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Genomic and Proteomic Biomarkers for Cancer: A Multitude of Opportunities

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Abstract

Biomarkers are molecular indicators of a biological status, and as biochemical species can be assayed to evaluate the presence of cancer and therapeutic interventions. Through a variety of mechanisms cancer cells provide the biomarker material for their own detection. Biomarkers may be detectable in the blood, other body fluids, or tissues. The expectation is that the level of an informative biomarker is related to the specific type of disease present in the body. Biomarkers have potential both as diagnostic indicators and monitors of the effectiveness of clinical interventions. Biomarkers are also able to stratify cancer patients to the most appropriate treatment. Effective biomarkers for the early detection of cancer should provide a patient with a better outcome which in turn will translate into more efficient delivery of healthcare. Technologies for the early detection of cancer have resulted in reductions in disease-associated mortalities from cancers that are otherwise deadly if allowed to progress. Such screening technologies have proven that early detection will decrease the morbidity and mortality from cancer. An emerging theme in biomarker research is the expectation that panels of biomarker analytes rather than single markers will be needed to have sufficient sensitivity and specificity for the presymptomatic detection of cancer. Biomarkers may provide prognostic information of disease enabling interventions using targeted therapeutic agents as well as course-corrections in cancer treatment. Novel genomic, proteomic and metabolomic technologies are being used to discover and validate tumor biomarkers individually and in panels.

Keywords

Diagnostic Biomarkers; Epitomics; Genomics; Mass Spectroscopy; miRNA; Proteomics; RNA Expression Profiling

Introduction

Tumor biomarkers are molecules often produced by the tumor itself or the host system in response to the tumor and provide the biological material to determine the risk of getting cancer, to detect cancer, to classify cancer, or to provide insight into prognosis and therefore a therapeutic advantage. Tumor biomarkers include cancer-specific mutations or changes in gene expression or promoter methylation, which can result in alterations in protein expression.

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Because cancer cells shed DNA and RNA in the circulation, a phenomenon rarely seen in healthy individuals, tumor-specific genetic changes such as promoter methylation, gene mutations or circulating small RNAs are detectable in nucleic acids prepared from plasma or other body fluids. Biomarker proteins, either overly abundant or variant proteins can be detectable in the circulation as the free, shed proteins or as novel autoantibodies to such proteins; the latter indicating that the host immune system can be exploited as biosensor of the disease. In addition, tumor-specific biochemical changes result in post-translational modification of proteins via glycosylation or phosphorylation providing a variety of biomarker molecules. Cancer-related biochemical changes often affect measurable metabolic variations within a cell or organism which may be powerful biomarkers.

Cancer biomarkers are discovered and utilized with a specific purpose in mind such as the (a) early detection of cancer, (b) diagnosis, (c) prognosis, (d) response to anticancer therapies or (e) cancer recurrence (Figure 1). Cancer cells provide the biomarker material that can lead to their own detection, which then provides the opportunity for their non-invasive detection in body fluids and tissues so as to reveal the presence of tumors or the level of tumor burden. Biomarkers for cancer diagnosis, prognosis, and response to therapy have become possible through elegant studies of specific carcinogenic mechanisms [1-3] leading to the development of clinical tests capable of predicting the optimal or targeted therapeutic approach for a given cancer. Alternatively, well-powered biomarker discovery projects have also led to clinical tests for therapeutic decisions for some cancers [4-10].

There is an emerging expectation that panels of biomarker analytes rather than single markers will be required to have sufficient sensitivity and specificity for the diagnosis or prognosis of cancer. This realization became apparent from the failure of single biomarkers to be adapted for clinical practice. The need for panels of biomarkers, specific for each cancer, is based on the experience from decades of molecular oncology research that has demonstrated molecular complexity of human carcinogenesis even within single cancer sites.

Biomarkers hold promise not only for diagnostic devices and to improve the effectiveness of clinical interventions but also to stratify oncology patients to the most appropriate treatment. However, early detection must provide a patient with more efficient delivery of healthcare, which ultimately should translate to better survival. For example, early detection technologies applied to population-based screening for cancer of the cervix, colon, and breast have resulted in reductions in disease-associated mortalities from cancers that are deadly if allowed to progress to an advanced stage. These screening approaches are technology driven but have proven that early detection will reduce the morbidity and mortality from cancer.

Biomarkers can provide prognostic information of disease enabling intervention with the appropriate therapeutic agent and early decisions for corrections of cancer treatment. There has been a burst of novel technologies that are being used to discover and validate tumor biomarkers individually and where necessary in panels or combinations of biomarkers. Although biomarkers have the potential to provide personalized diagnostic, prognostic and therapeutic information for the individual patient, the devices to implement them clinically must ensure that healthcare providers have clear algorithms for step by step implementation to their patients.

Analytes for Cancer Biomarkers

There are numerous approaches of analytical biochemistry to identify and validate biomarkers. In the context of this article, we will focus on three general areas of analytes, nucleic acids, proteins and metabolites.

Nucleic Acids Biomarkers

RNA Expression Profiling—The identification of gene expression profiles for stage or prognosis specific classification of cancer patients has become a useful tool in biomarker-driven patient management. Gene expression profiles containing panel of mRNAs can be more effective than more standard methods of patient stratification such as histopathology biomarkers. The discovery of the most useful gene expression biomarkers usually begins with a large scale gene profiling screen on microarrays with a technological progression for clinical application to simple microarrays with fewer probes or multiplexed quantitative RT-PCR. To validate such biomarkers clinically using fresh or archived clinical samples, the RNA preparations must have minimal degradation or alternatively use gene measurement technologies that are not overly sensitive to some reduction in the RNA size. Gene expression profiling coupled with well designed bioinformatics methods have greatly facilitated the identification of genes and pathways that regulate cellular changes critical to cancer development and molecular targets for personalized medicines. The microarray screening procedures are quite reproducible but require biological replicates or multiple samples from a particular class of sample to eventually be able to use the biomarkers for class prediction on an independent validation set. In addition, microarray technologies tend to underestimate the true changes in gene expression so that additional validation using RT-PCR or northern blot analysis is necessary to have confidence in the results. In principle the particular changes in gene expression between classes of samples may be less informative than the pathways they impact. There are numerous software approaches to pathways analysis for a given gene expression dataset. Those that weight the impact of the particular genes provide additional guidance to the identification of shorter lists of biomarkers [11-14].

DNA Sequencing—DNA sequencing is commonly used in research to identify genetic changes in candidate genes. However, the process of discovery of DNA sequence variants with clinical significance has been catapulted ahead by next-generation sequencing technologies. The use of DNA sequence of diagnostic markers is entering into clinical practice for the detection of DNA sequence variants, or small insertions or deletions in genes. Somatic changes in DNA sequences are present in tumor tissue but not present in the normal tissue from the same person. The selective advantage provided by somatic DNA sequence alterations is thought to be a critical part of the carcinogenic process resulting in changes in important growth regulatory genes. Oncogenic DNA sequence variants are useful diagnostic biomarkers and have provided specific molecular targets for cancer therapies, such as the mutant oncoproteins as pharmacologic targets or biomarkers of response to novel targeted agents. Large scale sequencing of numerous candidate genes is providing new biomarkers for studies of diagnosis and prognosis of lung, germ cell, colorectal and breast cancers [15-19]. Somatic DNA sequence analysis has expanded recently from such candidate gene studies to genome-wide high-performance DNA sequencing leading to whole genome analysis of DNA sequence variations in tumors [20].

Epigenetics—There are several normal cellular processes that are regulated in part by epigenetic modification through DNA methylation including developmental imprinting, X-chromosome inactivation and tissue-specific gene expression. Aberrant chromatin-remodeling mechanisms such as DNA methylation and histone acetylation are epigenetic modifications that have been observed in tumor tissues leading to the inhibition of tumor suppressor gene expression. Understanding these mechanisms may provide biomarkers of response cancer chemotherapy. Drugs targeting such epigenetic changes have not yet been developed into gene specific therapies. DNA methylation, histone deacetylase inhibitors, and poly-ADP-ribosylation inhibitors are in clinical trials and may provide novel tools to enhance classical chemotherapies or other targeted therapies, potentially activating tumor suppressors or other key checkpoint genes.

