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The development of molecular diagnostics for breast cancer

Anna Henrik Israyelyan
Louisiana State University and Agricultural and Mechanical College, aisray1@lsu.edu

THE DEVELOPMENT OF MOLECULAR DIAGNOSTICS FOR BREAST CANCER

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ABSTRACT

Breast cancer is one of the most common malignancies in women. It continues to be a major burden and cause of death among women worldwide. Molecular oncology is now one of the most promising fields that may contribute considerably to diagnosis of breast cancer and its metastases addressing major problems with early detection, accurate staging, and monitoring of breast cancer patients. The overall objective of these feasibility studies was to contribute to improved diagnosis, prognosis, and prediction of breast cancer disease through the development of reagents and protocols for the use of molecular biological advances and the assessment of the relative potential of these diagnostic procedures for the detection and quantification of multiple specific mRNA tumor markers. Newest molecular technologies such as real-time quantitative TaqMan RT-PCR assays, microarray analyses, and production of "in-house" arrays were included in the study. Tissue, blood, and bone marrow samples were obtained from surgeries of confirmed and suspected breast cancer patients. TaqMan assays were performed for six mRNA markers: MAGE 3, HER2/NEU, MGB 1, CK 20, PSA, and HPR. Low-density nylon arrays with 265 immobilized genes included in cell to cell interactions were used for microarray analyses. Three highly overexpressed genes from microarray analyses and negative controls were selected for custom spotting on nylon membranes to produce "in-house" arrays. It was concluded that TaqMan assays can be easily designed and implemented for the screening of a large number of clinical specimens when including carefully selected controls, high purity RNA from samples, and a set of mRNA markers. Custom arrays can be produced incorporating multiple selected mRNA markers. It is suggested that the initial screening of biological samples could be done by microarray

analyses and individual positive samples could be confirmed by additional tests using real-time quantitative TaqMan assays.

INTRODUCTION

Breast cancer is the formation of a malignant tumor that has developed from cells in the breast.

Breast Cancer Current Statistics

Breast cancer continues to be one of the most common cancers and a major cause of death among women worldwide. According to the current statistics of the Centers for Disease Control and Prevention, breast cancer is the most common cancer in women in the United States (excluding skin cancer) accounting for 32 percent of all female cancers. Breast cancer is responsible for 18 percent of cancer deaths in women and is second only to lung cancer. The National Cancer Institute estimates that about 1 in 8 women in the United States (approximately 13.3 percent) will develop breast cancer during her lifetime. This estimate is based on cancer rates from 1997 through 1999 (1). The American Cancer Society estimates that in 2003 approximately 211,300 new invasive cases of breast cancer will be diagnosed among women in the United States (3,800 of those cases will be diagnosed in Louisiana). An estimated 39,800 women will die from breast cancer (700 women will die in Louisiana). It is estimated that 1,300 men will be diagnosed and 400 men will die of breast cancer during 2003 (2).

Breast Cancer Types

Each breast has 15 to 20 sections called lobes, which have many smaller sections called lobules. The lobes and lobules are connected by thin tubes, called ducts (Figure1). The most common type of breast cancer is ductal cancer. It is found in the cells of the ducts. Cancer that starts in lobes or lobules is called lobular cancer. It is more often found in both breasts than other types of breast cancer. Cancers also are classified as non-

invasive (in situ) and invasive (infiltrating). The term *in situ* refers to cancer that has not spread past the area where it initially developed. Invasive breast cancer has a tendency to spread (metastasize) to other tissues of the breast and/or other regions of the body. A less common type of breast cancer is inflammatory breast cancer characterized by general inflammation of the breast. Other rare types of breast cancer are medullary carcinoma (an invasive breast cancer that forms a distinct boundary between tumor tissue and normal tissue), mucinous carcinoma (formed by the mucus-producing cancer cells), tubular carcinoma, etc. (3).

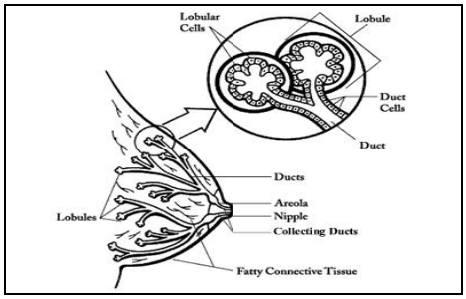


Figure 1: The structure of the female breast

Historical Overview

While the incidence of breast cancer as well as the recovery rate continues to rise, breast cancer is hardly a new affliction (4-8). The recorded history of breast cancer traces back thousands of years. It is no surprise that from the dawn of history doctors have written about cancer. Incidents of breast cancer have been documented back to the early Egyptians when the popular treatment was cautery of the diseased tissue. Surgery was practiced but it was an extremely radical treatment considering there was no anesthesia or antisepsis available.

The oldest description of cancer (although the term cancer was not used) was discovered in Egypt and dates back to approximately 1600 B.C. The Edwin Smith Papyrus describes 8 cases of tumors or ulcers of the breast that were treated by cauterization, with a tool called “the fire drill”. The writing says about the disease, “There is no treatment”.

The origin of the word cancer is credited to the Greek physician Hippocrates (460-370 B.C.), the "Father of Medicine." Hippocrates used the terms carcinos and carcinoma to describe non-ulcer forming and ulcer-forming tumors. In Greek these words refer to a crab, most likely applied to the disease because the finger-like spreading projections from a cancer called to mind the shape of a crab. Carcinoma is the most common type of cancer.

According to the doctrines of the Greek physician Caudius Galen (130-200 AD), whose works on physiology and anatomy dominated medical thought until the Middle Ages, melancholia was the chief factor in the development of breast cancer. Special diets were the recommended treatment. However, other treatments included exorcism and the use of topical applications which were seldom preferred by patients.

During the Renaissance, Andreas Vesalius recommended mastectomy as well as ligatures (sutures) to control the bleeding rather than cautery. Recognition that breast cancer could and did spread to the regional auxiliary nodes was first recognized by the physician LeDran (1685-1770). Dr. LeDran was likely the first to associate poor prognosis with the spread of breast cancer to the lymph nodes.

The famous Scottish surgeon John Hunter (1728-1793) suggested that some cancers might be cured by surgery and described how the surgeon might decide which cancers to operate on. If the tumor had not invaded nearby tissue and was “moveable”, he said, “There is no impropriety in removing it”.

During the mid 1800's, surgeons first began to keep detailed records of breast cancer. Those statistics indicate that even those treated by mastectomy had a high rate of recurrence within eight years—especially when the glands or lymph nodes were affected. Nevertheless, the common treatment was to remove the breast and the surrounding glands in an effort to stave off any further tumor development.

In 1894 William Roentgen discovered X-rays. This paramount discovery shed light on the detection of many diseases as well as breast cancer. Some years later, in 1913, Albert Solomon, a pathologist in berlin, produced images of 3,000 gross mastectomy specimens. He observed black spots at the centers of breast carcinomas (microcalcifications).

Between the 1930's and the 1950's treatment and detection improvements were noticeable. This was the time when Stafford Warren (Rochester memorial Hospital, New York) developed a stereoscopic system for tumor identification. Also, doctors started classifying the stage and progression of breast cancer. In 1949 Raul Leborgne (Uruguay) emphasized breast compression for identification of calcifications. In 1940s-1950s breast self-examinations were advocated.

In 1960 Dr. Robert Egan (Houston) adapted high-resolution industrial film for mammography, allowing simple and reproducible mammograms with improved image detail. And in 1963 the first randomized controlled trial of screening by the Health Insurance Plan of New York found that mammography reduced the 5-year breast cancer mortality rate by 30 percent. Major improvements in mammography equipment, such as reduced radiation dosage, digital imaging, and computer-aided diagnosis, improved detection of breast cancer.

Breast Cancer Risk Factors

Every woman is at risk for developing breast cancer. Several relatively strong risk factors for breast cancer that affect large proportions of the general population have been known for some time. However, the vast majority of breast cancer cases occur in women who have no identifiable risk factors other than their gender (9).

The "established" risk factors for breast cancer are female gender, age, previous breast cancer, benign breast disease, hereditary factors (family history of breast cancer), early age at menarche, late age at menopause, late age at first full-term pregnancy, postmenopausal obesity, low physical activity, race/ethnicity and high-dose exposure to ionizing radiation early in life.

The "speculated" risk factors for breast cancer include never having been pregnant, having only one pregnancy rather than many, not breast feeding after pregnancy, use of postmenopausal estrogen replacement therapy or postmenopausal hormone (estrogen/progestin) replacement therapy, use of oral contraceptives, certain specific dietary practices (high intake of fat and low intakes of fiber, fruits, and vegetables, low intake of phytoestrogens), alcohol consumption, tobacco smoking, and abortion.

Although men can and do develop breast cancer, the disease is 100 times more likely to occur in a woman than in a man (10). Women are at a higher risk of breast cancer because they have much more breast tissue than men do. Also, estrogen promotes the development of breast cancer.

The risk of breast cancer is higher in middle-aged and elderly women than in young women (10; 11). This risk increases as a woman ages, rising sharply after the age of 40. In

the United States, more than three-fourths of all breast cancers occur in women aged 50 or older.

A woman who has previously had breast cancer has a three- to four-fold increased risk of developing a new cancer in the other breast. Women who have had benign breast problems are also at increased risk but to a lesser extent (12; 13). The risk of breast cancer is higher among women who have a close blood relative (mother, sister, or daughter) who have had the disease. The increase in risk is especially high if the relative developed breast cancer before the age of 50 or in both breasts (14). However, most women who get breast cancer (approximately 80 percent) have no such family history of the disease (15). The effect of family history on breast cancer risk is believed to be due primarily to genetic factors. As much as 5–10 percent of all breast cancer cases are attributable to specific inherited single-gene mutations, and many other cases have some genetic component. The evidence from individual families in which breast cancer occurs very frequently and from large epidemiological studies has shown that some women have a familial predisposition to breast cancer. The evidence includes the pedigree of Broca's family (16). He was a famous French surgeon (1824-1880), and in his family tree (comprising over five generations) 10 out of 24 women died of breast cancer.

Epidemiological studies have shown that in women with a family history of breast cancer, the risk of breast cancer is increased two- to threefold. Studies have also shown that there are families in which breast cancer risk is inherited in an autosomal-dominant fashion ('hereditary breast cancer'). Recently, it has been shown that germline mutations in the BRCA1 and BRCA2 genes account for a large proportion of cases of hereditary breast cancer (17). Histopathological findings and careful autopsy examinations have played a

major role in the recognition of many familial cancer syndromes (18). In addition to mutations in the BRCA1 and BRCA2 genes, there are as yet unidentified genetic defects that predispose to breast cancer development (19), and additional studies may help in identifying these genes in the future.

Women who reach menarche at a relatively early age (12 or younger) and those who reach menopause at a relatively late age (55 or older) are slightly more likely than other women to develop breast cancer (13). These relationships are believed to be mediated through estrogen production (20). During the reproductive years, a woman's body produces high levels of estrogen. Women who start to menstruate at an early age and/or reach menopause at a late age are exposed to high levels of estrogen for more years than are women who have a late menarche or early menopause.

Age at first pregnancy is another aspect of reproductive history that is associated with breast cancer risk. Women who have their first full-term pregnancy at a relatively early age have a lower risk of breast cancer than those who never have children or those who have their first child relatively late in life (13). The biologic basis for this relationship is not entirely clear.

Obesity has been consistently associated with an increased risk of breast cancer among postmenopausal women (21; 22). This relationship may be mediated again by estrogen production. Fat cells produce some estrogen and obese postmenopausal women, therefore, tend to have higher blood estrogen levels than lean women.

Studies have consistently shown that the risk of breast cancer is lower among physically active premenopausal women than among sedentary women (23; 24). Physical activity

during adolescence may be especially protective, and the effect of physical activity may be strongest among women who have at least one full-term pregnancy.

Studies of racial/ethnic characteristics of breast cancer reveal that non-Hispanic white, Hawaiian, and black women have the highest levels of breast cancer risk. Other Asian/Pacific Islander groups and Hispanic women have lower levels of risk. Some of the lowest levels of risk occur among Korean and Vietnamese women (25).

