

Review

Biomarkers for early detection of breast cancer: What, when, and where?

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Abstract

Early detection of breast cancer reduces the suffering and cost to society associated with the disease. A sensitive assay to identify biomarkers that can accurately diagnose the onset of breast cancer using non-invasively collected clinical specimens is ideal for early detection. The earlier and more accurate the diagnostic biomarker can predict disease onset, the more valuable it becomes. Here, a brief review of existing and emerging approaches for breast cancer biomarker identification and analysis is presented. Those biomarkers found in biological fluids, blood in particular, apparently hold the best promise for fast development of screening assays. Autoantibodies and abnormal tumor-specific DNA methylation found in cell-free plasma DNA may provide the best opportunity for constructing multiplexed and highly redundant tests, which will be sufficiently specific and sensitive for early detection of breast cancer. It is expected that technologies developed for breast cancer detection will be useful for other types of cancer.

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Keywords: Early detection; Breast cancer; Biomarker; DNA methylation; Autoantibody**1. Introduction**

The burden of cancer involves not only the affected patients and their loved ones, who suffer physically, emotionally and financially, but society in general. The constant drain of socioeconomic resources by the ever-increasing health care costs is well established. The loss of human potential due to cancer-related morbidity and mortality is more difficult to estimate, especially if the ripple effect of the disease on patient's family and friends is also included. Since the incidence of cancer is highest among older people [1], and the population is getting older, the overall costs to society will most likely escalate in the near future. The important question is what can be done to limit the human suffering associated with cancer and to reduce the burden on society. One solution is early detection.

2. Early detection

When the cancer is discovered early, patients live longer, require less extensive treatment, and, in general, fare much

better than patients with more advanced cancer [2]. Data collected by the Surveillance Epidemiology and End Results (SEER) Program of the National Cancer Institute suggest that early detection of cancer still depends on how noticeable is the tumor. The 5 year survival rate of skin cancer patients approaches 95%, hovers between 75 and 90% for breast cancer patients, but is a dismal 15% for lung and 6% for pancreatic cancer (<http://seer.cancer.gov>). The differences in outcomes between cancer in observable sites and in internal organs indicate how frequently tumor detection depends on either direct inspection (in case of skin cancer) or enhanced imaging (e.g., mammography for breast cancer). The better clinical outcomes associated with early detection highlight the need for and the potential benefit of more sensitive techniques for early detection of many types of cancer.

3. Biomarkers

Cancer detection infers that certain characteristics of the tumor are different from corresponding normal tissue and can be seen and/or measured as biomarkers of tumorigenesis. Appropriate biomarkers may be able to define risks and identify the early stages of tumor development, assist in tumor detection and

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diagnosis, verify stratification of patients for treatment, predict outcomes of the disease, and help in surveillance for disease recurrence (Fig. 1). In most cases, different biomarkers will probably be important for different tasks. For example, CA125 is a useful serum biomarker for monitoring ovarian cancer patients and possibly for prediction of the patient’s response to therapy, but has insufficient sensitivity for diagnosis (recently reviewed in [3]). Prostate-specific antigen, on the other hand, has been successfully used both for early detection of prostate cancer and for monitoring disease relapse after surgery, setting an example of a more general biomarker (reviewed in [4]). An excellent discussion of biomarker development for different diseases can be found in the review by Chatterjee and Zetter [5].

4. Biomarkers for early detection

Detection of disease is not synonymous to its diagnosis. In medical practice, identification of disease requires not just recognition of symptoms but also detection of specific features that would unquestionably indicate the presence of a particular disease. Very often, initial symptoms point to a possible group of diseases with similar features, and additional targeted assays typically have to be performed before a diagnosis is established. Diagnoses based on symptoms are unacceptable for cancer because symptoms usually appear when tumors are sufficiently large. As a consequence, if detection of cancer is indeed to be *early*, it has to uncover tumors in *asymptomatic* individuals, so that the tumor-specific assay rises to the level of a *screening* test with associated very specific requirements. To begin, early detection in asymptomatic populations requires a non-invasive (or a minimally invasive) procedure for the assay, which has to be performed using a small amount of starting material. Furthermore, an optimal screening assay has to be able to detect cancer in different organs and establish site of tumor formation (to be *site-specific*). An effective assay also has to rule out confounding non-cancerous events in the same organ or tissue. Additionally, the assay needs to operate at the level of *differential diagnosis*, and has to be observer-independent, with objective measurements of disease biomarkers. Finally, the screening test has to be sufficiently specific to produce an acceptable limit of false-positive results, depending on the