Groups of CpG methylation events in promoters are easily detected by PCR-sequencing of bisulfite-treated DNA from cells or tumors or by methylation specific PCR techniques which permits the interrogation of a few CpG positions. Next-generation DNA sequencing is providing whole genome approaches to discover novel epigenetic changes at the level of DNA methylation. Aberrant promoter methylation has been found in growth suppressive genes in human tumorigenesis [21-24]. CpG dinucleotides are typically methylated in repetitive DNA elements and intergenic regions. In CpG islands, which are CpG rich stretches of DNA ranging from 200 to 2000 bp in length, in the regulatory regions of genes aberrant methylation leads to gene silencing. The transfer of a methyl group from S-adenosyl-L-methionine to the cytosines in CpG sites is catalyzed by DNA-methyltransferases (DNMT) [25]. There are three well-characterized DNMTs, DNMT1, DNMT3A and DNMT3B. DNMT1, the most abundant DNMT, is mainly responsible for maintenance methylation while DNMT3a and DNMT3b are responsible for *de novo* methylation [25].

In human cancers the silencing of tumor suppressor genes through aberrant DNA methylation of a CpG island(s) in the promoters in these genes is a common epigenetic change [22]. There are an assortment of pathways from which genes have been shown to be hypermethylated in cancer cells including DNA repair, cell cycle control, invasion and metastasis. The tumor suppressor genes *BRCA1*, *p16^{INK4a}*, *p15^{INK4b}*, *p14^{ARF}*, *p73* and *APC* are among those that are silenced by hypermethylation although the frequency of aberrant methylation is somewhat tumor type specific [23]. Aberrant hypermethylation of DNA can be reversed with chemical agents that inhibit DNMTs, which in effect ‘demethylate’ DNA. A commonly used inhibitor of DNMT is 5-aza-deoxycytidine (5-aza-dC), a cytosine analog. 5-aza-dC and related drugs work by substituting for cytosine during replication. DNMTs recognize and covalently bind 5-aza-dC in DNA. The covalently bound DNMT1 is unable to catalyze the transfer of methyl groups to the cytosine analog because the substituted nitrogen base cannot be methylated. Consequently DNMT1 is depleted within several rounds of replication. This in turn results in DNA hypomethylation and the re-expression of genes that were silenced by DNA methylation [26-28]. Higher throughput methods are being developed to identify larger panels of methylation biomarkers for disease detection and tumor progression, [29-31]. From such studies, panels of biomarkers for individual cancers are being developed for early detection and response to chemotherapy [32-46]. There are going to be difficulties in laboratory developing standards for implementation in a clinical setting, [47] particularly as a major direction of this field is the early detection of cancer using methylation of circulating tumor DNA in plasma, [48-51] [36].

MicroRNA—Another form of epigenetic modification of gene expression and post-translational alteration of protein expression is through microRNA (miRNA) related mechanisms. Microarray technology can provide the analysis of all known miRNAs similar to that for mRNA profiling. However, procedures for RNA preparations for this purpose must be performed under conditions that include small RNAs. Standardization and clinical testing could be implemented relatively easily using RT-PCR for quantitation of miRNAs. MiRNAs are synthesized by RNA polymerase II as longer transcripts that are processed to pre-miRNAs. Some miRNAs are transcribed by RNA polymerase III [52]. Two RNase III-related enzymes, Drosha and Dicer, process the maturation of the miRNAs [53]. In the cytoplasm, Dicer processes the pre-miRNA into a 22-nt double-stranded miRNA and then one strand of the RNA is degraded. The other strand, as part of the RNA-induced silencing complex (RISC), targets 3' untranslated region of specific mRNAs, destabilizing the target mRNA(s) or repressing their translation [54]. Screening for miRNA expression levels is routinely performed using array technologies to obtain a miRNome profile and validation/confirmation using Northern blot, RNase protection assay, or primer extension assay. Quantitative RT-PCR, in situ hybridization [55] and serial analysis of gene expression (SAGE) have also been applied to these small RNAs [56].

Although the existence of miRNAs in humans has only been recognized for less than a decade, the flurry of activity has identified roles for them in normal development and numerous diseases including cancer leading to a Nobel Prize in 2006. Currently, more than 500 human miRNAs have been identified and they often are found in chromosomal clusters. There is substantial evidence for differential expression of miRNAs in a variety of cancers. MiR-15a and -16-1 are found within a cluster in chromosome 13q14, which is frequently deleted region in B cell chronic lymphocytic leukemia [57]. The miRNome may provide a better classifier of cancer type than the mRNA expression profile and therefore the miRNome appears to be a useful technology for the diagnosis and prognosis of cancer [57]. miRNAs have been found to be repressed by epigenetic mechanisms in cancers [58,59] and consistent with that observation more miRNAs are downregulated than upregulated in cancer when compared to normal tissues [57] [60]. Because of their small size, miRNAs have tremendous potential as biomarkers because they are easily quantified in normal and cancer tissues as well as body fluids [61-64]. However, this field is in its infancy and no miRNA biomarkers are in clinical use.

Protein Biomarkers

Protein biomarkers hold a strong opportunity to be converted into clinical diagnostic tests. Protein biomarkers are often identified in basic science studies of cancer cells as overexpressed proteins. Given the proven ability of most manufacturers of clinical laboratory tests to adapt a protein-based immunoassay on to a standard clinical platform, the expectation of a rapid translation of protein discoveries to a clinical test should be quite rapid and efficient. The difficulty in establishing a clinical test is often at the level of developing antibody pairs for sandwich immunoassays when multiple protein analytes must be assayed in a body fluid such as serum, plasma or urine. If these panels of tumor biomarker proteins form a unit that is used together to classify test subjects for early detection or recurrence and if each member of the panel is required for sufficient accuracy, failing to develop an antibody pair for any single member of the panel will nullify the effectiveness of the clinical test [65]. Cancer-specific alterations in proteins may occur at the level of protein abundance or post-translational protein modification such as glycosylation or phosphorylation. If the protein being developed as a biomarker is present in a body fluid but only the post-translational modification is cancer specific, then antibodies to these specific changes represent a formidable challenge for the development of the antibody pairs. In addition, the issue of matrix complexity is formidable. The concentration range of plasma proteins comprises about nine orders of magnitude [66]. Given that the abundant plasma proteins may be functionally related to the disease processes, depleting abundant plasma proteins may cause the unintentional but direct removal of some important protein analytes or indirectly remove key biomarker proteins that were associated with the abundant protein thus intentionally removing them from the serum or plasma.

There are a number of direct approaches to identify cancer specific changes in proteins including abundance or post-translational modifications. Proteomic changes in cancer can be discovered by a combination of two-dimensional gel electrophoresis for separation of the proteins with a variety of potential methods for their visualization such as direct radioactive labeling, covalent attachment of fluorescent tags, and silver staining.

Mass spectrometric analysis can then be used for the sequence identification of each protein spot [67,68]. This technology is reliable but lacks in the ability to characterize low abundance elements of the cancer proteome. Using two-dimensional gel electrophoresis, approximately, 2000-4000 proteins cancer be resolved and identified from a complex matrix such as serum or tissue. Because of redundancy of multiple isoforms of proteins, the actual identification abundant proteins by two-dimensional gel electrophoresis with mass spectroscopic identification results in as much as 10-fold less actual protein determinations. The complexity can be diminished using enrichment procedures such as immunoaffinity chromatography for

the removal of the most abundant proteins [69] or using less complex matrices such as urine or lavage fluids. These latter body fluids offer a better source of tumor specific proteins if the organ being tested for cancer is bathed in these fluids. However given the low sensitivity of the detection methods, many have migrated to mass spectroscopy-based protein biomarker discovery for undirected high-throughput searches.

Proteomic mass spectroscopy—Proteomic mass spectroscopy-based platforms have provided the ability to identify large numbers of novel proteins with the potential to be biomarkers. These studies are being performed in various biological matrices such as cultures of normal and tumor cells or human clinical samples (serum, plasma, and urine). Mass spectroscopy platforms are generally limited by 1) quantitation of low abundance proteins, 2) whether a protein ion will be able to fly in the electric field and 3) whether the protein or peptides are unchanged in abundance after immunodepletion of high abundance proteins. Proteomic biomarker discovery strategies involve the identification of markers using multi-dimensional protein identification technology coupled with mass spectroscopic identification. Next the biomarkers must be prioritized based on criteria such as cancer relevance and the likelihood for clinical assay development. Lastly, validation of prioritized markers using multiple sample sources is critical as some proteomic mass spectroscopy studies have been biased by study design issues such that day to day laboratory variation or that the samples' origins can be more easily be classified than the essential biological variation such as cancer versus healthy. Proteomic mass spectroscopy-based methods hold promise for the discovery of novel biomarkers that might provide new clinical tests, but to date their contribution to the diagnostic armamentarium has been disappointing [70]. The importance of experimental study design to overall process of biomarker discovery and validation has been underestimated and led to overestimation of accuracy for a set of small protein fragments in an ovarian cancer study using SELDI technology [71-74]. Effective studies using SELDI will require thorough attention to study design and independent validation in order to develop successful protein or peptide biomarkers for non-invasive testing.