Women who were exposed to high doses of radiation, especially during adolescence, have an increased risk of breast cancer. This association has been observed both among atomic bomb survivors and among women who received high-dose radiation for medical purposes (26; 27).

Parity (having children) and the age of the woman at the birth of her first offspring are other endogenous hormonal factors that influence breast cancer. Women who have never had children (nulliparous) are at greater risk for the development of breast cancer than women who have had children (parous). There is also consistent evidence that first pregnancy completed before age 30-35 lowers risk of breast cancer, and that first full-term pregnancy after age 30-35 raises risk. More limited evidence suggests that women who have many pregnancies may be less likely to develop breast cancer than those who have only one pregnancy (13).

Some studies have shown that women who breast-feed their babies may be less likely to develop breast cancer than those who have children but do not breast-feed (28). Other studies, however, indicate that there may be little or no relationship between breast feeding and breast cancer risk. If breast-feeding does protect against breast cancer, it may

do so by delaying the resumption of ovulation (with its accompanying high estrogen levels) after pregnancy.

The long-term (more than five years) use of postmenopausal estrogen therapy (ERT) or combined estrogen/progestin hormone replacement therapy (HRT) may be associated with an increase in breast cancer risk (29).

The associations between the use of oral contraceptives and breast cancer have been studied. Many studies attempting to link oral contraceptives with increased breast cancer have been inconclusive (30). But these studies have shown that oral contraceptives do not have a large effect on breast cancer risk. Whether they have a small effect on risk is less clear.

A possible relationship between breast cancer and diet has been suggested due to the variation of breast cancer in societies with different national diets (the high rates in Western industrialized nations and the low rates in Asia, Latin America, and Africa). A comparison of vegetarian versus meat-eating women produced inconclusive results. No relation between breast cancer risk and total fat, saturated fat, or cholesterol was found. Some of the effects that were once attributed to dietary fat intake were probably due to obesity (which is often linked with high fat intake) rather than to fat intake *per se*. And the effects of fiber, fruits, and vegetables now appear to be small, at best. Diets high in fruits and vegetables and low in fat and calories are healthful for many reasons, and they may indirectly reduce the risk of breast cancer by helping to prevent obesity.

Plant substances called isoflavones (sometimes referred to as phytoestrogens) are most commonly found in soy products. It has been speculated that these substances may be

protective against breast cancer (31). They appear to have effects similar to those of estrogen in some tissues while antagonizing the effects of estrogen in other tissues.

A positive, but modest association between alcohol use and breast cancer risk is seen in most studies (32; 33). There is also some evidence that cigarette smoking may be associated with a small increase in breast cancer risk. However, epidemiological studies have variably shown positive, inverse, or null associations (34). Among women who have already been diagnosed with breast cancer, smoking may be associated with an increased risk that the cancer will progress more rapidly. In some studies, premature termination of pregnancy appears to increase breast cancer risk (35). In incomplete pregnancy, the breast is exposed only to the high estrogen levels of early pregnancy and thus may be responsible for the increased risk seen in these women. However, some other studies found no association between abortions and increased risk of breast cancer (36).

Stages of Breast Cancer

The staging systems currently in use for breast cancer are based on the clinical size and extent of invasion of the primary tumor (T), the clinical absence or presence of palpable axillary lymph nodes and evidence of their local invasion (N), together with the clinical and imaging evidence of distant metastases (M). This is then translated into the TNM classification which has been subdivided into Stage 0 called carcinoma in situ (lobular carcinoma in situ (LCIS) and ductal carcinoma in situ (DCIS) and four broad categories by the Union Internationale Centre Cancer (UICC), which are the following.

Stage I – early stage breast cancer where the tumor is less than 2 cm across and hasn't spread beyond the breast.

Stage II – early stage breast cancer where the tumor is either less than 2 cm across and has spread to the lymph nodes under the arm; or the tumor is between 2 and 5 cm (with or without spread to the lymph nodes under the arm); or the tumor is greater than 5 cm and hasn't spread outside the breast.

Stage III – locally advanced breast cancer where the tumor is greater than 5 cm across and has spread to the lymph nodes under the arm; or the cancer is extensive in the underarm lymph nodes; or the cancer has spread to lymph nodes near the breastbone or to other tissues near the breast.

Stage IV – metastatic breast cancer where the cancer has spread outside the breast to other organs in the body.

The Past and the Future of Breast Cancer Detection

Increased breast cancer awareness with breast self-examinations and major improvements in routine breast cancer screening had a paramount effect on early detection of breast cancer. Improvements in conventional mammography (an x-ray technique to visualize the internal structure of the breast) such as the low radiation dosage, enhanced image quality, development of statistical techniques for computer-assisted interpretation of images, long-distance, electronic image transmission technologies (telemammography /teleradiology) for clinical consultations, and improved image-guided techniques to assist with breast biopsies (the removal of cells or tissues for examination under a microscope) continue to lower the morbidity and mortality of breast cancer. The support of research on technologies that do not use x-rays and are not used for routine breast cancer screening, such as magnetic resonance imaging (MRI), ultrasound, and breast-specific positron emission tomography (PET) may play a considerable role in further improvements of

breast cancer early detection. In most cases, the earlier breast cancer is detected, the better the survival rate. Today mammography is the best available method to detect breast cancer in its earliest, most treatable stage - an average of 1.7 years before the woman can feel the lump. Generally, treatment is most effective before the disease spreads. When breast cancer is diagnosed at a local stage, the 5-year survival rate is greater than 90%. This rate decreases to less than 50% when the disease has spread to the lymph nodes and less than 20% when it has spread to distant organ sites.

Despite recent progress in early detection and surgical therapy, the mortality due to breast cancer has changed little over the past decades, primarily because the occult dissemination of cancer cells can occur at an early stage of carcinogenesis. Occult dissemination of tumor cells in patients with operable cancer can subsequently lead to formation of metastasis, yet it is usually missed by conventional tumor staging. The success of routine mammography screening for breast cancer is that it involves increasingly more patients with small primary tumors formerly thought to have an overall excellent prognosis. Yet, only approximately two thirds of these patients actually have this favorable prognosis, while the remaining third develops metastatic disease. Thus, there is emerging evidence that tumor cells can disseminate into secondary organs at an earlier stage of primary tumor development than appreciated by current risk classifications. There are several challenges that must be addressed in an effort to continue to lower the mortality associated with this disease.

Molecular oncology is currently one of the most promising fields, which may address the major problems with early detection and accurate staging of women with breast cancer. The advent of highly sensitive, molecular techniques, such as the polymerase chain

reaction (PCR), enables the detection of circulating tumor cells and small metastasis at the molecular level. PCR-based assays are used for the detection of tumor cells in lymph nodes, resection margins, bone marrow and blood. Methods to detect metastatic disease and circulating tumor cells at a molecular level are of two types: those that detect somatic events such as point mutations or chromosomal rearrangements, and those that detect expression of tumor specific mRNAs. Both methods have been applied for the detection of many different tumor types (37). The main limitation of the first method is that not all tumors contain mutations suitable for PCR amplification. For the second method to work, the molecular marker must be transcriptionally elevated in malignant cells and not in the surrounding cells or tissue.

Gene amplification/overexpression is a common event in the progression of human cancers, and amplified genes have been shown to serve as molecular markers and have diagnostic, prognostic and therapeutic relevance. Currently, molecular markers offer the unique opportunity to identify occult metastases in early stage cancer patients not otherwise detected with conventional staging techniques. The completion of the human genome as well as an enormous amount of information on the transcriptional activities in cancer cells enable the selection of specific markers for the detection of cancer cells. The ideal prognostic marker is one that clearly delineates a particular prognostic group, is 100% specific, highly sensitive, inexpensive and easy to perform on a small quantity of fresh or fixed tissue. No such marker exists but a number of potential prognostic markers have been extensively investigated. Multiple proteins have been found to be specifically overexpressed in certain types of tumors (i.e. Her2neu, PSA, p53, pRB, melanoma antigens, etc.). Detection and quantification of potential tumor markers using sensitive

molecular methods could assist in the early diagnosis of cancer disease as well as in the efficacy of anti-cancer therapy. The clinical application of molecular markers in the diagnosis, staging, and management of breast cancer continues to expand. The molecular detection of circulating tumor cells and micrometastases may help develop new prognostic markers. Extensive work by various groups has been done on minimal residual disease (MRD) detection in blood, bone marrow and lymph nodes in cancer patients with different types of cancer. In the past 10 years, numerous investigators have attempted the detection of occult tumor cells in malignancies using the highly sensitive reverse transcriptase polymerase chain reaction (RT-PCR) technique. This is a particularly sensitive technique for the purpose of detecting occult breast cancer cells in the blood, bone marrow, and lymph nodes of breast cancer patients (38-40). It is simple, rapid, and semi-automatic, and is an alternative method to fluorescence *in situ* hybridization and immunochemistry. RT-PCR has produced sensitivity levels of 1 tumor cell in 1,000,000 normal cells. It can detect micrometastases based on amplification of mRNA expressed exclusively in the cancer cells of interest or in significantly larger amounts in cancer versus non-cancer cells localized to the lymph node, other distant organs or circulating in the blood. RT-PCR may be a powerful tool for large randomized, prospective cooperative group trials and support future tumor-marker based biological and gene-therapy approaches. The labor intensive and time consuming pathological investigations can be minimized or substantially assisted using automated RT-PCR assays. These assays, in the vast majority, have been directed against tissue specific markers. In most studies on prostatic carcinoma, RT-PCR was able to specifically detect prostatic tissue specific markers in the peripheral blood, bone marrow and lymph nodes of patients with localized and metastatic disease. Melanoma related

transcripts were detected by RT-PCR in the peripheral blood, bone marrow and lymph nodes of patients with localized and advanced tumors. Many authors have shown a statistically significant correlation between RT-PCR positivity and a poorer outcome in both melanoma and prostatic carcinoma. In breast carcinoma, all markers that have been extensively tested were shown to be non-specific.

The presence or absence of axillary lymph node metastasis is still the single most reliable predictor of the final outcome in breast cancer and the primary determinant for the use of systemic therapy in patients with newly diagnosed cancer confined to the breast. Although these patients are considered as potentially curable, a substantial number of them develop recurrent carcinoma and die of their disease in 5 to 10 years, including nearly 30% of patients with negative axillary lymph nodes (41). As mentioned earlier, occult dissemination of tumor cells in the patients with operable cancer can subsequently lead to formation of metastases, yet it is missed by conventional tumor staging with histopathology and immunohistochemistry in the lymph nodes. The blood and bone marrow are not currently routinely examined for tumor cells in women with breast cancer. However, it is likely, that if these sites were routinely assessed, tumor cells would be identified. Ultimately, knowledge of tumor cells' presence in the blood or bone marrow may impact the survival of breast cancer patients via earlier detection and initiation of adjuvant therapy. A reliable RT-PCR assay has not been developed for breast cancer. The targeted tumor markers for detection by RT-PCR in breast cancer patients have not been identified as in melanoma. In addition, because of the extreme sensitivity of RT-PCR technique, an accepted cut-off value that defines tumor presence or absence has not been established. However, it is widely accepted that the detection of mRNA tumor markers and

well-defined experimental protocols can greatly improve the sensitivity, specificity, and reliability of the RT-PCR assay system.

Breast tumors are composed of a heterogeneous collection of cells with differing levels of individual gene expression. Therefore, the predominant cell type or its metastasis may not express a particular marker (42; 43). Therefore, it is believed that multimarker approaches with a panel of tumor-specific mRNA markers may improve the sensitivity and specificity for the detection of tumor cells over single marker assays in breast cancer patients. Furthermore, tumors continuously evolve genetically over time in response to host pressures and treatment interventions, which suggests that single marker testing may not be able to effectively monitor cancer progression. Simultaneous detection of such markers by newly developed methodologies such as real-time quantitative RT-PCR will enable the accurate monitoring of the level of mRNA markers as well as the precise comparison to known internal mRNA standards.