Table 1

Requirements for the early detection assay

Requirement	Consequence
(1) Early detection	Screening of asymptomatic population
(2) Minimally invasive procedure	Body-wide screening Biological fluids Minimal amount
(3) Site-specific detection	Organ/tissue specific
(4) Diagnostic value	Cancer-specific
(5) Observer-independent	Objective measurement of biological feature(s)
(6) Specific	Low number of false-positive
(7) Simple, low cost	Population-wide screening

prevalence of the disease in the population. Key features of an early detection assay are summarized in Table 1.

The progress in the development of early detection of breast cancer makes it a case study to identify the tools that will be effective for detection and diagnosis of other types of tumors, when these tools can be expected to work best, and how to improve their sensitivity and specificity for the earliest possible detection. Taken together, the successes and failures of techniques for detection of breast cancer may reveal promising directions for improvement in biomarker selection and assay development that will be relevant to other cancers. This review discusses different tactics applied to breast cancer detection and provides a broad view of the field. For obvious reasons, detailed description of each technique is beyond the scope of this paper; however, the provided references should serve as a first step for a more in-depth research to the interested reader.

5. Currently available diagnostic techniques

5.1. Imaging

The targeted location of resident breast cancer makes visualization of tumors with different imaging techniques relatively simple. With detection of ductal carcinoma *in situ* at 67.8% sensitivity and 75% specificity [6], mammography sets the standard for cancer detection methods, requiring any newly developed technique to be at least as sensitive and specific. Even so, the accuracy of detection is influenced by different factors, including the density of the breast tissue. For women with dense breasts the sensitivity of mammography is low (45.8%–55% [6]) and is even worse for women undergoing hormone replacement therapy (25% [7]). Moreover, the reading and assessment of mammograms is highly subjective. Experienced radiologists from high-volume hospitals have better success and make fewer mistakes compared to their colleagues from less intensive practices [8]. Finally, imaging-based tumor detection depends on the presence of a certain volume of tumor cells in a specific location, and will likely miss a smaller tumor, which has not yet grown to the required size, or a diffuse tumor, which is not concentrated in one specific site. These limitations of mammography indicate that it does not meet certain of the provisions listed in Table 1; namely, its identification of cancer only in the breast and not

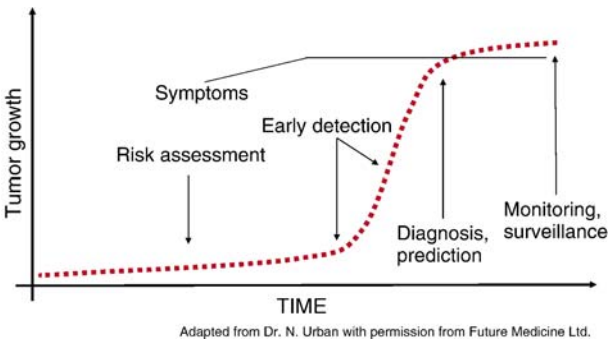


Fig. 1.

the whole body (conditions 1–3); its misidentification of cancer in dense breast tissue (not cancer-specific, condition 4); its subjective interpretation (condition 5), its tendency toward lower sensitivity and higher number of false-positive for women with dense breasts (condition 6). One of the most important advantages of mammography is its relatively low cost and simplicity, which affords for wide screening of the population (condition 7).

