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GRANT NUMBER: DAMD17-94-J-4181

TITLE: Detection and Characterization of Genetic Alterations on Chromosome 16 for Breast Cancer

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REPORT DATE: March 1996

TYPE OF REPORT: Final

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	March 1996	Final (30 Sep 94 - 30 Jan 96)

4. TITLE AND SUBTITLE
Detection and Characterization of Genetic Alterations on Chromosome 16 for Breast Cancer

5. FUNDING NUMBERS
DAMD17-94-J-4181

6. AUTHOR(S)
Rosemary B. Duda, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Beth Israel Hospital
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200)*

This grant funded a Special Sabbatical for research in breast cancer for one year. There were two principle tasks in this grant; the first was to localize a potential tumor suppressor gene on chromosome 16 and the second was to further develop the molecular biology skills of the investigator. The research project continues to be in progress as we are at the stage of the secondary screening of a normal breast cell cDNA library and are preparing to sequence the DNA. In addition to this project, two additional studies were initiated, evaluating the effects of estrogen and progesterone on breast cancer cells.

14. SUBJECT TERMS
Breast Cancer
tumor suppressor genes, estrogen, progesterone

15. NUMBER OF PAGES
21

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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PI - Signature

4/16/96
Date

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A. Introduction

This grant funded a Special Sabbatical in breast cancer research for one year. There were two main tasks or goals for this year. The first was to identify a candidate tumor suppressor gene at chromosome 16q24 by scanning the region with specific DNA probes from a genomic cDNA library. Once the gene was identified, the plan was to determine its expression in breast cancer specimens and to compare the expresion or lack of expression with known prognostic factors in breast cancer. The second goal was for this investigator to learn several new techniques in molecular biology for the purpose of applying these skills to broaden the scope of current investigations.

The research project continues to be investigated. It has been a challenging, ambitious and time consuming project. The results to date will be reported in this document. The second goal has been met with all expectations fulfilled.

B. Background

The incidence of breast cancer is rapidly increasing in the United States, with an estimated 180,000 women diagnosed in 1995.[1] A heterogenous disease, the cause of breast cancer is most likely multifactorial and remains elusive. Genetic alterations found in both familial and sporadic breast cancer patients are felt to be important events in tumor initiation and progression.

The development of breast cancer may result from a multistage process that involves the acquisition of several genetic alterations, including the expression of oncogenes and the inactivation or loss of tumor suppressor genes.

Since tumor suppressor genes are thought to be involved in the development of breast cancer, chromosomal deletions in the tumor specimens could indicate the localization of these genes. Frequent loss of heterozygosity (LOH) in tumor DNA implies the presence of a tumor suppressor gene. LOH has been identified on chromosomes 1q, 3p, 7q, 11p, 13q, 17p, 17q, and 18q.[15-18] Somatic mutations detected in the p53 gene, RB gene and prohibitin gene make each a candidate for a tumor suppressor role.[19]

Frequent LOH on chromosome 16q in breast cancer specimens has been identified by several investigators, being cited as high as 51% for primary (sporadic) breast cancers.[18-22] In fact, LOH at 16q is thought to be more common in sporadic cancers than in the early-onset familial breast cancers.[20]

LOH on chromosome 16q has recently been associated with distant metastases in primary familial breast carcinoma.[20] This

study by Lindblom, et al., demonstrated that there was a highly significant association between LOH at chromosome 16q and the subsequent development of distant metastases, but there was no correlation with estrogen receptor (ER) status, lymph node positivity, or tumor size at the time of diagnosis. Nineteen of 67 patients evaluated had LOH at 16q. Eleven of the total number of patients had developed distant disease from 1 to 13 years later, and 8 of those 11 had LOH at 16q. The most frequent marker was D16S7, located at 16q24, and showed LOH in 18 of 57 (32%) informative cases. The APRT locus, also located at 16q24, revealed LOH in 3 of 25 (12%) informative cases. It was concluded that a tumor suppressor gene exists on chromosome 16q, that it facilitates hematogenous spread of breast cancer, and that LOH at this locus of 16q24 is an independent prognostic marker for patients at potential high risk of recurrence. This would be an invaluable aid to identify those patients who might benefit from adjuvant therapy in order to prevent a tumor recurrence.