Lymph node metastatic involvement is arbitrarily subdivided into micro- and macrometastases, usually according to the size of the tumor deposits, with the cut-off point ranging from 0.2 to 2.0 mm (44; 45). Serial sectioning and immunohistochemistry appeared to increase the detection rate by 9-33%. A definite survival disadvantage was noted for patients with such occult metastases. The use of extensive serial sectioning and immunohistochemistry on all axillary lymph nodes is too expensive and labor-intensive to be practical. Sentinel lymph node (SLN) is the first lymph node in the axillary basin to receive metastases from the primary breast cancer if they have occurred. The concept of SLN was introduced by Cabanas (46). The SLN accurately predicts the pathology of the remaining axillary basin allowing a focused pathologic analysis of it (47). If the SLN does

not contain tumor, the chance of tumor in the remaining axillary lymph nodes is less than 1%. Therefore, patients without tumor in the SLN can avoid unnecessary axillary lymph node dissections (ALND). A SLN biopsy in comparison to an ALND has significantly less morbidity in terms of lymphedema, numbness to the arm, and decreased range of motion. The assessment of the SLN at the time of initial diagnosis of breast cancer may improve upon the current staging molecular method - multiple mRNA marker RT-PCR analysis, which would identify the sub-group of patients with metastases in the SLN but thought to be free of disease by conventional pathologic examinations. Therefore, the analysis of micrometastatic cells opens a new avenue by which to assess the molecular determinants of both early tumor cell dissemination and subsequent outgrowth into overt metastases.

Tumor cell detection in the bone marrow is being regarded increasingly as a clinically relevant prognostic factor for breast cancer. Many studies suggest that tumor-cell shedding already occurs during the early stages of breast cancer and have demonstrated a significant correlation between tumor cell detection in the bone marrow and decreased disease-free and overall survival (48-50). Studies have shown that breast cancer patients may harbor bone marrow metastases alone or in conjunction with axillary metastases. These studies suggest that the tumor status of the bone marrow may be a better prognostic indicator than the axillary lymph node status (51). Since the primary breast tumor can spread by both the lymphatic and hematogenous route, it is possible that patients may have metastases to the bone marrow and not the axillary lymph nodes. It is now clear that bone marrow is one of the most prominent secondary organs to receive disseminated tumor cells and is an important determinant for micrometastatic organ involvement due to its ease of accessibility and normal physiological absence of epithelial cells. Bone marrow aspirates

can be easily obtained from breast cancer patients at the time of surgery. The technical feasibility and the potential prognostic significance of bone marrow metastases makes assessing this site for tumor spread clinically important. Therefore, the multiple-marker RT-PCR assays may be used to molecularly stage the bone marrow detecting micrometastases not identified with conventional pathology.

Increased accuracy in staging breast cancer patient disease and initiation of earlier therapeutic interventions unequivocally are beneficial consequences of technological advancements that identify high-risk patients early in their disease course. Blood testing provides a minimally invasive method to evaluate the presence of circulating tumor cells that may serve as indicators for assessing risk of recurrence. Current imaging techniques used to identify breast cancer metastases often require a significant tumor burden for detection. Furthermore, the procurement of sufficient tissue to confirm the diagnosis can be associated with significant morbidity and cost depending on the size and location of the lesion as mentioned previously. Therefore, the utility of detecting tumor cells in the blood potentially offers a practical, safe, and cost-effective alternative to traditional methods of diagnosing disease recurrence and/or systemic spreading. In prostate and colon cancer, prostate specific antigen (PSA) and carcinoembrionic antigen (CEA) which are measured in the blood have served as a tremendous tool in the management of these cancer types respectively. To date, well-characterized molecular tumor markers to detect occult breast cancer cells in the blood are limited. There is no breast cancer tumor marker that can be measured in the blood, used for screening, serve as prognostic indicator, measure response to therapy, or signal early recurrence of the disease. The advantage of tumor marker detection in the blood is that it can be serially measured throughout the course of the

disease, unlike lymph node and bone marrow biopsies. The development of a multiple-marker RT-PCR assay to detect micrometastases in the blood would considerably improve staging at the time of diagnosis of breast cancer patients enabling the early institution of the therapy that could lead to improved survival in patients that have disease relapse. A reliable breast cancer tumor marker that could be measured in the blood would potentially detect the disease before it becomes clinically visible on screening mammography or palpable on clinical exam. The increased levels of the tumor marker could lead to more frequent monitoring, further testing of patients, or earlier biopsy of suspicious lesions seen on mammography. It could also be used for postoperative monitoring, for determining the response to chemotherapy, and for prolonged post-treatment monitoring. The development of a multiple-marker RT-PCR assay that would be able to detect micrometastases in the blood identifying circulating tumor markers would have the potential to be used in above mentioned situations.

New prognostic markers can be tested in this very efficient way. If the study proves successful, the markers can be adopted for routine use either alone or, more probably, in combination with standard clinical assessment. It is believed that a number of molecular markers will make the transition from the laboratory to the clinic over the coming decades with the ultimate benefit being better prognostication and therapy of breast cancer patients.

Tumor Markers

A tumor marker is defined as a substance present/overexpressed in or produced by a tumor (tumor-derived), or the host (tumor-associated), that can be used for differentiating neoplastic from normal tissue. Tumor markers are found in cells, tissues, and body fluids such as cerebrospinal fluid, serum, plasma, and milk. The ideal marker would be useful in

diagnosis, staging and prognosis of cancer, provide an estimation of tumor burden, and serve for monitoring effects of therapy, detecting recurrence, localization of tumors, and screening in general populations (52). Most (if not all) tumor markers do not fit the ideal profile. The reason for this can be the relative lack of sensitivity and specificity of the available tests. It should be noted, that virtually any protein or chemical has the potential to be a tumor marker. As tumor cells grow and multiply, some of their substances increase in tumor tissues and/or leak into the bloodstream or other fluids. Depending upon the tumor marker, it can be measured in blood, urine, stool or tissue. Some widely used tumor markers include: AFP, Her2/Neu, beta-HCG, CA 19-9, CA 27.29 (CA 15-3), CA 125, CEA, and PSA. Some tumor markers are associated with many types of cancer; others, with as few as one. Some tumor markers are always elevated in specific cancers; most are less predictable. However, no tumor marker is specific for cancer and most are found in low levels in healthy persons, or can be associated with non-neoplastic diseases as well as cancer. Tumor markers have been categorized as enzymes, isoenzymes, hormones, specific cell membrane proteins, oncofetal and cell-specific antigens, carbohydrate epitopes, oncogene products, genetic changes, etc. There are only a handful of well-established tumor markers that are being used by physicians. Many other potential markers are still being researched. There are many studies now that are trying to find new genes involved in signaling molecules or proteins that "tell" cells to proliferate, invade or metastasize.

- AFP and CEA: There are two common oncofetal antigens, alpha-fetoprotein (AFP) and carcinoembryonic antigen (CEA). The oncofetal antigens are so named because they are normally produced during embryonic development and decrease soon after birth. Cancer cells tend to dedifferentiate, or revert to a more immature tissue and begin to

produce fetal antigens again. Oncofetal antigens are very non-specific and expressed by a wide number of cancer types. However, they are used both to monitor a patient's progress and their response to treatment over time. CEA is a cell surface glycoprotein and it is a marker for colorectal, gastrointestinal, lung, and breast carcinomas (53). CEA is most useful in monitoring therapy (as declining levels correlate with tumor burden) and has utility in detecting recurrence of colorectal cancer. High CEA levels in breast cancer do not correlate with grade of tumor but are useful for monitoring therapy and detecting recurrence. AFP is a marker for hepatocellular and germ cell (nonseminoma) carcinoma. For hepatocellular carcinoma (HCC), the sensitivity of AFP is 98% and the specificity is 65%, making it the most useful marker for HCC (54).

- Cancer antigen (CA) 27.29 is elevated in breast carcinoma, ovarian and lung cancer, in normal pregnancy (1st trimester), benign breast disease, cirrhosis and hepatitis (55). For recurrent breast carcinoma, CA 27.29 has a sensitivity of ~57% and a specificity of ~87% (56). It lacks the required sensitivity and specificity for routine detection of breast cancer and does not discriminate patients with early carcinoma from those with benign breast disease. CA 27.29 is associated with the early detection of recurrent breast carcinoma.

- HER-2/neu is an oncogene-encoded growth factor receptor (homologue of epidermal growth factor (EGF) receptor), also known as c-erbB-2. It is overexpressed in breast cancers as a result of HER 2 proto-oncogene amplification. It is measured in the tissue from a biopsy either by immunological assays of the protein or PCR. The presence of HER-2/neu is generally associated with a more aggressive growth and poorer prognosis for breast and ovarian cancer (57; 58). It can also help to determine treatment options,

predicting an enhanced survival benefit from the Her 2-targeted therapy (reviewed by Horton, 2002 (59), Herceptin (trastuzumab), a monoclonal antibody that can block the protein receptor and decrease cancer growth. It may also predict for resistance to some conventional therapies.

- Prostate specific antigen (PSA) is the most valuable tumor marker for the diagnosisand management of prostate cancer in terms of high specificity and utility (60), but it is now widely accepted that PSA is also present in many nonprostatic sources (61). PSA is a kallikrein-like serine protease first described in seminal plasma (62) and later found in prostatic tissue (63) and to a minor extent in milk (64) and amnionic fluid (65). It is a product of epithelial cells of the prostate and is secreted into the seminal fluid. The measurement of circulating PSA levels combined with digital rectal exam is recommended annually for all men over age 50. In addition to its use in screening, PSA is frequently used to monitor treatment of prostate cancer. Other factors affect the PSA level besides cancer. Older men tend to have a higher PSA normally. Also men with benign prostatic hypertrophy (BPH) have higher levels.

- Estrogen receptor (ER) and progesterone receptor (PR): In both pre- and postmenopausal women, levels of steroid receptors ER and PR can predict which women are likely to benefit from hormone treatment. Measurements of ER and PR are recommended to use in the diagnosis, prognosis, and treatment planning for women with breast cancer. ER gives an indication of responsiveness to therapy. Tissue from a biopsy is used to measure the estrogen receptor. Most breast cancers in post-menopausal women are ER-positive, meaning that they require estrogen to grow. These ER positive breast cancers

are less aggressive than ER negative breast cancers, which are found generally in pre-menopausal women.

- P53 is a tumor suppressor gene that is mutated or changed in more than 50 percent of tumors. Studying p53 as a tumor marker helped researchers understand how tumors form, but measuring p53 levels in cancer patients has not been shown to predict differences in survival or quality of life. p53 was indicated as responsible for tamoxifen resistance in breast cancer suggesting that it can interfere in treatment response (66).

- Cathepsin-D: High levels of this lysosomal enzyme may indicate breast cancer. There is not enough information to recommend using cathepsin-D levels to make treatment decisions for patients with primary or metastatic breast cancer and especially to diagnose the disease but studies have shown its association with reduced disease-free and overall survival of breast cancer patients (67).

Researchers continue working on specific molecular pathways involved in oncogenesis, tumor response, tumor progression, etc. to discover new molecular markers that can have a potential to be routinely used in medical practices of breast cancer. Laboratory techniques for the study of potential prognostic markers are rapidly developing at both the gene and protein level. Most techniques now allow the analysis of fresh or archival tissue. Some of the newly discovered markers are markers involved in cell cycle dysregulation (cyclin D1, p16INK4a, p14ARF), tumor invasiveness (VEGF, factor VIII related antigen, Cox-2), stromal-breast epithelium interactions (uPA and related proteins, E-cadherin, b1 integrin), etc. The documented list of potential breast cancer markers is expanding every day promising valuable discoveries of new markers and their use in clinical settings.