Novel proteomic biomarker discovery technologies are being developed using quantitative isotopic labeling, improved mass spectroscopy algorithms and addressing the problems of sample preparation. Abundant proteins are being depleted and multidimensional pre-fractionation is being used to identify low abundance protein biomarkers. Isotopic labeling with deuterated or ^{13}C isotopes of acrylamide to cysteine residues is being exploited for relative quantitation of proteins by MALDI-TOF or LC-MS/MS high-resolution mass spectrometry [75,76]. These and similar mass spectrometry-based technologies generate enormous data sets from complex patient discover studies that challenge current computational analysis procedures [77,78].

Antibody microarrays—Multianalyte protein detection in complex matrices is also being approached using highly parallel immunoassays on antibody microarrays that resemble highly parallel ELISAs. There are a few approaches for this type of biomarker discovery tool [79, 80]. One strategy is to utilize previously validated antibody pairs from sandwich ELISA tests and multiplex the spotted capture antibodies on a surface and then label the detection antibodies for the parallelized assay. As with other ELISA tests, assay interference must necessarily be addressed [81]. Another strategy is to choose candidate overexpressed proteins from other studies and develop pairs of antibodies suitable for antibody microarrays [82,83]. The protein analytes can be measured in serum, plasma, or urine for non-invasive biomarker assays or in liquefied extracts of tumor tissue for evaluation of prognostic biomarkers or potential therapeutic targets [80].

Cancer Autoantibodies—Antibodies in the serum of cancer patients are induced by the humoral immune response to overexpressed or mutant tumor proteins. Cancer autoantibodies

have been found to HER2 protein [84,85], but because these antibodies have been detected in only 5-10% of cancer patients [86,87] their sensitivity for diagnosing cancer is too low. In fact, the sensitivity for p53 autoantibodies is also low even if the patient has p53 missense mutation. Autoantibodies to MUC1 have been found in the sera of women with benign breast disease, as well as invasive breast cancer at early and advanced stage [88]. MUC1 autoantibodies also are found in patients with other cancers also indicating a lack of their specificity [89]. A series of lung cancer antigens was identified by fractionating the proteins from lung cancer cell lines on 2D gels and western blotting with autoantibodies to those proteins from patients' sera [67].

Directly screening for autoantigens can be performed to discover the autoantibodies using the serologic identification of antigens by recombinant expression (SEREX) technology. This method has resulted in the discovery of numerous antigens for breast cancer including annexin XI-A, RPA32, NY-ESO-1, and many other proteins [90-94]. SEREX technology is based on screening tumor derived cDNA libraries made from a particular patient's tumor tissue with serum obtained from autologous patient. As a result, the identified tumor associated antigens, (TAAs) in most cases do not show high reactivity with allogeneic patients' sera. SEREX cloning tends to identify antigens that were overexpressed in the tumor and highly abundant in tumor-derived cDNA libraries. Thus low abundant mRNAs that may be coding for tumor relevant antigens are generally missed. As a prokaryotic expression system is used in this immunoscreening, this approach misses autoantibodies against tumor specific post-translational modifications such as glycosylation, which is frequently part of the epitopes recognized in TAAs. The cDNA clones derived from these studies were not only found expressed in tumors but also in normal testis tissues so they have been called cancer testis (CT) antigens. Several modifications have been introduced to improve SEREX technology. The first variation involves established tumor cell lines instead of fresh tumor specimen as a source of cDNA, thus avoiding contaminating tumor specimens with normal cell RNAs so that they won't be included in cDNA preparation. Also this avoids cDNA cloning of immunoglobulin sequences expressed by tumor infiltrating B cells giving rise to false-positives in the library screening [95].

Second, the screening of sera from cancer patients on allogeneic cDNA libraries including testicular cDNA libraries may result in identification of TAAs and CT antigens that will show higher reactivity with serum IgGs obtained from many different cancer patients. A third modification involves a combination of representational difference analysis (RDA) and SEREX. RDA is a PCR-based subtractive hybridization method which can effectively isolate differentially expressed genes from a given cDNA populations (tester) compared with another (driver) [96]. This combined technology of RDA and SEREX has been applied for the identification of additional CT antigens in melanoma [97]. However, these antigens have not yet provided sufficient sensitivity across a large enough cross-section of cancer patients to provide clinically useful early detection markers.

SERPA, a combination of serology and proteomics technology, is considered to be a "Top-Down" or intact protein analysis approach that involves separation of proteins on two-dimensional gel electrophoresis (2-DE), followed by western blotting, and finally identification by mass spectrometry. Klade *et al.* have combined 2-DE and serological analysis to develop the SERPA technology for the discovery of tumor biomarkers that have mounted humoral immune responses in cancer patients [98]. Using this technology, Hanash *et al.* have reported humoral immune response to calreticulin in pancreatic cancer [99]. In SERPA, three 2-DE gels are run with equal amounts of cell lysates that are made from fresh tumor specimens or cancer cell lines. Two gels are blotted onto nitrocellulose membranes and are processed with serum IgGs obtained from cancer patients and healthy individuals. The third gel (the preparative gel) is stained with coomassie blue. After comparing the immunoreactive spots from the blot of the

cancer patient with that of the control individual, spots that appear brighter or unique to patient are excized from the coomassie blue stained gel. Next, peptide mass fingerprinting analysis is performed using MALDI-TOF MS. The advantage of this technology is that it is less labour intensive procedure than SEREX. Although SDS/urea is used for protein denaturation, post-translational modifications are still intact. Therefore SERPA provides more immunoreactively antigenic epitopes for serological testing than SEREX. Despite the success, this technology has its shortcomings. 2-DE is only effective in identifying highly abundant proteins in patients compared to controls. Also, 2-DE is unable to separate different proteins that can co-migrate on gels due to certain post-translational modifications. With the use of high performance liquid chromatography (HPLC) to resolve tumor cell lysates into several thousand individual protein fractions in combination with multiplex Luminex xMAP technology for serological screening, SERPA can be modified to identify newer tumor diagnostic biomarkers in cancer patients.

Higher throughput tumor antigen cloning can be accomplished using phage display technology coupled with serological identification of tumor antigens and profiling humoral immune responses in cancer patients on protein microarrays [100-02]. Differential biopanning strategy was employed to immunoselect T7 phage tumor-derived cDNA libraries first with control serum IgGs (pooled from different age-matched normal healthy individuals) for the removal of common, non-tumor antigens that bind to IgGs in normal sera. Next, the T7 phage remaining from the cDNA libraries are incubated with serum IgGs from cancer patients to enrich for phage clones bearing tumor antigens. These phage clones are then robotically printed on protein microarrays for immunoassays to identify circulating serum antibodies in those cancer patients mounting a humoral response against cancer antigens. These antigen arrays are then further processed with Cy3 labeled T7 monoclonal antibody that recognizes phage capsid protein, and Cy5 labeled secondary goat anti-human IgG that recognizes IgG from either patient or healthy control bound to the antigens on the arrays. After quantification of the fluorescent signals, statistical analysis is applied to the dataset of dye ratios and then further validated with an independent set of patients and controls [101,102] using immunoassays on the antigen microarrays to validate the biomarkers as a diagnostic predictor of cancer. This approach has been adopted by others in the field of antigen biomarkers [103,104]. The strength of this approach is the development of large panels of biomarkers from the antigen space within the human proteome that are less sensitive to inter-patient variations in the population. This methodology is capable of accurate detection of antibodies in the sera of test subjects. Like SEREX, a limitation is that the phage display technology cannot identify cancer-specific posttranslational modifications such as glycosylation. Therefore, the serum antibody repertoire that this technology identifies is limited to amino acid epitopes of TAAs.

