, by analysing the effects of combination chemotherapeutic modalities on lung cancer cells, investigators were able to use functional genomic approach to define new apoptotic targets (Taxman et al. 2003).

Proteomics in the biomarker discovery

Cancer proteomics includes the identification and quantitative analysis of differentially expressed protein relative to healthy tissue counterparts at different stages of disease, from preneoplastic to neoplastic. Proteomics complements genomic-based approaches in the study of cancer. The inherent advantage afforded to proteomics is that the identified protein is itself the biological endpoint. Protein profile of a cell reflects both the intrinsic genetic programs of the cell and the impact of its immediate environment. Protein expression and function are subjected to regulation through transcription as well as through post-transcriptional and translational events. Different splicing can yield more than one RNA from one gene. Additionally, there are more than 200 post-transitional modifications that proteins could undergo that affect function, protein–protein, and nucleic acid–protein interactions, stability, targeting, half-life and so on (Banks et al. 2000), all contributing to a large number of protein products from one gene. Identifying and understanding these changes are the underlying themes in the cancer proteomics. Studies indicate that up to six different proteins forms per gene in humans (Wilkins et al. 1996), and understanding their functional status in non-diseased and various stages of disease progression can provide insights into designing strategies for prevention, diagnosis, and therapeutics. The rapid development and integration of analytical instrumentation combining reproducibility and sensitivity have accelerated the field of proteomics. Table I describes some biomarkers identified by various proteomic tools.

Proteomics technologies

Two-dimensional gel electrophoresis (2-DE)

2-DE has been the workhouse in quantitative proteomics (Hanash 2000). In this technology, proteins are extracted from chosen specimen and then separated according to their isoelectric points (pIs) in the first dimension and their molecular

Table I. Summarizing a few biomarkers identified by various proteomic tools.

2-DE:
Bladder cancer: cytokeratins, psoriasin, galectin 7 and stratifin ↓
Colorectal cancer: calgranulin B ↑
Renal cell carcinoma: ubiquinol cytochrome C reductase ↑
Breast cancer: proliferating cell nuclear antigen ↑
Lung adenocarcinomas: napsin ↑
Ovarian cancer: proliferating cell nuclear antigen, OP18, pHSP60, HSP90, calreticulin ↑ and tropomyosin-1 and -2 ↓
Prostate cancer: oncoprotein 18(v), elongation factor-2, glutathione *S*-transferase p, superoxide dismutase, triosephosphatase isomerase ↑ and cytokeratin 18 ↓
Neuroblastoma: protein p19/nm23-H1 ↑
MS:
Renal cell carcinoma: multimeric isoforms of manganese superoxide dismutase
Primary breast carcinoma: 14-3-3σ ↓, nuclear matrix, redox, and cytoskeletal proteins ↑
Fibroadenoma: DJ-1 ↑
Ovarian carcinoma: retinoic acid-binding protein, carbohydrate-binding protein and certain lipoproteins ↑
Lung adenocarcinomas: cathepsin D ↑

2-DE, two-dimensional electrophoresis; MS, mass spectroscopy; ↓, a decrease in the expression level of protein; ↑, an increase in the expression level of protein.

weights in the second dimension to generate protein profiles. Use of immobilized pH gradients has greatly enhanced the reproducibility in resolving almost complete spectrum of basic to acidic proteins allowing both analytical and preparative amounts of protein to be resolved (Gorg et al. 2000, Hanash 2000). More than 1000 protein spots can be resolved within just 1 unit pH range (Tonella et al. 1998). Silver staining of the gel can resolve approximately 3000 spots of proteins on a single gel. Fluorescent dyes are also being used to stain the gel for further analysis of proteins by mass spectroscopy (Steinberg et al. 1996, Chambers et al. 2000). Stained gels are then scanned with laser densitometers at different resolutions and the data obtained are then analysed with the help of software such as PDQUEST (Bergman et al. 2000) for the quantification and detection of particular protein resolved on a gel. Ratio analysis is used to detect the quantitative changes in proteins between the two samples, i.e. one from a diseased and other from a healthy control. 2-DE has been widely used to identify the biomarkers specifically detected in various types of malignancies (Table I). Recently some automated versions of 2-DE have been established. For example, 2-D membrane electrophoresis. This invention provides for a high speed, high-resolution 2-D method for separating proteins on high protein-binding membranes such as polyvinylidene fluoride (PVDF) or nylons, at room temperature with no cooling effect required. The separation in both dimensions is performed on the same membrane, which just has to be rotated in the electric field after the buffer change. The whole process take only 20 min as compared with 1–2 days for conventional 2-DE polyacrylamide gel electrophoresis (PAGE).