In an additional report by Lindblom et al.[22], this study of LOH in familial breast cancer demonstrated allele losses at 8p, 16q, 17p, 17q, and 19p. It revealed from their study population, that tumors from breast cancer families with early onset (<50 years) lost heterozygosity on 17q more frequently than other tumors and that there was a tendency for tumors from this group to show less LOH at 16q than other tumors. This difference was striking when comparing families diagnosed as predominantly early onset breast cancer to those families with only a single case of later onset (>50 years), sporadic, breast cancer. There were 3 of 4 cases (75%) with LOH at 16q24 for the sporadic families compared to 7 of 29 cases (24%) with LOH at 16q24 for early-onset families. The observed difference could therefore indicate that LOH at 16q is a more common finding in sporadic breast cancers than in familial ones. This study also suggested that since breast cancer genes have been shown to exist in the regions 17p and 17q, that it would be interesting to focus on the remaining three regions (16q, 8p, and 19p) to search for additional breast cancer genes. Since familial breast cancer only comprises 5% of all breast cancers, investigations at other loci, such as 16q24, may be more pertinent to the general sporadic breast cancer patients.

In the study by Sato et al [21], LOH on chromosome 16q24 identified at the locus D16S7 was reported in 45% (19 of 42) of primary (sporadic) breast cancer specimens with a trend toward an association with lymph node metastasis. Tumors showing LOH at 16q had frequent lymph node metastasis (67%) compared to the group of tumors in which chromosome 16 was not lost. This study suggested that one of four significant tumor suppressor genes exists on chromosome 16q.

Other studies by Sato et al. [18] suggest that the accumulation of genetic alterations, including loss of function of tumor

suppressor genes on chromosome 13p, 13q, 16q and 17q and the amplification of c-erbB-2 may contribute to tumor development and progression in primary breast cancer. In the case of chromosome 16q, the most frequent sites of LOH to date was observed at the *HP* locus 16q22.1 in 12 of 21 cases (57&), D16S157 locus 16q22-23 in 4 of 9 cases (44%), D16S156 locus 16q23-24 in 30 of 61 cases (49%) and D16S7 locus 16q22-24 in 59 of 138 cases (43%). There was a total LOH at chromosome 16q in 78 of 153 patient specimens evaluated (51%).

Takita et al [19], in an analysis of 17 specimens found a weak association with LOH at chromosome 16q and lymph node metastasis. In this study, LOH at chromosomes 11p and 17p had a stronger correlation with regional lymph node metastasis.

C. Methods: Analysis of Chromosome 16q24

Bacteriophage P1 Library Screening

The DNA segment D16S7 (located at 16q24) was purchased from ATCC, Rockville MD. A plasmid prep was performed and the insert removed from the pSP65 vector. The DNA insert was labeled with ^{32}P to a specific activity of $1X10^9$ and hybridized to a bacteriophage control to prescreen for vector contamination and repetitive sequences. The repeat sequences were removed under stringent conditions and the radiolabelled DNA insert was hybridized to a P1 library. Two positive clones are identified and confirmed by repeat hybridization to the labelled probe. The confirmed positive clones are grown in medium and the DNA is isolated. A P1 maxi preparation protocol (Qiagen) was used to increase the yield of the DNA. The presence of the DNA clone was confirmed on a 0.7% agarose gel. The DNA was digested using the BAMHI enzyme and the DNA cut from the gel.