Metabolites and Metabolomics

Metabolomics is the global analysis of the small molecule metabolites produced by normal or abnormal cellular processes. In general metabolomics is focused on chemical profiles using a variety of analytical technologies. The separation technologies involve gas chromatography, high performance liquid chromatography and capillary electrophoresis with detection and identification employing mass spectroscopy and nuclear magnetic resonance spectroscopy. The human metabolome however cannot be defined by any single analytical method [105]. There appears to be greater than 2000 metabolites that are accessible using current technologies which results in a smaller range of potential molecular targets than genomics, transcriptomics or proteomics [106]. Metabolic patterns and biomarker classifiers for tumor staging and stratification have been developed for breast cancer [107-108], prostate [109], and renal cell carcinoma [110]. Although in its infancy and not yet applied to clinical practice, the field of metabolomics should provide a readily accessible and easily measured set of analytes for the classification of cancers for early detection and prognostication. Given the well-known changes in the glycolytic pathways, apoptosis, and phosphometabolic changes that occur upon

carcinogenic progression, the changes in the metabolic profile of adenine nucleotide profiles provides a rich source for biomarker discovery [111,112]. The integration of metabolomics with other omics-related technologies may provide more specific classifiers of alterations related to cancer stage, grade, response to therapy, and prognosis [113-116] [107].

Risk Prediction

Cancer is a disease of somatic cell genetic damage resulting from the failure to recognize and repair mutations in DNA. Many germline mutations that predispose individuals and their relatives to cancer occur in genes that function in maintaining the integrity of the genome. The genes often code for proteins that are involved in DNA damage recognition or repair and also in genes involved in cell cycle checkpoint control. Those individuals born with one mutant allele of such genes are more prone to additional DNA damage and genomic instability and therefore accumulate these events leading to carcinogenic conversion of cells. Frequently, the remaining wild-type allele of the cancer predisposing gene is lost in the tumor cells. Evaluating the likelihood of developing cancer through these genomic biomarkers of risk is usually performed by DNA sequencing of candidate genes. Examples of such genes are shown in Table 1.

Genetic risk factors occur as mutations in DNA repair and cell cycle control genes leading to somatic genetic mutations and cancer. However, less than 10% of cancers can be traced to such Mendelian inheritance. Studies of genes responsible for this enhanced risk of developing certain cancers have led to a series of genetic biomarkers as germline mutations in cancer causing genes (Table 1). The majority of inherited cancer syndromes results in particular types of cancers. These cancers are the indirect result of inherited germline mutations, due to the failure to repair somatic mutations in precursor cells leading to cancer development. The patterns of certain cancers within families with inherited predisposition to cancer will trigger genetics professionals to determine the appropriate genes for clinical testing. The clarity of these observations and determining the gene that should be tested is often complicated by the incomplete penetrance of these genetic factors meaning that not all individuals who carry a germline mutation in a cancer predisposing gene will develop cancer.

Genetic testing is generally performed on peripheral blood lymphocyte DNA because large amounts of DNA can be obtained relatively non-invasively. The mutations when identified are in one of the two alleles of that gene and often the wildtype allele undergoes loss of heterozygosity in the tumor tissue indicating a total loss of function in the tumors. Those individuals who test positive for a germline mutation in a cancer predisposing gene are in need of enhanced medical surveillance and screening using biomarkers for the early detection of cancer.

The identification of genetic alterations that increase the risk for developing cancer are useful for predisposition testing with mutational screening and counseling as part of the established subspecialty of cancer genetics. Despite the promise of this predisposition genetic testing for inherited cancer risk, this information could lead to unforeseen negative consequences unless genetic analysis data information is used properly. Not all health care professionals are knowledgeable in the interpretation of genetic testing results to cancer susceptibility. The complexities include interpreting family history data and choosing the correct gene to be sequenced.

In addition to well-executed molecular technologies for DNA testing, an accurate and detailed family history is the essential risk-assessment tool in the evaluation of a hereditary cancer syndrome. Family history data include details of all cancers on both maternal and paternal sides within the family pedigree. Cancer-free relatives are included because the frequency of both

normal and affected relatives within a pedigree is a key factor in the evaluation of inheritance patterns. Family history data should be confirmed with medical and/or death records.

Early Detection Screening

In order to detect cancer at its earliest stages when it is most curable there is a need for biomarkers to detect individuals harboring occult cancers. The expectation is that early detection tests will be pursued with particular targeted subsets of patients in mind who need more intensive diagnostic imaging after a positive test. One approach for accomplishing this objective is to detect molecular fingerprints of an organ in the process of developing a cancer and then to define biomarkers suitable as targets for treatment prior to a significant tumor burden. Effective biomarker identification depends on multiple levels of study design, each of which must be optimized to ensure the utility of a biomarker or panel of biomarkers for this specific clinical application, early detection. The success of biomarkers for the early detection of cancer is measured by the fact they should not only detect disease early but also reduce the mortality from that cancer.

The choice of cellular tissue or body fluid for the early detection of cancer is clearly dictated by the breadth of the assay sought. A biomarker that can detect one cancer and rule out others will necessarily require a systemic body fluid such as plasma, serum or urine. Alternatively, if a biomarker is to have high sensitivity for a single cancer such breast cancer, then breast nipple aspirate fluid or ductal lavage fluid may provide a very specific matrix in which one would be less likely to find false positive results from other tissues. Likewise a test for a bladder cancer biomarker may reach a higher specificity if measured in urine rather than blood. Whether testing these matrices for early detection, diagnosis or recurrence of cancer, a variety of types of analytes and technologies are available, such as proteins, nucleic acids or metabolites.

Technological Issues

Non-invasive diagnostic approaches to detect early stage cancers may provide the clinician with evidence of cancer, but the criteria for a useful test depend on sensitivity and/or specificity of the test and how the results will affect the pathway of clinical intervention. The consequence of a positive result from an in vitro diagnostic cancer test may involve relatively invasive procedures to establish a true cancer diagnosis. Therefore the in vitro diagnostic test must be both specific, i.e. rarely produce false positive results due to unrelated conditions, and relatively sensitive, i.e., rarely produce false negative results, and only then such screening tests will offer life-saving potential for early detection and possibly a mechanism for personalized therapeutics using disease-related targets.

Various scenarios of diagnostic tests for the early detection of cancer have emerged using genomic, proteomic and metabolomic technologies as discussed above. Because screening tests might involve panels of analytes, these technologies have opened a “Pandora’s Box” of questions on the steps in development of multianalyte clinical tests. Developing the reagents into a format that is amenable to a clinical laboratory is a rather daunting barrier to such a test. Even the development of a generic approach to measure dozens of analytes will require solutions to some unique optimization challenges not the least of which is the informatics leading to a clinical decision. To ensure tests work properly during research and development, during production and in customer laboratories, kits require controls and calibrators for each analyte. A major barrier in multianalyte diagnostics will be the large number of controls and standards required for such a test. For nucleic acids-based tests these controls are more easily prepared. Controls are particularly difficult for protein-based diagnostics. Controls for this approach may consist of cancer cell extracts, cancer patients’ body fluids, or panels of human recombinant proteins for each biomarker. Because patient material containing these biomarkers could be in short supply, the alternative approach of using recombinant proteins for each

biomarker is preferable. However, preparing such each of the recombinant proteins as controls although feasible is clearly a substantial technical challenge in that recombinant proteins will be missing critical cancer-specific post-translational modifications such as glycosylation or phosphorylation. A set of individual standards for multianalyte assays may not be possible, whether they are serum autoantibodies, circulating proteins, or plasma genomic DNA or RNA targets. The danger is that the absence of true molecular standard calibrators may result in a test difficult to implement in clinical laboratories.

The true utility of a diagnostic test requires that the appearance of a positive test result must precede the development of late stage or incurable cancer. However, there is no established method to define early detection. What constitutes early detection may vary among the different types of cancer. Diagnostics for any particular cancer may require either high specificity or high sensitivity depending on the clinical pathway beyond the test. The prevalence of early stage disease and the costs of false positive and false negative result must be weighed against the benefits of early diagnosis. In balancing specificity vs. sensitivity the intended goal of the diagnostic application must be part of the study design from discovery to implementation. An ideal screening test would have very high sensitivity, identifying nearly all individuals with disease. To accomplish this, the test may falsely identify many individuals who do not have cancer, resulting in lower specificity for the panel of biomarkers, leading to unnecessary, invasive, medical testing. Thus it is important that in vitro screening tests have both high sensitivity and high specificity. If test sensitivity is valued over test specificity, significant misclassification in the case of low prevalence cancers may result in unnecessary and expensive medical follow-up. Conversely, valuing specificity over sensitivity may fail to detect cases of cancer. In light of all of these factors, “a one size fits all” approach to diagnostic standards for a multianalyte early detection screening test for cancer may be impossible. The balancing of specificity and sensitivity will depend on the nature of the clinical follow-up for each cancer. A high specificity imaging test following a high sensitivity biomarker screening test may be the best compromise solution.