Microarrays

Survival rates of breast cancer improved during the 1900's. They are steadily increasing now probably as a result of earlier detection, better staging and improved treatment. It is thought that the recurrence and mortality rate was high due to poor early detection techniques. According to the American Cancer Society, mortality rates of breast cancer declined during the past decade with the largest decreases in younger women (68). These somewhat encouraging trends are primarily associated with improved screening techniques and the subsequent increase in diagnosis at an early stage when most cancers are more successfully treated. Unfortunately, most current therapies have limited efficacy in curing late-stage disease. Therefore, there continues to be a need to develop new approaches to diagnose cancer early in its clinical course, more efficiently treat its advanced stages, predict tumor's response to therapy, and ultimately prevent cancer disease. A better understanding of how certain genes and their encoded proteins contribute to disease onset and tumor progression and how they influence the response of patients to therapies would be the only way to accomplish these goals. Our era of genetic, biological, and biochemical innovations gives prominent opportunities to address these questions uncovering molecular basis of cancer. DNA microarrays are one of the most promising and powerful technologies in this field becoming a major tool in biomedical area and reshaping molecular biology. Partial sequence data for thousands of genes have been generated due to the human genome project's large-scale sequencing efforts (69; 70). The roles these genes play in various biological processes have yet to be elucidated. Defining gene expression profiles, i.e. comparing patterns of expression in different tissues and developmental stages, in normal and disease states, or in distinct in vitro conditions, is a

big step toward understanding these roles. RT-PCR, RNase protection assays, and Northern blot analysis can accomplish the above mentioned goals but these methods focus on only a few genes at a time. Using microarrays, a single hybridization experiment can generate an expression profile for hundreds-thousands of genes at once. The ability to analyse the expression level of thousands of genes in a single assay using DNA microarrays is transforming the way we do research (71; 72).

The history of microarrays began 25 years ago with the Southern blot, which introduced the basic technique of anchoring nucleic acids to a solid support for analysis by hybridization. A DNA microarray is an orderly arrangement of known or unknown DNA samples attached to a solid support. Each DNA spot on the microarray is usually less than 200 μM in diameter and an entire array typically contains thousands of spots. Many different design formats are possible (73). The samples attached to the solid support can be small oligonucleotides, cDNAs or genomic sequences. RNA is isolated from samples, reverse transcribed into cDNA and labeled. Then it is hybridized on a microarray and visualized by different techniques.

The range of microarray technology applications is already enormous. While gene-expression profiling is currently the dominant microarray application, microarrays are also increasingly being used in pharmacogenomics and molecular diagnostics research. The development of DNA microarrays (or "DNA chips") in conjunction with human genome studies has promise to be used in development of a new taxonomy of cancer (74), including major insights into the genesis, progression, prognosis, and response to therapy on the basis of gene expression profiles. Genome-wide expression profiling of disease states opens up a new window for discovery of molecular disease markers and clinical

monitoring of patients. Major refinements of the technology underlying DNA libraries, PCR, and hybridization have come together in the development of DNA microarrays.

Currently available DNA microarrays are carefully designed to include genes that are of interest to researchers in breast cancer field. The use of DNA array techniques now allows for the simultaneous analysis of the mRNA expression levels of thousands of genes in mammary tumor cell lines and breast tumors to address different aspects concerning breast cancer. Using DNA microarrays researchers have attempted to identify clusters of genes not recognized by the currently available pathological techniques (75), to elucidate gene expression patterns and survival of breast cancer patients (76), to investigate DNA copy-number variations in breast cancer cell lines and tumors (77), to examine *in vivo* molecular events of breast cancer progression (78), to determine the global impact of gene copy number variation and reveal amplified novel genes (79), and predict breast cancer response to therapy (80). There are many other studies that used DNA microarrays to reveal questions of breast cancer phenomenon but it is impossible to refer to them all in this chapter. It is becoming clear that continued advancements in the comprehensive analysis of protein products in conjunction with already confined methods of measurement of mRNA expression may ultimately uncover the molecular basis of different cancers as well as breast cancer shedding light on uncountable questions about this problematic disease.

Objectives of the Project

The overall objective of this research was to contribute to improved diagnosis, prognosis, and outcome prediction of breast cancer disease through the use of molecular biological advances in the detection and quantification of multiple mRNA tumor markers.

Our hypothesis is that the use of well-developed real-time PCR technique will improve the detection of cancer cells. In addition, the use of real-time PCR in conjunction with molecular profiling of cancers through the use of DNA microarrays should ultimately provide a complete suite of molecular tools for the detection as well as characterization of breast metastatic tumors.

The first goal of this project was to develop and evaluate RT-PCR assays based on fluorescent TaqMan methodology for the early detection and quantification of multiple mRNA tumor markers in the blood, bone marrow and lymph nodes of breast cancer patients. The use of automated, real-time PCR allows for the detection as well as quantification of the relative levels of multiple tumor mRNAs in comparison to invariable "housekeeping" mRNAs. This work is a direct continuation of previous work performed by Dr. Peter Bostick (81-84). In initial studies, Dr. Bostick and colleagues showed the potential of detecting specific mRNA markers by conventional RT-PCR and Southern Blot analyses of breast cancer samples obtained from sentinel lymph nodes and blood. Initial markers included carcinoembryonic antigen (CEA), cytokeratin 19 (CK 19), CK 20, gastrointestinal tumor-associated antigen 733.2 (GA 733.2) and mucin-1 (MUC-1). CEA, CK 19, and MUC-1 have been efficiently detected by immunohistochemistry, however, detection of these mRNAs had no diagnostic value as mRNA markers for the detection of micrometastases by the RT-PCR assay, because they were expressed in relatively large amounts in both the blood and lymph nodes of individuals without cancer. Data about CK20 were inconclusive and additional experiments were warranted to establish whether CK20 could be used as a diagnostic mRNA tumor marker (81). The levels of expression of mRNA tumor markers C-Met, beta1→4GalNAc-T, and P97 were also compared. Whereas

all three tumor markers were expressed in 43% of histopathologically tumor-free sentinel nodes, the mRNA levels were significantly higher in patients with a family or prior history of breast cancer, infiltrating lobular carcinoma, estrogen receptor-negative tumor, or a higher Bloom-Richardson score. It was concluded that the multiple-marker RT-PCR and Southern blot assays improved the occult metastases detection in the sentinel node when compared to conventional hematoxylin and eosin staining and immunohistochemistry analysis. Expression of all 3 tumor mRNA markers in the sentinel node correlated with poor prognostic clinico-pathologic factors (82). In melanoma studies, Dr Bostick and colleagues compared detection of occult metastases in the sentinel node of melanoma patients by the hematoxylin and eosin staining and immunohistochemistry to detection of metastases by the multiple-mRNA marker RT-PCR assay. The levels of MAGE 3, MART-1, and tyrosinase mRNA detected by RT-PCR were correlated with hematoxylin and eosin staining and immunohistochemistry assay results, standard prognostic factors, and disease-free survival. Patients with histopathologically melanoma-free sentinel nodes who were multiple-mRNA marker positive were at increased risk of recurrence, while patients who were multiple-mRNA marker positive with histopathologically proven metastases in the sentinel node were at greatest risk of disease relapse, concluding that hematoxylin and eosin staining and immunohistochemistry underestimate the true incidence of melanoma metastases. Multiple-mRNA marker expression in the sentinel node more accurately reflected melanoma micrometastases and was also a more powerful predictor of disease relapse than the hematoxylin and eosin staining and immunohistochemistry alone (83). In another multiple-marker melanoma RT-PCR assay, the number of RT-PCR markers detected in blood was an independent prediction factor of disease recurrence significantly

predicting disease recurrence in 2, 3, and 4 years of follow-up (84). Molecular detection of circulating tumor cells, especially as multi-mRNA marker approach, has significant prognostic value in determining early disease recurrence and might be useful for stratifying patients for adjuvant therapy. Newer molecular methodologies such as real-time quantitative TaqMan RT-PCR and cDNA microarrays have been used in these feasibility studies for assessing the relative potential of diagnostic procedures for the detection of mRNA tumor markers in breast cancer patient samples.

The following mRNA tumor markers, some of which were used also by Dr. Bostick, were selected for multi-marker TaqMan analysis: (I) MAGE 3 (melanoma antigen 3); (II) HER2/NEU (human epidermal growth factor receptor 2); (III) MGB 1 (mammoglobin 1); (IV) CK 20 (cytokeratin 20); (V) PSA (prostate specific antigen); and (VI) HPR (heparanase). The set of markers was selected for multi-marker assay on the basis of the available published data of expression and specificity of each marker in primary and metastatic tumors.

MAGE (melanoma antigen) proteins are normal tissue antigens compartmentalized in testicular cells that play an important role in the early phase of spermatogenesis. Demethylation induces MAGE antigens in cells, suggesting that MAGE genes are important developmentally regulated genes under methylation control. Thus, genetic instability in cells causing loss of this methylating control could result in the preferential expression of MAGE genes in cancer cells. MAGE genes are preferentially expressed in many different cancers and detected at both the mRNA and protein levels (recognized by autologous cytolytic T lymphocytes (CTL). MAGE 3 gene is a member of MAGE gene family of tumor antigens expressed in many tumors of several types, such as melanoma

(85), ovarian carcinoma (86), hepatocellular carcinoma (87), head and neck squamous carcinoma (88), lung carcinoma (89) and breast carcinoma (90), but not in normal tissues except testes.

The connection of HER 2 to breast cancer outcome was noted relatively long ago (91). As mentioned earlier, in breast cancer, HER 2 amplification/overexpression correlates with earlier relapse, shorter disease free- and overall survival predicting for a poor clinical outcome (91; 57; 58). HER 2 is considered to be a clinically important molecule and testing for HER 2 abnormalities is already part of routine patient assessment in many parts of the world. Immunohistochemistry (IHC) and fluorescence *in situ* hybridization (FISH) are the most common HER 2 tests used and they guide breast cancer therapy. They show a high level of concordance, but there is currently no gold standard for HER 2 testing. The review by Dowsett et al (92) summarizes different assays used for HER 2 testing discussing their advantages and disadvantages. HER 2 testing approaches based on RT-PCR are very promising for the routine detection and quantification of this oncogene expression for primary and metastatic breast cancer diagnosis as well as for patient monitoring (93; 94). A well-developed and validated RT-PCR assay can be a major tool for HER 2 testing with defining cut-off values and other standards for the assay.

Mammoglobin (MGB) is a member of the uteroglobin gene family. It is homologous to a family of secreted proteins that includes rat prostatic steroid-binding protein subunit C3, human Clara cell 10-kilodalton protein, and rabbit uteroglobin. Mammoglobin is a relatively recently discovered gene but it has already shown promise for breast cancer detection. Its expression is limited to the adult mammary epithelium and it is frequently upregulated in human breast cancer cell lines and primary (95; 96) as well

as metastatic breast cancer (97). Mammoglobin could be a potential serum marker for breast cancer diagnosis because it is secreted and is specific to breast tissue.

Cytokeratines (CK), which comprise a multigene family of 20 related polypeptides, are constituents of the intermediate filaments of epithelial cells, in which they are expressed in various combinations depending on the epithelial type and the degree of differentiation. CK 20 is essentially confined to gastrointestinal epithelia, the urothelium and Merkel cells of epidermis. Sparse CK 20 positive epithelial cells have been noted in the thymus, bronchus, gall bladder, and prostate gland. CK 20 is expressed in endometrial (98) and hepatocellular carcinoma (99) tumors but not in the endometrium of patients with benign diseases or in the blood cells.

As discussed earlier, PSA is a product of epithelial cells of the prostate secreted into seminal fluid. For some time it was believed that PSA was exclusively expressed in the prostate and that PSA in the circulation must be prostatic in origin. This has been the basis for the use of PSA as a tumor marker for prostate cancer and the detection of this cancer by measuring circulating PSA levels (100; 101). As mentioned before, later it became apparent that PSA is expressed in nonprostatic tissues. Also, since PSA was originally identified in the prostate, it has long been assumed that women, lacking a prostate, would have no circulating PSA. However, with the advent of highly sensitive assays it has become clear that there are low but detectable levels of PSA in the circulation of women (102). PSA expression has also been reported in a wide variety of tumors. It has been detected immunocytochemically in many primary and metastatic melanomas (103), in primary ovarian carcinoma (104), etc. Many of the studies on extraprostatic PSA were

inspired by reports that PSA could be detected in breast tumors and in serum of patients with breast cancer (105; 106).