Mass spectroscopy (MS)

MS has provided a powerful means for obtaining peptide mass fingerprints for protein resolved on 2-DE gels. Before MS analysis, proteins are subjected to 'in gel' digestion with a protease and the proteolytic peptides are extracted from the gels. These are then ionized by applying them to MS. Availability of the unique ionization techniques

such as electrospray ionization (ESI) and matrix-assisted laser-desorption ionization (MALDI) has facilitated the characterization of the proteins by MS (Fenn et al. 1989, Hillenkamp et al. 1991, Karas & Hillenkamp 1998). These ionization techniques usually transfer the proteins in to their gaseous phase, which enables their analysis by MS conducive. Peptides, produced by the digestion of proteins by sequence specific proteases are co-precipitated with a light-absorbing matrix, which is then subjected to short pulses of ultraviolet light radiation under reduced pressure. Some of the peptides are ionized and accelerated in an electric field and subsequently turned back through an energy-correction device (Andersen & Mann 2000). Peptide mass is derived through a time-of-flight (TOF) measurement of the elapsed time from acceleration-to-field-free drift or through a quadrupole detector. Hence, a spectrum is generated with the molecular mass of individual peptides, which are used to search databases to find matching proteins. ESI involves dispersion of the sample through a capillary device at high voltage (Andersen & Mann 2000). The charged peptides pass through a MS under reduced pressure and are separated according to their mass-to-charge ratios through electric fields. Another frequently used MS is nano-electrospray ionization tandem MS (ESI MS/MS) (Yates et al. 1997, 1999). In this, a microcapillary tube containing 1 μl of peptide solution sprays a fine mist of charged droplets generated from a potential difference between the capillary and the inlet to the mass spectrometer. Desolvated peptides ions are formed as the solvent evaporates in a high vacuum chamber, and are resolved to produce the first MS scan. From the MS scan a peptide ion (parent ion) is selectively transmitted into a collision chamber where the peptide is fragmented by interactions with an inert gas. The fragments of the peptide ion are then resolved based on their m/z ratio to generate the second MS spectrum with a series of small peptides that differ only by a single amino acid. MS-MS can accurately identify post-translational modifications, such as phosphorylation and glycosylation, through the measurement of mass shifts. Liquid chromatography coupled with tandem mass spectrometry detection (LC-MS/MS) or the 'short-gun' approach (McCormack et al. 1997, Peng & Gygi 2001) uses reversed-phase LC to separate the tryptic digests of entire proteins followed by online ESI tandem MS for peptide sequencing. The generated MS spectra of whole cellular fraction are carefully analysed and protein identification is performed through peptide assignment and database searching. Matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS) has been used in the analysis of the expression patterns of various proteins differentially expressed in various malignancies (Table I), like renal cell carcinoma (Steinberg et al. 1996, Sarto et al. 1997), breast carcinoma (Bergman et al. 2000, Vercoutter-Edouart et al. 2001) as compared with normal tissues. Imaging MS has been also used for direct mapping and imaging of biomolecules present in tissue sections. Frozen tissue sections or individual cells, mounted on a metal plate, are coated with a UV-absorbing matrix and placed in the MS. MS images are generated at specific mass values (Chaurand et al. 1999) with the use of an optical scanning roster over the tissue specimen and the measurement of peak intensities over thousands of spots. Imaging MS has a tremendous potential in biomarker discovery, biomarker tissue localization, understanding of the molecular complexities of tumour cells, and in intraoperative assessment of surgical margins of tumours. Imaging MS has been used to identify protein biomarker in glioblastomas (Stoeckli et al. 2001).

Surface-enhanced laser desorption/ionization (SELDI)

It is a newly developed proteomics platform using patented technology to separate, profile, and analyse proteins at femtomole level (Jr et al. 1999). The principle behind the surface-enhanced laser desorption ionization is surface-enhanced affinity capture through the use of specific probe surfaces or chips. Chips with usually broad binding properties, including immobilized metal affinity capture, and with biochemically characterized surfaces, such as antibodies and receptors, form the core of SELDI (Merchant & Weinberger 2000). As little as 0.5 μl of sample volume can be processed. Once captured on the SELDI-protein chip array, proteins are detected through the ionization-desorption, TOF-MS process. Individual proteins are displayed as separate peaks on the basis of their mass and charge (m/z) and a retentate map is generated. The protein biochip is the counterpart of the assay technology in the genomic field and also forms the platforms for Ciphergen's ProteinChip® assay SELDI-MS system (Merchant & Weinberger 2000). The ProteinChip SELDI-MS system has been used to enhance the detection rate of low-grade bladder cancer to 75% relative to the 30% detected by traditional urine cytology (Vlahou et al. 2001). This system has also been used in identifying protein biomarkers expressed by prostate cancer, lung cancer, ovarian cancer and breast cancer.