5.2. Direct analysis of tumor cells

Breast cancer detection can be done by the direct examination of the cytomorphology of exfoliated cells [9–11], and the molecular analysis of tumor biomarkers [12–14] in nipple aspirate fluid (NAF) or in ductal lavage. The cytomorphology-based assay depends on subjective interpretation by a trained pathologist, whereas, in contrast, the biomarker-based detection is observer-independent. Regardless of the target, a broad screening assay for breast cancer based on NAF is unlikely, since many women fail to produce NAF at all [11,15] (see, however, [16]). To date, performance of NAF-based assays for screening of asymptomatic women has not been tested. It is uncertain how well would such assays perform for detection of lobular carcinomas, which are expected to contribute a smaller amount of measurable biomarker to ductal lumen. In the case of ductal lavage, each individual duct requires separate cannulation, hence the procedure can be very time-consuming. Despite the extensive sampling of ductal epithelium and/or biomarkers in the entire duct rather than in the immediate vicinity of the nipple [17], only a few of existing ducts can be successfully cannulated [18,19]. Recently reported improvements in cannulation for *in situ* visualization of ductal tumors by fiberoptic ductoscopy may overcome some of the technical problems of the procedure [20]. However, even with the best techniques, regular population-wide screening of asymptomatic women by direct visualization of tumor (i.e., ductoscopy or cytomorphology) will remain problematic because of the subjectivity of detection (condition 5). Furthermore, poor correlation has been observed between cytomorphology of the ductal lavage fluid and histological findings in mastectomy specimens [19], indicating that direct examination of exfoliated cells should not be used for early detection of breast cancer at this time. While the sensitivity and specificity of biomarker measurement in ductal lavage or NAF can probably be improved, they will most likely remain labor-intensive and time-consuming and therefore too expensive for population-wide screening of asymptomatic women (condition 7).

However attractive is the direct analysis of tumor cells, indirect diagnostic biomarkers from blood may offer the best potential for use in population-wide screening of cancer. Blood is the inimitable unifying substance that interacts with every cell in the body and integrates all metabolites into a unique and unrivaled system. Tumor cells [21], tumor-specific antigens [22], autoantigens [23], tumor RNA and DNA [24–28] can be recovered from blood, so that it can become the medium for a comprehensive screening test that can detect any type of cancer if the correct test is available.

5.3. Indirect analysis

Considering that *any* analyte can be used for discrimination of normal and abnormal specimens, it is inevitable that many classes of biological molecules have been tested as potential biomarkers of breast cancer: lipids [29,30], carbohydrates (e.g. sialyl Lewis X and sialyl Lewis a) [31,32], polyamines (e.g. N^1 , N^{12} -diacetylspermine) [33], proteins (for a recent review see [22]), RNA [26], and DNA [27,28]. While the first three have not yet been extensively developed, data on nucleic acids and especially proteins as biomarkers for breast cancer are voluminous. In the following sections the discussion of biomarkers will be limited to those used for *early detection*. Outcome prediction and disease monitoring reside outside the scope of this paper and a number of excellent reviews have recently addressed these issues [22,34–37].

5.3.1. Proteins

Circulating proteins have been tested as biomarkers for tumor detection. Carcinoembryonic antigen (CEA), described in 1965 [38], was among the first identified tumor antigens. CEA is a glycoprotein belonging to the immunoglobulin family of genes [39] which is detected in the serum of cancer patients using radioimmunoassay or enzyme-linked immunosorbent assay. The clinical value of CEA detection is limited by a high false-positive rate in normal populations [40], and by low diagnostic sensitivity and specificity [41,42]. An elevated level of CEA is not specific for breast cancer, since CEA can be found in many different types of neoplasia [43]. In breast tumors, CEA is more prevalent in ductal compared to lobular carcinomas [44]. CEA was found in patients with ductal carcinoma *in situ*, suggesting that CEA is an early marker of the tumorigenic process [45].

Glycoproteins of the MUC family have been used to develop several tests for cancer detection. Mucins are components of mammary cell–cell junctions [46] and mediate ICAM-1-initiated signal transduction [47]. Assays based on MUC1 (different epitopes CA 15.3 and CA 27.29) and MUC16 (CA 125) are probably the best known [48,49], although the latter is more frequently used for ovarian than for breast cancer [50]. Other tested members of the MUC family (e.g. CA 549) [51] have insufficient sensitivity for proper diagnosis [52]. Considering their roles in cell–cell interactions and cancer development, it is possible that other mucins may eventually emerge as diagnostic biomarkers [53].