The D16S7 probe was radioloabelled and hybridized to a primary and secondary normal breast cDNA library. Hybridization revealed several positive plaques. These plaques were lifted, placed in SM buffer, processed with E.coli and plated on LB plates with ampicillin. Multiple colonies were identified on the plates, several were then incubated in LB broth with ampicillin for 14 hours. Twenty colonies were selected for further evaluation. A mini plasmid preparation was performed and the DNA was digested with ECOR-1 and XhO1 enzymes and loaded onto a 1% agarose gel. The filter was hybridized with the ^{32}P labelled probe. The results revealed binding to D16S7 DNA in some samples, with nonspecific binding to the vector and to the lambda control. This portion of the experiment had been repeated several times as initially there was no binding identified to the DNA. After repeat digestion experiments, lane 14 revealed binding to the D16S7 clone. (Figure 1) Another gel was performed and the DNA was extracted from the gel. The DNA sample is being prepared for sequencing.

D. Results

Project 1: Chromosome 16q24

There are no definitive results yet from this experiment. As noted in the above section, after a secondary screening of the normal breast cDNA library, we have isolated a DNA fragment that binds to the D16S7 clone. (Project 1/Figure 1) This experiment is still in progress. Because of the slow progress of the above experiments, two other experiments were initiated.

Project 2: Identification of novel genes induced by either estrogen or progesterone

This study addressed the problem of identifying estrogen and/or progesterone induced genes in several breast cancer cell lines (MCF-7, T-47D) by the differential display PCR technique.

The hypothesis of this investigation is that differentially expressed genes exist in the pathogenesis of breast cancer and that they may be induced by estrogen or progesterone stimulation. Estrogens and progesterones are important for the growth and development of normal breast tissue, are mitogenic for normal and malignant breast epithelial cells, and may also facilitate carcinogenesis. The estrogen induced mRNA differential display studies were performed in the MCF-7 human breast cancer cell line, which expresses high levels of the estrogen receptor and low levels of the progesterone receptor. The progesterone induced studies were performed in the T47D breast cancer cell line, which has low levels of estrogen receptors but high levels of progesterone receptors. These differentially expressed genes may contribute to or facilitate carcinogenesis or may serve as a prognostic variable for breast cancer patients.

The first estrogen regulated gene product characterized was the progesterone receptor (PR).[23] The action of progesterone is mediated by the PR and estradiol was considered to be the primary up-regulator of PR in human breast cancer cells.[24] The PR has been proven to be a significant indicator of anti-estrogen therapy in PR positive breast cancer patients as well as being an important prognosticator for disease free survival in these patients.[25-27]

Use of the subtractive hybridization technology in estrogen stimulated MCF-7 breast cancer cells resulted in the identification of the estrogen regulated gene pS2.[28] The induction of pS2 is a primary transcriptional event directly controlled by estradiol through the estrogen receptor.[29-30] In addition, stimulation of pS2 transcription can also occur indirectly through growth factor mediated mechanisms.[31-32] Expression of pS2 has been found in some studies to afford a favorable prognostic significance for survival and disease recurrence and pS2 also serves as a predictor for response to anti-estrogen therapy.[33-37] A third example of an ERG is

cathepsin D, a ubiquitous lysosomal protease whose transcription is induced by estradiol within 24 hours of treatment. A functional estrogen receptor is required for transactivation. Estradiol induces the expression of the cathepsin D gene in estrogen responsive breast cancer cells by interacting with the transcription machinery at the promoter level. Although the precise role of this protein in the development of breast cancer has not been determined, cathepsin D has been shown clinically to be a negative prognostic factor for disease free survival for breast cancer patients.[38-40]

Previously, the best method of differential expression cloning was subtractive hybridization.[41] Subtractive hybridization is a powerful tool that has been used to identify the differential expression of critical genes and subsets of genes. This specific methodology is technically difficult and results in the incomplete recovery of genes and selects only for either underexpressed or overexpressed genes and screening is laborious.[42]

To facilitate the identification of genes that are differentially expressed in tumor cells in comparison to normal cells, a method was developed called mRNA differential display that involves the reverse transcription of mRNA followed by the PCR reaction.[42-43]

Differential display is a technique whose key element is to use a set of oligonucleotide primers, one anchored to the polyadenylate tail of a subset of mRNAs, the other being short and arbitrary in sequence so that it anneals at different positions relative to the first primer.