Isotope-coded affinity tags (ICAT)

ICAT (Gygi et al. 1999) depends upon the chemical labelling of any pair of protein samples with two identical reagents isotopically different in mass, allowing the relative amount of protein to be quantitatively compared in the subsequent mass spectral determination. A pair of protein samples are labelled on their cysteine residues, respectively, with either light or a heavy form of ICAT chemical reagents and then mixed together for proteolytic digestion. The digested mixture is purified through avidin affinity chromatography using biotin tags on ICAT reagents to isolate the ICAT-labelled peptides. MS analysis of these peptides produces peak ratios for different protein expression and subsequent MS/MS peptide sequencing results in the identification of proteins in altered expression levels. This is especially important to biomarker discovery because expression analysis of all proteins and identification of changes that are a function of a disease process give us molecular handles to target intervention strategies.

Proteins and antibody microarrays

Protein arrays using ordered arrays have been explored through the development of multipin synthesis (Geysen et al. 1984). Assays of clones form phage-display libraries are probed with antibody-coated filters for high throughput antibody screening (De Wildt et al. 2000). Proteins covalently attached to glass slides through aldehyde-containing silane reagents have been used to detect protein–protein interactions, enzymatic targets, and protein-small molecule interactions (Arenkov et al. 2000). Other methods of generating protein microarrays are by printing the proteins or antibodies using a robotic arrayer and a coated microscopic slide in an ordered array. Protein solution to be measured is labelled by covalent linkage of a fluorescent dye to the amino groups on the proteins (Haab et al. 2001). Protein arrays consisting of immobilized proteins from pure populations of microdissected cells have been used to

identify and track progression (Paweletz et al. 2001a). The availability of large antibody arrays can enhance the discovery of differential biomarkers in non-diseased and cancer tissue. An antibody microarray experimental format can be broadly categorized into two classes: (1) direct labelling experiments in which proteins present in a mixture are labelled covalently providing a means for detecting bound proteins after incubation on an antibody microarray; if the proteins are labelled with a tag, such as biotin, the signal from bound proteins can be amplified; and (2) dual antibody sandwich assay in which proteins captured on an antibody microarray are detected by a cocktail of detection antibodies, each antibody being matched to one of the spotted antibodies. These microarrays increase the number of proteins that can be conveniently measured, therefore taking advantage of the benefit of using combined markers in cancer diagnosis. The QudraSpec 'Bio-CD' (Quadraspec, Inc., West Lafayette, Indiana) array is one such array which can perform 1000 unique tests on as many as 100 samples of antigens, biomarkers or other molecular species. Recently, many antibody arrays for specific detection of different cytokines have been established. For example, TranSignal^{TM} (Panomics, Inc. Frement, California) cytokine antibody array, which can profile multiple cytokines in one hybridization experiment. These arrays can detect soluble cytokine proteins at concentrations in the pg ml^{-1} range with array consistency of zero and 10% between the same spots on two of the same type of membrane.

Tissue arrays

A typical array is constructed by arranging cylindrical biopsies from multiple individual tumour tissues in to a tissue array block, which is then sliced in to $\geq$200 identical slides for probing RNA or protein targets. A single immunohistochemistry or *in-situ* hybridization experiment provides information on all specimens on the slides, whereas subsequent sections can be analysed with other probes or antibodies. Cancer specific tissue array slides with various kinds of subsets can be generated (subsequent cancer cases, preneoplastic lesions, metastatic lesions, synchronous cancers, metachronous cancers, young patients, and familial cases) for further analysis (Kim 2001).

Biomarker assay development

The ability to use molecular tools to analyse samples, especially body fluids, for cancer screening and surveillance has a great future because of the technical simplicity of sample collection and the potential for easy assay automation and large-scale population-based analysis. However, one critical issue in the selection of an appropriate clinical and analytical assay for biomarker discovery and detection is the ability to detect low expression levels of mRNA or protein and even to identify them with a high degree of statistical confidence. This issue is more acute especially in the case of the detection of biomarkers in various body fluids such as serum, plasma, and sputum as a vast dynamic range of protein concentrations are encountered in these body fluids in spite of other interfering compounds. However, there can be various approaches to develop a simple clinical assay. For example:

- Assays based on various body fluids, such as sputum, plasma/serum, blood, urine, nipple aspirate fluid, etc.