Cytokeratins of intermediate filaments, in particular cytokeratins 8, 18 and 19, have also been tested as cancer markers. Fragments of cytokeratin 19 (CYFRA 21.1 test) [54], cytokeratin 18 (TPS test) and cytokeratin 8, 18 and 19 (TPA test) [22] are proposed as markers for early stages of breast cancer [55], although low sensitivity makes their use problematic. Several other proteins (e.g. MCA, MSA, CA M26, CA M29 and BCM) have been tested for breast cancer detection, although none of them has emerged as a useful biomarker [55–58].

Mass-spectrometry combined with chromatographic separation techniques has revealed several potential protein biomarkers in serum, including the heat shock protein HSP27 and the

transcriptional regulator 14-3-3 sigma, and the derivatives of the complement component C3a [59–61]. Once validated, these biomarkers may become useful tools for breast cancer detection.

Antibodies in the serum of breast cancer patients represent a separate class of potential biomarkers for cancer detection, especially once early tumor antigens are identified and appropriate autoantibodies evaluated. Autoantibodies to HER2/neu protein are present in the bloodstream of breast cancer patients [62], although they are detected in ovarian and other types of cancer as well [63]. Autoantibodies to MUC1 have been reported to be elevated in 14 out of 40 patients (37.5%) with benign breast tumors, 35 of 140 (25.7%) breast cancer patients before treatment, and 11 of 61 patients (18%) with advanced metastatic disease [64], and appear to be related to early stages of breast cancer, although they are also found in ovarian cancer patients [65]. High titer of autoantibodies to endostatin correlates with clinical outcomes [66], suggesting their potential value not only for detection but also for prognosis. Other autoantibodies (e.g. to annexin XI-A, the p80 subunit of the Ku antigen, ribosomal protein S6, RPA32, NY-ESO-1, and other proteins) have been identified using the serologic identification of antigens by recombinant expression (SEREX) procedure [67–72]. The original SEREX screening involved cDNA cloning into a phage vector, followed by infection of a suitable bacterial host, and then screening of lytic plaques on a solid support with sera from cancer patients [67]. To overcome problems with abnormal modifications and folding of selected antigens, they can be expressed on surface membrane of yeast [73]. Another modification of SEREX takes advantage of T7 phage display, in which peptides up to 1200 amino acids can be displayed on the surface as fusion products with the T7 capsid protein [74]. The epitopes reacting with autoantibodies can be enriched by high-throughput biopanning, so that cloning and identification of multiple reactive epitopes is facilitated [23,75]. High-throughput screening is especially important in this case because it allows rapid selection of the most effective epitopes suitable for cancer detection. A recent review of techniques for discovery of cancer-specific autoantibodies (CSA) provides additional in-depth information [76].

The use of CSAs as biomarkers for cancer diagnosis has several advantages, including their early origination, the general methods for analysis, and the potential for high throughput analysis. Numerous CSAs can be incorporated into a multiplexed protein microarray-based assay and analyzed simultaneously in each specimen [75,77] to select the most informative set for cancer detection. Since the immune system responds to tumor emergence very early, CSAs can potentially be detected in patient's serum much earlier than any other tumor biomarkers. These early responses can also be more relevant to *risk evaluation* than to *detection* because presence of CSAs may indicate successful elimination of emerging cancer cells by the immune system rather than its failure to destroy these cells. If that is indeed the case, the presence of CSAs can be expected in every individual, with the repertoire increasing as the person grows older and

new tumor antigens are presented. By the same token, a high titer of a specific CSA may better correlate with robust anti-tumor response, signaling *reduced* rather than *increased* risk of cancer emergence.

5.3.2. Peptides

Analysis of serum peptides by mass-spectroscopy has yielded encouraging results for detection of ovarian cancer [78,79] with a reasonable expectation that the technique can be extended to other types of tumors. Unfortunately, systematic differences in sample preparation rather than biological differences between cancer patients and healthy controls seem to account for observed phenomena [80]. Additional concerns regarding the technology have also been expressed [81], so the full potential of this technique requires further exploration. It is also unclear whether the same technique can be applied to breast cancer detection.