Briefly, a modification of the originally described procedure uses four (rather that 12) 3′ primers that divide the expected 10,000-15,000 different mRNAs into four groups and anchor the PCR products to the 3′ end of the mRNA.[41-43] The 5′ primers are arbitrary 10-mer oligonucleotides that hybridize with complementary sequences located at varying distances from the 3′ end of each newly synthesized cDNA strand. Twenty different 5′ primers used with four different 3′ primers divide the mRNA population into 80 groups.[41] The amplified cDNA subpopulations of the 3′ termini of mRNAs subpopulations defined by these primer pairs are amplified after reverse transcription and resolved on a DNA denaturing sequencing gel.

When multiple primer sets are used, reproducible patterns of amplified cDNA fragments are obtained that show strong dependence on sequence specificity of either primer. Unfortunately PCR-DD also generates a large number of spurious sequence fragments, irrelevant genes, high abundant genes or small cDNA fragments that do not represent differentially expressed genes.[44] Modifications of the technique have been described to reduce the number of false

positive cDNA bands detected.[45-46]

As this is a relatively newly described technique, only a few studies have reported identification of candidate genes in breast cancer. Liang et al[45] identified cDNA bands in a comparison of RNA from human breast cancer cells and normal epithelial cells that were differentially displayed. Of these 15 cDNA bands analyzed by northern blot analysis, 5 were confirmed to be differentially expressed, another 6 failed to detect any signals and 4 were false positive. Sager et al[41] identified a candidate tumor suppressor gene by mRNA-DD that is confirmed by sequencing analysis to be integrin alpha-6, a component of heterodimeric integrin receptors.

Three micrograms of purified total RNA from untreated and estradiol treated MCF-7 cells were reverse transcribed with HT11G, HT11C, and HT11A 3′ primers using the Gen Hunter kit (Brookline, MA.) and amplified by the polymerase chain reaction (PCR) using an arbitrary 5′ primer (H-AP4). The reactions were performed in PCR buffer containing 10 uCi of ^{35}S dATP (New England Nuclear).[42-43] The PCR products were displayed on a 6% sequencing gel to identify and select genes that may be differentially expressed. The gel was dried without fixation, exposed to Kodak XAR film overnight, and an autoradiograph developed overnight. A total of four cDNA fragments were identified in the HT11C and HT11G groups treated with estradiol for 48 hours that were either not present in the untreated group or were represented as very faint bands and appeared to represent differentially expressed genes. The cDNA fragments were dissected from the gel, precipitated with ethanol and reamplified by PCR using the same primers. The PCR products were displayed on a 1.5% agarose gel, cDNA bands were present for all four samples. The cDNA was recovered by placing the gel samples in an ultra-free-mc 0.45 um filter unit, stored at -20^0C for 30 minutes then centrifuged for 15 minutes at 6300 rpm to extract the DNA from the gel. Ten microliters of each probe was labelled with ^{32}P dCTP by the random primer method and hybridized to the filters. All four probes hybridized equally to the RNA from the estradiol treated MCF-7 cells as well as the untreated and ethanol treated controls. (Project 2/ Figure 1)

This experiment was repeated in MCF-7 and the immortalized, nontumorigenic human mammary cell line 185B4, using different primers and combinations of primers. No novel genes were identified after many candidate genes were radiolabeled and hybridized to cell lines and tissue specimen RNA and DNA for Southern and northern blots.[47]

T47D cells were grown in culture and the medium was stripped of endogenous steroids as described. The T47D and MCF-7 cells were treated with 8 X 10^{-8} M of progesterone (Sigma) for 30 minutes, 1, 6, 12, 24, and 48 hours, RNA was extracted and a northern blot made. The reverse transcriptase reaction was performed as described for

the previous study. The PCR products were displayed on an 8% sequencing gel. Six cDNA fragments appeared to be differentially expressed in the progesterone treated cells in comparison to the untreated T47D cells. None were identified for the MCF-7 cells. The cDNA was recovered from the gel and the radiolabelled probes hybridization to the northern blot. There was no difference in expression of the candidate genes in treated or untreated cell lines and tissue specimens. Project 2/ Figure 2 depicts the results of these studies in the T47D cell line.