- Assays based on the detection of antibodies/autoantibodies in the serum of the cancer patient.
- Breath analysis to identify specific biomarkers present in the exhaled air from the cancer patient.
- Assays based on RNA or protein-based multiple markers to decipher the different pathways by which the tumour progresses from benign to malignant stages.

Table II summarizes some of the biomarkers detected in various body fluids taken from the cancer subjects. Obtaining tumour tissue for genetic analysis may not always be logistically accessible and thus such an approach would be difficult for assessing serial tumour genetic events that occur during cancer progression or treatment. That is the advantage of the samples collected non-invasively, like sputum and urine, or with minimally invasive technique, such as blood. Although, the examination of sputum cytologically has not shown any improvement in the early detection of lung cancer or decreasing lung cancer mortality; protein and/or nucleic acid based analysis show promise in improving early detection rates. In a recent study, about an eightfold high odds ratio in sputum of lung cancer patients has been detected demonstrating the aberrant methylation in any one gene from an eight-gene panel in individuals with moderate sputum atypia or worse (Hirsch et al. 2003). Similarly, the discovery of biomarkers and biomarker-based assay for nipple aspirate fluid in breast cancer (Paweletz et al. 2001b) may lead to a potential non-invasive method in the diagnosis of the disease. The quantification of free circulating DNA and detection of the

Table II. Potential biomarkers detected in various cancer specimens.

Sputum: Mutations of K-ras and p53 (Zhang et al. 2003, Keohavong et al. 2004); epigenetic changes (Chen et al. 2003, Liu et al. 2003, Palmisano et al. 2000); methylayion of p16 and MGMT (Palmisano et al. 2000, Gilliland et al. 2002, Pulling et al. 2003); overexpression of hnRNP A2/B1 and other members of hnRNP family (Pino et al. 2003, Tominaga et al. 2003)
Circulating genes in plasma/serum: Mutations of K-ras, p53 (Andriani et al. 2004) and β-tubulin genes (Rosell et al. 2000); LOH of FHIT (Andriani et al. 2004); promoter hypermethylation of TMS-1, RASSF1A, DAPK, APC genes (Bearzatto et al. 2002, Usadel et al. 2002, Ramirez et al. 2003); detection of hTERT mRNA (Sozzi et al. 2003); presence of elevated cell-free circulating DNA and RNA levels in cancer as compared with healthy controls and patients with benign diseases (Bermnes et al. 2005); detecting abnormal proteins\peptides, for example CEA and NSE (Kulpa et al. 2002, Molina et al. 2003)
Autoantibodies in serum: Detection of antibodies against p53 (Iizasa et al. 1998, Mitsudomi et al. 1998, Laudanski et al. 1998, Mack et al. 2000, Neri et al. 2003); glycosylated annexins I and/or II (Brichory et al. 2001); anti-p40 (Yamaguchi et al. 2000); antineural and antinuclear antibodies (Blaes et al. 2000); MUC1 (Hirasawa et al. 2000); livin and survivin (Yagihashi et al. 2005); c-Myc and L-myc (Yamamoto et al. 1996, 1999)
Breath analysis: Detection of volatile organic compounds (VOCs), mainly alkanes and aromatic compounds (Di Natale et al. 2003, Phillips et al. 2003, Giardina & Olesik 2003)

MGMT, methylguanine methyl-transferase; hnRNP, heterogeneous nuclear ribonucleoprotein; LOH, loss of heterozygosity; FHIT, fragile histidine triad; TMS-1, target of methylation inducing silencing; RASSF1A, ras association domain family 1A gene; DAPK, death-associated protein kinase; APC, adenomatous polyposis coli; hTERT, human telomerase catalytic subunit; CEA, carcinoembryonic antigen; NSE, neuron-specific enolase.

mutations, epigenetic changes and microsatellite alterations in the serum/plasma of lung cancer patients has been used for the early detection of lung cancer (Bermnes et al. 2005). Allan et al. (2001) detected loss of heterozygosity (LOH) in plasma DNA of 12 out of 13 individuals who subsequently developed lung cancer. Detection of the autoantibodies in the serum can provide new approach for the early detection of some malignancies. These autoantibodies originate because of autoimmune reaction against the abnormally expressed proteins or altered protein structures. Many of these studies indicated low sensitivity when an antibody against single molecule was detected. Combining multiple targets, thus, may improve value of the approach as observed by Fernandez-Madrid et al. (1999). They detected lung cancer with 63% sensitivity and 89% specificity rates by using 12 antigens. Breath analysis is another upcoming technique for biomarker screening. Presently in its earliest stages of development, breath analysis may help in the early detection of roentgenographically occult lung cancer. The concept behind this assay is that volatile organic compounds (VOCs), mainly alkanes and aromatic compounds, are preferentially produced and exhaled by lung cancer patients and can be used as accurate markers of malignant disease (Di Natale et al. 2003, Phillips et al. 2003, Giardina & Olesik 2003).

