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Juliet Karen Bailey

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JULIET KAREN BAILEY

DISSERTATION

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

1999

MAJOR: PATHOLOGY

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ABSTRACT

IDENTIFICATION OF DIFFERENTIALLY EXPRESSED GENES
IN BREAST CANCER TISSUES AND CELLS

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JULIET KAREN BAILEY

May 1999

Adviser: Dr. Fazlul H. Sarkar

Major: Pathology

Degree: Doctor of Philosophy

Breast cancer, is the second leading cause of cancer related deaths among women in developed countries, and the incidence of morbidity and mortality is rising in developing countries. The purpose of this project was to utilize the differential display technique to identify genetic changes in normal versus malignant breast tissue. It was also used in a defined cell culture system having differential cellular characteristics, to identify genes that may be responsible for different biological behavior of these cell lines.

Messenger RNA from normal, breast cancer tissues, and breast tissues from reduction mamoplasty yielded fifty-nine differentially expressed bands representing differentially expressed genes. Northern hybridization analysis proved negative, suggesting that these genes may represent low abundant message. mRNA from two clones; one tumorigenic, and the other non-tumorigenic in nude mice; obtained by stable transfection of galectin-3 gene in a non tumorigenic BT 549 breast cell line, was analyzed by differential display. Galectin-3, a calcium independent carbohydrate binding protein has been shown to be involved in many biological processes, but its exact function is still unclear. A 607 bp fragment was differntially expressed by the tumorigenic clone, and DNA sequence of

which revealed a 93% homology with the human Line 1 retrotransposon (L1). L1 is a poly-A mobile element, and its insertion into functional genes has been implicated in human diseases, including breast cancer, however its role in breast cancer is not clear. To determine the locale and expression of galectin-3 and L1 in normal versus tumor tissues, immunohistochemical analysis of breast carcinoma specimens, fibrocystic, normal breast tissues, and the tumorigenic clone of BT 549, 11-9-1-4, was performed. L1 and galectin-3 was found to be co-localized, and the immuno-staining was most intense in tumor tissue, and was minimal in normal tissue. Staining was significantly correlated with disease progression and tumor recurrence, suggesting that the expression of galectin-3 and L1 may represent a new mechanism by which breast tumor cells acquire aggressive phenotype. However, the interaction of L1 and galectin-3, if any, and their influence on tumor development and progression remains to be determined.

DEDICATION

I would like to dedicate this work to my son Jeremiah Andrew, and to my parents Eric and Agnes. The times when I felt that I couldn't go on, each of you in your own unique and special way provided inspiration and motivation. Jeremiah, as I looked into the future and I saw you, I knew I had to go on; and Mum and Dad, your hopes, prayers, and sacrifices, were spent in this endeavor; this work is as much your work as it is mine. Thank you.

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I would like to thank my mentor, Dr. F. H. Sarkar, the members of the pathology graduate committee, and my Ph.D. committee for their help and suggestions, for their patience and understanding regarding the extended time it took to complete this project; to Karen and Amy for their untiring help and encouragement. I would also like to thank my parents and other family members, and friends for their support and encouragement through the years; and last but not least, I would like to thank the creator and the sustainer of all life for this privilege.

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INTRODUCTION

Historical Perspectives:

Breast cancer has been and continues to be a major problem for thousands of women and their families, around the globe. It is estimated that in the United States of America alone 178,700 new cases will be diagnosed in 1998, and 43,900 individuals will die from this disease. Cancer of the breast is the second leading cause of death among women in industrialized countries, and the incidence of morbidity and mortality from this disease is also increasing in developing nations (1).

What is breast cancer, and is it a new phenomenon? The American Cancer Society's working definition of breast cancer is a disease characterized by uncontrolled growth and spread of abnormal cells, which can result in death. The first written record of the disease is found on an Egyptian papyrus dated about 2000 BC. The physicians at the time recognized their inability to deal effectively with this disease, and they suggested it be left alone since the treatment offered may be worse than the disease (2). We next hear about breast cancer during the time of the ancient Greeks. They believed that an excess of black bile caused cancer. Breast cancer was therefore a coagulum of black bile within the breast, the monthly menstrual flow would naturally relieve women of having any excess, and this explained why breast cancer was most common in women who had experienced menopause. Therapy was therefore aimed at getting rid of this excess black bile "by diets, purgation, venesection, cupping and leaching". Their teachings were codified by Gallen around 200 AD. (2).

The nineteenth century brought a burst of scientific knowledge. In 1810, Muller described the cellular nature of cancer (2), and in 1840, Virchow published the result of

several decades of study of breast cancer. He proposed that breast cancer was not a systemic disease, but rather a disorder of the local cellular economy. The tumor, which arose in the breast epithelial cells, invaded locally in all directions, and spread via lymphatic and along fascial planes. The disease usually overcame the patient before it had time to spread to the distal limbs. With the advent of adequate anesthesia and antiseptic techniques in the 1890s, radical mastectomy became the treatment of choice for early breast cancer. In spite of the many meetings and debates, radical surgery continued to be the treatment of choice until the 1960s (2).

Several scientists, among them Bernard and Ed Fisher, Devitt, and Crile, based on results from animal experimental breast cancer studies found that the teachings did not hold true; the spread of the disease was not in a predictable centrifugal fashion along the lymphatic and fascia planes. They concluded that micrometastases were often present at the time of diagnosis, presumably due to a long subclinical history during which invasion had taken place via the blood vessels. It became clear why radical surgery did not improve the overall survival; the horse had already bolted when they attempted to close the door (3). These findings resulted in more conservative surgery of the breast and the addition of adjuvant endocrine therapy and chemotherapy. This improved the overall patient survival and reduced the risk of relapse, however even this has proven to be inadequate. "Cytotoxic chemotherapy produces anything up to 80% of objective remission in patients with advanced breast cancer, whereas endocrine therapy produces about 30% remission under similar circumstances (4)." Given this scenario, an 80% reduction in the risk of relapse and death after appropriate systemic treatment is predicted. Presently only a 30% reduction in the relative risk of relapse over a ten year period has been achieved (5).

Epidemiology and Risk Factors:

Global Trends: Since the 1960s, breast cancer incidence has been increasing all around the world, with western Europe and North America showing the highest rates; these increases are thought to be due primarily to changes in screening methods. Factors yielding higher recorded incidences include: mammographic screening in developed countries, earlier diagnoses, and a decrease in deaths in the United States and the United Kingdom. In the United States, the rates rose by twenty four percent between the years of 1973 and 1991. Japan and Singapore experienced the largest increase. In Japan the incidence rate for women aged 35-44 doubled between 1960 and 1985 to about two thirds the rate of North American rate. Although screening has contributed to this increase, it is believed that it is due to changes in risk factors (6). Even within countries where the incidence is relatively low, urban dwellers have a higher incidence than those in non-urban areas; and in the United States southern dwellers have a lower risk than those dwelling in the north, especially the northeastern part of the country. Individuals who migrated to western Europe and North America from areas with a low incidence of breast cancer, after a few generations, their life time risk became the same as the general population. This suggests the presence of environmental, or life style factor(s) which strongly influence the onset and the progression of this disease. It is estimated that there will be one million new patients per year globally, by the year 2000. More than fifty percent of these cases would have occurred in developing countries, with an increasing proportion of death (7).

Present Status: Currently, breast cancer is the most frequent cancer of women in developed countries (8). Between 1940 and 1980 the incidence of breast cancer rose about 1% each year according to the data in the Connecticut Tumor Registry. Between

1980 and 1987 the Surveillance Epidemiology and End Results (SEER) program of the National Cancer Institute reported a sharp increase of 32.5%, or more than 4% each year. It is felt that this increase was due to an increase to the early detection of small lesions, localized lesions, and carcinoma in situ (9). The American Cancer Society estimates that in 1998, 178,700 new cases of invasive breast cancer will be diagnosed in women in the United States, and about 1600 new cases in men. They estimate that about 43,900 deaths will occur, 43,500 women, and 400 men (1).

Lifetime Risks: Women in the United States have a 1/8 life time risk of developing breast cancer, while women in the United Kingdom have a 1/12 life time risk of developing this disease. In America, breast cancer is the second leading cause of cancer deaths among women; lung cancer being first. It is the leading cause of cancer deaths among women between the ages 40 to 55. The trend is similar in the United Kingdom where it is the leading cause of death among women between the ages 35 to 54. Although the 1990s have seen a decline in the rate of increase and in the mortality rates, this decline has been so slight that for all practical purposes it has remained essentially the same. This slight change can probably be attributed to the increased use of mammography among older women and regular screening by younger women (8).

Risk and Ethnicity: In the United States, white women are more likely to develop this disease, with an incident rate of 113.1 per 100,000, when compared to African American women with an incident rate of 101.0 per 100,000. African American women, age less than 45, are more likely, than white women, age less than 45 to develop this disease; and African American women have a higher mortality rate 31.2 per 100,000, than white women, 26.0 per 100,000. In the UK this disease is more common among minority

ethnic groups than indigenous Britons. The UK has the highest mortality rate in the world from this disease, although not the highest incidence. Over one million women die annually from this disease worldwide, and in the US, one woman dies every twelve minutes(8, 9).

Risk factors: The risk is highest in women who have a personal family history of breast cancer, especially a first degree premenopausal relative with bilateral cancer. Other known factors include some forms of benign breast disease; early menarche; late menopause; lengthy exposure to postmenopausal estrogens; recent use of oral contraceptives; never having children or having the first live birth at a late age; higher education and socioeconomic status, and an advanced age. Additional factors which are being studied are pesticides and other chemicals, alcohol consumption, induced abortion, diet and obesity, race, physical inactivity, and genetic makeup (10). Most women have one or more risk factor for breast cancer, however these risks are a very low levels and only partially explain the high frequency of the disease in the population; in fact the majority of cases are presently without specific risk factors. The etiology of this disease is obviously not well understood, and what is known has not been translated into practical ways of prevention (9, 10).

Dietary Factors: In the 1980s, dietary fat received much attention as contributing to the etiology and progression of this disease. This was the case with colon cancer, and women in Western Europe and North America consumed much larger amounts of fat, especially animal fat, than their counterparts in Asia and other less developed regions of the world. The link between dietary fat and breast cancer has been controversial, and the evidences are conflicting. Recent studies of the essential fatty acids suggest that omega-3 fatty acids found in fish oils and omega-6 fatty acids found in vegetable oils may play a

role in the etiology of breast cancer. Spanish and Italian studies of olive oil consumption and breast cancer have found that large consumption of this mono-unsaturated oil offers some protection against breast cancer development (11). A study done at three Spanish hospitals found that breast cancer patients had a significantly higher intake of fats, oils, lipids and meat, when compared to the control group who had a higher intake of cereals, legumes, carbohydrates, fiber and proteins. They found that these foods were protective against the disease while intake of poly-unsaturated fatty acids (higher than 15g daily) would be considered a risk factor for breast cancer (12). The role of dietary fat in breast cancer is still unclear.

Environmental factors: Another questionable risk factor is cigarette smoking. A recent report has contradicted the previously held belief that cigarette smoking was linked to breast cancer. The study found that there was no correlation between smoking, heavy smoking, or long time smoking and breast cancer (13); there is however some evidence that passive smoking may be linked to breast cancer (14), and this is quite contradictory. Attention has also been focused on the environment, particularly the synthetic estrogens. There is evidence for strong correlation between these compounds and this disease (15).

Purpose and Objectives of Research:

How these risks factors influence the genetic changes observed in breast cancer is still unclear; it could be, especially in the case of environmental factors, that they cause damage to genes, or they influence the regulation of the damaged genes. These are questions that are being addressed by other researchers.

The question of which genetic changes are translated into differential gene

expression in the cascade of breast cancer development, and progression [normal → atypical hyperplasia → carcinoma in situ → invasive cancer] will be investigated using the differential display technique. The objectives of this project are based on the hypotheses that (1) the heterogeneity in clinical breast cancer reflects the presence of multiple mechanisms involved in its genesis and progression, and that (2) there are genes which are differentially expressed at different histological stages of breast cancer development and progression. Based on these hypotheses, novel genes which are differentially expressed at different stages of breast cancer development and progression will be explored to identify and characterize them in an effort to increase, and improve the present knowledge of breast cancer, and to aid in the development of more effective therapeutic interventions.

The specific aims of this project are:

1. To identify the differences in gene expression among different stages of human breast cancer specimens using differential display of RNA prepared from microdissected breast lesions, and

2. To clone and separate the differentially expressed genes and identify their novelty by screening Gene Bank data sequences.

Mechanisms of Carcinogenesis:

What causes breast cancer? Who gets this disease? Cancer is caused by external (chemicals, radiation, and viruses) and internal (hormones, immune conditions, and inherited or developed mutations) factors. Causal factors may act together or in sequence to initiate or promote carcinogenesis. Ten or more years often pass between exposures or

mutations and detectable cancer. A consequence of this is that most cancers develop late in life. Cancer is a disorder of cells and although it appears as a tumor made up of a mass of cells, the visible tumor is the end result of a whole series of changes. The development of this disease is a multistep process. The first essential step or event in this process is initiation. This step occurs in the DNA, resulting in destruction or damage, or deregulation of some gene. More than likely this occurs in the tissue where the cancer will develop; for this discourse, the breast. The agent responsible for this event is a carcinogenic agent. This initial event causes transformation in the cell, but this transformation, may or may not lead to cancer, and if it does lead to cancer, the process may take years. For the development of cancer to occur a promoting agent must act on this transformed cell inducing it to divide, and develop into a tumor. These growth promoting agents are usually under tight control, and therefore an imbalance between growth promotion and growth inhibition must exist for tumor development (16).

