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Our research proposal is on developing highly sensitive, rapid, cost-effective molecular assays for quantitating the transcripts of prognostic factors in breast cancer. In the first granting period, our goals were to establish the conditions for quantitating the transcripts of wild type-, and variant types of estrogen receptor (ER) using breast cancer cell lines in culture. We have designed PCR primers which can amplify various portions of ER mRNA. We tested the primers first on a plasmid cDNA (pIC-ER-F) which contains full length coding sequences of ER to establish the optimum PCR conditions. We have then used them to amplify various exons of ER cDNA prepared by reverse transcribing the RNA from MCF-7 cells. We are currently in the process of quantitaing the ER transcripts in MCF-7 cells by competition assay.

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Robert E. Taylor, M.D., Ph.D. & Indra Poola, Ph.D. — 8/28/95

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GRANT REPORT

Robert E. Taylor, M.D., Ph.D
and
Indra Poola, Ph.D

A. INTRODUCTION

Breast cancer is a tumor of the mammary gland epithelium affecting about 176,000 women in the western hemisphere and killing about 50,000 people every year in the United States. A substantial body of epidemiological, experimental, and clinical evidence indicates that exposure to the natural hormones, estrogen, progesterone and prolactin, which are important for the normal development and function of the breast tissue, play a major role in the growth of breast cancer cells and may even contribute to the development of breast cancer.

Prognostic factors in breast cancer . Once diagnosed with breast cancer, therapeutic approaches, survival rate and disease recurrence depend upon the expression of the prognostic factors, hormone receptors, growth factor receptor superfamily and proteins involved in invasive metastatic phenotype in the tumor tissues.

1. Hormone receptors. The most important among prognostic factors is the estrogen receptor (ER). The presence of ER in tumors indicates a good prognosis and the patients respond to anti-estrogen therapies. The expression of various molecular forms of ER was studied in several breast cancer cell lines and tissues. The results have revealed very interesting findings. Analysis of the estrogen receptor mRNA has shown that it undergoes alternate / inaccurate splicing in the tumor tissues giving rise to several aberrant (variant) forms of the receptor molecules[1]. These studies raise the possibilities of various species of ER which have mutations/truncations in the 1) estrogen binding region (exons 4-8), 2) DNA binding region (exons 2-3) and 3) other portions of the receptor molecule. In T47D cells, several variant mRNA species and their protein products lacking exons 2, 3, or 7 representing 25% of the ER mRNA were recognized[2]. **To study the effect of the above mutated\truncated (variant) species, cDNAs corresponding to wild type and variant forms were expressed in cell lines and the estrogen-, and DNA binding properties were evaluated. It was found that the variant species interfere with the normal wild type ER for its binding with estrogen response elements (ERE) on the DNA- and hormone binding and estrogen induced gene transcription presumably by heterodimer formation[3].**

2. Growth factor receptor superfamily. In the last five years, it was shown that several other proteins participate in the aberrant growth of breast cancer cells and influence disease prognosis. These include EGF-receptor, IGF-I receptor, and erbB2.

3. Proteins which are involved in invasive or metastatic phenotype. In addition to hormone and growth factors receptors several other gene products are implicated in the

prognosis of breast cancers. Important among them are nm 23, laminin receptor and p53.

4. Current prognostic factor evaluation and their disadvantages. The presence of ER and other prognostic factors in the tumor tissues are currently evaluated by immunochemical methodologies in clinical laboratories. While these provide information on the presence of ER, they are 1) very cumbersome, 2) time consuming, 3) not highly sensitive, 4) require a large sample to evaluate all prognostic factors, 5) **do not yield quantitative information**, 6) not suitable to evaluate in fine needle aspirations and 7) very expensive to the patient. **Most importantly, immunohistochemical assays cannot distinguish between the wild type and variant forms of ER, therefore, cannot predict hormone therapy responders precisely. Because of the therapeutic considerations, there is an urgent need to rapidly quantitate and get a profile of wt and vt of ER to predict hormone therapy responders and disease prognosis.**

The essence of our proposal is to develop highly sensitive cost effective quantitative PCR assays to measure a multitute of hormone receptors and other diagnostic and prognostic factors in breast cancers.