More often, the use of a single marker does not provide enough information regarding the mechanism behind the cancer progression as the tumour continuously evolves genetically in response to host pressure and treatment interventions. Also due to the heterogeneity associated with tumour cells, diagnosis of cancer with only one marker by nucleic acids or protein-based assays may not provide enough sensitivity and specificity. In that condition, assays based on multiple markers must be designed to decipher different pathways associated with cancer progression. For example, the multiple-marker nested real-time quantitative polymerase chain reaction (PCR) assay was developed for the prognosis of NSCLC by detecting circulating cancer cells in the blood (Sher et al. 2005). The frequency of the detection of NSCLC was found to be 72% as compared with 41% using a single marker. Similarly, at least one gene was detected in 71% of lung cancer patients using five markers as compared with highest sensitivity rate of 41% achieved using only a single marker (Hangai et al. 2000). Furthermore, analysis of a panel of protein-based markers can also prove very useful in the diagnosis of various malignancies.

Biomarkers in cancer screening

Screening has been defined as 'the presumptive identification of unrecognized disease or defect by the application of tests, examinations, or other procedures that can be applied rapidly' (Miller 1985). In other words, it is the application of diagnostic tests or procedures to asymptomatic people for the purpose of dividing them into two groups: those who have a condition that would benefit from early intervention and those who do not (Wallace 1998). For a screening programme to be useful, it should meet some important criteria's (Table III).

Whether a screening test is effective depends on whether the 'critical point' occurs before, during or after the asymptomatic period. The critical point is the point in the natural history of the disease before which therapy is relatively effective and after which therapy is relatively ineffective. In the case of cancer, the critical point would represent the time at which regional or distant metastasis occurs. If the critical point

Table III. Characteristics of an ideal screening programme.

- Should be able to detect disease at an early, asymptomatic stage when it is more likely to be amenable to treatment and cure
- Ultimate goal of the screening test should be to reduce morbidity and mortality
- Screening test must be specific enough to minimize false-positive results
- Overall justification of the screening programme should be included in the measurable improvement in outcome of the disease, not in the early diagnosis itself
- Must be cost-effective, should not miss too many cancers and should not say you have a cancer if you do not
- Should be easy to apply and ethically true
- Should be a high prevalence of the disease in the screened population, with patients willing to take part in the further work-up and treatment, and medical care should be easily accessible (Mulley 1995)

occurs early, screening will not be effective because the disease will have 'escaped from cure' before it was detectable by screening. If the critical point is late, screening it is unnecessary because the disease is curable even when it presents with clinical symptoms. Screening will have a potential effect on the natural history of the disease only when the critical point occurs sometime during the asymptomatic period. Screening of the cancer with the help of various biochemical or molecular test also depends upon many other factors such as sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV), which will be discussed later in this review.

In a recent screening study (Henschke et al. 1999), low-dose helical computed tomography (LDCT) detected almost six times as many stage I lung cancers as chest radiography and most of these tumours were 1 cm or less in diameter. However, the effectiveness of screening with LDCT has not yet been evaluated in a controlled clinical trial. In the USA, the Early Lung Cancer Action Project was designed to evaluate whether annual CT screening is useful for detecting early lung cancer in high-risk individuals. There have been two reports from this study suggesting that CT screening allows for the diagnosis of lung cancer at substantially earlier and more curable stages when compared with no screening (US Preventive Task Force 2004, Strauss et al. 2005). The Early Lung Cancer Action Project is currently performing a randomized trial of CT screening versus no screening in high-risk individuals and the results of this trial will probably not be known for at least 10 years. The US Preventive Services Task Force (USPSTF) concluded that current data do not support screening for lung cancer with any method. Lung cancer detection using chest X-ray is one of the most widely used technology, although lately it has been replaced by fluoro-2-deoxyglucose-positron emission tomography (FDG-PET) scanning which to some extent is more specific for differentiating lung cancer from other causes of pulmonary nodules by detecting metabolic processes in proliferating lesions. However, many of these techniques have not been used frequently for cancer screening. For example, detection of prostate cancer is usually done by: (1) digital rectal examination, (2) serum acid phosphatase assay, and (3) prostate-specific antigen in the follow-up of prostate cancer. Similarly, bladder cancer is diagnosed by flow cytometry, cytoscopic examination, intravenous pyelography, computed tomography, and magnetic resonance imaging (Ross et al. 1988, Stein 1989, Melamed 1990, Piccoli & Rifkin 1990).