An understanding of the events in the cell cycle is necessary to appreciate the changes that would lead to tumor development. The events in the cell cycling are divided into mitosis (M), gap 1, DNA synthesis (S) and gap 2, (M-G1-S-G2). These are not discrete events, but the divisions allow for study and understanding. During the *S phase* DNA is synthesized so that the normal amount is doubled, other components are also synthesized. During the *gap* that follows the integrity of the DNA and other components are checked, before the cell enters into mitosis, with the formation of two identical cells. There are many molecules that function during this cycling, and deregulation or mutation in any one of them could result in tumor development. Many of these molecules bind to DNA to regulate gene transcription; some are inducers and others inhibitors. There are also molecules, which bind to these transcription regulators to control their

activities. The whole process is very complex with several levels of control.

There are basically two types of genetic damage seen in cancer cells: dominant and recessive. Dominant damage usually occurs in proto-oncogenes, resulting in a net gain in function, while recessive damage occurs on tumor suppressor or growth suppressor genes which function as negative regulators of the cell cycle. Proto-oncogenes are usually normal cellular genes, which play a role in the cell cycle. These normal cellular genes may become oncogenic, resulting in a malignant neoplasm. They provide the cell with constitutive growth promoting signal. When there is damage to the gene, its expression may change or its product may change. Either a change in expression or a change in product may result in malignancy. Dominant proto-oncogenes do not need both alleles to show damage for transformation to result. The normal allele may continue to be expressed, but the phenotype of the mutated allele dominates. One reason for this could be that there is a certain threshold for expression to be of any consequence; and secondly, the product of the gene acts on targets some distance away. In many cases, the protein product of these genes must form oligomers in order to exert an influence, and oligomers with mutated products may interfere with normal functioning. In recessive mutations, usually both alleles show mutations. In some cases where there is a dose dependency, mutation in one allele is sufficient for transformation. (17).

While the search goes on for the environmental agent or agents that cause(s) breast cancer, intense efforts are being made to understand the genetics, with the hope of better predictive, prognostic, controlling, and curative procedures.

Genetics of Breast Cancer:

Familial breast cancer: Breast cancer is a complex disease genetically. There are many factors implicated in the pathogenesis of this disease. Except in a very few cases,

mostly familial, each case of the disease shows a different genetic profile. Many growth factors and nuclear regulatory proteins play a role in the development and progression of this disease. To date, the only genes found to have clear connections with breast cancer development are BRCA1 and BRCA2. BRCA1 is a large gene located on chromosome 17q21. It encodes a 190kDa zinc finger protein. This protein has sequence homology and biochemical homology to the granins. In normal cells, including alveolar and ductal epithelial cells of the mammary gland, it is a nuclear phosphoprotein. However, in malignant cells it seems to be localized mainly in the cytoplasm, rather than the nucleus. The granins, which share sequence homology with BRCA1, are secreted proteins, which are posttranslationally glycosylated and are responsive to hormones. Some members of this family are triggered by cyclic AMP and are regulated by estrogen. During pregnancy BRCA1 mRNA is elevated in mammary epithelial cells. There is evidence suggesting that pregnancy and lactation are protective against breast cancer development. In individuals with germline mutations in, or loss of, the BRCA1 gene there is a high risk of ovarian cancer, and up to ninety four percent risk of breast and or ovarian cancer by the age of seventy. Abnormality in this gene is present in approximately forty-five percent of early onset, or familial, breast cancer. Thus, it seems possible that the product of this gene plays a role in growth regulation in mammary epithelial cells, most likely to inhibit proliferation. In ninety percent of the families with germline mutations in this gene, there is a prematurely truncated protein, resulting from one allelic loss by somatic deletion (18).

The other gene showing direct correlation to breast cancer development and progression is BRCA2, localized to chromosome 13q12-13. In approximately forty percent of hereditary early onset breast cancers this gene is mutated. Mutation in BRCA2 confers a high risk for breast cancer but not ovarian cancer. This is especially

true for male breast cancer, and there is an elevated risk for prostate cancer in carriers of the mutated form of this gene. Almost all cell lines or tumors showing a loss of BRCA2, simultaneously show loss of the RB1 gene on chromosome 13q14. It is thought that these two genes may act in concert. The mutations in these two genes are usually loss or deletions, resulting in loss of function. In their normal state, their function would prevent tumor development, and so they are designated tumor suppressor genes. Thus five to ten percent of breast cancers which show a familial pattern and early onset are attributable to mutations in these susceptible genes. The remaining ninety to ninety five percent show no consistent pattern and are referred to as sporadic or nonfamilial breast cancer (17, 18, 19, 20).

Sporadic breast cancer: Individuals with breast cancer who have no family history of this disease through two generations, from both the maternal and the paternal lineages are categorized as sporadic cases. The following is a discourse on some of the genetic anomalies observed in sporadic breast cancer. Many nuclear regulatory genes show mutations and other aberrations in breast cancer; among them are P53, MYC, ERBB-2 BRCA-1, RB1, 11p, and 3p. MYC is a nuclear phosphoprotein located on chromosome 8q24, which seems to have many functions. In normal cells it is tightly regulated, and functions as a transcriptional regulator, controlling cell proliferation, cell differentiation, and apoptosis by binding to specific regions on DNA, and dimerizing with the nuclear regulatory protein, MAX. Expression of c-myc is necessary, and in some cases sufficient, for entry into S phase of the cell cycle. It is usually found in a heterodimeric complex with MAX. MYC also associates with the transcription initiator TFII-I to repress transcription from basal promoter elements. MAD, is another nuclear regulatory protein which appears to antagonize MYC. It is induced in differentiated cells, and MYC is down-

regulated in these cells. MYC induces apoptosis under conditions in which it is not normally expressed, such as serum deprivation and growth factor withdrawal, however, the bcl2 product can overcome this. When c-myc expression is shut off, the cell is quiescent or cellular differentiation occurs. Structural alterations to MYC may occur through amplification, chromosomal translocation, retroviral insertion, retroviral transduction and other means; and these are always associated with tumorigenesis. In the normal mammary gland there is increased expression with the proliferative phases, and with the normal apoptotic involution. Experimentally, c-myc expression can be induced in normal and tumorigenic breast cells by several growth factors, hormones and other growth promoting substance such as epidermal growth factor, TGF-α, IGF-I, estrogen, insulin, and linoleic acid. The TGFs are all able to suppress c-myc expression induced by EGF, and thus epithelial cell proliferation. MYC is able to induce ornithine decarboxylase, cyclin A, and cyclin E. Ornithine decarboxylase regulates polyamine biosynthesis and is essential for S phase progression. MYC can transform and immortalize cells in association with ras, and bcl-2. The c-myc gene is often amplified in breast cancer, and amplification is associated with poor prognosis. In the MDA-MB-231 estrogen receptor negative cell line there is a constitutively high level of myc expression, which seems to be caused by a threefold increase in the half-life of the mRNA (21).

Jun and fos are two other nuclear phosphoproteins that are involved in the regulation of transcription. Fos is a 55kDa protein, which is activated after induction of differentiation or cell division. Mutations occurring in the 3' region, both coding and noncoding, result in instability of the fos mRNA, conferring a longer half life. Substitution of the normal promoter for a viral promoter, so that the serum response element is no longer adjacent to the gene, results in a change in the specificity of response to

mitogenic factors. The protein $p39^{jun}$ is related to activator protein 1 (AP-1). Jun forms a complex with fos and is involved in the regulation of transcription. Jun participates in cell proliferation, differentiation, response to toxic agents and apoptosis. This diversity of action may come from its association with self, with fos and with other activating transcription factor response element binding protein. Vitamin E succinate (VES) is a potent inducer of apoptosis in human breast epithelial cells. C-Jun seems to play some role in VES induced apoptosis. After VES treatment of MCF-7 cells c-jun mRNA and protein are elevated and this elevation is prolonged and there is increased AP-1 binding activity (22).

ERBB-2 (HER-2/neu), located on chromosome 17q12 , is another gene that is often amplified, twenty to thirty percent in invasive breast cancer, and there is strong correlation with relapse and with poor prognosis. The tumors show a poor response to hormone therapy. This gene codes for a 185 kDa transmembrane glycoprotein; it is a receptor protein related to the epidermal growth factor receptor. It has intrinsic tyrosine kinase activity, and carries out autophosphorylation. The ligand for this receptor is unknown (23).

Cyclin D1: The gene ccnd1 is located on chromosome 11q13, and encodes cyclin D1. Cyclin D1 is bound by a cyclin dependent kinase, forming a complex which phosphorylates the retinoblastoma protein, P^{RB}/P^{107} allowing for progression from G1 to S in the cell cycle. Cyclin D1 appears to be a positive growth regulator, and its synthesis in the cell cycle is highly dependent on.the growth factor state. The D cyclins bind P^{RB} and P^{107}, they also bind viral oncoproteins E1A and E7. Cyclin D1 overexpression is found in approximately fifty percent of primary breast cancer, and thirty three percent of these are linked to amplification of the gene. The majority of these tumors are positive for the

estrogen receptor, making cyclin D1 expression a good marker for response to hormone therapy (17).

Apoptosis or programmed cell death, has recently gained a lot of attention. This is a physiological process, which is genetically determined and is essential for normal development and maintenance of tissue homeostasis. All cells in their normal state are programmed to die. A malignant state generally confers immortality on these cells. Apoptosis is induced by many toxic substances, radiation DNA damage, and by insufficient nutrients. The cell must have the ability to detect these toxic substances, to assess the nutrient state, and to respond by turning on or off the genes necessary for apoptosis. Cells undergoing programmed cell death have their DNA fragmented into 180 bases (nucleosome), and these are compacted into sharply delineated masses. The cytoplasm is condensed, and blebs or apoptotic bodies are pinched off and phagocytozed. There is no inflammation associated with this cell death. Tumor suppressor genes are thought to play a major role in these responses. These genes would be responsible for arresting the cell cycle, until the noxious stimulus is removed, or activating the processes necessary for cell death (24).

P53 and apoptosis: The tumor suppressor gene p53 localized on chromosome 17p13 is detected at very low levels in all normal cells. The protein blocks cell proliferation by arresting the cell at the G1-S transition. This is a checkpoint in the cell cycle, where the integrity of the genome is assessed, When DNA damage occurs, p53 synthesis is increased, resulting in its accumulation and the halting of DNA replication and cell division so that DNA repair may occur. If repair is not successful, then the cell's suicide machinery is triggered. This is especially true after ionizing radiation, and other types of DNA damage; it enhances sensitivity to ionizing radiation and to anticancer drugs. P53 is

regulated by phosphorylation; it binds specific sequences in promoters and activates target genes. In order that it might bind these response elements p53 must form tetramers. The majority of observed mutations in this gene occurs in the mid region affecting the protein protein interaction and the DNA binding ability. Mutant p53 cannot achieve the appropriate configuration and it blocks the activity of wild type p53 by forming oligomers with it, giving a dominant negative effect. There are many target genes for this protein, and many of these are associated with DNA replication, for example, myb, histone H3, DNA polymerase-a , growth arrest DNA damage inducible genes, GADD45, MDM2, $p21^{waf}$, and CDC2. MDM2, the mouse double minute gene encodes a 90kDa phosphoprotein, which copurifies with p53; it binds to p53 and blocks its transactivation. Overexpression of MDM2 increases tumorigenic potential and inhibits G1 arrest and the apoptotic function of the p53 protein. This overexpresson could result from gene amplification. The overexpression seen in breast cancer cell lines results from an increase in transcription without gene amplification. Wild type p53 induces MDM2, indicating that a feedback loop exists between the two. P53 is also necessary for cell death resulting from viral infection. Several viruses have evolved a protein, which can prevent or delay the onset of apoptosis. The E1A gene product of the adenovirus is necessary for the lytic life cycle and is responsible for much of its transforming properties. Some studies show that the E1A gene product allowes the cells to overcome p53 G1 arrest after ionizing radiation, and continues to synthesize DNA. Other studies show that wild type p53 induced apoptosis in the presence of the E1A gene product. E1B gene products are proteins produced by the virus. The 55kDa product interacts with p53 and prevents it from transactivating other genes, preventing its ability to induce apoptosis; the 19kDa product appears to exert its influence downstream of p53. P53 is mutated in forty

percent of breast cancers, and most of these mutations are missense mutations. In fact mutation in the p53 gene is the most frequent genetic alteration detected in cancers (25).

Bcl-2 and apoptosis: Another very important gene in the apoptotic process is Bcl-2, this appears to act downstream of p53 to prevent apoptosis. Bcl-2 is located on chromosome 18q21, and encodes two proteins, a 26kDa bcl-2a, and a 22kDa bcl-2b. These two proteins are formed as a result of alternative splicing of the mRNA. Bcl2 belongs to a family which includes bcl-xl, bcl-w, mcl-1. All these along with bcl-2 act to prevent apoptosis. Other members of the family include bik, bak, bad, bax, bcl-xs; these activate apoptosis. The members of this family dimerize with each other resulting in antagonism or enhancement the activities of one by another. The protein products of bcl-2 and bcl-x appear to be localized in the outer membrane of mitochondria, the endoplasmic reticulum and the nuclear membranes. It is thought that in these positions they stimulate and antioxidant pathway where oxygen free radicals are produced; they also modulate the cytoxicity of some anticancer agents, thus inhibiting apoptosis. Although many groups of epithelial cells expressing bcl-2 are in the proliferating zone, it appears that bcl-2 act to inhibit cell death, rather than to stimulate cell proliferation. P53 down regulates the bcl-2 gene, however, coexpression of bcl-2 with c-myc can overcome p53 induced apoptosis. Bcl-2 and bcl-xl can also inhibit Fas mediated apoptosis. Bcl-2 is overexpressed in many breast cancers, and when this is the case there is an inverse relationship between its expression and the apoptotic index. Bcl-2 expression in mammary tumors is associated with a positive estrogen receptor status and a negative epidermal growth factor receptor status. An inverse relationship is also seen with c-erb-B2. It seems possible that bcl-2 is involved in growth, morphogenesis, and evolution of breast cancer. Experimentally, bcl-2 enhances tumorigenesis and metastatic potential in

MCF-7 cells. When cultures are infected with bcl-2, they show significant increase in the concentration of MMP2 and MMP9 in the supernatant. These two molecules facilitate invasion by mediating the degradation of the extracellular matrix components. When compared with prostate cancer, overexpression in breast cancer is associated with a better prognosis (26, 27).