Project 3: Effect of progesterone on breast cancer cell growth

This study also addresses the issues of the specific role of progesterone on breast cancer cells. This subject appears to be controversial as to whether or not progesterone increases proliferation or decreases cell growth in culture.

There is substantial experimental, epidemiologic and clinical evidence that breast cancer is influenced by ovarian hormones.[1,48] Growth of the normal mammary gland involves the process of proliferation and differentiation which is regulated by a cyclic balance between the actions and interactions of estrogen and progesterone.[49]

Estrogen has been demonstrated to have mitogenic effects on normal and malignant breast cells.[50-51] This effect represents either an increased or altered sensitivity of the breast epithelium to estrogen.[52] There is speculation that this altered sensitivity may be related to progesterone.[49] The progesterone receptor (PR) is an estrogen responsive gene (ERG) and under normal conditions, progesterone down regulates the estrogen receptor (ER).[53]

Progesterone induces and activates the processing of the PR and the regulation of the PR responsive gene fatty acid synthetase.[54] The PR-B isoform inhibits the expression of the ERGs (pS2 and cathepsin D).[55] The mechanism of inhibition of ERGs is most likely not by inhibiting the ER gene transcription as previously hypothesized, but perhaps by squelching a transcription co-factor that is absolutely required for selective ERG responses.[55]

The role of progesterone in breast carcinogenesis is less apparent than that of estrogen and remains controversial. Confounding the issue is that the biologic activity of progesterone on normal and malignant breast cells has not been clearly elucidated. Progesterone may be even more important than estrogen as a hormonal stimulus in driving the proliferation of normal human mammary epithelium.[49] In vivo, the highest proliferation of histologically normal mammary epithelium occurs in the progestogenic phase of the menstrual cycle as well as under the progesterone influence of oral contraceptives.[56] While progesterone is beneficial and protective against endometrial carcinoma,[49]

exogenous progesterone in the form of hormone replacement therapy has been associated with an increased breast cancer risk.[57] High doses of both hormones had been used with efficacy as therapeutic modalities in breast cancer patients.[55]

There is conflicting data regarding in vitro cellular proliferation in response to progesterone, with results of these studies ranging from no effect, an inhibitory effect, to a stimulatory effect. It has been established that progesterone does stimulate the expression of mitogenic growth factors and/or their receptors in steroid positive breast cancer cells.[49] Rationale for the lack of consistent data on cell proliferation studies may include the fact that the cells required estradiol for expression of the progesterone receptor (PR). It therefore is difficult to distinguish the primary effects of progesterone from antiestrogenic effects. In addition, a number of growth factors and hormones affect the progesterone response through regulation of the progesterone receptor (PR) level making it imperative that the culture medium be sufficiently stripped of the steroids and growth factors that support cellular growth.

MDA-MB231 (ATCC, Rockville, MD.) is an ER, PR negative human breast cancer cell line. We have transfected MDA-MB231 with the phPR-B plasmid containing the progesterone receptor B gene using the hMT-II zinc inducible promotor by the lipofectin method.[58] We have isolated a number of stable transfectants in G418 selection medium (neomycin resistant) which have been confirmed to express PR RNA and protein. (Project 3/Figure 1) Initial cell proliferation studies in Eagle's Minimal Essential Medium/10% fetal calf serum performed by the direct cell count method and the MTT assay revealed a decreased rate of growth of the PR-B gene transfected cells in comparison to the parental MDA-MB231 cell line and the phPR-B transfected cells.

While the first task of identifiyng a tumor suppressor gene at chromosome 16 in this sabbatical has not been completed, the second of developing new skills has been successful. During this sabbatical, several new techniques, including sequencing, DD-PCR and cell transfection, and their applications have been learned.