5. Development of new prognostic assays. In our grant, we proposed to develop highly sensitive, rapid, cost-effective PCR based methods to quantitate all the prognostic factors in a small amount of clinical samples. The developed assays could be used virtually in every clinical lab to diagnose and evaluate prognosis of breast cancers. The significance of this assay is that patients could then be identified who are most likely to respond to hormone therapy and that the overall prognosis may be assessed on the basis of many indicators. At present, only one or a few, prognostic markers are tested on a given patient sample.

B. SPECIFIC AIMS.

The specific aims of our proposal are to develop highly sensitive PCR methods for rapid, specific, diagnosis of breast cancers on the basis of their estrogen- and progesterone receptor expression. To achieve these goals our proposed to conduct the following.

1. Quantitation of wild type and variant forms of estrogen receptor in estrogen responsive cell lines: a) RNA extraction, b) reverse transcription, c) primer selection, d) PCR, e) relative quantitation and f) absolute quantitation.
2. Correlation of the transcript number with functionally active estrogen receptor molecules in estrogen responsive cell lines: a) estrogen binding activity and b) DNA (ERE) binding activity
3. Quantitation of wild type and variant types of estrogen receptor in breast tumor tissues
4. Comparison of estrogen receptor mRNA levels and functionally active protein levels in patient samples
5. Quantitation of other prognostic factor transcripts and
6. Application of RTP asssay to predict prognosis in patients.

C. BODY OF THE REPORT (STUDIES AND RESULTS).

During the first year of granting period, we have focussed on achieving our first goal: quantitating the wild type and variant forms of estrogen receptor. In the first granting period we have 1) achieved in procuring and culturing the estrogen receptor positive breast cancer cell lines 2) establishing the PCR conditions for amplifying the various portions of estrogen receptor mRNA in estrogen responsive cell lines and 3) started quantitating estrogen receptor transcripts from MCF-7 cell lines.

1. Procuring and culturing of estrogen receptor positive and negative cancer cell lines. We have obtained estrogen responsive cell lines, MCF-7 and T47D from ATCC and established them in culture in our laboratory. As well, we have obtained estrogen receptor negative cell line from ATCC, and HCD-57, a murine erythroleukemia cell line from Dr. D. Hankins of Johns Hopkins University Medical School.

2. RNA isolation from tissue culture cell lines. In order to quantitate RNA transcripts by PCR, which amplify DNA, it is critical that both the RNA extraction and reverse transcription of RNA are quantitative as well. We have extensively tested various RNA isolation procedures and found the procedure described below to be most quantitative. We found that it is extremely reproducible and accurate with small number of cells. The procedure we have standardized is essentially the modification of procedure published by Gouch[4]. Briefly, 5 X 10^8 cells from culture were rinsed with phosphate buffered saline pH 7.4 (PBS) and resuspended by gentle mixing in 200 ul of solution A (10 mM Tris (pH 7.5), 0.15 mM NaCl, 1.5 mM $MgCl_2$, 0.65 % Nonidet P-40) then incubated on ice for 5 min to allow cells to lyse. Samples were vertexed, pelleted and the supernatant transferred to a fresh tube containing 200 ul of solution B (7 M urea, 1% SDS, 0.35 mM NaCl, 10 mM Tris pH 7.5 and 10 mM EDTA). Samples were then extracted with 400 ul of PCI (one part isoamy alcohol to 24 parts chloroform mixed equally with phenol), once with 400 ul of Cl (one part of isoamyl alcohol to 24 parts of chloroform) then ethanol precipitated by standard methods[5]. The isolated RNA was tested for on 1% agarose gels.

3. Reverse transcription of RNA. To make cDNA from RNA samples, 1 ug of total RNA was reverse transcribed using random hexamers as primers in a 20 ul of reaction mix (10 mM Tris-HCl, pH 8.3, 5 mM $MgCl_2$, 50 mM KCl, 1 mM each dNTP, 2.5 uM random hexamer, 1 U/ul RNAse inhibitor, 2.5 U/ul reverse transcriptase (Perkin Elmer Cetus, Norwalk CT). This was incubated at room temperature for 10 min at 42^0 C for 15 min and 4^0 C for 5 min using the thermal cycler for all but the room temperature incubations (Mj Research, Inc. Programmable Thermal Controller Model PTC-100).