P^{RB} and P^{107}: The retinoblastoma protein, and p107, both tumor supressor proteins, binds to myc/max complex and prevents transactivation. E1A inactivates pRB and p107. PRB does not bind myc, but p107 seems essential for its regulation. Overexpression of p107, results in cell cycle arrest before the S phase (28).

Growth Factors:

There are also many growth factors which are implicated in breast cancer. They play a major role in tumor progression. Many tumors seem to have an autocrine growth response, where the production and target site is the same, making the tumor cells less dependent on the surrounding environment. Many growth factors have cell membrane anchored receptors. These receptors are generally transmembrane glycoproteins, with an extracellular ligand binding domain and an intracellular ligand binding domain. Signal transduction is usually mediated by a tyrosine, threonine or serine residue, which function as kinase. Many protein tyrosine kinases have substrates which function in cell adhesion, such as integrins and vinculin. Abnormality in expression or function would have implications, not only for transformation but also for metastasis. Some of these growth factors interact with nuclear proteins; for example, insulin like growth factors I and II (IGF-I and IGF-11) appear to bind p107 and pRB (retinoblastoma) proteins, both of which are nuclear proteins. There are some evidences showing that the stromal cells surrounding the breast secrete IGF-I and IGF-II, FGFI and FGFII, and other peptide

growth factors, and these would exert their influence in a paracrine manner. Sixty percent of the primary breast tumors with detectable estrogen receptors respond positively to hormone therapy. The antiestrogen , tamoxifen is the main drug used for this purpose. The belief is that this drug mediates its action through the growth factor TGF-β, which has an autocrine growth inhibitory effect on most human breast cancer cells (29).

TGF-β is a multifunctional growth factor with three isoforms, β1, β2 and TGF-β3. TGF-β1 and β2 are potent inhibitor of epithelial cell growth, while being stimulatory in other cell types. TGF-β inhibits c-myc induction, and may induce P15 and P27. Experiments show that there is an increase in the secretion of biologically active TGF-β in cell lines in response to antiestrogens, that are inhibitory. The exact mechanism of action is not clear. Experimental results from patients studies show two patterns; an initial increase in the plasma concentration of TGF-β in response to antiestrogen therapy, and a later increase or decrease which is dependent of the tumor size. This suggests that the tumor is secreting TGF-β2 (30).

EGF: The epidermal growth factor receptor, a transmembrane glycoprotein, whose overexpression is associated with a negative estrogen receptor status, failure to respond to hormone therapy, and poor prognosis. Gene amplification seems not to be the reason for the overexpression, there is an increased amount of mRNA, but not protein. Epidermal growth factor (EGF) has a transmembrane protein receptor with tyrosine kinase activity. There are four members of this receptor, ErbB1, ErbB2, ErbB3 and ErbB4. The binding of the ligand to the receptor results in the activation of the receptor, which eventually results in the synthesis of DNA and cell proliferation. The receptor erbB2 is frequently overexpressed in breast cancer patients, and the prognosis is poor in each case (31).

FGF: Fibroblast growth factors are potent stimulators of endothelial cell proliferation. They are important in angiogenesis, the formation of new vascular channels, an essential step in the establishment of solid tumors. Many other growth factors participate in this process (31).

There are also G-proteins which active the exchange of GTP for GDP in response to receptor ligand complex. The RAS protein falls into this category. The members of the ras family are membrane associated G proteins with GTPase activity. They exchange GDP for GTP and a signal is transduced. Mutations in the encoded protein p^{21ras} are usually point mutations which result in a change in the oncogenic potential, keeping the protein in the active GTP form. Many mammary carcinomas have mutations in cHa-ras at codon 12 (32).

Metastasis:

Metastasis is a major aspect of tumor biology, and it is responsible for most of the mortality associated with cancer. Metastasis is the distant spread of the tumor from the original site, and the formation of colonies in secondary sites.

Epithelial cells rest on a basement membrane which separates them from the surrounding connective tissues and the vasculature. Surrounding the basement membrane is the connective tissue of the extracellular matrix, forming barriers to invasion.

Many tumor cells secrete proteases which are able to cleave components of the basement membrane and the extracellular matrix; or they are able to stimulate neighboring cells to secrete these substances. Part of the metastatic phenotype is the cells ability grow without neighboring cells, and to be uninhibited by confluence or contact inhibition factors. Some metastatic cells have invadopodia, and these have integral membrane proteases on their surface. These membrane associated proteases allows for

degradation of and adhesion to extracellular matrix components. Some of these proteases are secreted, and are soluable. The matrix metalloproteases (MMPS) are among those that seem to participate in this process. There are collagenases and gelatinases that are able to degrade collagen fibers, elastase which degrades elastin, and stromelysin. Matrix metalloproteases are regulated by tissue inhibitors of metalloproteases (TIMPs). TIMPs form complexes with MMPs and regulate their activites. TIMPS are down regulated in many metastatic tumor cells. Cathepsin B and L are proteases that are associated with the cell surface. They degrade extracellular matrix components both at acidic and neutral pH (33).

Serine proteases such as urokinase plasminogen activator (UPA) also play a role in this process. UPA and tissue plasminogen activator, tPA, cleave plasminogen to form plasmin which is a broad specificity protease. The receptor for uPA, uPAR is a 55-60 kDa cell surface glycoprotein. Comparisons of tumor infiltrate and normal cells, show many more tumor cells to be positive for uPAR than normal cells. UPAR is also deteced in malignant epithelial cells. It appears that uPA functions to release cells from the substratum during migration rather than mediating extracellular matrix degradation (34). There are other molecules that have been found to be involved in this process. Annexin I is a calcium binding protein involved in membrane cytoskeletal associations. This molecule is produced at the surface of the highly metastatic rat mammary adenocarcinoma cells, MTLn3. A metastatic associated gene, mta1, was found to be increased fourfold in this cell line, when compared to the nonmetastatic cell line, MTC4. Expression of mta1 correlated positively with the metastatic potentials in two human breast systems, MDA MB 231 (35).

Galectin-3 is an animal lectin with specificity for b-galactoside. It is a 29-30

kDa, calcium independent carbohydrate binding protein, with a single carbohydrate binding domain. Although its exact role in the body is unknown, there have been several experimental results that showed it be involved in many processes, including cell growth, adhesion, differentiation, inflammation, transformation, morphogenesis, and metastasis. Galectin-3 also acts as a substrate for human and bacterial metalloproteinases 2 and 9. Galectins represent a family of 8 carbohydrate-binding proteins identified by characteristic amino terminal sequence and affinity for P-galactoside containing simple sugars and glycoconjugates. Galectin-3 is expressed in a wide range of neoplasms including spontaneous, viral, chemical, and UV induced tumors. Among some related experimental tumor cell variants such as melanoma, fibrosarcoma and angiosarcoma, its expression correlates with the experimental metastatic capability of the cells (36). Recently a large number of tissue specimens were surveyed and the expression of galectin-3 in the human colon was related to the neoplastic transformation, and progression towards metastasis. In steroid sensitive breast carcinoma cells, it was suggested that estradiol and progestin might act as coordinates regulating specific genes, including up-regulation of galectin-3 expression, leading to the acquisition of the metastatic phenotype (37).

Histopathology of the Breast:

The breast which is under the influence of the ovarian and pituitary hormones undergoes cyclical changes each month until menopause, when they atrophy and involute. Each breast has fifteen to twenty five glandular units or lobes, and each lobe has a compound tubulo acinar gland. A fiberous septa separates each lobe. The nipple or lactiferous duct drains each lobe by a separate opening on its surface. Each lobe is divided into lobules each with a system of ducts, the alveolar ducts. The functional secretory units of the breasts are the distal termini of the network of tubes, and they are referred

to as terminal duct lobular units (TDLU). Dense interlobular connective tissue separates these lobules, and the ducts are lined by cuboidal or by low columnar epithelial cells.

Like any other organ the breast can experience pathological conditions based on the tissue present. Because of its anatomic position it is subject to trauma, which could cause bruising and scarring. It is a very fatty tissue, and fat necrosis is possible, so are hematomas and ruptured cysts. The breast can also have infections, which could result in galactocele in nursing or recently nursing women; then there is the comedomastitis, a periductal inflammation of unknown etiology. Fibrocystic disease, or disorders of normal breast development, are very common, greater than forty percent of the population present with these. Papillomatous growth, solitary or multiple are also possible. All of these are considered benign, and most of them do not predispose to invasive carcinoma (38).

Ductal carcinoma in situ (DCIS), arises in the TDLU and is considered the true precursor of invasive cancer. It is often seen around invasive cancer. DCIS is usually presented as microcalcifications, or as soft tissue comedocarcinoma. The comedo type of DCIS has a necrotic center, and high grade pleomorphic nuclei. Mammography shows coarse granualar calcification of the necrotic center, and casting (out lining of the duct). Many of these tumors are positive for Her-2-neu, and have microinvasion to axillary region. The prognosis is poorer than for non comedo DCIS. Non comedo DCIS, is negative for high grade pleomorphic nuclei, they lack necrotic centers, and have microcalcification. They have a more favorable outcome.

There is a group of carcinomas referred to as special case: infiltrating ductal carcinoma, mucinous, tubular and papillary. Of these, infiltrating ductal carcinoma is the most common, 50 to 75% of cases; it also has the worst prognosis. The others are well differentiated tumors with an improved rate of survival (39).

Lobular carcinoma in situ LCIS, or lobular neoplasia, is clinically a disease of premenopausal women. Sixty to seventy percent of the lesions are mutlticentric and are found in one breast; 35% are found bilaterally. These are nonpalpable microscopic lesions that are distributed through the breast, and are discovered incidentally, because of biopsy or aspiration for other reasons, ie, fibrocystic disease. In these women the risk of invasive carcinoma is 7 – 12 times higher than in the age adjusted population; or a twenty five percent chance of developing an invasive cancer in either breast in twenty five years. After treatment for the initial finding, a majority of patients remain disease free for twenty years or longer (40).

Ductal and lobular epithelial hyperplasia may be linked over time to development of carcinoma in situ. There is a morphologic continuum from hyperplasia to mammary carcinoma, and there is a morphologic continuum from epithelial atypia or carcinoma in situ to invasive carcinoma, however, most patients with atypia or carcinoma in situ do not develop invasive breast cancer within ten to twenty years after local resection. Both hyperplasia and carcinoma in situ begin in the terminal ductal lobular units (38).

MATERIALS AND METHODS

Differential Display Technique: Messenger RNA differential display was described by Ling and Pardee in a 1992 Science article (41). It was an alternative to subtractive hybridization. It is a technique for identification and analysis of altered gene expression in eukaryotic cells at the level of the messenger RNA. Unlike more conventional techniques , mRNA differential display does not require prior knowledge of the gene of interest. Genes that are known and unknown can be studied by this technique, since both upregulated and down regulated gene can be identified simultaneously.

This technique utilizes two different primers in a sequential manner. The first primer is an anchored oligo-dT ($T_{11}MN$), which anneals to the poly(A) tail of the untranslated 3' sequence of the gene. The enzyme reverse transcriptase uses this to generate cDNAs. The second primer is an arbitrary primer, with a defined or known sequence; usually a tenamer (10 nucleotides), is added to the cDNA and the enzyme taq polymerase makes double stranded DNA, and amplifies it using the polymerase chain reaction. This is done in the presence of a radioactive nucleotide. These amplified DNA fragments represent the 3' termini of mRNAs. These fragments are separated on denaturing polyacrylamide gel, and exposed to X-ray film. The film is used to identify differences in expression between two or more samples. The genes or bands of interest are cut and purified from the gel, reamplified, cloned, and sequenced either before or after Northern blot confirmation (Fig. 1,2).