Heparanase (HPR) is an enzyme expressed by various cells such as platelets, leukocytes, endothelial cells and smooth muscle cells. It is an endoglycosidase (heparan sulfate-specific endo-beta-D-glucuronidase) that cleaves heparan sulfates (HS). Heparan sulfate proteoglycans (HSPGs) play a key role in the self-assembly and barrier properties of basement membranes and extracellular matrices. Hence, cleavage of heparan sulfate (HS) affects the integrity and functional state of tissues and thereby fundamental normal and pathological phenomena involving cell migration and response to changes in the extracellular microenvironment. The heparanase mRNA and protein are preferentially expressed in metastatic cell lines and human tumor tissues (107; 108). Enhanced heparanase expression correlates with increased chance of tumor metastases with tumor vascularity and poor prognosis of cancer patients (109-111).

The next objective was to focus on the use of low-density DNA arrays containing cell interaction genes for discovering additional tumor markers. This molecular detection technology would help to identify multiple tumor associated mRNAs overexpressed in breast cancer patients in a short time in contrast to time-consuming other different techniques. It was also anticipated that custom made microarrays could be made to include the newly discovered and promising markers. For the initial microarray feasibility studies it was decided to use Atlas Human Cell Interaction array from Clontech, a nylon cDNA array with 265 immobilized genes on it. Cell interaction molecules such as cell adhesion proteins, extracellular matrix proteins, proteases, etc. play key roles in mediating cell-cell, cell-tissue and cell-extracellular matrix interactions and are involved in the normal

processes of cell growth, division, differentiation, migration, as well as apoptosis. These genes are also important in many diseases and pathophysiological processes, including tumor invasion and metastasis, rheumatoid arthritis, cardiovascular disorders, wound healing, inflammation, and some central nervous diseases. Considerable research has been done to discover the particular role these proteins play in cancer diseases (112-116). The list of cell interaction molecules that could serve as potential tumor markers for early diagnosis of tumors and their metastases is continuing to expand.

MATERIALS AND METHODS

Sample Acquisition, Storage, and Identification

A limited number of tissue, bone marrow and blood samples were obtained via collaboration with surgeon oncologist Dr. Peter Bostick and the Baton Rouge General Hospital. Samples were stored at –80°C after arrival at LSU. RNA was isolated immediately upon arrival from a portion of the blood samples. The remaining blood samples were aliquoted into 2ml cryo vials and stored at –80°C. Large tissue samples were fragmented into smaller samples for ease of storage and application of RNA extraction procedures and stored at –80°C. Bone marrow samples were aliquoted into smaller samples within cryo tubes for future manipulations. The list of the samples stored in the GeneLab freeze, is shown in Table 1. All the samples were obtained from Dr. Bostick's surgeries of confirmed or suspected breast cancer patients.

Table 1. List of specimens received at LSU School of Veterinary Medicine

Tissue samples	Blood samples	Bone marrow samples
5062801Tln	5062801BD	5057331BMl
00716247Tln	506322BD	713328BMl
5061542Tln	5061542BD	713328BMr
00657403Tbts	657403BD	313163BMr
00716247Tbtm	05065155BD	5057331BMr
0Tun	5057331BD	05062801BMl
5057331Tbtm	8/02/00BD	05062801BMr
00497241Tbtm	00408233BD	5061542BMr
05071355Tbtm	05077954BD	5056311BMl
05065155Tun	42578906BD	053145BMr
		5056311BMr
		5056242BMl
		053145BMl
		5056242BMr
		323496BMr
		323496BMl
		313163BMl
		5053504BMr
		05063173BMr
		05063173BMl
		42578906BMr
		42578906BMl

Samples are identified by a number assigned by the Baton Rouge General Hospital, which relates to each patient. An upper case T denotes tissue samples, ln denotes lymph node samples, un is for unidentified samples, bts is for breast tissue, btm is for breast tumor. An upper case BM denotes bone marrow samples, l is for left and r is for right aspirates. An upper case BD is for blood samples. For example, bone marrow sample number 713328 right is identified as 713328BMr.

Table 2 includes the list of samples from patients confirmed to have metastatic breast cancer.

Table 2. Samples of known origin

Sample number	**Histological type of cancer**	**Sample types**
5056242	infiltrating ductal carcinoma	bone marrow
313163	infiltrating ductal carcinoma	bone marrow
5056311	infiltrating ductal carcinoma	bone marrow
713328	intraductal and infiltrating carcinoma (mucinous type)	bone marrow
05057331	poorly differentiated mammary carcinoma	tissue, blood, bone marrow
5062801	infiltrating ductal carcinoma	tissue, blood, bone marrow
5061542	infiltrating ductal carcinoma	tissue, blood, bone marrow
506322	infiltrating ductal carcinoma	blood

RNA Extraction

Total RNA was isolated from 0.65 cm cubes of tissue samples, from 0.25 ml blood and bone marrow samples using Absolutely RNA RT-PCR Miniprep Kit from Stratagene (La Jolla, California). The Absolutely RNA system simplifies the traditional guanidine thiocyanate method by using a silica-based matrix in a spin-cup format. Following lysis and homogenization of the clinical samples with Lysis Buffer-β-ME mixture, homogenates were passed through Prefilter Spin Cups by centrifugation at maximum speed to remove particulates and much of the DNA contamination. The filtrates were mixed with 70% ethanol, transferred to Fiber-Matrix Spin Cups (RNA binding spin cups) and spun. The bound RNA was washed with Law-Salt Wash Buffer and DNase treated by DNase solution (contained DNase Digestion buffer and RNase-Free DNase I). After multiple washes with High-Salt and Low-Salt Wash Buffers, RNA was eluted from the spin cups by Elution Buffer (a low ionic strength buffer). RNA in the Elution buffer was stored at –80°C for future use.

RT-PCR Assays

The Real-Time Quantitative One-Step RT-PCR TaqMan assays were performed on the Perkin Elmer ABI PRISM 7700 Sequence Detection System equipment (Applied Biosystems, Foster City, California), which provides product verification with the highest stringency and sensitivity permitting continuous automated reading of fluorescence intensities during PCR. Signal production is directly proportional to the hybridization of a fluorescent probe, which serves to authenticate the PCR product as well as quantify its relative amount in comparison to known internal controls (GAPDH, 18S, etc.). A total of six mRNA markers were tested. Those were: MAGE 3 (melanoma antigen E 3), HER

2/NEU (human epidermal growth factor receptor 2), MGB 1 (mammoglobin 1), CK 20 (cytokeratin 20), PSA (prostate specific antigen), and HPR (heparanase). Dilutions of RNA samples from a cell line were used to construct standard curves for target genes and endogenous reference (GAPDH or 18S). RNA from negative breast cancer cell line Hs578Bst (American Type Culture Collection, Manassas, Virginia) was used as a calibrator. Primers and probes were chosen using Primer Express software (Applied Biosystems). Probes were labeled at the 5' end with the reporter molecule 6-carboxyfluorescein (FAM) and at the 3' end with the quencher 6-carboxytetramethyl-rhodamine (TAMRA) (Applied Biosystems) (Table 3).

Table 3. Primer and probe sequences of each marker gene for real-time PCR amplification

Gene	**GenBank accession number**	**Primer or Probe**	**Sequence**
MAGE 3	U03735	Forward	GGTGAGGAGGCAAGGTTCTG
		Reverse	TCTGCTCAAGAGGCATGATGA
		Probe	ACTGGCAGATCTTCTCCTTCAGTGCTCCT
HER2/NEU	X03363	Forward	AGTGTGCACCGGCACAGA
		Reverse	TTGTGAGCGATGAGCACGTA
		Probe	AGCCTGTCCTTCCTGCAGGATATCCAG
MGB1	AF015224	Forward	CAAGACAATCAATCCACAAGTGTCT
		Reverse	AACACCTCAACATTGCTCAGAGTT
		Probe	CTTCTTCAAGAGTTCATAGACGACAATGCCACTACA
CK 20	X73502	Forward	TGCGAAGTCAGATTAAGGATGCT
		Reverse	CCACTGTTAGACGTATTCCTCTCTCA
		Probe	CATACTTCAGTCTGAAGTCCTCAGCAGCCAGT
PSA	NM-001648	Forward	CATTGAACCAGAGGAGTTCTTGAC
		Reverse	TCCAGCACACAGCATGAACTT
		Probe	AACTTGCGCACACACGTCATTGGAA
HEP	AF144325	Forward	TCGTGGACCTGGACTTCTTCA
		Reverse	ACAAGCCTCTGGCCAAGGTA
		Probe	CCACGGACCCGCGGTTCCT

Amplifications of an endogenous control (GAPDH or 18S) were performed to standardize the amounts of sample RNAs added to reactions. Commercially available primers and probes for the housekeeping genes, GAPDH and 18S, were obtained from Applied Biosystems. The TaqMan One-Step RT-PCR Master Mix Reagents which combine the key components into a master mix designed for one-step RT-PCR were obtained from Applied Biosystems. The same universal thermal cycling parameters were used for all quantitative TaqMan assays. The reverse transcription step was carried out at 48°C for 30 minutes. The polymerase (Amlitaq Gold) was activated at 95°C for 10 minutes. Forty cycles of RT-PCR were done, each consisting of 95°C for 15 seconds and 60°C for 1 minute.

Data Analyses

The levels of marker gene expression in tissue, blood and bone marrow samples, as well as in Hs578T positive cell line (American Type Culture Collection), were quantitated. Relative quantitation with data from the ABI PRISM 7700 Sequence Detection System (SDS) was performed using the SDS 1.7 Software (Applied Biosystems). The initial SDS software analysis of the acquired fluorescent data include normalization of the reporter dye signal to an internal passive reference, calculation of the ΔRn and Ct values, and standard curve construction. Normalization was necessary to correct for fluorescent fluctuations due to changes in concentration or volume. Normalization was accomplished by dividing the emission intensity of the reporter dye by the emission intensity of the Passive Reference to obtain a ratio defined as the Rn (normalized reporter) for a given reaction tube. The Passive Reference was a dye included in the 10X TaqMan Buffer and did not participate in the 5'-nuclease assay. ΔRn reliably indicated the magnitude of the signal generated by the

given set of PCR conditions. The value was calculated as the difference between the Rn^+ value, the Rn of a reaction containing all components including the template, and the Rn^- value, the Rn value of an unreacted sample, as for example that obtained from a reaction not containing template, a No Template Control. To ensure statistically high confidence levels, at least three No Template Controls per microplate were used. The threshold cycle or Ct value was the cycle at which a statistically significant increase in ΔRn was first detected, in other words, the increase in signal associated with an exponential growth of PCR product.

Relative quantitations were performed using the standard curve method. Below is a sample example of a standard curve for MAGE 3 and formulas used for calculations (Figure 2).

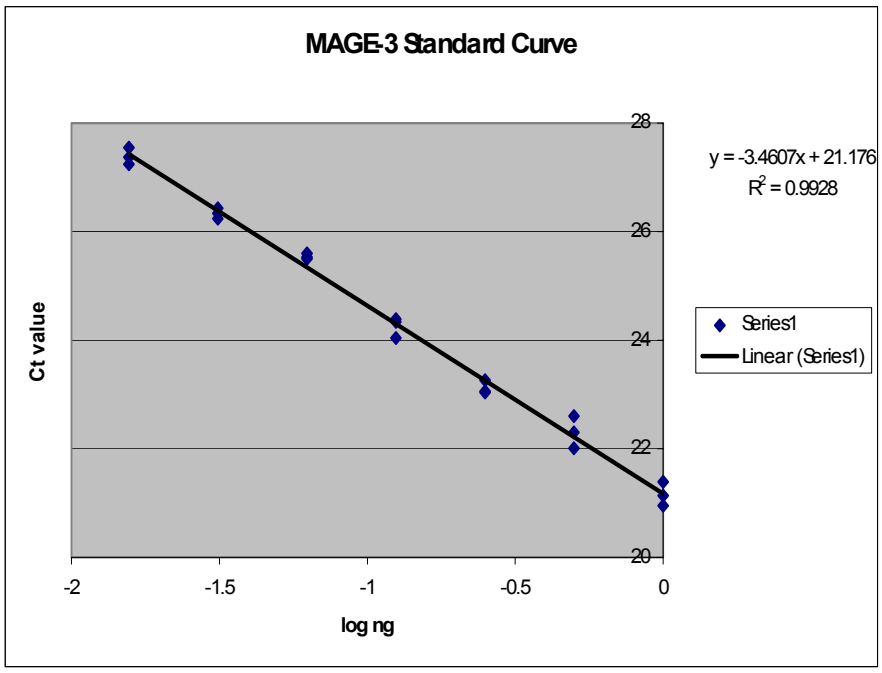


Figure 2. MAGE 3 standard curve and quantitation formulas

The construction of standard curves was based on the linear relationship between the cycle threshold (Ct) and the log of concentration (ng) of a sample RNA. The input amount was calculated by using m and b values, then was normalized against the endogenous control (quantitation of an mRNA target normalized for differences in the amount of total RNA added to each reaction). Subsequently, it was calibrated using the Hs578Bst values (negative cell line from normal breast tissue).