5.3.3. Nucleic acids in circulating cells

Techniques for cancer detection by targeting circulating tumor cells and cell-free nucleic acids have been developed. Usually tumor cells are found in blood during later stages of the disease, so they are considered primarily as a target for detection and monitoring of metastatic disease rather than for early detection (recently reviewed in [82–84]). Considering that circulating cells have been detected in less than 10% of patients with M(0) disease [21], it seems unlikely that they will significantly contribute to early detection of breast cancer. It is possible, however, that development of new techniques for abnormal cell detection, which are unrestricted by the amount of blood drawn (currently 7–20 ml per assay) may open new opportunities for cell-based cancer detection.

5.3.4. Cell-free circulating nucleic acids

Cell-free DNA [85,86], and RNA [87] have been detected in serum and plasma. It is unclear how stable RNA is in the bloodstream, especially considering the high levels of serum ribonuclease in some cancer patients [88]. Cell-free RNA may be protected by association with other macromolecules, so that its stability and resistance to nuclease attacks is augmented. If cell-free RNA is associated with proteins it may present a separate group of epitopes, which may be undetectable by regular techniques. Another unexplored issue is potential specificity in RNA protection, which can either improve plasma RNA-based detection (e.g. if only cancer-specific sequences are preserved) or reduce its accuracy (e.g. if cancer-specific sequences are preferentially destroyed). With only a few studies on cell-free tumor-specific plasma RNA available (reviewed in [89]), its clinical utility remains mostly unknown.

Cell-free plasma (cfp) DNA is present in clinically healthy individuals at a concentration of approximately 10 ng/ml [90–92]. This level can fluctuate 50-fold in the course of 24–30 hr [93], which can probably explain why attempts to use total cfp-DNA concentration for diagnosis have been unsuccessful [85]. Interestingly, fetal cfp-DNA can also be detected in the bloodstream of pregnant women as early as the 30th day of gestation [94]. Both maternal and fetal cfp-DNA increase in maternal

bloodstream in certain cases of pathology [95], and the fluctuations of maternal and fetal DNA appear independent of each other [93]. Since fetal markers are often found in tumor cells, fetal cfp-DNA may represent a unique biomarker for early detection of breast cancer in non-pregnant women.

The origin of cfp-DNA remains unknown; however, it may be released from dying cells via apoptosis or necrosis. Cell-free plasma DNA is increased in tumor-bearing animals after treatment with anti-CD95 antibodies or with acetaminophen, even when no lysis of infiltrating T cells or destruction of endothelial cells was found [96]. Apoptotic cell death may explain the large size of cfp-DNA fragments [96–98]. Alternatively, serum DNase [99] could probably generate similar fragments from high molecular weight DNA. Necrotic cell death, which is postulated to occur from reduced oxygenation of cancer cells in the interior of the tumor, can produce high molecular weight DNA [96,97], and both apoptotic and necrotic pathways may be active at different time points in the same tumor [96]. Interestingly, similar mechanisms are suggested for appearance of fetal cfp-DNA in maternal bloodstream [100]. In this respect, it is tempting to speculate that cfp-DNA is produced by the immune system in the course of elimination (e.g. via induced apoptosis) of foreign or damaged tissues. This mechanism would explain the presence of basal levels of cfp-DNA in healthy individuals [90], its increase in plasma of pregnant women [93], and cancer patients [101], in blood of athletes after excessive training [102], patients with trauma [103] and stroke [104]. It appears that active cell destruction is required to generate cfp-DNA. The simple presence of a tissue with a different genotype is insufficient for the production of cfp-DNA, since placental volume does not correlate with the amount of cfp-DNA released [105]. At least some of the cfp-DNA is present as nucleosomes [106]; whether all cfp-DNA is histone-associated is unknown. It has been reported that large fractions of cfp-DNA are associated with cell surface in healthy individuals but not in breast cancer patients [107], possibly suggesting differences in origin that can be used for more specific detection.