However, neither of the above methods was found to be suitable for cancer screening nor do they lead to reduce cancer-related moralities. There has been lot of development in the molecular and imaging technologies, but none has focused on the screening of cancer. Theses technologies have just begun to explore the utility of spiral compute tomography (CT), fluorescence bronchoscopy, positron emission tomography (PET) imaging (Gohagan et al. 1999), and proteomic and genomic analysis of tumours and other specimens in lung cancer (Verma et al. 2001). These approaches have the potential to identify biomarkers for small and early lesions that have not been readily accessible in clinical practice through more conventional detection methods. Stool screening for colorectal cancer is vigorously used in search of biomarkers and it provides clear advantage over other screening methods, such as colonoscopy and flexible sigmoidoscopy. A faecal occult blood test (FOBT) has been used to screen colorectal cancer for more than three decades in the USA. It is the only protein biomarker shown to decrease cause-specific mortality in cancer screens (Ahlquist et al. 2000). A guaiac-impregnated Hemoccult card, which detects blood in the faecal smear, is the most frequently used FOBT type. But low sensitivity (26%) and specificity (88–98%) are limiting factors of this technology when screening cancer populations. Recently, James et al. (1999) reported that X-ray diffraction of hair taken from women diagnosed with breast cancer (and those at high risk by virtue of a proven *BRCA1*/*BRCA2* mutation) showed a diffuse ring. They claimed a 100% correlation rate with the disease, advocating the use of pubic hair as a simple non-invasive screening method for breast cancer. They did not detect any false-negatives in the preliminary reports. However, there were some false-positive cases and the efficacy of this test is still to be evaluated to replace mammography. Very recently a new blood test has been developed for the screening of ovarian cancer (Mor et al. 2005). This blood test is based on the detection of four proteins: leptin, prolactin, osteopontin, and insulin-like growth factor-II. In a test group of over 200 ovarian cancer patients and healthy women, the test showed 95% sensitivity and specificity rates.

There are, however, several issues that can have potential harms and which must be considered against any potential benefit of screening for cancer. These involve small risks of serious complications that may be immediate (e.g. complication with colonoscopy, bronchoscopy) or delayed (e.g. potential carcinogenesis from radiation); the false-positive test result, which may lead to anxiety and unnecessary invasive diagnostic procedures; a false-negative screening test, which may falsely confirm an individual with subsequent clinical signs or symptoms of cancer and hence can delay the diagnosis and effective treatment; and overdiagnosis, i.e. the diagnosis of a condition that would not have become clinically significant had it not been detected by screening. Overdiagnosis is becoming more common as screening tests become more sensitive at detecting tiny tumours. However, an idea of using a group of markers to complement the sensitivity and specificity of each other should prove logical and extremely beneficial. This can be possible in two ways: (1) in series testing, various tests are performed one after the other depending upon the results of the previous test and a test-positive patient would be one who has scored positive in all tests; and (2) in parallel testing, all tests are performed upon all patients and a test-positive patient would be positive for at least one of all tests performed on him.