The projects of evaluating novel gene expression by DD-PCR and evaluating the effects of progesterone on a transfected cell line are directly portable to my existing laboratory and we will continue to pursue these studies.

E. Conclusions

This has been an extremely beneficial and educational oppportunity for me, one for which I am truly grateful to the U.S.Army Research and Materiel Command. If the sabbatical had been

two years, I am confident we would have had sufficient results from these studies for at least two or three publications. Since this sabbatical was performed in my home location, we will continue to pursue them as long as possible and should realistically publish the results in the future.

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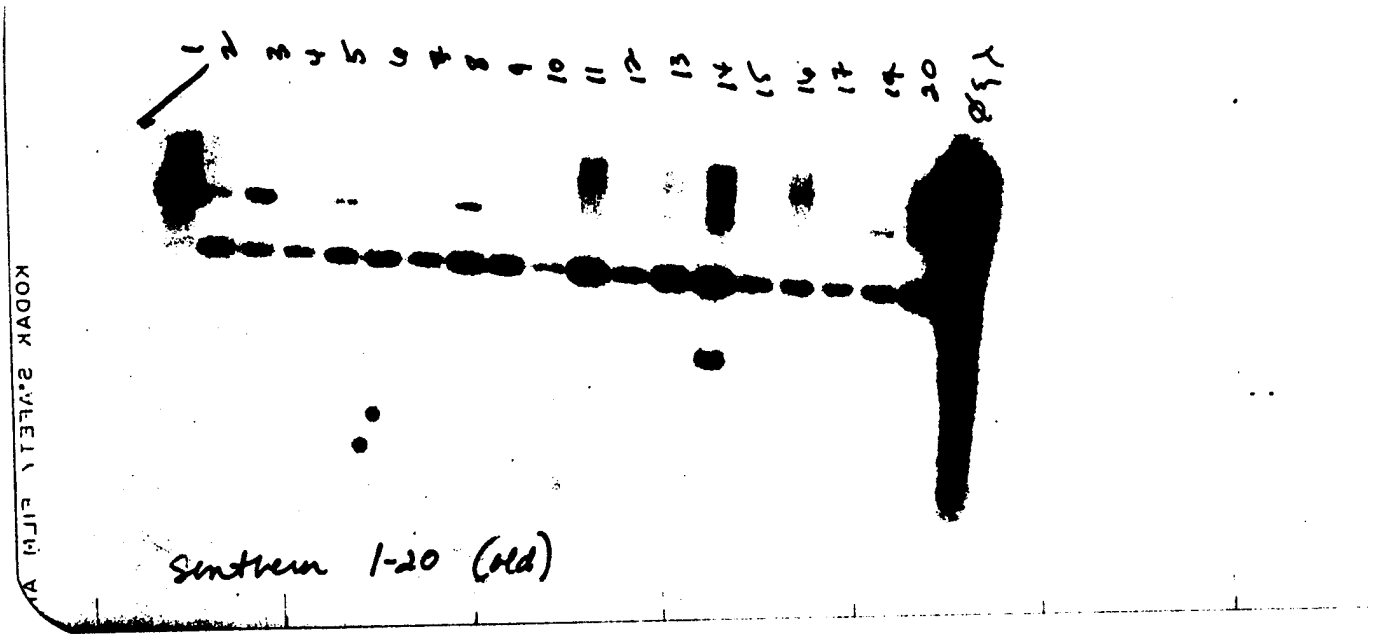
G. Publications and Personnel

There are no publications to date.
The only person who received salary support was myself.

Project 1 / Figure 1



This figure represents a Southern blot hybridization of DNA obtained from the secondary screening of the normal breast cDNA library to a ^{32}P labelled D16S7 clone. There is binding to the DNA in sample 14 as well as non-specific binding to the vector and control lambda.

Figure 1.