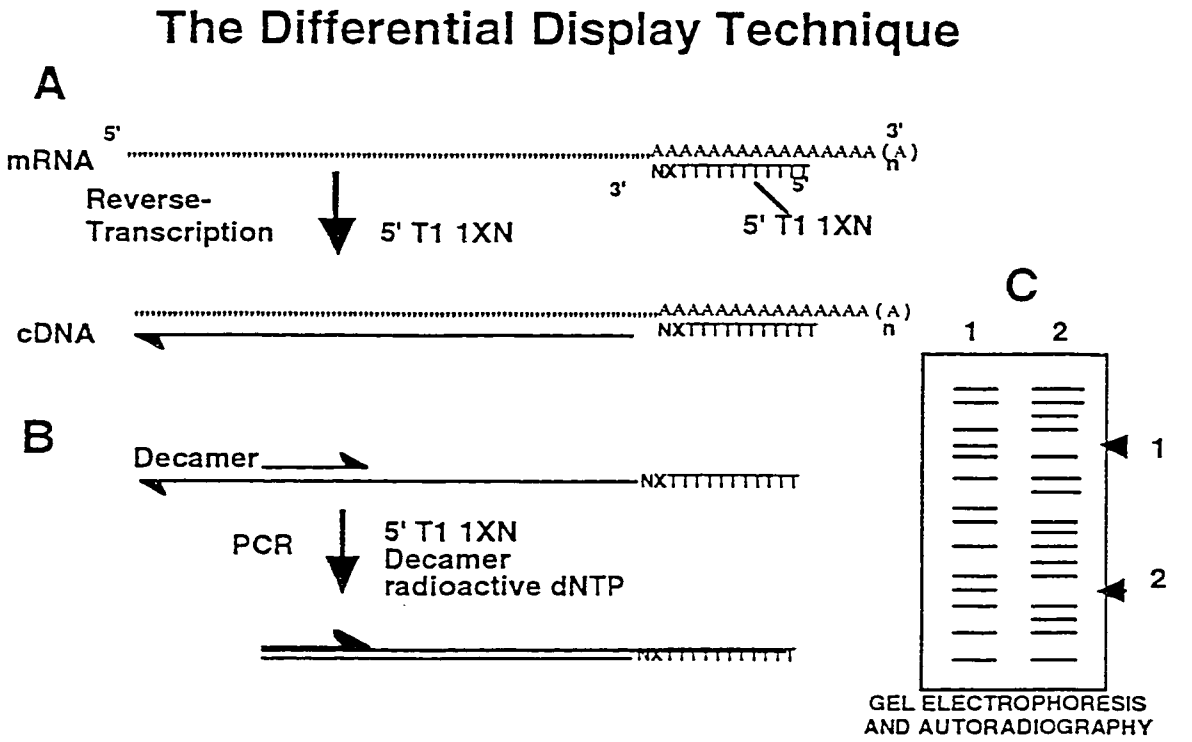

Figure 1. A schematic outline of the differential display process. Obtained from Dr. Reddy.

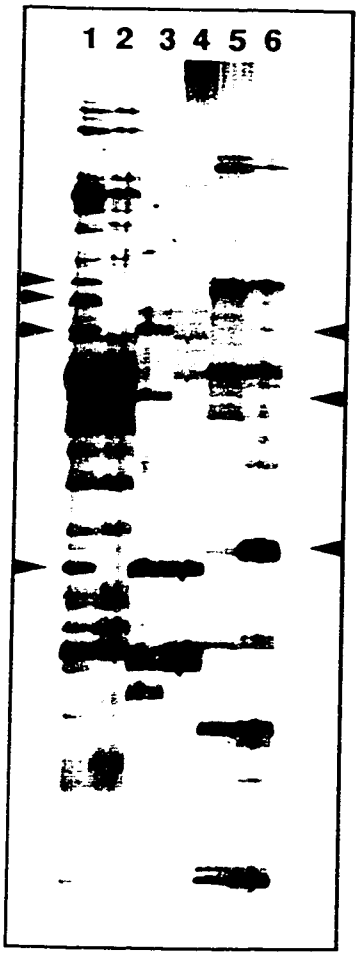

Figure 2. A typical example of a differential display gel. Here lanes 1 and 2 AP-1 primer, lanes 3 and 4 AP2 primer, and lanes 5 and 6 AP3 primer. Lanes 1, 3, and 5 represent mRNA differential display of 11-9-1-4 cells, and lanes 2 4, and 6 represent 11-YX-1 cells. Arrowheads on the left indicate some of the overexpressed sequences in 11-YX-1 using AP-3 primer. The top two arrowheads on the right indicate overexpressed sequences in clone 11-9-1-4 using AP-2 primer.

mRNA Isolation from breast tissue: Several samples of breast cancer tissue were identified as normal, intermediate, or invasive cancer according to histology. Twelve from different samples and patients were grouped accordingly, and the total RNA extracted from them using the TRIzol method. These were paraffin embedded tissues. Total RNA was also isolated from fresh frozen breast tissue, normal and cancerous. The Trizol

reagent from Gibco BRL was used for this extraction. Briefly, 20 ml of Trizol was added to each tube containing 10mg or less of tissue. The mixture was homogenized until the tissue was in solution. The sample was kept on ice throughout the entire process. After incubating at room temperature for 5 minutes, 0.2ml of chloroform:isoamyl alcohol/1ml of Trizol was added, and the mixture shaken vigorously. Again it was incubated at room temperature for 3 minutes, and centrifuged at 8000xg/15 minutes at 4^0C. The upper aqueous phase was transferred to clean tubes , and 0.5ml isopropyl alcohol/1ml Trizol added, mixed by shaking and incubated at room temperature for 10 minutes. After incubation, the sample was centrifuged at 12000 x g/ 10 mins. At 4^0C. The supernatant was removed, and the pellet washed with 75% ethanol (at least 1ml /1ml Trizol). Vortexing was used to break up the pellet and the sample was centrifuged at 7,500 x g/5min at 4^0C The sample was either air dried or vacuum dried (incompletely to facilitate dissolving). The pellet was dissolved in RNase free water, aliquoted , the concentration determined by optical density, and the sample was stored at -70^0C.

Differential Display on breast tissue: The RNAmap Kit for GenHunter Corporation was used for this differential display experiment. Briefly, 0.2 mg of total RNA was heated to 65^0 C for 5 mins., then to 37^0C for 60 mins., 95^0C for 5 mins and then 4^0 C. This reaction was performed in an environment containing TRIS-CL, KCl, $MgCl_2$, DTT, dNTP, the polyT-XX primer and MMLV reverse transcriptase. All reagents except the RNA was supplied in the RNAmap Kit obtained from GenHunter Corporation. Two microliters of this reaction product was used in the subsequent polymerase chain reaction. The PCR has a volume of 20mL, with buffer containing Tris-Cl, Kcl, MgCL, and gelatin.

Each of the four RT reactions were mixed with the appropriate polyT-XX

primer and one of ten nonspecific tenamer primer (Ap 1 - 10), dNTPs and a ^{35} S-dATP or a ^{33} P-dATP, Taq polymerase and mineral oil. The reaction parameters were 94^{0}C 30 sec. ----> 40^{0}C, 2 min. -----> 72^{0}C, 30 sec. for forty cycles -----> 72^{0}C 5 min. -----> 4^{0}C. Again, all the reagents were supplied in the RNAmap Kit. The PCR products were electrophorosed on a 6% denaturing polyacrylamide gel in TBE buffer for at least three and a half hours at 100W. The gel was covered with plastic wrap and dried on a gel dryer, and exposed to Kodak X-OMAT film overnight with intensifying screens. A total of 59 differentially expressed bands were cut from sequencing gels. 10mL of the reamplified bands were run on agarose gel and their size determined. The band sizes ranged from 700 - 140 MW according to DNA marker 8. Fourteen bands were used to probe the stock cDNA in reverse hybridization assay. (Table 1 & Fig. 3).

BAND ID	TISSUE TYPE	PARTNER STATUS	MOL. WEIGHT.	BAND CHARACTER
AP1 AN4	NORMAL	MISSING	370	SINGLE FAINT
AP3 CN2	NORMAL	MISSING	370	SINGLE FAINT
AP5 CT3	TUMOR	MISSING	370	MEDIUM
AP5 AN2	NORMAL	MISSING	370	MEDIUM
AP5 AN3	NORMAL	MISSING	320	MEDIUM
AP1 AN1	NORMAL	MISSING	501	FAINT
AP1 AN2	NORMAL	MISSING	404	FAINT
AP3 CN1	NORMAL	MISSING	501	FAINT
AP4 CN1	NORMAL	MISSING	320	FAINT
AP5 CT2	TUMOR	MISSING	320	MEDIUM
AP9 GN1	NORMAL	MISSING	320	SINGLE DARK
AP8 TNI	NORMAL	FAINT	489 -501	SINGLE DARK
AP6 CT2	TUMOR	MISSING	380	MEDIUM
AP6 CT3		FAINT	390	MEDIUM

Table 1. Characteristics of differentially expressed bands.

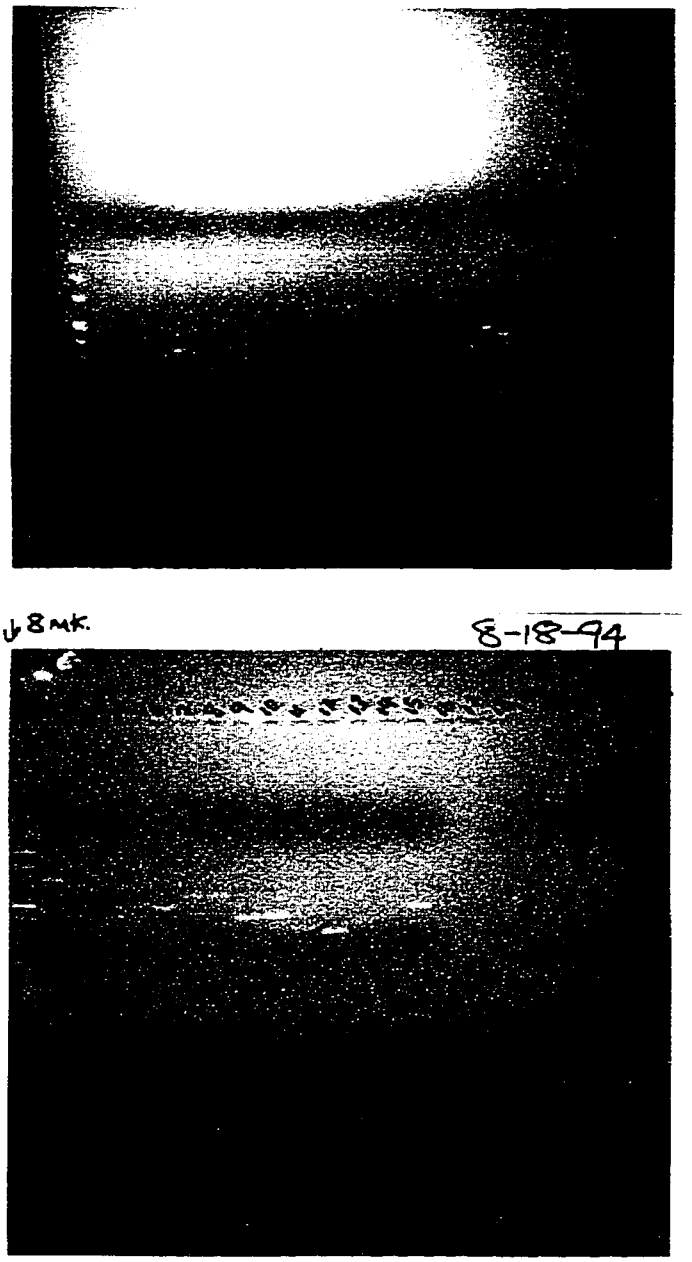

Figure 3. Agarose gel with bands which were reamplified. DNA marker 8 was used to determine the approximate size of the bands which were reamplified.

Reverse Hybridization: The reamplification products of the bands were prepared for slot blotting. The bands were hybridized to a nylon membrane and cross-linked. The membrane was prepared in triplicate. Three radioactive cDNA probes were made from the stock RNA; one probe from each sample pool. The probes were allowed to hybridized

to the membrane bound bands overnight. The membranes were washed and exposed to x-ray film. The bands that showed further differential expression in this reverse hybridization process were selected for cloning. The membranes were cut to fit the slot device, and presoaked in 10 X SSC. The samples were prepared as follows: 20µl sample, 10µl ddH_20, 0.04M NaOH, 25mM EDTA to 5X volume (150µl). This mixture was incubated at 95^{0}C for two mins. And immediately placed on ice. 2M Tris-HCl pH 7.4 at 5 X volume (150µl). The sample was mixed, and again placed on ice. 20µl of 6X dye was added, the tubes spun briefly at 4^{0}C, and placed on ice. Two pieces of filter paper, the size of the slot device was soaked in 10X SSC and place on the base of the slot device. All air bubbles were removed. The presoaked membrane was added on top of the filter paper, and again the air bubbles removed, the well plate put in place and secured. The slot device was attached to the vacuum source, and the samples loaded at 100µl/well. The vacuum, was turned on and the liquid siphoned off. Each well was washed twice with 200µl of 5 X SSC, and the excess fluid siphoned off by the vacuum. The unit was disassembled, the membrane, marked and air dried, and then UV cross linked. The membrane was wrapped and stored at 4^{0}C until time for hybridization. The probes were prepared by random primer labeling. CDNA from the reverse transcriptase reaction was evaporated in a speed vacuum for 30 mins. To approximately 0.5□l, to this 9.5□l of ddH_2O was added . The Stratagene random labeling kit was used, and the reagents added in the following order and amounts: 5µl random primer, 200ng cDNA,and 9.5µl of ddH_2O. Heated at 95^{0}C for 10 mins, and immediately placed on ice. After this 5X primer buffer without dCTP was added 5ml, and 5ml of dCTP (10mCi/ml), 5U/ml of klenow enzyme (.05µl). The mixture was incubated at 37^{0}C for 10 mins, and 1ml of stop solution added. Finally 44µl of STE was added and the probe purified by column. The column was wetted with 70µl

STE and the excess fluid and air was removed. The probe was added to the top of the column and pushed through. 2µl of purified probe was added to 10ml of scintillation fluid and the count taken. The membranes were prehybridized, and hybridized in the following solution; 6X SSC, 5X Denhardt's, 0.1% SDS, 0.1mg/ml sperm DNA. The membranes were prehybridized at 37°C for four hours. For hybridization, an identical solution was used with the probe added, and hybridization took place overnight, at 37°C, for 15 -18 hours. Once the hybridization was completed, the membranes were washed according to these times and temperatures, in a wash solution of 1X SSC and 0.1% SDS; 3 x at 42°C for 20 mins. each; 1 x at 50°C for 20 min; and 1 x at 55°C for 20 mins. The membranes were air dried and a film exposed to them and incubated at -70°C for 1 hour, and then developed.