Microarrays

Atlas Nylon Human Cell Interaction Arrays containing 265 genes encoding for cell-cell interaction molecules were purchased from BD Biosciences, Clontech (Palo Alto, California). The membranes also contained 9 housekeeping genes, such as ubiquitin C, tubulin alpha 1, cytoplasmic beta-actin, etc, and 3 negative controls (M13 mp18(+) strand DNA, lambda DNA, and pUC18 DNA). Atlas SpotLight Probe Labeling Kit (Clontech) was used for probe labeling and SpotLight Chemiluminescent Hybridization and Detection Kit (Clontech) was used for hybridization and detection procedures as specified by the manufacturer. Briefly, probe mixtures were synthesized and directly labeled by reverse transcribing the total RNA (extracted from 00716247Tln and 5062801Tln samples) using the cDNA synthesis (CDS) Primer Mix and the SpotLight Labeling Kit which contained a labeling mix with biotinylated dCTP. Labeled cDNAs were purified from unincorporated biotin-labeled nucleotides and small cDNA fragments using the Atlas Nucleospin Extraction Kit. Membranes were prehybridized at 42°C for 2 hours, then they were hybridized with the biotin-labeled probes overnight at 42°C using the SpotLight Chemiluminescent Hybridization and Detection Kit. After high stringency washes hybridization patterns of probes were detected and signals were visualized both by

exposing the membranes to X-ray films and by scanning them using the AlphaEaseFC Imaging System equipment (FluorChem IS-8800, Alpha Innotech, San Leandro, California).

Cloning and Spotting of Overexpressed Genes

Three of the overexpressed genes from microarrays (EMMPRIN_extracellular matrix metalloproteinase inducer, DSH homolog 1-like; DVL1L1_dishevelled homolog 1-like protein, and TIMP1_ tissue inhibitor of metalloproteinase 1) were cloned into the pcDNA2.1 vector (Invitrogen, Carlsbad, California) for E. coli propagation and confirmed by sequencing using T7 primer. The cloned fragments, about 500 base pairs each were amplified by PCR. 1µl of each PCR product and negative controls (H_2O, λ DNA, and pUC19) were spotted in triplicate on nylon membranes (Zeta-Probe Membranes (Bio-Rad, Herculaes, California). Membranes were placed in UV Stratalinker 2400 equipment (Stratagene) to crosslink and immobilize the DNA samples. Membranes were prehybridized at 42°C for 2 hours, then they were hybridized with the biotin-labeled probes from 5062801Tln and 00716247Tln samples overnight at 42°C using the SpotLight Chemiluminescent Hybridization and Detection Kit. X-ray pictures were taken.

RESULTS

Development of TaqMan Assays for Selected mRNA Tumor Markers

Detection and quantification of the relative expression levels of multiple tumor mRNAs requires high quality, contamination-free RNA with a satisfactory concentration for sensitive molecular methods such as RT-PCR. During the developmental stage of the breast cancer project, multiple methods and equipment were used to purify RNA until methodologies were developed to assure a high degree of reproducibility of real-time PCR results. The best results were obtained when RNA was extracted using the Absolutely RNA RT-PCR Miniprep Kit (Stratagene, La Jolla, California) specifically optimized for RT-PCR incorporating effective on-column DNA removal specifically for RT-PCR applications. This system simplifies the traditional guanidine thiocyanate method by using a silica-based matrix in a spin-cup format. All the data presented here were derived from RNA samples purified with the above mentioned methodology (see Materials and Methods).

TaqMan real-time quantitative RT-PCR assays were developed for six mRNA tumor markers: (I) MAGE 3 (melanoma antigen E 3); (II) HER2/NEU (human epidermal growth factor receptor 2); (III) MGB 1 (mammoglobin 1); (IV) CK 20 (cytokeratin 20); (V) PSA (prostate specific antigen); and (VI) HPR (heparanase). PCR primers and TaqMan probes were designed through the use of Primer Express software and empirical observations of the oligonucleotide sequences (see Materials and Methods). In all instances the targeted nucleotide sequence was less than 200 bases long. The expression levels of the marker genes relative to standard curves of cell lines were calculated, normalized against the endogenous control and calibrated to the negative breast cancer cell

line Hs578Bst as detailed in Materials and Methods. Figure 3 shows a typical real-time PCR amplification reaction as visualized and graphed by computer-assisted software in the Perkin Elmer ABI PRISM 7700 Sequence Detection System. Typically, robust amplifications of targets were obtained for both selected mRNA tumor markers as well as endogenous controls (GAPDH or 18S).

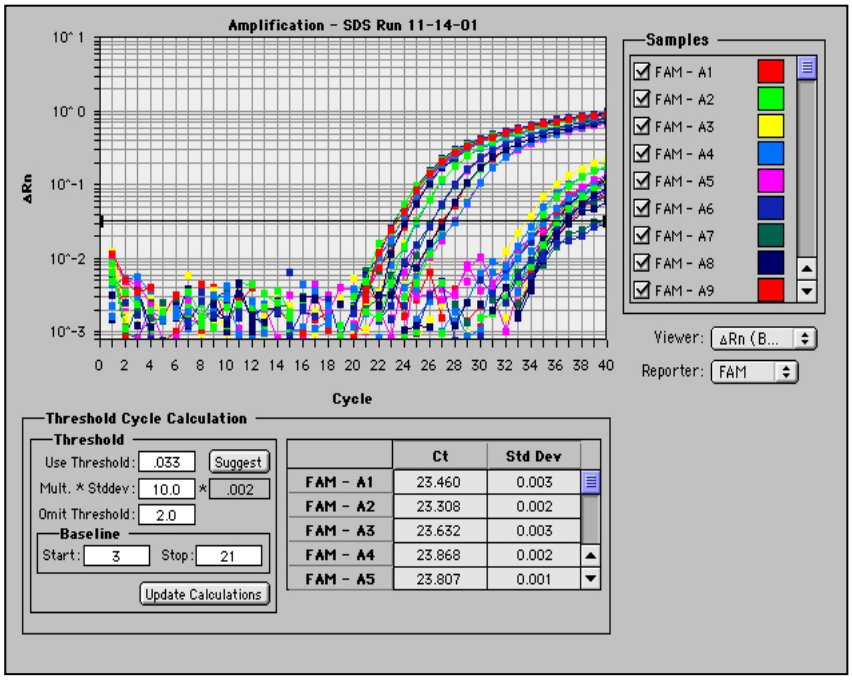


Figure 3. Amplification plot of a real-time TaqMan RT-PCR experiment for MAGE 3 in bone marrow samples

The tables below show the relative quantities for each of the six marker genes for the individual tissue, blood and bone marrow samples from the breast cancer patients (Tables 4; 5; 6; 7; 8; 9).

Table 4. Relative quantities of MAGE 3 marker gene expression in tissue, blood and bone marrow samples of breast cancer patients

Tissue

Sample Name	Calibrated to Hs578Bst
5062801Tln	2964
00716247Tln	19200
00657403Tbts	1163
00716247Tbtm	103944
0Tun	4
5057331Tbtm	7010
05065155Tun	16218316
Hs578Bst	1
Hs578T	152

Blood

Sample Name	Calibrated to Hs578Bst
5062801BD	2
506322BD	0.2
5061542BD	28
657403BD	119
05065155BD	50
5057331BD	130
8/02/00BD	4261
Hs578Bst	1
Hs578T	0.03

Bone Marrow

Sample Name	Calibrated to Hs578Bst
5057331BMl	73800
713328BMl	0.004
713328BMr	2090
313163BMr	295
5057331BMr	2400
05062801BMl	44200
05062801BMr	1390000000
5061542BMr	27
5056311BMl	1680
053145BMr	2070000000000
5056311BMr	22500
5056242BMl	5780
053145BMl	4650000
5056242BMr	65300000
323496BMl	1394808925
313163BMl	157610
Hs578Bst	1
Hs578T	34827

Table 5. Relative quantities of HER2/NEU marker gene expression in tissue, blood and bone marrow samples of breast cancer patients

Tissue

Sample Name	Calibrated to Hs578Bst
5062801Tln	308
00716247Tln	387
00657403Tbts	1
00716247Tbtm	62
0Tun	0.4
5057331Tbtm	0.1
05065155Tun	36
Hs578Bst	1
Hs578T	0.0002

Blood

Sample Name	Calibrated to Hs578Bst
5062801BD	0.001
506322BD	0.02
5061542BD	0.0004
657403BD	0.06
05065155BD	0.01
5057331BD	0.009
8/02/00BD	14
00408233BD	4670
Hs578Bst	1
Hs578T	0.0002

Bone Marrow

Sample Name	Calibrated to Hs578Bst
713328BMl	894
713328BMr	89
313163BMr	5
5056311BMr	6
053145BMl	8
5056242BMr	1
323496BMr	0.5
323496BMl	2
313163BMl	4
Hs578Bst	1
Hs578T	0.0002

Table 6. Relative quantities of MGB 1 marker gene expression in tissue, blood and bone marrow samples of breast cancer patients

Tissue

Sample Name	Calibrated to Hs578Bst
5062801Tln	16556
5061542Tln	2332
00657403Tbts	2350
00716247Tbtm	9906
0Tun	7088
5057331Tbtm	4544
05065155Tun	5351
Hs578Bst	1
Hs578T	1066325

Blood

Sample Name	Calibrated to Hs578Bst
5062801BD	33071
506322BD	4
5061542BD	2
657403BD	1039101
05065155BD	5095152568
5057331BD	46029599
8/02/00BD	0.04
Hs578Bst	1
Hs578T	1.7

Bone Marrow

Sample Name	Calibrated to Hs578Bst
5057331BMl	4950582
713328BMl	105614
713328BMr	66677
313163BMr	2231328
5057331BMr	45462
05062801BMl	467876522709
5061542BMr	4324264
5056311BMl	11400000000000
053145BMr	1427667908
5056311BMr	5717382771
5056242BMl	26256465
053145BMl	4759889
5056242BMr	313464
323496BMr	80100000000000
323496BMl	16416569953
313163BMl	1411368915
5053504BMr	4733029432
05063173BMr	320991
05063173BMl	781037
Hs578Bst	1
Hs578T	1.7

Table 7. Relative quantities of CK 20 marker gene expression in tissue, blood and bone marrow samples of breast cancer patients

Tissue

Sample Name	Calibrated to Hs578Bst
5062801Tln	4889029
00716247Tln	97564
5061542Tln	70451855
00657403Tbts	13429158
00716247Tbtm	4214750
0Tun	33008
5057331Tbtm	17200073
00497241Tbtm	1470000000000
05071355Tbtm	3182792
05065155Tun	6601348
Hs578Bst	1
Hs578T	2038

Blood

Sample Name	Calibrated to Hs578Bst
5062801BD	367204206
506322BD	845000000000
657403BD	317000000000
05065155BD	45964232658
5057331BD	24330876750
8/02/00BD	12550537
00408233BD	212000000000
Hs578Bst	1
Hs578T	2038

Bone Marrow

Sample Name	Calibrated to Hs578Bst
5057331BMl	2167966
713328BMl	1428841363
713328BMr	41930002922
313163BMr	71726552
5057331BMr	626636
05062801BMl	6526575
05062801BMr	70172857165
5061542BMr	31445
5056311BMl	1086430
053145BMr	715024
5056311BMr	9705186
053145BMl	6667135148
5056242BMr	765139883
323496BMr	302212
323496BMl	62909073
313163BMl	5371481
5053504BMr	27545799
05063173BMr	11527
05063173BMl	38606
Hs578Bst	1
Hs578T	2038