Factors affecting performance of a biomarker

Biochemical assays used in cancer screening and detection vary not only in accuracy, precision and reliability, but also in performance characteristics, e.g. sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV). The sensitivity of a biomarker refers to the proportion of case subjects (individuals with confirmed disease) who test positive for the biomarker. Specificity refers to the proportion of control subjects (individuals without disease) who test negative for the biomarker. Ideal biomarkers should have a 100% specificity and sensitivity rate, i.e. everyone with cancer would have a positive test for a particular biomarker, and everyone without cancer would have a negative test. The lower the sensitivity, the more often individuals with cancer will not be detected; the lower the specificity, the more often individuals without cancer will test positive. It is the lack of sensitivity and/or specificity that cause many potential biomarkers to be discarded. In addition, the requirements for the performance characteristics of a test vary with the intended use. For diagnostic and monitoring following treatment, high sensitivity is important, whereas for screening, high specificity is required. For example, a test that was 100% specific and 99% sensitive seems to have impressive credentials, but the actual scenario is dismal when the test is used for screening. Screening 100 000 people would yield 99% of the positives, which is an acceptable 'pick-up rate', but would yield 1% false-positives, i.e. 1000 non-diseased. Therefore, it is important to be careful while evaluating the performance of biochemical test and, whenever possible, the receiver operating characteristics (ROC) plots must be generated. ROC curves are used to measure the test accuracy with regard to sensitivity and specificity. The overall performance of the test is measured by the position of the ROC line. While the line for the perfect test will rise rapidly and reach close to the top left-hand corner where both the sensitivity and specificity are located, a test with poor performance will have a line close to the rising diagonal (Figure 3). As with other diagnostic methods, sensitivity and specificity tell us the accuracy of test but not the probability of disease, which is

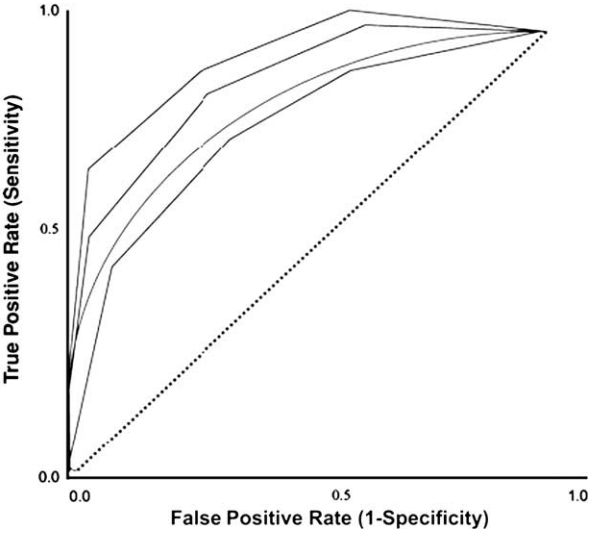


Figure 3. Receiver operating characteristics curve.

Table IV. Factors affecting the performance of biomarkers and biomarkers-based assays.

- Experimental designing
- Quality and source of cancer specimen
- Progressive biological heterogeneity
- Pre-analytical factors such as age, sex, dietary status, smoke exposure, use of tobacco, geographical and environmental factors
- Analytical factors like errors during sample collection and processing, dilution errors, purity of reference standards, cross contamination of selected biomarker, fluctuations in temperature and instrument performance, contamination in purity of chemicals, and calculation errors
- Social and economical issues

governed by predictive values. PPV tells about the percentage of people with a positive test that actually has the disease, while NPV is the percentage of people with a negative test who do not have the disease. PPV provides the information about the likelihood of the disease being present if the test is positive. Therefore, it is clear that in cancer screening there is a need to establish a statistical and inferential framework for evaluating candidate markers in confirmatory clinical trials. It is not a wise step to replace an existing technology unless a biomarker provides adequate information regarding the screening of the disease.

Other than these, there are various other factors (summarized in Table IV) that can affect the performance of a biomarker or biomarker-based assay.

Challenges and futuristic approaches

Challenges to develop ideal cancer biomarkers (characteristics mentioned in Table V) include a better understanding of biological heterogeneity, including host/tumour heterogeneity; analytical factors, such as interference and analytical sensitivity; clinical pathological factors, such as current histopathologic standards; and health service and market factors. Advances in technologies such as genomics, proteomics, and bioinformatics have recently grown exponentially, which have helped greatly in the discovery of new biomarkers for cancer research and detection by unwinding the possible mechanism behind the progression of disease and identification of key growth regulators, which check the cell cycle. Yet in the past 10 years this has resulted only in the discovery of ten new US Food and Drug Administration (FDA)-approved biomarkers (Anderson & Anderson 2002) (Table VI). It is clear that a new approach

Table V. Characteristics of an ideal biomarker:

- Should be highly sensitive and specific for a particular disease (even better organ specific as well) and should be able to reproduce and standardize across different clinical laboratories
- Should be able to detect almost all tumour cells, preferentially at an early stage (i.e. useful for screening)
- Level of its expression, synthesis and release must correlate with the tumour load
- Inherent error in the technical measurement itself (coefficient of variation) should be sufficiently low over the entire spectrum of values for the biomarker, so that small changes in the level of the biomarker reflect true changes in the clinical status of the patient
- Changes in the level of a biomarker for cancer should accurately reflect changes in the patient's clinical status, as well as changes in the patient's prognosis
- Benefits obtained with the use of an ideal biomarker should be applicable to men and woman of all ages, and of all different racial backgrounds
- Detection method should be non-cost intensive

Table VI. Ten new protein analyte biomarkers approved by the US Food and Drug Administration (FDA) under Clinical Laboratory Improvement Amendments (CLIA) regulations since 1993.