Ligation and TA Cloning: Eight bands were selected for cloning. The one shot TA cloning procedure from Invitrogen was followed. Each reaction tube contained 1µl 10X ligation buffer, 2µl vector, 7µl DNA (PCR product), and 1µl ligase. The tubes were incubated at 12°C overnight, 15 -18 hours. Several 10cm diameter LB agar plates with ampicillin were prepared, and made ready. The ligation reactions were spun briefly and placed on ice. From previously thawed 0.5M β-mercaptoethanol, 2µl was taken and added to each 50µl vial of competent cells (E.coli INVaF'), gentle tapping on the vials allowed mixing. One ml of the ligation reaction was added to the vials of competent cells, and mixed by gentle tapping. The vials were placed on ice for 30 mins, then in a 42°C water bath for exactly 60 seconds, then immediately back on ice for 2 mins. To each vial was added 450µl of pre-warmed SOC media. They were placed in a 37°C gyrator incubator, with setting at 225 rpm, for one hour. During this time 25µl of a stock solution of

40mg/ml of X-gal was added to the LB agar plates. Once the hour of incubating was completed, the vials were placed on ice immediately. From each vial 25µl, and 100µl was spread on the prepared LB plates. The plates were placed in 37^0C incubator for overnight growth. The white colonies were selected for further growth, plasmid isolation, and sequencing .

Plasmid Isolation and Digestion: Transformed cells were grown overnight in terrific broth or LB broth with ampicillin. 1.5 ml of bacteria suspension was placed in a microfuge tube and the bacteria pelleted by centrifugation. The supernatant was discarded and the pellet resuspended (vortexing) in 100µl of the following ice-cold solution 50 mM glucose, 10 mM EDTA, and 25 mM Tris.Cl (pH 8.0). The mixture was incubated at room temperature for 5 minutes and 200µl of freshly prepared 0.2 N NaOH, and 1% SDS was added. The top of the tube was secured, and contents of the tube were mixed by inverting. This mixture was then incubated on ice for 5 minutes. To this was added 150µl of potassium acetate (pH 4.8), and with the cap closed the tube was vortexed gently in an inverted position, then incubated on ice for 5 minutes. After this there was a 5 minutes centrifugation, and the supernatant transferred to a fresh tube. Equal volumes of phenol/chloroform were added, mixed by vortexing, and pelleted by 2 minutes centrifugation, the supernatant was transferred to a fresh tube. Two volumes of ethanol was added, and the contents of the tube mixed by vortexing, then incubated at room temperature for 2 minutes, the supernatant was discarded, and the tube inverted on a paper towel to drain away all fluid. The pellet was washed briefly with 1 ml of 70% ethanol, a brief vortex and a brief centrifugation. The supernatant was removed and the pellet dried in a vacuum desiccator, and resuspended in 50 µl of TE (pH 8.0)

containing DNase free RNase 20 mg/ml. Ten µl of this solution of plasmid was incubated at 12°C with 1.2 µl of buffer H and 1 unit of EcoR1 restriction enzyme overnight. The following day the fragments were analyzed on 1% agarose gel.

Cell and Culture Conditions: The parental human breast cancer cell lines, T47D and BT-549, were a generous gift from Dr. Erik Thompson (Lambert Cancer Research Center, Washington, DC). Human bladder carcinoma cell line, 582, and human fibrosarcoma cell line, HT-1080, were purchased from ATCC (American type tissue culture). Human colon carcinoma (K12M), and human melanoma (A375) cell lines were originally obtained from Dr. I. J. Fiddler (M.D. Anderson Cancer Center, Houston,Texas). All of the cell lines and the established galectin-3 transfected BT-549 cell clones 11-9-1-4, 11-8-1-1, 11-YX-I were maintained in 150mm culture dishes in Dulbecco's modified Eagles medium supplemented with 10% heat inactivated fetal bovine serum, penicillin, streptomycin, vitamins, and essential and non-essential amino acids in a humidified chamber with 95% air and 5% CO_2, at 37°C.

Isolation of RNA from culture cells: Total RNA was extracted using Catrimox-14, which is a cationic surfactant solution capable of lysing the cells and inhibiting ribonucleases. The cells were grown to 80% confluency, washed with PBS and scraped in 5 ml of Catrimox-l 4 surfactant. After 10 mins. incubation at room temperature, the lysate was centrifuged at 1000xg for 10 mins. The pellet was vortexed with 200µl of guanidinium solution (3M guanidinium isothiocyanate, 200 mM sodium acetate, pH 4.0). Phenol chloroform extractions were performed and the RNA was precipitated with an equal volume of isopropanol and resuspended in DEPC (diethyl pyrocarbonate) treated water. Total RNA extracted from the two clones were treated

with RNase free-DNase 1, at 37^{0}C for 45 mins. in the presence of RNAsin . After phenol-chloroform extraction and ethanol precipitation, the pellet was dried and resuspended in 10ml of DEPC treated water containing RNAsin and quantitated by spectrophotometer.

Differential Display on cell lines: Differential display of total RNA extracted from clones 11-9-1-4 and 1-YX-I was performed using one-base anchored primers as described above. One µl of total RNA was heated for 5 min at 65^{0}C and reverse transcribed for 1 hr at 40^{0}C in 50µl of a buffer containing 10 mM Tris pH7.5, 50 mM KCI, 3mM $MgCI_2$, and 25 mM dNTPs (deoxyribonucleotide triphosphates) in the presence of 1 mM of polyT-X primer and 4 U of reverse transcriptase.

Polymerase chain reaction: Two µl of the above described reverse transcribed products were amplified in 20µl reaction volume in a buffer containing 10mM Tris pH 7.5. 50 mM KCI, ImM $MgCl_2$, 2.5 mM of the appropriate polyT-X primers T_{11}-G, T_{11}-A and T_{11}-C). 0.6 mM of the non-specific random primer (AP-1 to AP-8), 2.5mM of dNTPs, and 10 mCi of ^{32}PdATP (3000 mCi/mmol). Using three 3' primers and eight 5' random primers, all-in-all twenty four reactions were run in parallel for all mRNA samples. After heating the samples at 94^{0}C for 2 min for initial denaturation, 2.5 U of Tag polymerase was added and subjected to PCR amplification using the following parameters: 94^{0}C 30 sec.. 42^{0}C 1 min, 72^{0}C 30 sec, with a final elongation step of 5 min at 72^{0}C. Four ml of each PCR product was loaded on a 5% long ranger gel and ran at 100 watts until the xylene cyanol marker dye reached at the bottom of the gel. The dried gel was exposed to Kodak X-OMAT film overnight with intensifying screens.

Differentially expressed cDNA was recovered from dried sequencing gels

and reamplified by PCR to obtain optimal yields of candidate genes. This PCR amplified cDNA was cloned using the TA cloning system, version 1.3, from InVitrogen as described earlier. These clones were used as probes on Northern blots to ascertain their expression in BT-549, 11-9-1-4 and II-YX-I cell lines.

Northern blot analysis and cDNA sequencing: Total cellular RNA (10 μg per lane) from BT-549 and its galectin-3 expressing clones1 1-9-1-4 and 11-YX-I, was separated by electrophoresis on a 1% formaldehyde agarose gel and blotted onto nitrocellulose membrane. The membrane was prehybridized at 42^{0}C with a solution containing 50% formamide, 0.5% dextran sulfate, 5x Denhardt's solution, 0.05M sodium phosphare, 5 x SSC (saline sodium citrate) and 300 mg per ml denatured salmon sperm DNA. Hybridizations were performed with randomly primed ^{32}P-labeled cDNA probes which were cloned by differentially display or β-actin cDNA probe at the same temperature. The membranes were washed at 55^{0}C with 2x SSC-0.1% SDS (sodium dodecyl sulphate) for 30 mins., 2 x SSC-0.1% SDS for 30 mins., and 0.1% SDS for 20 mins., and autoradiographed.

One of the differential display probes, c4a., showed expression only in clone 11-9-1-4 This clone was sequenced using T7 and SP6 primers by chain termination DNA sequencing method using ABI DNA sequencer. Sequence analysis comparison was performed with other sequences from the gene bank.

Electrophoresis and immunoblotting: SDS-polyacrylamide gel electrophoresis was performed under reducing conditions, using a 12.5% polyacrylamide separating gel and a 3.5% stacking gel. Low range prestained protein standards were used to assess the

molecular weight of the proteins. The samples from the slab gels were electroblotted onto PVDF+ (polyvinylidene flouride) membranes. The membranes were quenched in 5% non-fat dried milk in PBS (phosphate buffered saline) for 4 h and then incubated with the first antibody [rat anti-galectin-3 monoclonal antibody, ATCC TIB-166; or polyclonal anti-p40 (first open reading frame, ORF 1, of L1 retrotransposon), raised in a rabbit (obtained as a generous gift from Dr. T.G.Fanning) for 1 h at 23^{0}C in the same quenching solution at a dilution of 1:200 and 1:1000 respectively. The membrane was washed 5 times (10 mins. each) with the quench solution containing 0.1% Tween-20 and subsequently incubated with the secondary antibody (horse radish peroxidase conjugated rabbit anti rat and goat anti rabbit, respectively) for 1 hr at 23^{0}C and washed as above. The bands were visualized using enhanced chemiluminescence kit from Amersham following the manufacturer's protocol.

Immunofluorescence: The cells were grown on coverslips (to identify the cytoplasmic expression of the proteins), fused and permeabilized with cold methanol (-20^{0}C) for 20 min, and washed with PBS. 0.1% BSA (bovine serum albumin) three times. They were blocked with 1% BSA in PBS for 30 min and reacted with the primary antibody (anti p40 ;1:200 or anti galectin-3; 1:2) for I h. The cells were washed again with PBS , 0.1% BSA three times and incubated with the secondary antibodies [TRITC (tetraethyl rhodamine isothiocyanate) conjugated anti-rabbit for p40 and FITC (fluorescene isothiocyanate) conjugated anti-rat for galectin-3] for 30 mins. After this, the cells were washed with PBS three times and mounted in slow fade (Molecular Probes). The slides were observed under Zeiss 310 Laser Scanning Microscope.

Immunostaining of breast tissues: Freshly frozen breast tissue specimens

were used from the frozen tumor bank of Harper Hospital, Detroit. MI. Cryostat 5mm sections from 34 breast carcinomas and 9 benign breast tissues were fixed for 10 minutes in cold acetone and allowed to air dry. Individual sections were stained for L1 (p40), galectin-3 or both using both antibodies as described below.

For L1, immunoperoxidase staining was performed on the Ventana Automated Immunostainer. The air dried slides were incubated with anti p40 antibody at 1:400 dilution for 20 mins., incubated with secondary antibody (peroxidase conjugated goat anti rabbit) and detected with DAB (diamino benzidine). After staining, slides were removed from the instrument and counter stained with hematoxylin. For galectin-3, the slides were rehydrated in modified phosphate buffered saline for 10 minutes, and incubated with anti galectin-3 antibody diluted at 1:400 for 20 mins. Slides were then washed in three changes of modified PBS, incubated with biotinylated rat secondary antibody (1:200) for 10 mins. Staining was completed on the automated stainer using Alkaline Phosphatase Red for detection and then counter stained with hematoxylin.

For dual staining, staining for L1 was performed as described above. Slides were then removed from the stainer, washed, incubated with Primary and secondary antibodies as described above (galectin-3) and then replaced on the stainer for the Alkaline Phosphatase Red detection. Slides were then counter stained with hematoxylin.

The immunostaining was semi-quantitated and scored in tumor cells as well as in host derived stromal cells (fibroblast and endothelium). Cases with diffuse strong staining were designated "2 +", cases in which staining was heterogenous or, were designated "1+" and tumors exhibiting staining in less than 10% of area were designated "0/+/-".

RESULTS

Differential display of RNA from breast tissue: Initially, samples were cut from a group of eleven paraffin embedded breast tissues. These tissues were grouped and labeled carinoma, carcinoma *in situ*, and fiberous, according to their histological appearance. The total RNA was isolated from each group and subjected to differential display analysis. The first five primers in RNAmap Kit 1 (GenHunter) yielded fifty-nine differentially expressed bands. Fourteen of these were selected based on estimated size and band characteristics, whether faint or bold, whether the partner was present or missing. (see table 2 & fig.3). These were isolated, reamplified and used to probe the stock RNA in a reverse hybridization reaction. Another set was isolated, and used as probes in a reverse hybridization reaction, and in both cases there were no positive results.

Differential display of RNA and Northern blot analysis of cell lines: Differential display analysis of total RNA obtained from clones resulted in the identification of several differentially expressed cDNA clones. A total of 53 differentially expressed messages were identified between tumorigenic 11-9-1-4, and non-tumorigenic 11-YX-1 cell clones. Initially, Northern analysis was performed using cDNA probes derived from 12 clones for which the signal was high. Of these, only one clone (c4a of about 600 bp) was unequivocally expressed in the 11-9-1-4 clone as revealed by mRNA expression. The results of a Northern blot analysis of RNA extracted from parental BT-549, and its galectin-3 expressing clones, hybridized with cDNA clone c4a, is demonstrated by Figure 4. The results depicted the presence of a hybridized band of about 6.5 Kb only in 11-9-1-4 cells. In the parental BT-549 clone no signal was detected, even after prolonged exposure. The blots were reprobed with β- actin cDNA to determine that RNA loading

variability and integrity had not contributed to the observed results (Fig.4) and the results showed that the differential expression of 6.5 Kb RNA band in 11-9-1-4 cells RNA was not an artifact.

BAND ID	SIZE	R. HYB.	CLONED
AP1 1 A-3	700	X	X
AP1 3 A-4	620	X	X
AP1 3 A-5	620	X	X
AP1 3 C-1	650	X	X
AP2 3 G-1	700	X	
AP2 1 T-1	630	X	
AP1 1 C-3	650	X	
AP1 3 C-2	400	X	
AP2 1 C-1	270	X	
AP4 1 A-1	570	X	
AP4 1 A-2	460	X	X
AP4 1 C-3	200	X	X
AP5 1 G-1	500	X	X
AP5 1 G-2	400	X	X

Table 2. Bands used in reverse hybridization.