Table 8. Relative quantities of PSA marker gene expression in tissue, blood and bone marrow samples of breast cancer patients

Tissue

Sample Name	Calibrated to Hs578Bst
5062801Tln	194413963
00716247Tln	10367891
5061542Tln	42253609027
00657403Tbts	45996208
00716247Tbtm	308727899
0Tun	2994075
5057331Tbtm	9943595532
00497241Tbtm	203934000000000
05071355Tbtm	132404068
05065155Tun	549137203
Hs578Bst	1
Hs578T	552304

Blood

Sample Name	Calibrated to Hs578Bst
5062801BD	24515292365
506322BD	2281920000000000
657403BD	50809400000000
05065155BD	324658000000000
5057331BD	570793000000000
8/02/00BD	834879459
00408233BD	229069000000000
Hs578Bst	1
Hs578T	552304

Bone Marrow

Sample Name	Calibrated to Hs578Bst
5057331BMl	50256
713328BMl	15377518
713328BMr	9839563168
313163BMr	901129
5057331BMr	91037
05062801BMl	2090891
05062801BMr	3619288989
5061542BMr	8884
5056311BMl	40619
053145BMr	8497
5056311BMr	142148
323496BMr	2775
323496BMl	68251876
313163BMl	2559204842
5053504BMr	4099839
05063173BMr	29517
05063173BMl	1858
Hs578Bst	1
Hs578T	552304

Table 9. Relative quantities of HPR marker gene expression in tissue, blood and bone marrow samples of breast cancer patients

Tissue

Sample Name	Calibrated to Hs578Bst
00716247Tln	311249
5061542Tln	127561335
00657403Tbts	61928266888
00716247Tbtm	379353478
0Tun	94145598765
5057331Tbtm	557200971
05065155Tun	29940940539
00497241Tbtm	3131
05071355Tbtm	255363302
42578906Ttm	9321463717
Hs578Bst	1
Hs578T	29

Blood

Sample Name	Calibrated to Hs578Bst
5062801BD	1274055
506322BD	56447
657403BD	132809939
05065155BD	171114
5057331BD	406816
8/02/00BD	735192
00408233BD	1525775
05077954BD	224575511
42578906BD	3847460
Hs578Bst	1
Hs578T	13

Bone Marrow

Sample Name	Calibrated to Hs578Bst
5057331BMl	11870
713328BMl	2
713328BMr	118
313163BMr	21
5057331BMr	1
05062801BMl	308
05062801BMr	0.2
5061542BMr	12
5056311BMl	67
053145BMr	407
5056311BMr	152
5056242BMl	130
053145BMl	1524
5056242BMr	0.3
323496BMr	1423
323496BMl	139909
313163BMl	470
5053504BMr	667198
05063173BMr	41
05063173BMl	74968
42578906BMr	17810643
42578906BMl	203346
Hs578Bst	1
Hs578T	37

Only three of the patients had all three types of samples (tissue, blood, and bone marrow) available. Table 10 shows the results of MAGE 3 relative expression for that three breast cancer patients with available tissue, blood, and bone marrow samples.

Table 10. MAGE 3 expression in tissue, blood, and bone marrow samples of patients known to have breast cancer

Sample number and types	Relative expression of MAGE 3
5057331	
breast tumor tissue	high
blood	high
bone marrow (r)*	high
bone marrow (l)*	very high
5062801	
lymph node tissue	high
blood	low
bone marrow (r)	very high
bone marrow (l)	very high
5061542	
lymph node tissue **	-
blood	high
bone marrow (r)	high

* r and l – right or left bone marrow aspirates
** no available sample

Selection of Additional mRNA Tumor Marker Genes through the Use of Micro-arrays Spotted With Genes Involved in Cell-cell Interactions

A subset of the initial set of tumor markers represented cell surface expressed protein and glycoprotein antigens. Therefore, it was of interest to test additional cell surface expressed antigens as potential mRNA tumor markers. For this purpose, commercially available microarrays containing genes coding for proteins involved in cell-cell interactions were tested using labeled mRNA from specific tumor samples. Two tissue

samples, which had high relative expression levels of MAGE 3 and HER2/NEU genes were used in the microarray experiments with Atlas Nylon Human Cell Interaction Arrays containing 265 genes (Clontech, Inc). The arrays contained multiple housekeeping genes serving as internal controls, as well as genes serving as negative controls (see Materials and Methods). Biotin-labeled probes were prepared from 00716247Tln and 5062801Tln lymph node samples of two breast cancer patients. Figure 4A shows the photograph of the microarrays after hybridization using labeled cDNA probe obtained from the 0071624Tln tumor sample. Figure 4B shows the X-ray picture of the microarray hybridized with a probe made from the 5062801Tln sample. Both figures show a number of relatively overexpressed genes as dense dots pointed by arrows.

A

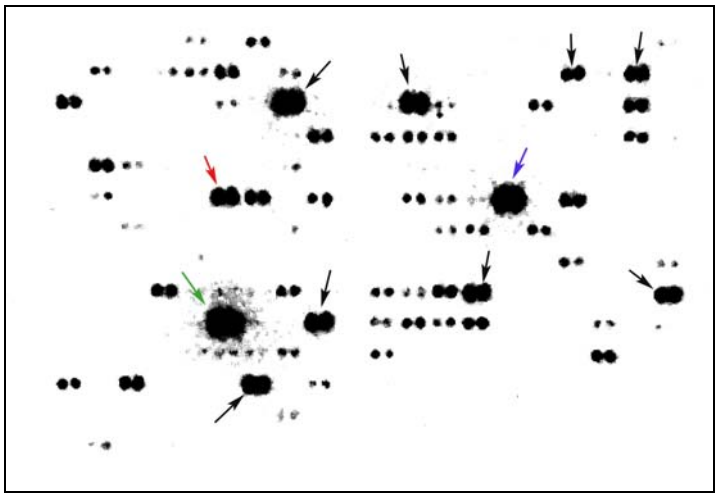
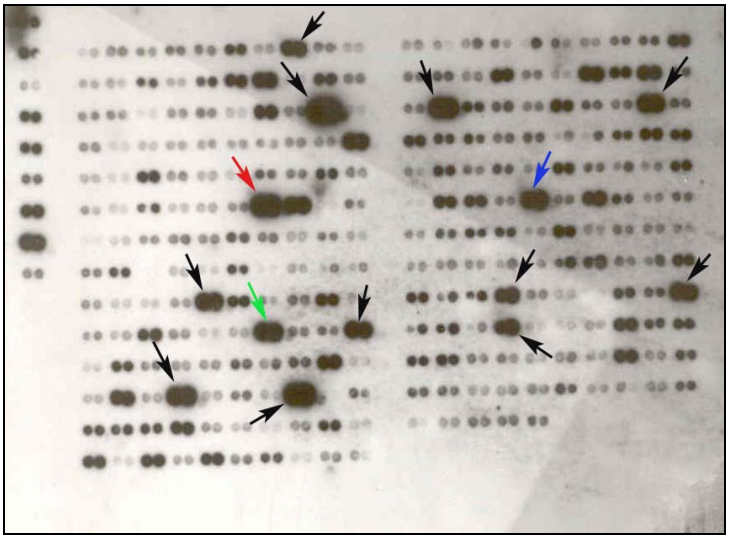


Figure 4. Microarrays hybridized with lymph node samples. A. cDNA microarray was hybridized with biotin-labeled probe prepared from RNA of 0071624Tln sample. B. cDNA microarray was hybridized with biotin-labeled probe prepared from RNA of 5062801Tln sample. The arrows indicate the relatively overexpressed genes. The locations of overexpressed EMMPRIN, DVL1L1, and TIMP 1 genes are indicated by green, blue, and red arrows respectively (full names of the genes in Table 10) (Figure continued)

B



The top six of the highly overexpressed genes discovered as a result of these microarray experiments are listed in table 11. These six genes were found to be overexpressed in both microarray hybridization experiments.

Table 11. The full and abbreviated names of some genes highly overexpressed on both microarrays

GenBank Accession #	Gene/protein name	Gene/protein classification
L20471	basigin (BSG); leukocyte activation antigen M6; collagenase stimulatory factor; extracellular matrix metalloproteinase inducer (EMMPRIN); 5F7; CD147 antigen	Other Enzymes Involved in Protein Turnover Cell Surface Antigens
U46461	dishevelled homolog 1-like protein (DSH homolog 1-like; DVL1L1)	Other Intracellular Transducers, Effectors and Modulators
M63928	tumor necrosis factor receptor superfamily member 7 (TNFRSF7); CD27L antigen receptor	Death Receptors Growth Factor & Chemokine Receptors Other Receptors (by Activities) Cell Surface Antigens
D13866	alpha 1 catenin (CTNNA1); cadherin-associated protein	Matrix Adhesion Receptors
X03124	tissue inhibitor of metalloproteinase 1 (TIMP1); erythroid potentiating activity protein (EPA); collagenase inhibitor (CLGI)	Protease Inhibitors
M12807	CD4 antigen; T-cell surface glycoprotein T4/leu3	Cell Surface Antigens

Production of In-house Macroarrays for Selected Genes

As part of these feasibility studies for assessing the relative potential of diagnostic procedures for the detection of mRNA tumor markers, it was essential to assess whether custom made macro and microarrays could be developed. For this purpose, three genes, which were highly overexpressed in both hybridization experiments were chosen for custom spotting on nylon membranes. A target nucleotide sequence of approximately 500 bases from each gene was amplified using specific PCR primer sets, cloned into plasmid vectors for E. coli propagation and confirmed by DNA sequencing. Individual gene segments were re-amplified from their respective plasmids and spotted onto nylon

membranes (see Materials and Methods). The three genes selected for these experiments are shown on Table 12.

Table 12. The full and abbreviated names of three overexpressed genes selected for spotting on nylon membranes

GenBank Accession #	**Gene/protein name**	**Gene/protein classification**
L20471	basigin (BSG); leukocyte activation antigen M6; collagenase stimulatory factor; extracellular matrix metalloproteinase inducer (EMMPRIN); 5F7; CD147 antigen	Other Enzymes Involved in Protein Turnover, Cell Surface Antigens
U46461	dishevelled homolog 1-like protein (DSH homolog 1-like; DVL1L1)	Other Intracellular Transducers, Effectors and Modulators
X03124	tissue inhibitor of metalloproteinase 1 (TIMP1); erythroid potentiating activity protein (EPA); collagenase inhibitor (CLGI)	Protease Inhibitors

Genes were spotted in triplicate onto nylon membranes and hybridized with biotin-labeled probes prepared from 00716247Tln and 5062801Tln lymph node tumor samples. Figure 5 shows the hybridization pattern of the arrays containing the three genes as well as control DNA samples. The selected overexpressed genes appeared as triplets of dense-stained dots. Negative controls did not show any hybridization signals. These experiments verified relative overexpression of EMMPRIN, DVL1L1, and TIMP 1 genes in tumor samples of breast cancer patients.

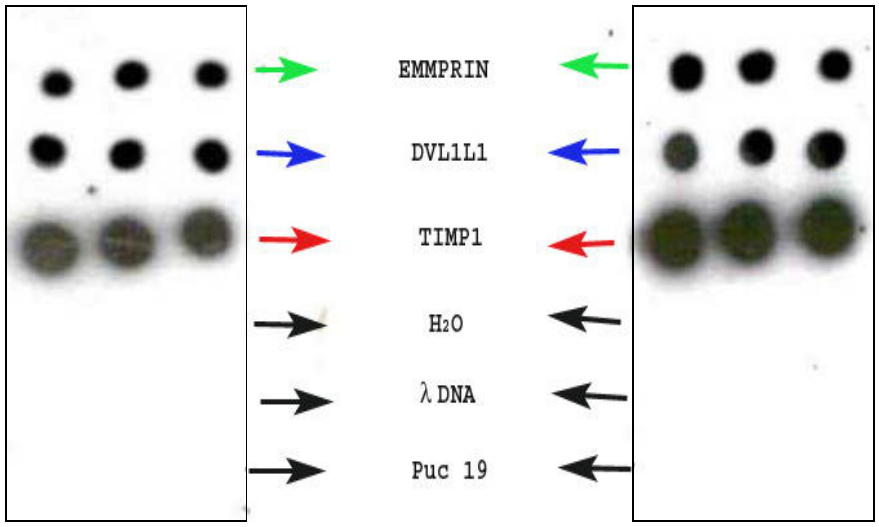


Figure 5. Hybridization pattern of three overexpressed genes (EMMPRIN, DVL1L1, and TIMP 1) and negative controls spotted on nylon membranes as triplets and hybridized with 00716247Tln (left) and 5062801Tln (right) lymph node samples

DISCUSSION AND CONCLUSIONS

The overall goal of these investigations was to assess the potential of real-time quantitative RT-PCR based on fluorescent TaqMan methodology and microarray analyses as tools for the detection and quantification of multiple mRNA tumor markers of breast cancer. Previous work (81-84) showed the potential of detecting specific mRNA markers by RT-PCR and Southern Blot analyses of breast cancer and melanoma samples obtained from patients. It was concluded that the multiple-marker RT-PCR and Southern blot assays improve the occult metastases detection in patient samples when compared to conventional hematoxylin and eosin staining and immunohistochemistry analysis, which alone underestimate the true incidence of metastases.