4. Design and testing of PCR primers which can specifically amplify various portions of estrogen receptor mRNA. The genomic and cDNA sequences of the human estrogen receptor have been published[6]. Because of the observations that estrogen receptor is expressed as truncated and mutated (variant) forms in breast cancer tissues, we have designed several primer sets encompassing exons 2-8, so that all the variant forms can be amplified and

quantitated. The primer sets are given in the Table.1. A probe was also synthesized (Table.1), which has the sequence in the exon 4 region, so that it can recognize the PCR products of all the primers. We have tested the above primers first with cloned human estrogen receptor cDNA gene, pIC-ER-F, which contains the full length coding sequences (obtained from ATCC). After establishing the optimum conditions we have tested the primers on cDNA prepared by reverse transcribing the RNA isolated from MCF-7 and T47D cell lines.

TABLE 1: PCR PRIMERS FOR THE HUMAN ESTROGEN RECEPTOR (hER)

PRIMER PAIRS	**LOCATION**
ER1S 5'ATGACCATGACCCTCCACACC3' ER1A 5'GGTCAGTAAGCCCATCATCG3'	Exons 1-4
ER2S 5'GGAGACATGAGAGCTGCCAAC3' ER2A5'CCAGCAGCATGTCGAAGATC3'	Exons 4-6
ER3S 5'GCCCGCTCATGATCAAACGC3' ER3A 5'TCAGACTGTGGCAGGGAAACC3'	Exons 4-8
PROBE 5'CTGGCCTTGTCCCTGACGGCCGACCA- GATGGTCAGTGCCTTGTTG 3'	Exon 4

a. Testing the primers on plasmid (pIC-ER-F) cDNA. All the above three pairs of primers were first tested to optimize the PCR conditions.

a1. Testing the primer set ER1S and ER1A. We have tested this set of primers which can amplify in the region of the exons 1-4 on pIC-ER-F for optimum Mg^{++} concentration, primer concentration, denaturation-, annealing-, and extension conditions. We have found that the optimum Mg^{++}ion concentration is between 0.75 -1 mM, denaturation at 94^0C for 1 min, annealing at 45^0C, and extension at 72^0C for 3 min for 35 cycles. The resulting PCR product of size 1041 bp could be visualized on agarose gels (shown in Fig.1).

a2. Testing the primer set ER2S and ER2A. As described in the above table this set amplifies the segment of mRNA in the region of exons 4-6. We have tested this set for optimum PCR conditions as described above for ER1S and ER1A. We found that the optimum Mg^{++} ion concentration is between 2-2.5 mM, denaturation at 94^0C for 1 min, annealing for 2 min at 58^0C , and extension at 72^0C for 3 min. The resulting PCR product of size 438 bp can be seen in Fig.2

a3. Testing of primer set ER3S and ER3A. This primer set amplifies the mRNA in the region of exons 4-8. The optimum PCR conditions we have obtained are Mg^{++} ion concentration at 1 mM, denaturation at 94^0C for 1 min, annealing for 2 min at 45^0C , and extension at 72^0C for 3 min. The resulting PCR product of size 920 bp can be seen in Fig.3

b. Testing of the primers on cDNA prepared from reverse transcription of MCF-7 cell RNA. After standardizing the PCR conditions for the above primers using the plasmid cDNA, we went on to test them on the cDNA prepared by reverse transcribing the RNA. Total RNA was isolated and reverse transcribed as described. We found that using the same PCR conditions described as above, we could amplify the sequences of reverse transcribed RNA of the exons 1-4, 4-6 and 4-8. We have obtained similar results as seen in Figs 4-6 respectively. In each case we have included a primer set which amplified mRNA of constitutively expressed protein, glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

5. Quantitation of estrogen receptor transcripts. Our main goal of the proposal is to quantitate the transcripts of estrogen receptor and various other prognostic factors. Having established the PCR amplification of estrogen receptor, we began the experiments on the quantitation of wild type and variant types of estrogen receptor transcripts by competition assay. Briefly, we will quantitate the transcripts by precisely determining the number of specific RNA transcripts by template competition[7]. In this approach, two templates which are specific for the same primers are used in the PCR reactions. The templates are amplified at the same efficiency and the ratio of the two PCR products will reflect the starting ratio of the two template DNAs. This means that if the amount of a competitor DNA is known then the amount of unknown template can be precisely determined by measuring the two PCR products. This requires that the products generated by the two different templates must be distinguishable from each other.