Figure 4. Northern blot of total RNA Northern blot analysis of total RNA isolated from BT-549 (lane 1), and clones 11-9-1-4 (lane 2), and 11-YX-1 (lane 3). 15μg of total RNA was separated on 1% formaldehyde agarose gel and blotted onto the immobilon membranes. ^{32}P labelled probe was prepared by random labeling of cDNA obtained from clone c4a. The top arrow on the right indicates the expression of a 6 kb mRNA hybridized with the clone c4a present in clone 11-9-1-4, but completely absent in 11-YX-1 cells. The lower panel represents β-actin expression.

DNA sequencing of clone c4a: The clone c4a was characterized by DNA sequencing. The results of DNA sequencing of clone c4a revealed a fragment size of 607 bp (Fig.5). GenBank search revealed that clone c4a shared a 93% homology to second ORF of L1 retrotransposons (42) (Fig.5) from bp 1932 to 2541. L1 retrotransposons are a family of highly repeated long interspersed sequences which are dispersed in the mammalian genome. It was reported earlier that L1 retrotransposons are transcribed into a 6-7

Kb long transcript (42-44). The full length mouse or human L1 mRNA

showed two open reading frames which translated into two proteins, p40 and reverse transcriptase (45-48). Thus, Western blot analysis was performed using an available antibody against the p40 to establish the presence of this protein in the cell lines. In addition, a series of human breast tissue specimens, including benign breast and tumors were screened to evaluate the co-expression of p40 and galectin-3 by immunohistochemical studies.

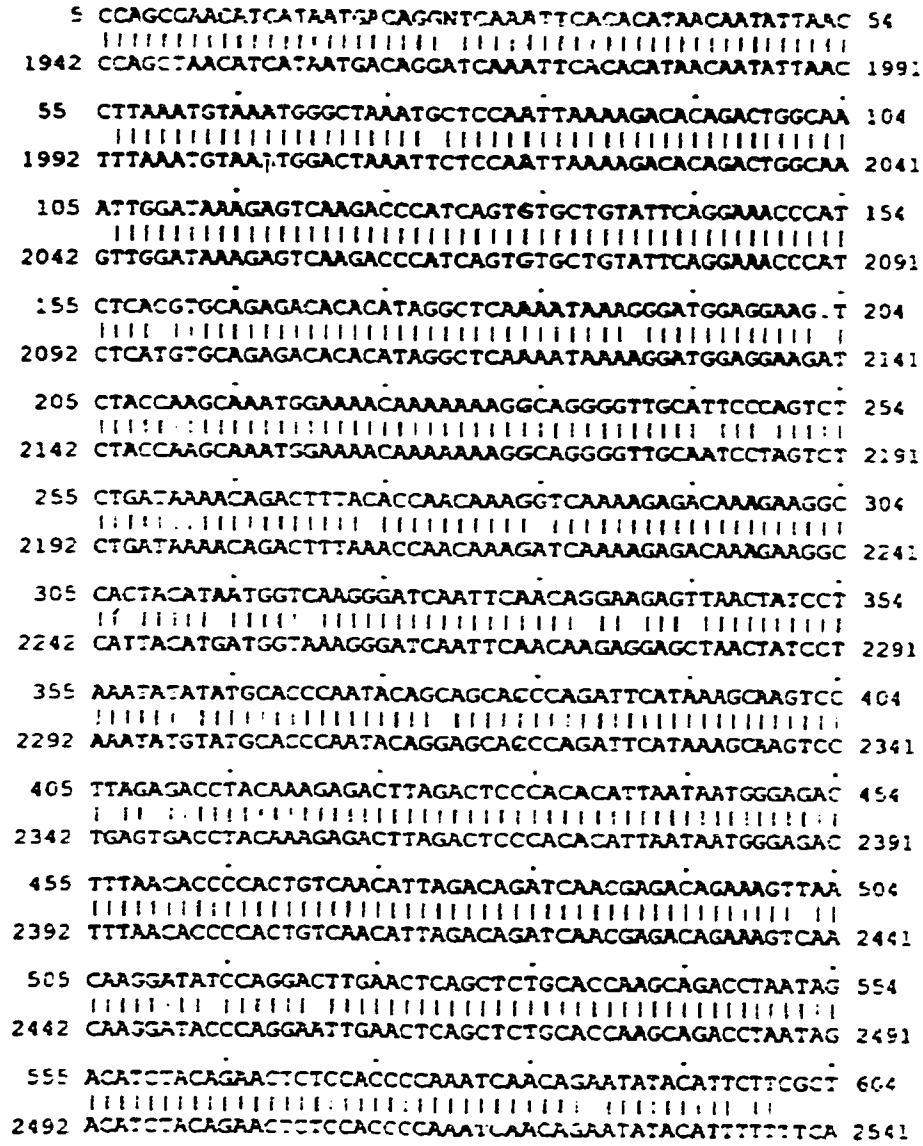

Figure 5. Nucleic acid sequence. Nucleic acid sequence comparison of 607 bp clone c4a showing 93% homology from base 1942 to 2541 ORF2 of the L1 retrotransposon. N represents bases not identified. The dots represent every tenth base.

Galectin-3 and Line-1 expression by immunoblotting: Western blot analysis of cell lysates was performed. As shown in Fig. 6 the expression of galectin-3 was observed in T47D and in clones 11-9-1-4 , 1 1-8-1-1 and 11-YX-I. Similarly, the expression of L1 in various clones was analyzed using a polyclonal antibody raised against p40 protein (48). Figure 6 showed the expression of L1 in the nude mice tumorigenic clones 11-9-1-4, 11-8-1-1 and T47D cell line. The non-tumorigenic parental cells BT549 and clone 11-YX-I did not express L1. SK-Br-3 cells, which are non-tumorigenic in nude mice did not show espression of galectin-3 nor p40 . To determine if the cell lines of epithelial origin were the only cell lines expressing p40, Western blot was performed with various cell lines of different origin. The results are shown in Figure 7; the co-expression of galectin-3 (A) and p40 (B) in human bladder carcinoma (J82), colon carcinoma (K12M). melanoma (A375) and fibrosarcoma (HT-l 080) cell lines. These results suggested that in this system cells which expressed L1 concomitantly expressed phenotype galectin3, and both gene products contributed to the tumorigenic phenotype.

Cellular distribution of galectin3 and L1: In order to ascertain the cellular distribution and localization of galectin-3 and L1, immunofluorescence studies were performed on 11-9-1-4 cells. When the cells were fixed and permeabilized by methanol and stained with anti-galectin-3 or anti-p40 antibodies, it was found that both the proteins were present in the cytoplasm and colocalized in the perinuclear region (Fig.8).

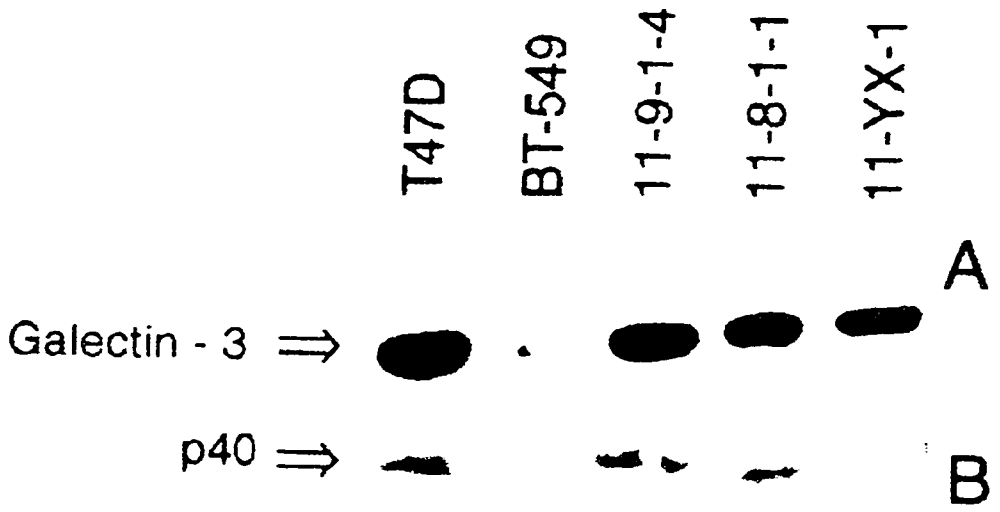

Figure 6. Immunodetection in various clones of BT-549. Immunodetectionof galectin-3 and L1 ORF1 protein, p40 in various clones of BT-549 transfected with galectin-3. T47D cell line was used as a positive control. Position of galectin-3 and p40 was determined by molecular weight marke proteins. Panel A: Galectin-3 and Panel B: p40.

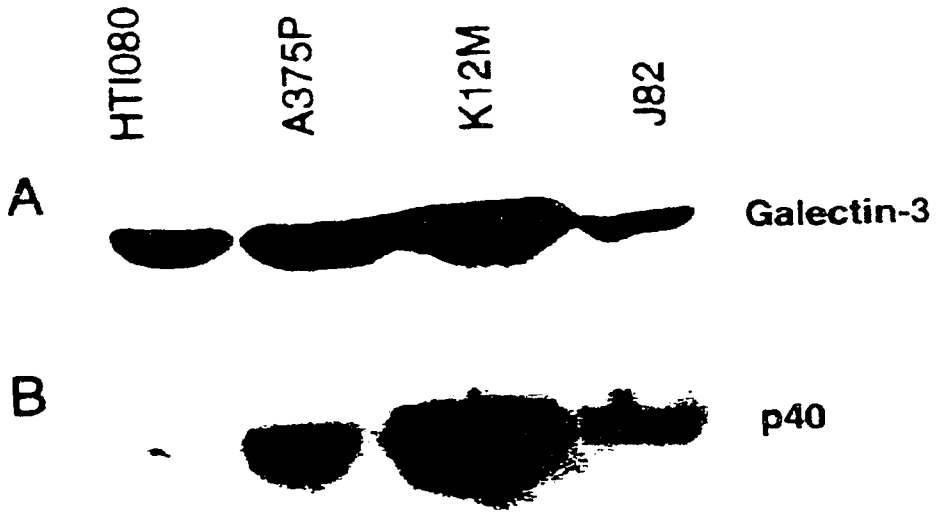

Figure 7. Immunodetection in various cell lines. Immunodetection of galectin-3 and ORF1 protein, p40 in various cell lines. Position of galectin-3 and p40 was determined by molecular weight marker proteins. Panle A: Galectin-3 and Panel B: p40.

Figure 8 Indirect immunofluorescence labeling of clone 1-9-1-4. Indirect immunofluorescence labeling of clone 11-9-1-4 with anti-galectin-3 monoclonal, and anti-L1 ORF1 polyclonal antibodies using confocal lasar microscope. The cells were fixed with cold methanol, incubated with primary antibodies, and stained with anti rat or rabbit FITC (green) and TRITC (red) conjugated secondary antibodies. Green color represents staining with galectin-3 and red represents p40 staining; yellow color represents colocalization of the two proteins (x630).

To further analyze the pathological relevance of the co-expression of both antigens in human breast cancer specimens and to establish that the above is not the reflection of the cells' adaptation to tissue culture conditions, immunostaining of the breast

tissues was performed using both antibodies.

Immunostaining of breast tissues with antibodies against galectin-3 and L1: Histologically normal breast tissues were weakly positive for L1 ORF 1 (p40, Fig.9A). Stromal cells were not stained in these specimens for either of the antigens. The immunostaining was limited to the apical portion of the epithelial cell cytoplasm, however, resolution in some of the cases was difficult to define. There were two cases of fibrocystic change with epithelial hyperplasia (Fig.9B). Both of these cases demonstrated significant staining of endothelium and or fibroblasts. They also demonstrated patchy strong staining of cyst epithelium or hyperplastic epithelium (Fig.9B,arrow). It is noteworthy that the intensity of staining was significantly greater than that observed in normal or atrophic lobules (compare 9A with 9B). The staining intensity with galectin-3 antibody was minimal, indicating very low levels of galectin-3 expression in histologically normal or fibrocystic breast tissues.

In carcinomas, the quantitation of the immunostaining was done separately for tumor cells as well as host derived stromal cells (fibroblasts and endothelium cells Fig.9C&D). The neoplastic populations demonstrated diffuse strong immunoreactivity (2+) with p40 in 47% of the tumors, heterogeneous immunoreactivity (1+) in 35% of the tumors, and they were essentially negative (+/-) in 18% of the cases. There was also significant immunostaining of the host-derived elements. This was predominantly patchy, or 1+, which was observed in 39% of the tumors. In 21% of the tumors, however, there was diffuse strong immunoreactivity of host- derived cells (Table 3).

	Overall Staining		Stromal Cell Staining vs Recurrence		Tumor Cell Staining vs Recurrence	
	Tumor Cells N=34 (%)	Stromal Cells N=33 (%)	No Rec. N=14 (%)	Rec. N=19 (%)	No Rec. N=14	Rec. N=20 (%)
0/+/-	6 (18)	13 (39)	7 (50)	6 (32)	3 (21)	3 (15)
1+	12 (35)	13 (39)	5 (36)	8 (42)	6 (43)	6 (30)
2+	16 (47)	7 (21)	2 (14)	5 (26)	5 (36)	11 (55)

Table 3. Immunostaining of human breast carcinoma with p40. Shows quantitation of immunostaining in carcinomas. Neoplastic cells had diffused strong reactivity (2+), with p40 in 47% of the tumors. Heterogenous immunoreactivity (1+) was seen in 35% of the tumors, and 18% were negative (+/-). The host derived stromal cells (fibroblast andendothelial cells) showed predominantly patchy (1+) immunostaining in 39% of the tumors. Strong, diffused immunoreactivity of host derived stromal cells was observed in 21% of the tumors.