TaqMan Assays

Newer molecular methodologies such as real-time TaqMan RT-PCR and cDNA microarrays have been used in these feasibility studies for assessing the relative potential of diagnostic procedures for the detection of mRNA tumor markers in breast cancer patient samples.

To develop a sensitive detection assay for selected mRNA tumor markers in tissue, blood, and bone marrow samples of breast cancer patients, a variety of RNA extraction methods were attempted. The best method of RNA extraction included the use of a commercially available extraction kit (Absolutely RNA RT-PCR Miniprep Kit) specifically optimized for RT-PCR. The Absolutely RNA RT-PCR kit is stringently qualified for RT-PCR incorporating effective on-column DNA removal specifically for RT-PCR applications. The Absolutely RNA system simplifies the traditional guanidine thiocyanate method by using a silica-based matrix in a spin-cup format. RNA was

extracted from all the samples using the mentioned kit. Six mRNA tumor markers were selected and real-time quantitative RT-PCR TaqMan assays were developed and applied to detect them as well as quantify their relative expression levels in tissue, blood, and bone marrow samples. The selected markers were: (I) MAGE 3 (melanoma antigen E 3); (II) HER2/NEU (human epidermal growth factor receptor 2); (III) MGB 1 (mammoglobin 1), (IV) CK 20 (cytokeratin 20); (V) PSA (prostate specific antigen); (VI) HEP (heparanase). Among these six markers, MAGE 3 was the prevalent mRNA tumor marker and was reliably amplified from different samples of patients confirmed with breast cancer as well as from some blood samples. 18S (18S ribosomal RNA) and GAPDH (catalytic enzyme involved in glycolysis and called glyceraldehyde-3-phosphate dehydrogenase) internal standards were used as endogenous references (controls). Substantial problems were experienced obtaining reasonable Ct values for the endogenous control GAPDH. Multiple comparisons of experimental data suggested that 18S was a better endogenous control, thus GAPDH was used only for the initial runs that were testing the HER2/NEU marker and 18S was used in TaqMan assays for the remaining five markers.

Ideally, negative controls should be obtained from patients and the data from these negative samples should be used in quantitations. Because of substantial difficulties in obtaining appropriate negative controls, attempts were made to derive meaningful data by comparing clinical samples to a negative cell line derived from breast tissue. A similar cell line, obtained from breast tumor tissue of the same patient was also available as a positive control. Many of the mRNA markers could be efficiently amplified from tissue, bone marrow, and blood samples of patients known to suffer with metastatic breast cancer (see Materials and Methods, Table 2).

The results from TaqMan assays showed that some of the samples obtained from the same patient with breast cancer had a good correlation for high expressions of some markers. For example, MAGE 3 was amplified from tumor tissue, blood, and bone marrow of patient number 5057331 (see Results, Table 4). It had high expression values in all the three samples compared to negative cell line values. It was also overexpressed and was amplified from blood and bone marrow of patient number 5061542 (28- and 27-fold overexpression, respectively). Unfortunately, the lymph node tissue sample of this patient was depleted in earlier experiments, thus it couldn't be tested for MAGE 3 gene expression. MGB1 had high expression values in lymph node and bone marrow of patient number 5061542 (2332- and 4324264-fold overexpression, respectively), although not in blood of the same patient (only 2-fold overexpression) (see Results, Table 6), which probably means that either there were no circulating tumor cells in the blood, or the marker MGB1 couldn't be detected in circulating cancer cells. Table 10 shows the results of MAGE 3 relative expression for breast cancer patients with available tissue, blood and bone marrow samples. There were only these three patients for whom tissue, blood, and bone marrow samples were available. MAGE 3 was readily amplified from all of the three mentioned patient samples. 10-fold and higher overexpressions were considered high expressions, less than that were considered low. More than 10,000-fold overexpressions were considered as very high expressions (see also Table 4). Generally, MAGE 3 was overexpressed in all samples obtained from the three patients shown in Table 10, with the exception of one blood sample, which showed a low expression value for MAGE 3 leading to the conclusion that, most probably, there were no circulating cancer cells in this patient's blood.

Overall, the results showed very high expression values for most of the markers. Some of these values were unreasonably high. For example, MAGE 3 was 207x 10^{10}-fold overexpressed in right bone marrow aspirate of patient number 053145BMr (see Results, Table 4, Bone Marrow), MGB 1 was 801x10^{11}-fold overexpressed in right bone marrow aspirate of patient number 323496 (see Results, Table 6, Bone Marrow), CK 20 was 845x10^{9} times overexpressed in blood sample of patient number 506322 and 147x10^{10} times overexpressed in breast tumor sample of patient number 00497241 compared to negative cell line (see Results, Table 7), etc. These results indicate that calibrations to a negative cell line control produced artificially higher values of certain marker potentially because cell lines have very low amounts of these mRNA tumor markers. The absence of reliable negative samples from patients as well as the limited number of samples obtained were major problems, which prevented any statistical determination of the relative importance of the developed real-time PCR diagnostics. Real-time PCR may be a highly efficient diagnostic tool if positive and negative tissue samples can be obtained from the same patient, and if with positive blood and bone marrow samples obtained from breast cancer patients negative blood and bone marrow samples could be obtained from healthy individuals. Similarly, real-time PCR may be effectively applied to follow the disease progression and response to therapy of an individual patient.

PCR methods have been shown to be superior in detecting minute amounts of tumor cells compared to other methods. The extreme sensitivity implies that false positive test results are likely to occur. The ultimate choice of molecular marker(s) and methodology to use in a clinical setting will be determined by its sensitivity and potential to discriminate between true micrometastases and background noise (117). The clinical

value of molecular detection of micrometastases will be detemined by its potential to increase prognostication of individual patients and by its predictive value of response to adjuvant treatment. For these purposes large trials are necessary in which the assay used to detect micrometastases gives unequivocal, reproducible results and is easy to use. For breast cancer, there is still a need for a cancer-specific marker which reliably can detect micrometastatic disease. Most probably a combination of carefully selected markers must be used to ensure a high detection rate. RT-PCR analysis is a particularly sensitive technique for the purpose of detecting occult breast cancer cells in the lymph nodes, blood, and bone marrow of breast cancer patients (38-40; 118).

Microarrays

The advent of microarray technology has revolutionized the molecular profiling of disease tissues and tumors. It is one of the most promising and powerful methodologies in molecular oncology. This newer technology was used to test additional molecules as potential mRNA tumor markers for breast cancer diagnostics. Cell interaction cDNA atlas nylon arrays were selected to pursue the goal of discovering additional markers, such as adhesion molecules, extracellular matrix proteins, etc. involved in cell to cell interactions. The basic assumption here was that cell surface proteins involved in cellular adhesion and cell-to-cell communication may be overexpressed in breast cancer samples. In this feasibility study, multiple overexpressed genes were identified from two lymph node samples of breast cancer patients as new potential mRNA tumor markers for diagnosis of breast cancer (see Results). Interestingly, genes for extracellular matrix metalloproteinase inducer (EMMPRIN), dishevelled homolog 1-like protein (DVL1L1), tissue inhibitor of metalloproteinase 1 (TIMP1), alpha 1 catenin (CTNNA1), tumor necrosis factor receptor

superfamily member 7 (TNFRSF7), and some other cell interaction molecules were highly overexpressed in the both lymph node samples of the breast cancer patients. Specifically, TIMP1 and EMMPRIN have been shown by others to be overexpressed in breast and some other cancers (119-123). There are many gaps in understanding the molecular basis of cancer metastasis formation, but it was shown that both EMMPRIN (inducer of MMPs) and TIMP-1 (inhibitor of MMPs) can be overexpressed in cancer patients playing crucial roles in cancer progression and predicting a poor prognosis. Invasion and metastasis of tumor cells involves the degradation of the basement membrane, caused by proteases derived from the tumor or adjacent normal cells or tumor-infiltrating immune cells. A class of proteases implicated in this process is the matrix metalloproteinases (MMPs), which are a family of zinc-dependent neutral endopeptidases. Aberrant MMP activity in tumor cells and surrounding stromal tissue is implicated in tumor progression, invasion, metastasis and angiogenesis. The activity of MMPs in the extracellular space is controlled by a family of specific inhibitors, the TIMPs. EMMPRIN is prominently displayed in human cancer tissue and plays an important role in cancer progression by increasing synthesis of MMPs (122; 123). EMMPRIN plays a role in tumor invasion, metastasis, and neoangiogenesis by stimulating extracellular matrix remodeling around tumor cell clusters, stroma, and blood vessels. The latest data suggest that dishevelled proteins organize dynamic subcellular signaling complexes functioning in signal transduction through Wnt pathways. Abnormal disheveled protein and Wnt signaling can cause a variety of diseases as well as cancer (reviewed by Wharton KA Jr. (124).

From the initial experiments, three genes, which were highly overexpressed in both hybridization experiments, were selected for further investigations including custom

spotting on nylon membranes. These experiments revealed that diagnostic macro- and microarrays can be produced for the rapid screening of biological samples for the overexpression of mRNA tumor markers. These experiments utilized PCR-derived gene fragments of about 500 base pairs. Alternatively, multiple synthetic oligonucleotides representing different parts of each target gene can be spotted onto nitrocellulose or glass slides. Rapid synthesis of oligonucleotides will enable the spotting of many gene targets onto arrays enhancing the potential for obtaining clinical relevant data. Finally, initial screening by microarrays can be followed by real-time PCR validation of individual mRNA results.

The present investigations constituted feasibility studies for the development of reagents and protocols for the use of TaqMan and microarray assays. Overall, it was concluded that such assays can be easily designed and implemented for the screening of large number of clinical specimens. TaqMan RT-PCR is an extremely sensitive technique. That's why the presence of carefully selected controls, high purity RNA from samples, and other important parameters of TaqMan runs are of great importance. The TaqMan assays developed for this project can be used for the detection of mRNA tumor markers in various samples from breast cancer patients and probably will have more significant results when used for bigger sized samples with negative controls taken from same patients' not diseased tissues or from healthy individuals. The set, rather than a single marker gene, may significantly decrease the likelihood of false positive results.

Microarray experiments revealed that custom arrays can be easily produced incorporating many potential mRNA tumor markers. Microarrays have a number of advantages over TaqMan assays, especially when many clinical samples are to be

screened. In addition, microarrays can incorporate multiple positive and negative controls as well as multiples of the same sample at different locations of the array to ensure reproducibility and statistical significance. Ideally, initial screening of biological samples could be done by microarray analysis and individual positive samples could be confirmed by additional tests using TaqMan assays.

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VITA

Anna Henrik Israyelyan was born in Yerevan, Armenia. She graduated with medical degree from the Yerevan State Medical University after Mkhitar Heratsi in 1999. The same year she started her narrow specialization in epidemiology of infectious and non-infectious diseases in the Department of Epidemiology, National Institute of Health of Armenia. In August 2001, Anna enrolled in graduate school in the Department of Pathobiological Sciences at Louisiana State University. There she began the work on the early diagnostics of breast cancer by mRNA tumor markers. After graduation, Anna plans to continue her studies toward a doctoral degree.