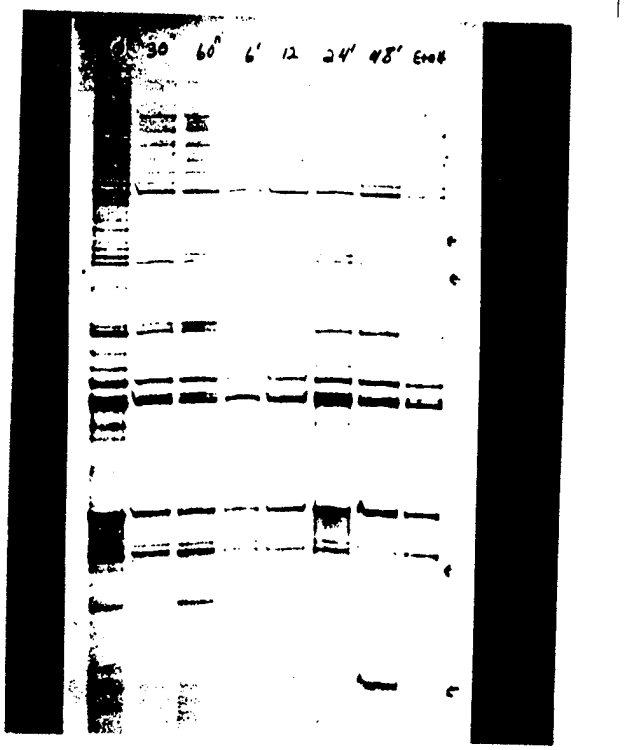


Figure 2.