Cancer antigen 19–9
B-type natriuretic peptide (BNP)
Cancer antigen 27.29/15–3
Lipoprotein (a) (Lp (a))
Transferrin receptor
Insulin-like growth factor (IGF-II)
Troponin T, cardiac
Troponin-I, cardiac
Interleukin-2 receptor
Insulin-like growth factor-binding protein (IGFBP)

is needed to distinguish accurately between normal cells, preneoplastic lesions, and neoplastic tissue growth. Furthermore, the validational studies using newly identified biomarkers in clinical trials are still to be evaluated. There are many problems still left in this area, such as the development of new analytical methods for cancer screening algorithms and issue such as selecting a panel of markers and how many of the markers should this panel contain for the specific and sensitive diagnosis of various malignancies. Other areas, such as new methods for evaluating biomarkers, the designing of classical population-based screening trials, the selection of an appropriate standard and control population, physician education and patient factors, and reimbursement policies are still to be addressed properly. A lack of awareness about clinical trials can hinder the enrolment of patients in various trials. However, generating awareness in the public through celebrity endorsements can draw people's attention towards cancer screening (Larson et al. 2005). According to Larson et al., at least one-quarter of respondents who had seen or heard a celebrity endorsement said that the endorsement made them more likely to undergo mammography (25%), prostate-specific antigen testing (31%), or sigmoidoscopy or colonoscopy (37%). Physician factors can also play an important role because lack of awareness of available protocols or their specific eligibility criteria preclude many from participating in research trials (Foley & Moertel 1991, Fleming 1994). Also, as the care of patients with cancer has become increasingly complex and multidisciplinary in nature, educating the physicians about the different issues involved in screening, diagnosis, patients care, and the management of treatment-related symptoms and therapeutic procedures can prove an important step in this direction as reported by Smith (2001). In addition to these important factors, a payer-reimbursement policy is a frequently cited barrier to recruiting patients into clinical studies (Fleming 1994). Many private insurers do not routinely cover patient care costs for 'investigational treatments', particularly for patients seeking to participate in phase I or phase II trials (Goodman 1998, Hutchins et al. 1999). The 1997 Board of Scientific Advisors Report of the National Cancer Institute Clinical Trials Program Review Group stated that a 'Lack of third-party reimbursement for clinical trials may be one of the most critical barriers to patient participation'. These are challenging problems that need to be addressed to facilitate the translation of laboratory-discovered biomarkers to valid clinical screening tools.

The National Cancer Institute's Early Detection Research Network (EDRN) (http://edrn.nci.nih.gov) has begun an innovative, investigator-initiated project to improve methods for detecting biomarkers of cancer cells. The EDRN is a consortium

of more than 32 institutions to link the discovery of biomarkers to the next steps in the process of developing early detection steps. The EDRN has three main components: Biomarkers Developmental Laboratories, Biomarkers Validation Laboratories and Clinical/Epidemiology Centers. The Biomarkers Developmental Laboratories conduct studies into the development and characterization of new or the refinement of existing biomarkers; the Biomarker Validation Laboratories serve as a EDRN resource for clinical and laboratory validation of biomarkers to include technological development, the standardization of assay methods and refinement; and the Clinical/Epidemiology Centers conduct clinical and epidemiological research based on the application of biomarkers. An auxiliary Data Management and Coordinating Center (DMCC) provides the statistical, logistics and informatics support. The DMCC also develops the theoretical statistical approaches to the simultaneous pattern analysis of multiple markers. These discoveries will lead to early clinical validation of tests with improved accuracy and reliability. Five years ago, the American Cancer Society (ACS) began publishing a yearly report on its cancer detection guidelines, current issues related to screening and/or testing for the early detection of cancer, and updates on cancer screening rates (Smith et al. 2000). This can help in simplifying the understanding of disease prevention, promoting the recommendations that can reduce an individual's risk for disease, in designing a cost-effective strategy for disease treatment and prevention, and in overall improvement in the health progress.

In summary, the value of biomarkers as an effective cancer-screening tool can be considered only when it is able to reduce the increasing cancer related mortalities by the early detection of precancerous lesions, which are amenable to surgical resection/cure and chemopreventive trials.

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