Current Screening Tests for Cancer

Carcinoembryonic antigen (CEA), first described in 1965 [117], was among the first identified tumor biomarkers. CEA is a biomarker that is elevated in a variety of cancers including colorectal, breast, lung, or pancreatic cancer. CEA in breast cancer detection has low diagnostic sensitivity and specificity [118,119] with frequent false positives in normal individuals. High levels of CEA occur in many other cancers as well. [120]. CEA is a better biomarker of ductal carcinomas than lobular carcinomas of the breast [121]. Because CEA can be found in the serum of patients with ductal carcinoma in situ, CEA may provide a biomarker indicative of an early stage of the cancer [122]. Mucins such as MUC1, or CA 15.3 and CA 27.29, MUC16 (CA 125) [123-125] have low sensitivity for presymptomatic diagnosis of breast cancer [126]. With the advent of mass-spectroscopic protein identification in complex matrices such as serum and plasma, new biomarker proteins have emerged, including HSP27 and the transcriptional regulator 14-3-3 sigma, and the derivatives of the complement component C3a [127-129]. None of these biomarkers has become a useful tool for breast cancer early detection.

A variety of ovarian tumor markers have been studied and the most extensively investigated of these is CA125. This antigen was first recognized in 1981, using a murine monoclonal antibody developed in response to immunological challenge with an ovarian cancer cell line [130]. CA125 levels were found to be increased in 50% of stage I and 90% of stage II ovarian cancers [131]. Although sensitivity for stage I disease using a simple cutoff of 30U/ml was limited, it was apparent that CA125 was capable of detecting ovarian cancer preclinically. The use of a combination of markers to increase sensitivity and specificity has been extensively investigated and the marker that appears to exhibit the most complementarity to CA125 is

OVX1, a monoclonal antibody developed using sequential immunization with three different ovarian cancer cell lines [132]. Increased OVX1 has been found to be elevated in 70% of patients with clinically evident ovarian cancer. In addition, 59% of patients with normal CA125 levels had increased OVX1, suggesting complementarity between the two markers. Although these results indicate improvement in sensitivity, preliminary data from different laboratories suggest that OVX1 may be unstable unless serum is rapidly separated, which could complicate its use in population screening if samples are sent by post. Another serum marker which exhibits complementarity to CA125 is macrophage colony stimulating factor (M-CSF). Among 25 patients with clinically evident tumors and a negative CA125, 56% had an elevated M-CSF serum level [133]. A variety of other tumor markers have also been studied in ovarian cancer. Many of these have been shown to be of insufficient sensitivity or specificity regarding epithelial tumors. Among them, carcinoembryonic antigen (CEA) has been reported to be elevated in 30%-65% of epithelial tumors, mainly in patients with advanced stage disease [134]. CA19-9 is another carbohydrate antigen that can be found elevated in only 17%-25% of patients with epithelial malignancies [135]. Lipid associated sialic acid (LSA) can be detected in serum of about 60% of patients with advanced stage disease [136]. The interleukins, IL-6 and IL-10, have been shown to be present in high levels in the ascites and serum of women with advanced stage epithelial cancer [137,138]. Measurement of serum levels of tumor-associated antigen CA125 [139], in conjunction with ultrasound screening as a second-line test, confers high specificity [140] but detects only approximately one half of early stage cases [141]. Use of multiple serum markers may provide a more sensitive test. Complementarity has been demonstrated between CA125 and two novel markers. Macrophage colony stimulating factor (M-CSF) [142] and OVX1 [132] identify a percentage of patients with persistent ovarian cancer who had normal CA125 levels prior to second look surgical staging procedures. Other new markers such as TN or CASA have not provided additional discriminative value [143]. Serum levels of EGF and its receptor are significantly different between ovarian cancer patients and healthy women and they may provide a potential diagnostic and/or prognostic marker useful for the management of recurrence and late stage cancer [144]. HOXB7 was recently found to be a tumor antigen whose up-regulated expression could play a role in promoting growth of ovarian carcinomas [145]. Urban et al. have characterized the behavior of five serum tumor markers in a large cohort of healthy women [146]. Serial measurements of CA125, HER-2/neu, urinary gonadotropin peptide, lipid-associated sialic acid, and Dianon marker 70/K during six years of follow-up of 1257 healthy women at high risk of ovarian cancer showed that the individual-specific tumor markers behaved independently with substantial heterogeneity among high-risk but cancer-free women. None of these research findings has yet translated into a reliable early detection clinical test.

Molecular Diagnostics for Cancer

Large scale gene expression profiling holds promise for developing molecular portraits of subtypes of human tumors with different clinical outcomes that could not be sub-classified upon initial clinical presentation. One of the possible problems in performing such studies is the number of samples as compared to the number of genes tested, the so-called “curse of dimensionality”. Without independent validation set, studies with many more genes than samples may result in over-fitting of the data thus leading to a gene panel that is only accurate on the discovery sample cohort or at the one site of sample collection. Another issue is that there may be small numbers of genes whose expression discriminate cancer subtypes but they may not be driving of the carcinogenic process and therefore may provide little survival information. Another not surprising issue is that independent studies can identify different panels of genes with similar discriminatory specificity and power. This is likely to be due to the technical differences such as the type of microarray platform used or the algorithm that identified the genetic classifier and because there is redundant information among the individual attributes of gene expression profiles. These points are illustrated below.

One of the earliest applications of gene expression profiling to finding biomarkers for clinical oncology was a project on diffuse large B-cell lymphoma. The International Prognostic Index (IPI) was established as a predictor of outcome in diffuse large-B-cell lymphoma based on clinical characteristics of age, tumor stage, serum lactate dehydrogenase levels, performance status, and number of extranodal disease sites [147]. However, the outcome in patients with diffuse large-B-cell lymphoma who have identical IPI values varies considerably. Gene expression profiling was pursued to develop risk-adjusted classifiers for possible therapeutic outcomes for diffuse large-B-cell lymphoma suitable for clinical practice. One group identified a panel of genes characteristic of normal germinal-center B cells whose expression when elevated indicated a significantly longer survival among patients with diffuse large-B-cell lymphoma than patients whose tumors had low levels of expression of these same genes [148]. Another group identified a panel of 13 genes that was independent of the IPI. Only three of these 13 genes were present in the data analyzed by Alizadeh et al. [148] and of those three, two were associated with survival [149]. Another group used supervised analysis of gene-array data from 160 patients with diffuse large-B-cell lymphoma and developed a 17-gene predictive model which when applied to a set of similar lymphomas from 80 other patients could predict survival [150]. However, there was no overlap with either of the other two classifiers or survival. Another report identified 36 genes that predicted survival in diffuse large-B-cell lymphoma previously discovered from either individual studies or from large published microarray data sets. Using RT-PCR they ranked the genes for their predictive power for longer overall survival. Using a weak z value of ± 1.5 (p -value=0.13) they found that six genes (*LMO2*, *BCL6*, *FN1*, *CCND2*, *SCYA3*, and *BCL2*) in a univariate analysis provided a predictor of survival. This six-gene model was as accurate as the 17-gene model of [150], and was independent of the IPI values in predicting outcome, [151]. However, as yet there is no test in clinical practice derived from these studies.

Progression and Cancer Treatment Selection

In the area of cancer prognosis, the challenge is to weigh the impact of a number of variables, including systemic versus tissue biomarkers that can be used to predict outcome in patients with early-stage cancer. The expectation is there is a subset of individuals who may not benefit from chemotherapy treatments and might be treated surgically with watchful waiting as a follow-up to surgery. Classical prognostic testing based on tumor tissue analyses was inexpensive and easy to perform. Some biological assays such as proliferative index, gene amplification and overexpression of oncogenes or their proteins are being employed in certain cancers as predictors of outcome. These tests have entered clinical practice but far more complex test data are now appearing often employing panels of genes whose expression levels are incorporated with use of algorithms into the test reports. Not only can such tests predict outcome [4-6] [152] [8-10] but also can identify the tissue of origin in metastatic cancers of unknown primary [153,154]. A clinical practitioner will have to identify the appropriate test, or tests, that can predict when a therapeutic approach can improve the patient's outcome or determine whether more classical diagnostic information alone is more clinically useful. A major goal of prognostic testing in cancer is to predict which patients will develop a recurrence of cancer and need treatment which patients do not. The prognostic value of a test is usually expressed in terms of relative risk (RR), which is the ratio of the risk of cancer recurrence in patients who have a positive test to the risk in those who test negative.