Both the tumor and host cell populations demonstrated diffuse cytoplasmic granular staining (Fig.9C). Similar immunostaining was also observed using galectin-3 antibody

(Fig.9D). Overall, the staining patterns of both p40 and galectin-3 appeared to be contributed by the same cell population (i.e. host stroma as well as tumor epithelium within the tumor areas, Fig.9E).

Immunoreactive host cells were generally present in close proximity to the neoplasm. This was observed predominantly at the "invasive front" (that is in sections where this was visible). Several of the sections also demonstrated an accentuation of immunoreactivity of p40 within the tumor cell populations at the "invasive front" (Fig.9C).

Each of these experiments demonstrate coexpression and colocalization of L1 and galectin-3; furthermore, the expression was most intense in neoplastic tissue.

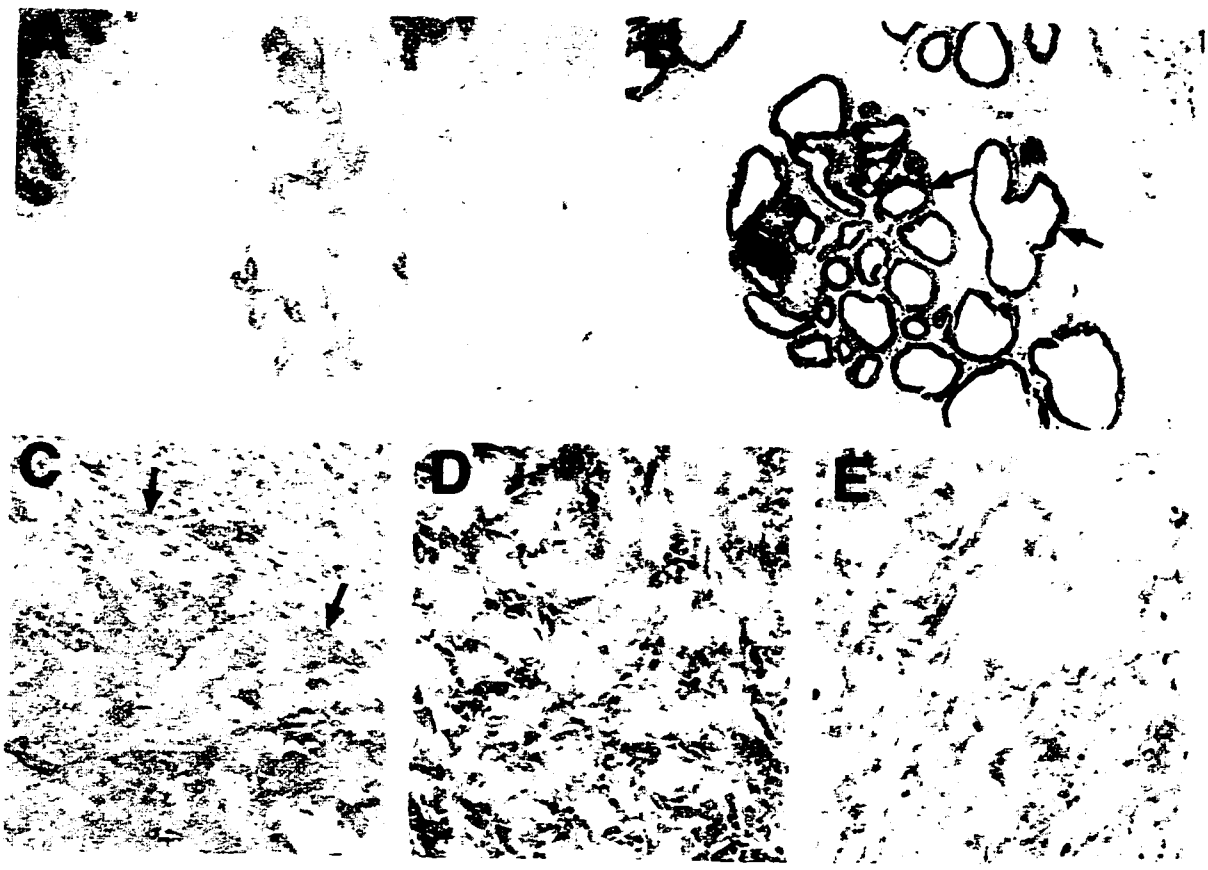

Figure 9. Immunostaining of benign and malignant breast tissue.

A. Normal terminal duct lobular unit, showing absent/minimal immunostaining for p40 (x150).

B. Fibrocystic change with epithelial hyperplasia (arrows) showing patchy strong staining of hyperplastic epithelium (x150)

C. Neoplastic epithelial cell immunoreactivity of p40 at the "invasive front" of an inflitrating carcinoma demonstrating strong cytoplasmic immunoreactivity of neoplastic epithelium (x200).

D. Immunostaining of host derived stromal cells in invasive tumor at higher magnification (x250), also showing some staining in tumor epithelium using galectin-3 antibody.

E. Two color dual immunoperoxidase stain for L1 (brown) and p40 (red), demonstrating simultaneous epitope in invasive tumor (x250).

DISCUSSION

The differential display technique presented quite a challenge, and several modifications were required; even so the results expected were not obtained. In the first round of experiments total RNA was extracted from paraffin embedded breast tissue. Areas were identified as tumor, intermediate (carcinoma *in situ*), and fibrous (normal). Several samples were pooled according to their classification, and analysis of these samples side by side using the first two random primers Ap1 and Ap2 resulted in fifty-nine differentially expressed bands cut from the sequencing gel.

In an effort to minimize the false positives the reverse hybridization step was included. The purified bands were used to probe the original RNA samples in a DNA /DNA hybridization, to ensure that their expression was not artifactual or contamination. There were no positive hybridizations.

After presentation of the first seminar where the proposed work and the progress to that date were presented, it was decided that some modifications were necessary. Because genetic and biochemical changes preceded histological changes, it seemed possible that the areas deemed intermediate (carcinoma *in situ*) and normal (fibrous) may already have had genetic and biochemical changes, and therefore would not show differential expression. Hoping to overcome this problem, it was decided that fresh breast reduction tissue be used and the normal control and the intermediate samples was eliminated. This presented a new problem, that of sufficient RNA recovery from the fresh breast tissue. Breast reductions are mainly fatty tissue with a high degree of protease activity, and very little fibrous tissue. In spite of the stringent precautions taken, very little RNA was obtained based on optical density measurements, and it was often degraded in a very short time, as

evidenced by the smears on the northern gels (see figs.10 A,B,C&D) To overcome this, various methods of RNA extraction and tissue handling were followed, but to little or no avail. The Trizol method was the simplest and worked as well as the others.

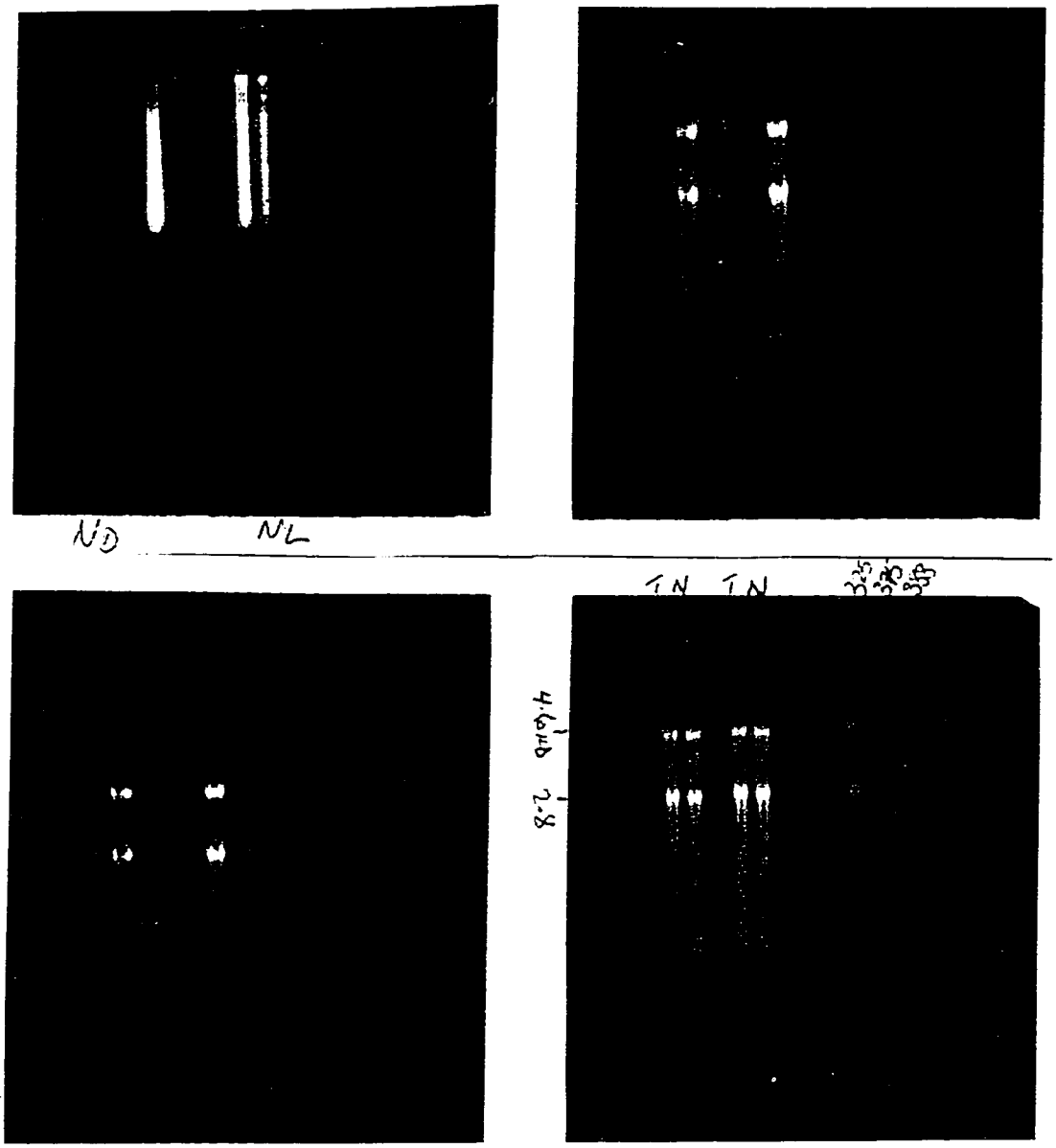

Figure 10. RNA from breast tumor and breast reductions Show nucleic acid degradation on formaldehyde agarose gel.

Collecting the sample in Trizol immediately, and processing before freezing or addition of other preserveing reagent, gave the best results; even so there was very often

insufficient RNA for polymerase change amplification and Northern blot analysis. The reverse hybridization step was deleted, and the Northern blot assay placed before the sequencing of selected bands. This would preserve the sample, while still allowing selection of true differentially expressed bands for sequencing, and Gene Bank search.

Theoretically, this technique seemed simple and a sure way of identifying differentially expressed genes. This in practice turned out not to be the case. Instead there was an unexpectedly high number of false positives, which were not confirmed by Northern blot analysis, or reverse hybridization. Since the original description of the technique, there have been many modifications. Callard et al (49) described a technique for the elimination of contaminating sequences. They found that many of the PCR products even after gel purification were contaminants rather than differentially expressed genes. The differentially expressed bands which were cut from the gel were dialyzed against 100mL of 10 mM Tris-HCl, pH 8.9, 0.1 mM EDTA for 4 - 16 hours at 37^0C in siliconized microfuge tube. The eluent was then filtered through glass wool. Liang et al (50) described a one base anchored oligo-dT primer (H-T_{11}C). They concluded that this minimized the redundancy, and reduced the number of reverse transcription reactions needed for each sample. The second primer was increased from a 10mer to a 13mer. This primer had a restriction enzyme site at the 5' end, which allowed for easier handling and manipulation of the amplified cDNA. Ayala et al (51) suggested that the low stringency conditions of the polymerase chain reaction were responsible for the high false positives, and unsatisfactory reproducibility. They changed the stringency conditions from 40^0C for annealing to 60^0C, the primers were also elongated. They obtained improved results with these changes.

Given the time constraints for this project, and the lack of satisfactory result, a new system was started, this time using cultured breast cancer cells, to look at galectin-3 expression.

Previously, the presence of galectin-3 in a spectrum of human and murine tumor cells, was established, and it was proposed that interaction of cell surface galectin-3 with complimentary serum glycoprotein(s) promote(s) cell-cell adhesion of tumor cells in circulation leading to tumor embolization, thereby contributing to the pathogenesis of metastasis (52-55). This system had less uncontrolled variables. The differential display technique could still be utilized, and results were expected in a relatively short time.