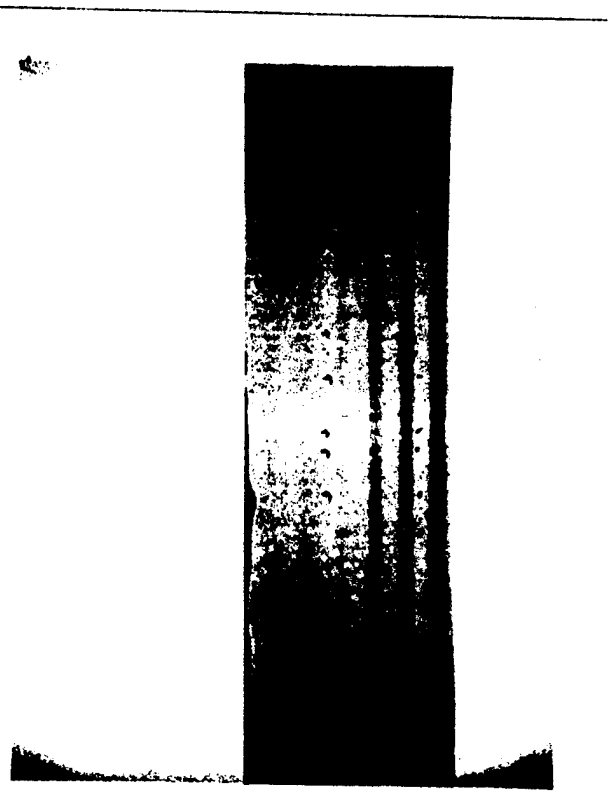
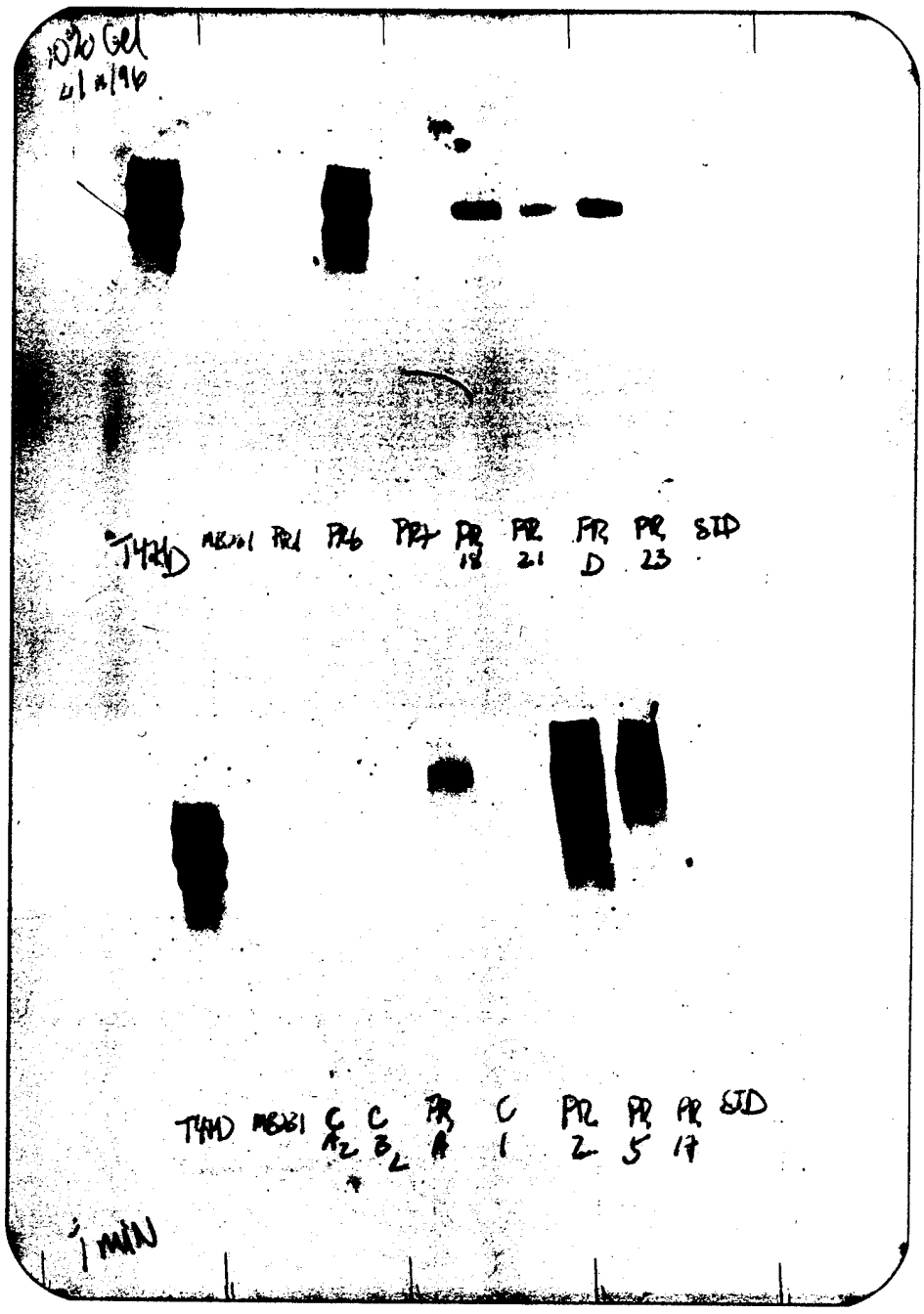


Figure 1 represents the results of a sequencing gel displaying the PCR products of estradiol treated and untreated MCF-7 cells using the primers HT11A, HT11C, HT11G and the arbitrary 5' primer H-AP4. There are four candidate differentially expressed genes identified in this section of the gel.

Figure 2 represents an 8% sequencing gel displaying the PCR cDNA products of progesterone treated T-47D breast cancer cells. Lane a is treatment for 30 minutes, lane b is treatment for 12 hours, lane c is treatment for 48 hours and lane d is no treatment. There are six candidate genes that are expressed in the progesterone treated cells but not expressed in the untreated cells.

Project 3 / Figure 1



This figure represents the western blot depicting expression of the progesterone receptor (PR) protein in the positive control T47D cell line, and the MB-231 cells transfected with the PR gene PR6, PR18, PR21 PRA, PR2 and PR5 cell lines. The control cell line MB-231 and the MB-231 cells transfected with the plasmid only do not express the PR.