Biomarkers of Prognosis

With the advent of whole genome expression profiling, the opportunity to identify features of the transcriptome that indicated a good or poor survival became feasible. Likewise proteomic profiling of primary tumor tissues could also provide a rich source of protein biomarkers that may discriminate differences in survival but the impact of proteomic biomarkers has yet to be

realized. Two gene-expression-based tests have been developed to determine the risk of recurrence in breast cancer patients with stage I or II node-negative breast cancer. The first test, Oncotype DX™, is an RT-PCR-based assay performed on RNA extracted from paraffin-embedded tumor tissue. This test determines the expression levels of 21 genes, 16 of which are cancer-related genes and 5 are control reference genes. The Oncotype DX™ test specifically was developed to predict the risk of breast cancer recurrence in women with ER-positive, node-negative breast cancer using the readily-available paraffin-embedded tumor tissue. The results are used to calculate a recurrence score that predicts the likelihood of cancer recurrence in patients treated with tamoxifen. Women with a low recurrence score need only treatment with tamoxifen, and those women with an intermediate- and high-risk score require additional treatment such as chemotherapy.

The second multianalyte test for breast cancer recurrence is called MammaPrint®. Biomarkers of breast cancer subtypes were identified using patterns of gene expression measured by microarrays [155]. Using hierarchical clustering of gene expression, large gene sets were able to identify five subtypes of breast cancer including basal-like, Her2-overexpressing subtype, two types of luminal cells, and a normal breast tissue-like subgroup. The basal cell type was often seen in carriers of BRCA1 mutations [155]. These molecular classifiers were able to show that patients with the basal and Her 2 subtypes had a good response to therapy. The genes associated with a complete response to chemotherapy in the basal subtype were different than those that defined the Her2-overexpressing subtype [156]. This work has led to the 70 gene panel in the MammaPrint® technology. MammaPrint® involves an oligonucleotide microarray assay to analyze the expression of a panel of 70 genes and is performed on fresh-frozen tumor tissue. MammaPrint® was developed to address early-stage breast cancers so as to evaluate them as having a high or low risk of future metastases. Those women categorized as having a high-risk of metastasis are treated with more aggressive chemotherapy while those categorized as low risk are spared unnecessary chemotherapy. The key to the successful development of these two tests was the establishment of a clear target population who would be candidates for each test and focusing on establishing the parameters of the algorithms for each test on that population. Other prognostic tests for breast cancer are being developed using IHC or gene expression but only MammaPrint® is currently approved by the FDA.

Biomarkers for Unknown Primary Tumors Presenting Initially with Metastases

About 10% to 15% of cancer patients present with metastases without an apparent site of origin at presentation with the cancer. In one third of these patients no primary tumor can be identified histologically and they are designated as having a cancer of unknown primary origin. This situation leaves tremendous ambiguity and often a failure to apply appropriate systemic combination chemotherapy. Clinical evaluation of metastatic lesions of unknown origin is unsuccessful in up to 60% of patients. In response to this clinical need, tissue of origin classification of uncertain primary cancers was developed microarray-based-gene expression profiling using a 1668 gene probe set to evaluate the similarity of tumor specimens to 15 known tissue profiles [154]. A molecular similarity profile of each test tumor specimen's expression pattern is compared to 15 patterns from unique tissue types including bladder, breast, colorectal, gastric, germ cell, hepatocellular, kidney, non-small-cell lung, non-Hodgkin's lymphoma, melanoma, ovarian, pancreatic, prostate, soft tissue sarcoma, and thyroid. For each test specimen, an algorithm reduces dimensionality of the expression data into 15 separate "Similarity Scores", one for each tissue type. This test referred to as Pathwork^{DX} is approved by the FDA for the application of tissue of origin testing of metastases from unknown primary cancers.

Circulating Cancer Cells

The presence of circulating tumor cells (CTC) in the blood of cancer patients was recognized in the 1950s but only recently has technology become available to exploit them as biomarkers for the detection or prognosis of cancer [157]. The detection of CTCs has become commercially developed as a device and can predict which women with metastatic breast cancer will have a better progression-free period and overall survival [158]. This technology has also been applied to the prognosis of prostate, colorectal, and gastrointestinal cancers [159-161]. Molecular events driving cancer development and likewise therapeutic targets such as EGF-receptor mutations are detectable in CTCs, [162] and CTCs provide a novel and very specific diagnostic tool for the future.

Targeted Treatment Selection

Targeted anticancer therapies have resulted from years of research focused on understanding the changes in molecular mechanisms and biomarker differences between cancer cells and normal cells. Classical chemotherapies have killed rapidly dividing cells, a feature of cancer cells. Because certain normal cells proliferate rapidly, chemotherapies often result in significant side effects. By targeting therapy to a molecular mechanism known to differ between normal and cancer cells, targeted therapies have been developed that attack the cancer cells without affecting the normal cells, thus reducing side effects. Targeted therapies by definition provide molecular biomarkers of efficacy, i.e. the mechanisms being targeted. Targeted therapies focus on the functions of the cancer cell predefined as dysregulated thereby providing biomarkers for the choice of the appropriate targeted therapy. These molecules get into the cell and disrupt the particular molecular function of the cells that caused them to be neoplastic.

Once individuals are detected with early disease and potentially gene profiling can indicate the dysregulation of particular pathways, there is a need to identify subsets of patients for specific targeted cancer therapy based on their molecular profiling. Because even within a single cancer histologic type, there are a variety of responses to therapy, identifying one or more critical biomolecules that drive the cancer phenotype in a patient will provide a personalized targeted therapeutic approach. These molecular targets for cancer therapy are the products of years of research. Given the rate of identification of candidate genes, the appearance of cancer specific mutations and potential therapeutic targets will rapidly expand due to emergence of next generation DNA sequencing capabilities as applied to tumor tissues [163-170]. The selection of target-positive patient populations provides efficiencies in more rapid assessment of drug performance. Also smaller patient populations can provide rapid objective evaluation of treatment outcomes.

EGF-Receptor Family—Biomarkers for targeted or personalized cancer treatment using small molecule therapies and antibody-based therapies by necessity requires years of research and development. For example, overexpression of the *HER2* receptor protein often resulting from gene amplification occurs in approximately 25% of breast cancer patients. Her2 overexpressing breast cancers are more aggressive often associated with shortened disease-free and poor overall survival [3] [171-172]. The therapeutic monoclonal antibody trastuzumab (Herceptin®) was directed against an epitope on the extracellular domain of the HER-2 receptor protein. This agent has clinical efficacy against HER-2-overexpressing breast cancers used as a single agent or when used in combination with chemotherapy [173-176]. An early obstacle to adoption of Her2 as a diagnostic target and ultimately of a molecular target of therapy was the development of reliable tests for its gene amplification and protein overexpression [177-178]. Clear Her2 biomarker guidelines are now available for using targeted therapies in breast cancer [179]. Trastuzumab, the commercial name for this monoclonal antibody targeting HER2 is now the first-line treatment of patients with HER2-positive breast cancer. Her2 can

also be targeted using small molecule inhibitors of its tyrosine kinase activity. A critical issue is now the optimal selection of patients for each type of therapy, monoclonal antibody versus small molecule inhibitor. The monoclonal antibodies do not cross the blood-brain barrier efficiently, and although the small-molecule inhibitors are able to cross the blood-brain barrier, they have a shorter half-life in the blood [180]. Lapatinib (commercial name Tyverb[®]) is a dual specificity tyrosine kinase inhibitor of both EGF-receptor and HER2 that inhibits the autokinase activity of these proteins by binding to the ATP-binding sites [181]. Lapatinib in combination with capecitabine in women with HER2-positive breast cancer has significant efficacy in metastatic breast cancers that progressed after failure on trastuzumab-based therapy plus an anthracycline and a taxane [182]. Unfortunately there is no clear set of serum biomarkers of response to trastuzumab plus chemotherapy to indicate when to change to the small molecule inhibitor plus capecitabine.

There are a series of approved therapies targeting the epidermal growth factor receptor (EGFR) protein. Rather than simple overexpression of the wildtype growth factor receptor, the biomarker of efficacy for these agents is a EGFR kinase domain mutation at amino acid 858 changing a leucine to an arginine (L858R) or less frequently, one of multiple possible in-frame deletions in exon 19 [183-185] [162] [186]. Tumors with these mutational biomarkers of efficacy are sensitive initially but acquire resistance to gefitinib and erlotinib due to second site mutations in EGFR. Somatic mutations in the tyrosine kinase domain of the epidermal growth factor receptor (EGFR) gene are associated with sensitivity of lung adenocarcinomas to the EGFR tyrosine kinase inhibitors, gefitinib and erlotinib. Acquired drug resistance is frequently (50%) associated with acquisition of a new biomarker of resistance with a secondary somatic missense mutation substituting a methionine for threonine at amino acid position 790 (T790M).