It has been shown that galectin-3 was present in all trophoblastic lineage in normal human placenta. The authors concluded that this was an indication that galectin-3 was involved in cell cell interactions, and in cell matrix interactions of trophoblast during placentation (56). Galectin-3 has been found to be expressed in embryonic macrophages; and was identical to the macrophage marker, Mac-2. It was found to be expressed in normal peripheral monocytes, and its expression was dramatically increased as monocytes differentiated into macrophages. Immunogold cytochemistry, electron microscopy, showed its expression on the surface. Immunogold labeling also showed its expression in mast cells and basophils, and it was localized both in the nucleus and the cytoplasm. In the nucleus, labeling was of the heterochromatin areas, and euchromatin areas were unlabelled. The cytoplasmic labeling showed it in secretory granules. The intensity of staining was greatest in mast cells from the skin, when compared to other sources. These results indicated a soluble form which could be released on degranulation. In confluent T84 cells, galectin-3 was localized at the apical membrane in large granular

inclusions. Galectin-3 bound IgE, and there was evidence that it activated neutrophils in a dose dependent manner, thus, participating in inflammation and host defense through modulation of neutrophil functions. An interesting observation was its involvement in the autoimmune process. Auto antibodies of the class IgG1 with epitopes for galectin-3 were found in an individual, who was later found to have adenocarcinoma of the colon. The titers of anti-galectin-3 IgG were sharply elevated after hemicolectomy. Other individuals with neoplasms have been shown to have anti-galectin-3 antibodies, at lower titers. The pathogenesis remained unclear, but it seemed to occur in older individuals (57-59).

Transfection of weakly metastatic UV-223 7-cl 15 cells with the murine galectin-3 cDNA resulted in its elevated galectin-3 expression and conversion into a highly metastatic cell clone (60). Virally transformed 3T3 cells expressed higher levels of galectin-3 when compared with the untransformed parental cells (60). In human tumors the role of galectin-3 in progression and metastasis was less clear. A direct relationship between galectin-3 levels in colon carcinoma cells and stage of tumor progression was shown (61,62); while others have found a decreased expression of galectin-3 in colon cancer tissues when compared with associated normal mucosa (63,64). In gastric carcinoma it was found that the tissue levels of galectin-3 were higher in certain primaries and their metastasis when compared with the adjacent normal mucosa (65). Galectin-3 was also found to be a marker of anaplastic large-cell lymphoma (66). In ovarian carcinoma, however, no correlation was observed between the galectin-3 expression and clinicopathological features (67). Galectin-3 is thought to be involved in several physiological processes through interation with specific ligands. These processes include cell growth and differentiation, adhesion, inflammation, transformation and metastasis.

To establish the possible role of galectin-3 in human breast cancer, its expression was examined in relation to the malignant phenotypes of five established and well characterized human breast carcinoma cell lines, namely, T47D, MDA-MB-331, MDA-MB--135, BT-549 and SK-Br-3. Out of the five, two cell lines (BT-549 and SK-Br-3) which are non-tumorigenic in nude mice (68-71) were found not to express galectin-3 (72).

The cell line BT-549 which was non tumorigenic in nude mice and negative for galactin-3 expression was used for the transfection of the galactin-3 cDNA. Six clones from this transfection were randomly selected, four sense transfected clones and two antisense transfected clones. Monoclonal antibody against galactin-3 was used to show its expression in these six clones. The four sense clones expressed varying amounts of galactin-3 while the two antisense clones were negative for its expression. The clones were plated in agar to determine their ability to proliferated in semi-solid medium. All the sense clones showed significant increase in their ability to proliferate in semi-solid medium when compared to the parent cell. Further work was done to examine the expression of galactin-3 and the growth of the nontumorigenic BT-549 cell in nude mice. The parental BT-549 and an antisense clone 4-1-4-2-1 developed no tumor in the nude mice 150 days after inoculation. Three of the four sense clones showed a tumorigenic phenotype. One clone (11-9-1-4) was highly tumorigenic with tumor diameter of 1 cm three to four weeks after injection and metastasis to the lymph nodes. One galactin-3 sense clone (11-YX-1) showed no tumorigenesis in nude mice up to six months after inoculation. What was the difference between these two galactin-3 expressing clones (11-9--4 and 11-YX-1); why was one highly tumorigenic and the other non-tumorigenic?

The introduction of recombinant human galectin-3 into the null BT-549 cells,

resulted in the acquisition of anchorage independent growth properties in all four, while tumorigenicity in nude mice occured only in three fourths sense-transfected cell clones (72). One of the clones (1 1-9-1-4) also showed lymph node metastasis. Here, the apparent lack of the relation between galectin-3 expression and tumorigenicity in one of the clones was questioned. To address this, differential display was performed. Of primary interest was the expression of galectin-3 in tumorigenic versus nontumorigenic; and analysis of the *in vivo* difference between the non-tumorigenic, and the tumorigenic cell clones, to determine whether it resulted from activation or suppression of other gene(s).

Fifty-three differentially expressed bands were identified using eight random primers. Northern hybridization was performed with RNA from parental BT-549 cells, and the clones 11-9-1-4 and 11-YX-1. On the basis of the initial screening, clone (band) c4a was selected for further studies (Fig.4), because of its overexpression in clone 11-9-1-4. The PCR amplified fragment was 607 base pair long and after sequencing it was found to share a 93% homology to the second open reading frame (ORF2) of line 1 retrotransposon. The 7% sequence discrepancy probably resulted from amplification errors or sequence variations (73).

The data presented herein suggested that a concomitant expression of galectin-3 and L1 retrotransposon is associated with tumorigenicity of breast cancer in an *in vivo* experimental system and may be associated with breast cancer pathobiology *in situ*.

Long interspersed nuclear element, LINE, retrotransposons, are a family of highly repeated long interspersed DNA sequences, dispersed in all mammalian genomes (74-79) constituting an estimated 10% of the mammalian genome (44). Most of the copies

are truncated from the 5' end and are thought to be non-functional. The 3'end is constant, and is followed by a poly -(A) run of variable length (44). Full length transcripts are 6-7 Kb and contain two long ORFs in the same reading frame (42-44). ORF 1 encodes a 40 kDa protein (p40) of unknown function (no cellular homologue is known), but is thought to be an RNA binding protein. The protein is 338 amino acids long, and it is usually found in a multimeric cytoplasmic complex. This complex can be dissociated by ribonuclease. Binding and competiton studies suggest that p40 binds to single stranded RNA containing a RNA binding site, but not single stranded or double stranded DNA, or double stranded RNA, or even a DNA-RNA hybrid containing a binding site sequence (100, 101). The amino acid sequence contains a leucine zipper motif which is usually seen in transcriptional regulators with transient nuclear localization. The sequence also suggest a-helical supercoiling, and participation in protein protein interactrrions of the coiled-coil variety (101). The ORF2 encodes a 150 kDa (p150) reverse transcriptase (45-47). No ORF1 and ORF2 fusion protein has been detected (48). Recent studies have demonstrated that ORF2 encodes an endonuclease also; this endonuclease needs divalent cations, preferrably magnesium or manganese, for its activity.

The results obtained showed the presence of a 6.5 Kb message in the tumorigenic cells, which seems to represent the full length mRNA of L1, containing both functional ORFs. A polyclonal antibody against ORFI (anti p40) is available and was used to study its expression in various cell lines and clones.

Among the proteins predicted by the open reading frame were regions with homology to reverse transcriptase and nucleic acid binding proteins. No specific transcripts were detected by Northern blot and primer extension methods; however expression of

L-1 elements were observed in cell lines derived from epithelial cancers. There was no expression seen in normal breast tissue. One tumor showed evidence of colocalization with galectin-3. Immunostaining of several breast cancer tissue was performed to observe the expression and distribution of galectin-3 and Line-1 retrotransposon, and to determine whether or not colocalization occurred. In some tumors expression of L-1 was found primarily in the stroma, and in others in the epithelial cells, and in some tumors there was expression in both stromal and epithelial tissue. This was true also for galectin-3 expression. The parental cell line BT549 and the two clones 11-9-1-4 and 11-YX -1 were studied using immunocytochemical techniques to determine the locale in the cells where the galectin-3 and L-1 proteins were expressed.

Previous studies had suggested that L1 was not active in most of the cells since specific transcripts were not detected by Northern blots and Primer extension methods (80, 81). The expression of L1 elements using p40 antibody was seen in human testicular cancer, breast cancer, and pediatric germ cell tumors (82-84). Lls were a specific group of transposable elements that could be transcribed into RNA, reverse transcribed into cDNA and then reintegrated as cDNAs into the genome at a new location. Although most integrations were probably only in nongenic sequences, some integration events may have involved genes important in the control of cellular proliferation, and their inactivation may have led to, or enhanced the neoplastic state. The examples observed were the de novo insertion of LI elements into myc allele in primary breast carcinoma (85) and into the APC (adenomatous Polyposis coli) tumor suppressor gene in a colorectal cancer (86). In addition, it has been proposed that L1 retrotransposon ORF1 encoded proteins which functioned as oncoproteins in some cancers (82). Recently, a role for endogenous

retroelements has been proposed in the repair of chromosomal breaks (87). It seems possible that these elements also play an important role in the evolution of the genome, especially in the exchange of information among acrocentric chromosomes.

The expression of L1 is not limited to epithelial cells, the analysis of several lymphoma and leukemia cell lines (88) have demonstrated that other tumor cell lines derived from non epithelial origin and non breast epithelial cells co-express L1 and galectin-3. The immunohistochemical studies shown here support and expand the published data on L1 expression in human breast cancers (88). Based on the study performed on 43 cases the following observations were made: 1) There was a weak, focal immunoreactivity for L1 and galectin-3 in histologically unremarkable benign breast tissue. 2) Fibrocystic change with hyperplasia appeared to be associated with increased immunoreactivity for L1 and galectin- 3, and 3) Breast carcinomas demonstrated significant expression of both the proteins in a majority of tumors both within the tumor cell population as well as within the host stromal cell populations. The colocalization of L1 and galectin-3 in the host stroma and accentuation of L1 immunoreactivity at the invasive front suggests that it might have some functional role in stromal remodeling and/or tumor cell invasion and that there might be an association between immunoreactivity for L1 and galectin-3, and aggressive behavior in breast carcinoma.

Accentuation of stromal expression of genes, have been documented in breast carcinomas. Previous studies have shown that the stromal expression of TIMP-2 (tissue inhibitors of metalloproteinases) correlated with disease recurrence; whereas the expression of matrix metalloproteinases-2 (MMP-2) and matrix metalloproteinases-9 (MMP-9) elaborated on stromal epitheial cells did not correlate with disease

recurrence (88). Even though MMP-2 and -9 were identified in tumor epithelial cells, the mRNAs for MMP-2 and TIMP-2 were reported to be expressed in desmoplastic fibroblasts of the stroma in breast carcinomas (89). Some other invasion related proteins that showed expression in host stromal cells were cathepsin D, urokinase- type plasminogen activator, and TGF□ (transforming growth factor alpha) (90,91). Collectively, these results indicated the role of host stromal cell gene expression in response to tumor epithelial cells, and may have represented extra cellular matrix remodeling and thereby invasive phenotype. The elaboration of gene expression by host stromal cells and tumor epithelial cells is due to complex processes of host tumor interactions.

The above studies, however, were preliminary in nature and did not explain the type of interactions that these two/three proteins, galectin-3 and p40 and/or p150 might have. It was suggested that p40 may be capable of homodimerization and may undergo heterodimerization with other proteins (92). Galectin-3 also undergoes homodimerization and has binding domains to other proteins (93). In addition in the mouse embryonal cell line F9, full length, sense strand L1 RNA was found in ribonucleoprotein (RNP) particles, which also appeared to include ORFI protein (94-96). Human L1 also appeared to be present in a high molecular weight complex with reverse transcriptase activity in Ntera2D cells (46); while galectin-3 may be found complexed with RNP particles (97), and is also involved in pre mRNA splicing (98). Whether LI-ORFI, ORF 2 and galectin-3 form the same RNP complex needs to be determined. It is obvious that the results depicted here and their interpretation are of preliminary nature. While, supporting independently the reports on the possible role of galectin-3 and LI in human tumors, the precise continuing

role of the interdependent expression of these two gene products to tumor progression in the breast remains to be established. Is the colocalization of these two protein coincidental or is one being regulated by the other, and in what way does their expression and possible interaction influence the deveiopment and progeression of the tumorigenic phenotype? These and other questions still remain to be answered.

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ABSTRACT

IDENTIFICATION OF DIFFERENTIALLY EXPRESSED GENES
IN BREAST CANCER TISSUES AND CELLS

by

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Breast cancer, is the second leading cause of cancer related deaths among women in developed countries, and the incidence of morbidity and mortality is rising in developing countries. The purpose of this project was to utilize the differential display technique to identify genetic changes in normal versus malignant breast tissue. It was also used in a defined cell culture system having differential cellular characteristics, to identify genes that may be responsible for different biological behavior of these cell lines.

Messenger RNA from normal, breast cancer tissues, and breast tissues from reduction mamoplasty yielded fifty-nine differentially expressed bands representing differentially expressed genes. Northern hybridization analysis proved negative, suggesting that these genes may represent low abundant message. mRNA from two clones; one tumorigenic, and the other non-tumorigenic in nude mice; obtained by stable transfection of galectin-3 gene in a non tumorigenic BT 549 breast cell line, was analyzed by differential display. Galectin-3, a calcium independent carbohydrate binding protein has been shown to be involved in many biological processes, but its exact function is still unclear. A 607 bp fragment was differntially expressed by the tumorigenic clone, and DNA sequence of

which revealed a 93% homology with the human Line 1 retrotransposon (L1). L1 is a poly-A mobile element, and its insertion into functional genes has been implicated in human diseases, including breast cancer, however its role in breast cancer is not clear. To determine the locale and expression of galectin-3 and L1 in normal versus tumor tissues, immunohistochemical analysis of breast carcinoma specimens, fibrocystic, normal breast tissues, and the tumorigenic clone of BT 549, 11-9-1-4, was performed. L1 and galectin-3 was found to be co-localized, and the immuno-staining was most intense in tumor tissue, and was minimal in normal tissue. Staining was significantly correlated with disease progression and tumor recurrence, suggesting that the expression of galectin-3 and L1 may represent a new mechanism by which breast tumor cells acquire aggressive phenotype. However, the interaction of L1 and galectin-3, if any, and their influence on tumor development and progression remains to be determined.

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