Designing the competitor for the absolute quantitation of estrogen receptor. We have designed a competitor plasmid as described in the Fig.7. As described in the Fig.7 each set of primers in Table. 1 flanks an unique HindIII site within exon 4. A 125 bp HindIII fragment from bacteriophage lambda DNA was inserted into the site. We will use this construct as a competitor to quantitate estrogen receptor transcripts. When this si used as a competitor template in a PCR reaction for the estrogen receptor, it will generate a product that is 125 bp larger than the normal product and easily distinguishable on an agarose gel. We are currently in the final stages of obtaining the competitor cDNA. We anticipate that in the next couple of weeks we will conducting the trancript quantification experiments.

D. CONCLUSIONS

The results we have obtained indicate the feasibility of developing the quantitative molecular assays for breast cancer. Estrogen receptor transcripts could be quantitated by PCR in breast cancer cell lines.

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APPENDIX

FIGURES

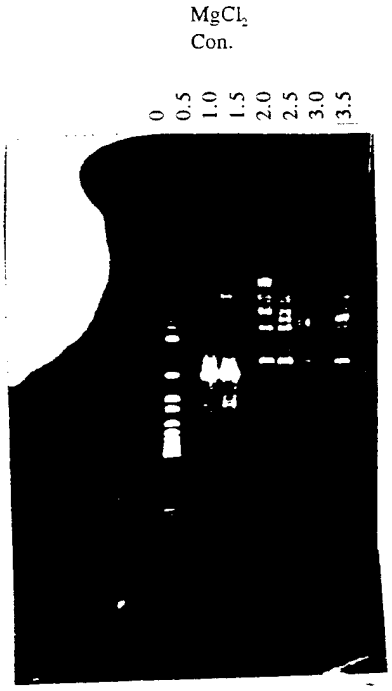


Fig.1.