Bcr-Abl Oncogene—The bcr-abl oncogene, is the result of a translocation of DNA sequences from human chromosomes 9 and 22, often called the Philadelphia Chromosome [187-188] which forms in a new fusion transcript producing a fusion protein from BCR and ABL genetic sequences [2]. This translocation and the bcr-abl tyrosine kinase are present in 95% of patients with chronic myelogenous leukemia (CML), and have been implicated as the cause of this disease. A chemical compound [189], designed to inhibit the Abl protein tyrosine kinase, was evaluated for its effects on cells containing the BCR-ABL fusion protein. Cellular proliferation and tumor formation by BCR-ABL-expressing cells were specifically inhibited by this compound. This compound (STI 571 or CGP 57148B [190] or commercially as Gleevec or Imatinib mesylate) was developed into a highly selective treatment of BCR-ABL-positive CMLs [191-196]. Mutations in the kinase domain of *BCR-ABL* gene alters drug binding, renders CML patients Gleevec-resistant and as such is a biomarker of resistance [197]. In other patients increased BCR-ABL expression is an excellent biomarker of Gleevec resistance [198]. Gleevec is also relatively non-toxic and effective in gastrointestinal stromal tumors with activation of the c-Kit receptor indicating that this receptor is a biomarker of efficacy for this drug [199-203].

Additional oncogene biomarkers resulting from chromosomal fusions of tyrosine kinases, such as ABL and PDGFRA/B, are associated with leukemias resembling CML and have potential for targeted therapeutics and personalized medicine. Gleevec has remarkably improved the outcome of bcr-abl-containing CML, but because some CML patients with a poor response to Gleevec, biomarkers of response beyond detection of the bcr-abl fusion transcript are necessary. Thus, cytogenetic and molecular testing for BCR-ABL and other biomarkers during treatment provides important prognostic information [204-206].

Estrogen and Progesterone Receptors—The expression of estrogen and progesterone receptors has long been known as excellent prognostic biomarkers for breast cancer [1].

Numerous biochemical studies preceded its implementation as a biomarker and therapeutic target [207-210]. As a biomarker, estrogen receptor positivity in breast cancers indicated that that cancer may respond to chemotherapy and chemoprevention with selective estrogen receptor antagonists Tamoxifen and Raloxifene [211-213] or respond in a post-surgical therapeutic setting. In women with advanced metastatic cancer, inhibitors of the enzyme aromatase, which is required for estrogen biosynthesis, can increase the time to progression as compared to tamoxifen [214-216]. In addition, most clinical trials revealed a survival advantage for treatment with aromatase inhibitors in early stage estrogen receptor positive breast cancer [217].

In summary, the use of biomarkers brings the era of personalized medicine for choice of cancer therapy is a new reality. It acknowledges the limitations of one size fits all chemotherapy and provides a rational basis for individualized cancer care.

Recurrence after Treatment

An important area of biomarker use is the surveillance of cancer recurrence after treatment. Early intervention after signs of recurrence provides a clinical advantage. The translocations associated with leukemias and lymphomas are particularly useful molecular markers to determine the presence of minimal residual disease and as quantitative PCR assays of DNA can be used to determine which the tumor burden is increasing. However, for most solid tumors there are few blood-based biomarkers of relapse. Some of the clinically utilized biomarkers are listed below including PSA, CA125, CEA, NMP22, and CA19-9. For additional, biomarkers of relapse see [218].

PSA

Prostate specific antigen, PSA, is naturally secreted from normal prostate cells, but higher levels of PSA in serum have a correlation with the existence of prostate cancer. PSA is probably the only serum biomarker used in a primary care setting. PSA was originally approved for the recurrence of prostate cancer but was used off-label so frequently that sufficient data arose to indicate its use pre-symptomatically. Other conditions can cause increased levels of PSA. Benign prostatitis, infections of the prostate gland, exercise that results in irritation of the surrounding tissues, and digital prostate examination by a doctor can result in a rise in PSA. The rate of PSA elevation and the fraction of free PSA often determine the clinical pathway. PSA elevation above normal will often result in additional diagnostic testing to identify prostate cancer at an early stage. The clinical follow-up is usually digital rectal exam of the prostate, prostate biopsies and imaging. Even with such comprehensive clinical follow-up the majority of treated cases would probably result is no clinical cancer [219]. The high rates of over-diagnosis are evident from the fact that the lifetime chance of prostate cancer diagnosis is 18% but the likelihood of death from prostate cancer is only 3%. This low specificity is causing a considerable amount of unnecessary treatment and better biomarkers for the presymptomatic detection of prostate cancer need to be found. Chromosomal translocations also involving ETS-family transcription factors have been observed in prostate cancer and should provide improvements in the diagnostic specificity for that disease using PSA [220,221].

Cancer antigen 125 (CA125)

Cancer antigen 125 (CA125) also known as muc16, is a protein that binds a monoclonal antibody used for its testing. CA125 is a useful indicator of ovarian cancer recurrence, but as a biomarker for presymptomatic detection of ovarian cancer it has low sensitivity and low specificity. CA125 levels were found to be elevated in 50% of stage I and 90% of stage II ovarian cancers [131]. However, multiple benign diseases both gynecological and non-gynecological conditions can elevate serum levels of CA125 [141] [222], and therefore false

positive rate of CA125 is high. CA125 levels are elevated in people who have pancreatitis, kidney or liver disease, indicating its limited utility as a cancer diagnostic tool. CA125 is a good biomarker of recurrence of a previously treated ovarian cancer.

Carcinoembryonic antigen (CEA)

Carcinoembryonic antigen (CEA) is synthesized during the development of the fetal gut, and is turned on in adult intestinal carcinomas and other cancers. CEA is a biomarker that is elevated in numerous cancers such as colorectal, breast, lung, or pancreatic cancer. Although sometimes used as a screening test, there are several sources of false positive tests such as smoking. CEA testing after surgery for colon cancer is an effective way of determining the reduction of tumor burden and the recurrence of cancer after postoperative chemotherapy. However, there is no survival advantage of metastatic disease colon cancer between patients who went to a second-look surgery based on elevated CEA levels versus other criteria [223].

NMP-22

NMP-22 is a specific nuclear matrix protein involved in DNA synthesis, RNA transcription, the regulation of gene expression, and mitosis. Bladder cancer results in elevation of intracellular NMP-22 up to 25-fold and the protein is shed into the urine. A urine test for NMP22 was approved in a number of formats first for bladder cancer recurrence and later for presymptomatic testing for bladder cancer. The commercial test was reported to be 68% sensitive and 65% specific [224]. Combining NMP22 with a telomerase enzyme assay, called TRAP, resulted in 100% sensitivity and 96% specificity, [225]. However, no combination of biomarkers demonstrated sufficient sensitivity to detection low-stage and grade bladder tumors or carcinoma in situ to replace standard urethrocystoscopy, [226].

CA19-9

CA19-9 is a tumor biomarker that like CEA or CA125 is not sufficiently specific for the presymptomatic detection of gastrointestinal cancers. Non-neoplastic conditions such as gallstones, cirrhosis, pancreatitis, and cholecystitis induce elevated levels of CA 19–9. Although CA19-9 was discovered in colorectal cancer patients, CA19-9 has also been detected in the serum of patients with pancreatic, stomach, and bile duct cancer [227]. Researchers have discovered that, in those who have pancreatic cancer, high levels of CA19-9 are associated with advanced pancreatic cancer. CA19–9 is a useful biomarker to evaluate whether a patient is responding to cancer treatment for bile duct and pancreatic cancers. CA19-9 is also an indicator of recurrence of pancreatic cancer [228].

Chromosomal translocations

Chromosomal translocations, particularly in leukemias and lymphomas can be used to determine the presence of residual disease. This approach was first applied to the early relapse or the detection of minimal residual disease in patients having chromosomal translocation for follicular lymphomas, t(14;18)(q32;q21) [229] allowing the detection of cells with the t(14;18) translocated DNA sequences at 1 cell in 100,000. PCR was also applied to acute T-cell leukemia/lymphoma using the t(10;14)(q24;q11) chromosomal translocation as the biomarker target [230]. The Philadelphia chromosome is a specific biomarker of chronic myelogenous leukemia and has been used to target therapy using the therapeutic tyrosine kinase inhibitor Gleevec. The presence of the translocation protein BCR-ABL-kinase is detected by PCR to monitor response to therapy and the presence of residual disease [231-234]. Likewise for Follicular lymphoma, a common type of Non-Hodgkin Lymphoma, PCR using the characteristic 14:18 translocation is used for diagnosis and the presence of residual disease [235,236] [229]. Interestingly, patients with evidence of minimal residual disease can remain in remission which indicates additional biomarkers are necessary to improve the predictive

values of these assays. For follicular lymphoma additional molecular markers such as ras oncogene mutations and p53 mutations may provide additional prognostic value [237-238].