Cell-free plasma DNA has also a mysteriously long persistence—male sequences have been detected in maternal cfp-DNA years after pregnancy [108] implying that this DNA is not cleared from circulation. Similar observations of cfp-DNA longevity have been made for breast cancer [26], although in this case, the presence of occult metastatic disease cannot be excluded. An alternative explanation for cfp-DNA longevity involves incorporation of these sequences into the genome of maternal cells similar to the process during naked DNA vaccination [109] and uptake of apoptotic bodies [110]. Once incorporated, these DNA fragments can then be released via an active process as described for other systems [111–113]. If indeed cfp-DNA is produced in this manner, the implications for cancer biology might be staggering because carcinogenesis via cell-free DNA transmission will need to be considered [114].

The analysis of cfp-DNA for detecting breast cancer can be done by measuring *genetic* and *epigenetic* modifications. Genetic analysis entails measurement of multiple loci for

detection of microsatellite instability, which was observed in only a small percentage of cases [115], or mutations in genes associated with cancerogenesis. For breast cancer the genes for BRCA1/2 [116], different kinases and phosphatases [117], and several others [118] have been tested. While microsatellite instability, as well as mutations, can be detected in cfp-DNA [27,119], the clinical value of the assay is limited by the nature of the analyte and the uncertainty in location of mutations. For example, mutations in p53 genes are distributed throughout several exons [120], so despite established mutational hotspots, the whole gene has to be sequenced; an endeavor impractical for screening. Moreover, only 22% of carcinomas *in situ* contain mutations [120], which are more frequent in higher grade tumors [121] and thus are of little value for early detection. Considering that cancer-dependent changes in gene functionality depend not only on mutations but also on gene regulation through the activity of different promoters, splicing events, or mRNA stability [122–124], a mutation-based approach has its main clinical value for hereditary cancers. Even then a mutation can be used only for risk assessment as has been shown for BRCA1/2 [116,125], but not for cancer detection. It is conceivable that continued development of whole genome sequencing techniques and accumulation of data on specific mutations linked to early cancers may change this outlook and provide a genetic test for early detection of cancer.

Epigenetic analysis of DNA methylation for early breast cancer detection has been pioneered by Evron et al. [126], in comparing the methylation of cyclin D2, RAR- β , and Twist promoters using cells from ductal lavage fluid. Besides detecting cancer-specific methylation in patients with DCIS, these investigators found abnormal methylation in cells from some of the healthy women, who later developed breast cancer. These results provided the first direct evidence that DNA methylation can be used to detect cancer in asymptomatic individuals with non-suspicious mammograms and normal breast examinations [126]. The findings of Evron et al. were confirmed and expanded in later work of the same group [127–129].

The first report of Evron et al. showed that methylation in DCIS is different from that in healthy breast and thus defined a critical shift from *whether* DNA methylation can be used for early detection to *what genes* have to be analyzed to make early cancer detection successful. Methylation of RASSF1A was found in DCIS specimens [130]. In this case, however, breast tissues and not blood plasma were used for analysis. Moreover, the frequency of methylation increased with progression to invasive cancer [130] raising doubts that methylation of RASSF1A would be useful for *early* detection. Jeronimo et al. explored methylation in CDH1, GSTP1, BRCA1, and RAR- β 2 [131] in fine needle aspirates. Although some differences in methylation of RAR- β 2 in benign as compared to malignant lesions were observed, the authors concluded that “the selected gene panel does not discriminate between benign and malignant breast lesions” [131]. Subsequent analysis of methylation in promoters of GSTP1, RAR- β 2, p16INK4a, RASSF1A, and DAPK using NAF showed that in 82% of cases, at least one promoter was hypermethylated both in tumors and in

corresponding NAFs. These results are tempered by the fact that only 7 samples of DCIS were analyzed [132]. At least one of the RASSF1A, APC, and DAPK promoters was found to be methylated in 7 samples of DCIS when tissues were analyzed. In contrast, the same gene was often scored as unmethylated in corresponding serum samples [133].