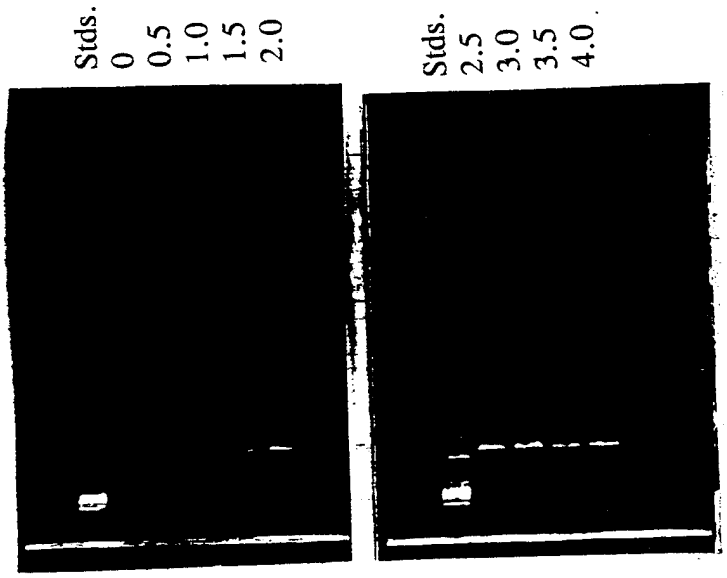


Fig.2

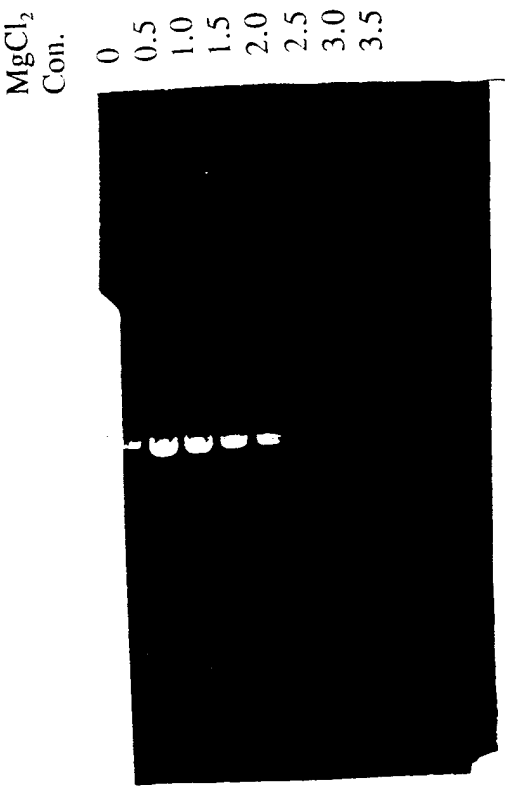


Fig.3

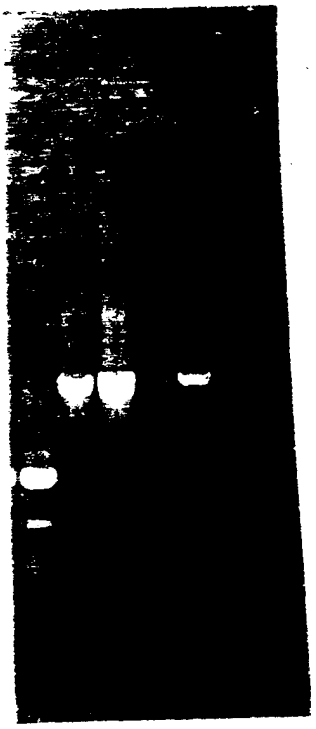

GAPDH
Plasmid cDNA
Plasmid cDNA
MCF-7 cDNA
No Template

Fig.4

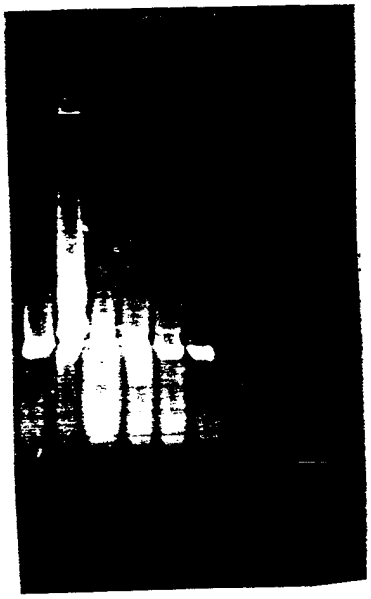

GAPDH
Plasmid cDNA
MCF-7 cDNA, 2.5 mM MgCl2
MCF-7 cDNA, 2.0 mM MgCl2
MCF-7 cDNA, 1.50 mM MgCl2
MCF-7 cDNA, 1.0 mM MgCl2
Stds.

Fig.5

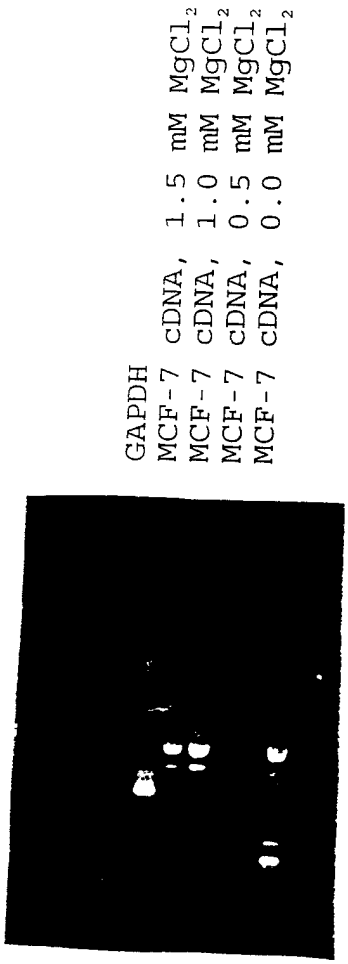
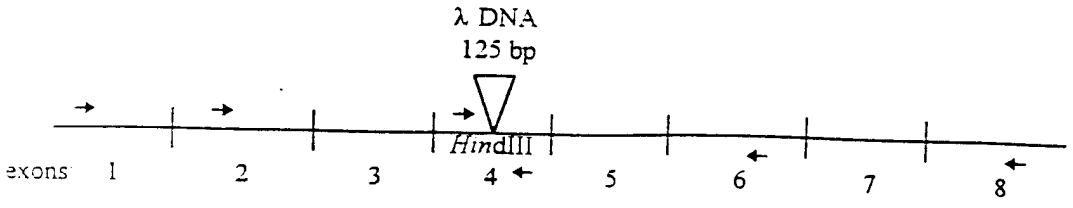


Fig.6



Estrogen Receptor cDNA

Fig.7