A summary of leukemia/lymphoma translocations commonly employed clinically as biomarkers of disease, response to therapy and relapse is shown in Table 2, [239].

Sarcomas are highly variable in cell-type of origin, growth properties, and response to chemotherapy. Sarcomas often arise with specific chromosomal translocations that can be used to differentiate various subtypes. For a review of chromosomal biomarkers in sarcomas see [240-243].

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Figure 1.

Table 1
Germline Mutations in Hereditary Cancer Syndromes: Cancer Causing Genes as Biomarkers of Risk

Familial Cancer Syndrome	Germline Mutation Gene Name (Chromosomal Locus)	Cancer Phenotypes
<u>Familial Breast Cancer Syndromes</u>		
Hereditary Breast and Ovarian Cancer	<i>BRCA1</i> (17q21)	Breast–female, Ovarian, Fallopian tube, Primary peritoneal, Prostate Reported: Breast–male, Colon, Pancreas
	<i>BRCA2</i> (13q12.3)	Breast–female, Breast–male, Ovarian, Fallopian tube, Primary peritoneal, Pancreas, Prostate Reported: Colon, Gastric, Gallbladder, Melanoma
Li-Fraumeni Syndrome/Li- Fraumeni-Like Syndrome	<i>p53</i> (17p13.1) <i>CHEK2</i> (22q12.1)	Adrenal cortical carcinoma, Brain (glioblastomas), Breast–female, Leukemia, Lymphoma-NH, Lung, Larynx, Sarcoma (chondrosarcoma, osteosarcoma, rhabdomyosarcoma)
Cowden Syndrome	<i>PTEN</i> (10q23.3)	Breast-female, Breast-male, Thyroid (non-medullary), Endometrium, Renal Benign Lesions: Hamartomatous polyps (stomach, small bowel, colon), Thyroid adenoma/cysts, Breast fibroadenoma, Fibroma, Lipoma, Cerebellar gangliocytomatosis, Hemangioma, Uterine leiomyoma, Neuroma, Papilloma, Trichilemmoma, Acrchordons
<u>Familial Colon Cancer Syndromes</u>		
Familial Adenomatous Polyposis	<i>APC</i> (5q21-q22)	Colon/rectum, Hepatoblastoma, Brain (medulloblastoma), Pancreas, Small bowel, Gastric, Thyroid (non-medullary) Benign Lesions: Desmoid tumor, sebaceous and epidermoid cysts, odontogenic cysts, Lipoma, Adenomatous polyps–large/small bowel, Osteomas, Gastric polyps (non –adenomatous), Dental anomalies, CHRPE
Hereditary Non-Polyposis Colon Cancer/ Lynch Syndrome	<i>MLH1</i> (3p21.3) <i>MLH3</i> (5q11-q12) <i>MSH2</i> (2p21-p22) <i>MSH6</i> (2p16) <i>PMS2</i> (7p22)	Colon/rectum, Endometrium, Ovarian, Gastric, Biliary, Pancreas, Renal–transitional, Small bowel, Brain (glioblastoma) Sebaceous gland Benign Lesions: Adenomatous polyps–large/small bowel, sebaceous adenoma, keratoacanthomas
MYH-Associated Polyposis	<i>MYH (MUTYH)</i> (1p32.1)	Colon/rectum, Small bowel (duodenal) Benign Lesions: Adenomatous polyps (colon, duodenal), Fundic gland polyps (gastric), Sebaceous gland adenoma, Pilomatricomas
Peutz-Jeghers Syndrome	<i>STK11/LKB1</i> (19p13.3)	Breast–female, Colon/rectum, Endometrium, Gastric, Lung, Pancreas Benign Lesions: Adenomatous and Juvenile polyps–large/small bowel, Ovarian sex cord tumor, Testicle sertoli cell tumor
Neurofibromatosis Type I	<i>NF1</i> (17q11.2)	Malignant peripheral nerve sheath tumor, Astrocytoma, Pheochromoctoma, Neuroblastoma, Ependymoma, Rhabdomyosarcoma, Glioma Benign Lesions: Neurofibroma, Pheochromocytoma, Meningioma, Intestinal polyps (hamartomatous), Gastrointestinal stromal tumor, Optic nerve glioma
<u>Familial Endocrine Neoplasia</u>		
Multiple Endocrine Neoplasia Type 1	<i>MEN1</i> (11q13)	Pancreas–islet cell, Amine precursor uptake and decarboxylation tumors, Adrenal cortical carcinoma, Carcinoid Benign Lesions: Adenoma-hyperplasia (parathyroid, pituitary, pancreatic), Sebaceous/Epidermoid cysts, Lipoma, Meningioma, Leiomyoma (uterus), Angiofibroma, Pheochromocytoma

Familial Cancer Syndrome	Germline Mutation Gene Name (Chromosomal Locus)	Cancer Phenotypes
Multiple Endocrine Neoplasia Type 2A, 2B, and Familial Medullary Thyroid Cancer	<i>RET</i> (10q11.2)	Pheochromocytoma, Thyroid (medullary) Benign Lesions: Adenoma-hyperplasia (parathyroid), Ganglioneuroma, Neuroma, Pheochromocytoma
Multinodular Goiter and Papillary Thyroid Carcinoma	<i>TTF-1/NKX2.1</i> (14q13.)	Papillary thyroid carcinoma Benign Lesions: Multinodular Goiter
von Lippel-Hindau disease	<i>VHL</i> (3p26-p25)	Renal (clear cell), Endolymphatic sac tumor, Pancreas- adenocarcinoma/islet cell, Pheochromocytoma Benign Lesions: Hemangioblastoma (CNS/retinal), Adenoma (broad ligament or epididymal) Adenoma –hyperplasia (parathyroid, adrenal cortex/medulla, pancreatic), Cysts (epididymal, hepatic, pancreatic, splenic, renal), Glomus tumor, Paraganglioma, Pheochromocytoma
Retinoblastoma	<i>RBI</i> (13q14.1-q14.2)	Retinoblastoma, Sarcoma (chondrosarcoma, fibrosarcoma, rhabdomyosarcoma, Ewing's sarcoma), Glioma, Lung, Pinealblastoma, Sebaceous Glands, Melanoma, Leukemia, Lymphoma Reported: Bladder, Breast–female Benign Lesions: Lipoma, Retinoma

Table 2
Chromosomal Translocations in Leukemias and Lymphomas

Disease	Genes in Translocation	Chromosomal Target	References
FL	IgH-BCL2	t(14;18)(q32;q21)	[244] Yunis et al., 1987 [229] Lee et al., 1987
ALL	MLL-AF4	t(4;11)(q21;q23)	[245] Kersey et al., 1998 [246] Uckun et al., 1998
PreB-ALL	E2A-PBX1	t(1;19)(q23;p13)	[247] Mellentin et al., 1990 [248] Nourse et al., 1990 [249] Kamps et al., 1990
CML (AML)	BCR-ABL	t(9;22)(q34;q11)	[250] de Klein et al., 1982 [251] Lugo et al., 1990
APL	PML-RARA	t(15;17)(q22;q21)	[252] de The et al., 1990 [253] Longo et al., 1990
AML	CBFB-MYH11	(inv)16p13q22	[254] Hébert et al., 1994 [255] Poirel et al., 1995
Pediatric T-ALL	SIL-TAL1	micro deletion 1p32	[256] Brown et al., 1990 [257] Bernard et al., 1991
Pediatric PreB-ALL	TEL-AML1	t(12;21)(p13;q22)	[258] Romana et al., 1995 [259] Raynaud et al., 1996
AML	AML1-ETO	t(8;21)(q22;q22)	[260] Tobal et al., 1996 [261] Muto et al., 1996

FL: Follicular Lymphoma; ALL: Acute Lymphoblastic Leukemia; PreB ALL: Pre-B cell acute lymphoblastic leukemia; CML: Chronic Myelogenous Leukemia; APL: Acute Promyelocytic Leukemia; Pediatric T-ALL: T-cell Acute Lymphoblastic Leukemia; AML: Acute Myeloid Leukemia