Recently, breast cancer detection using a specific DNA methylation profile identified in cf-DNA has been reported [134]. When measured in APC, GSTP1, RAR- β 2, and RASSF1A using DNA from tissues and from plasma, DNA methylation was shown to increase with tumor progression. For example, methylated APC promoters were detected in 1 of 24 (4%) subjects with stage I and II cancer, and in 13 of 66 (20%) subjects with stage III and IV cancer. It is unclear whether only patients with ductal carcinoma were considered [134]. Low sensitivity (4%) in patients with stage I and II disease suggests that additional genes may be required to increase the accuracy of the assay and its sensitivity for early stages of breast cancer.

To confirm that an accurate detection assay based on DNA methylation can be developed for ductal carcinoma we used tissue specimens from atypical ductal hyperplasia, DCIS, and invasive ductal carcinoma. The methylation of 56 different promoters was evaluated in each specimen using a methylation-sensitive restriction enzyme to differentiate methylated and unmethylated fragments [135]. When compared to normal breast tissue, the methylation of 20 promoters was substantially different, so the resulting profile identified abnormal specimens with 70–87% sensitivity and 73–95% specificity (Melnikov, submitted). Considering that the assay requires no more than 1 ng of DNA [136], the assay is now used with cf-DNA to generate a diagnostic profile for clinical application.

6. Conclusions and possible future directions

Early detection of cancer by definition must be employed in asymptomatic patients. Therefore, testing for cancer rises to the level of population screening. It is unlikely that a screening test will include imaging techniques, at least until fully automated image analysis is possible. More realistically, a highly redundant blood-based assay which measures many different components within each sample will be developed. Results of this single assay will focus on combinations of elements rather than on individual components, and these combinations (“signatures” or “classifiers”) will indicate not only the presence of a disease but also its localization. In certain ways this type of assay may be similar to the binary computer code where various combinations of multiple elements uniquely identify specific tasks.

Easy access, potential for repeated sampling, and a wealth of already discovered and as yet unknown biomarkers make indirect detection of breast cancer through blood-based assay feasible. What target is selected for such a test will depend on the sensitivity of the analytical technique and on the potential for signal amplification. Today, autoantibodies and DNA methylation-based biomarkers are probably the most attractive analytes for development. The advantage of DNA over autoantibodies as the basis for a singular technique is that it

can be uniformly amplified *ex vivo* to improve analytical sensitivity, whereas autoantibodies are amplified *in vivo* by the immune system, and this process cannot be regulated. The third potential biomarker, cancer-specific peptides, may be profiled from serum using the high sensitivity of mass-spectroscopy and the power of high-throughput proteomics. Such techniques may be sufficient for early detection even in the absence of any amplification. Whether a sufficiently specific peptide profile can be determined for breast cancer remains to be seen.

These three groups of biomarkers offer a possibility of a highly redundant assay. For DNA, every CpG site in the genome can become a separate biomarker. The number of potential tumor-specific peptides or proteins eliciting immune response is equally enormous. According to the current paradigm, abnormal DNA methylation and production of abnormal proteins (and corresponding autoantibodies) occur in asymptomatic patients before the first symptoms of cancer appear, so it is quite possible that either of them or their combination will be sufficiently accurate for breast cancer detection. Whether the same is true for low-molecular weight peptides is still unknown. Combinations of elements measured by autoantibody, peptide, and methylation assays can be easily modified to include additional or exclude uninformative components to form the proper diagnostic classifier. An additional advantage is the availability of multiple platforms for both DNA methylation analysis and for detection of autoantibodies, so the most sensitive, inexpensive, and simple technique can be selected. As a result, the throughput will be high while the cost of each assay is kept low, creating a distinct possibility of population-wide screening. It appears that once the bottleneck of appropriate specimens for assay development and validation is resolved, a working universal test for early detection of breast cancer will be created relatively quickly.

While still inadequate, screening for breast cancer is still much more effective than existing diagnostic tests for ovarian, pancreatic, lung, and many other types of cancer. Will the approaches developed for breast cancer work for other types? We believe that the general principles will be applicable because cancer-specific abnormal DNA methylation and autoantibodies have been found in different cancers (e.g. ovarian [137,138], pancreatic [139,140], lung [141,142], gastric [143,144]). Conceivably, experience accumulated with correlative biomarkers for early detection of breast cancer will serve as a starting point to develop diagnostic tests for other types of cancer as well.

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