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AWARD NUMBER DAMD17-94-J-4495

TITLE: New Therapeutic Approaches and Prognostic Assays for Breast Cancer: Radiolabeled Ligands and Antibodies and Quantitative PCR

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REPORT DATE: November 1997

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, 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
	November 1997	Annual (30 Sep 96 - 29 Sep 97)

4. TITLE AND SUBTITLE
New Therapeutic Approaches and Prognostic Assays for Breast Cancer Radiolabeled Ligands and Antibodies and Quantitative PCR

5. FUNDING NUMBERS
DAMD17-94-J-4495

6. AUTHOR(S)
Indra Poola, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Howard University
Washington, DC 20059

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, 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 words)*

14. SUBJECT TERMS
breast cancer ; hormone receptors, quantitative PCR

15. NUMBER OF PAGES
54

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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GRANT REPORT

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

1. 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.

2. Hormone receptors in the prognosis and therapy of breast cancers. The most important among prognostic factors are the hormone receptor, estrogen-, and progesterone receptors (ER and PgR). Estrogen receptor. 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 exon deletions/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 exon deleted\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].**

3. Current methods of estrogen receptor evaluation and their disadvantages. The presence of ER 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 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 multitude of hormone receptors and other diagnostic and prognostic factors in breast cancers.

4. Development of new prognostic assays for estrogen receptor. In our grant, we proposed to develop highly sensitive, rapid, cost-effective PCR based methods to quantitate ER and other 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.

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 we 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. Application of the molecular assay to predict prognosis and hormone therapy response in patients.

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

In the granting period, we have 1) developed quantitative PCR methodologies to quantitate the exact copy numbers of estrogen receptor in breast cancer cell lines and tumor samples and 2) collected breast tumor samples from African American women and studied the profiles of estrogen receptor in them.

Our work on the quantitation of estrogen receptor copy numbers is accepted for publication in Analytical Biochemistry. A copy of the paper is attached.

We studied the profiles of estrogen receptor exon deletion and truncated variants in the tumors of African American women because several recent reports indicate that 1) African American women experience three times higher mortality rate with breast cancer than others and 2) tumor biology also appear to be different. The tumors found in African American women are poorly differentiated with low frequency of hormone receptors. To gain an insight into the factors which may be responsible for these, we studied the alterations in the estrogen receptor gene, the most important prognostic factor in breast cancer. Our results indicate that only a fraction of the tumors which are typed as estrogen receptor positive by clinical laboratories contains full length transcripts for estrogen receptor. In addition to the detection of previously identified exon deletion variants, we identified the receptor transcripts which have truncations in both exons 1 and 8 or only in exon 8.

This work is being prepared for publication. We enclose here a draft of the manuscript as a part of the grant report.

CONCLUSIONS. A highly sensitive molecular biological approach has been developed to quantitate the exact copy numbers of estrogen receptor mRNA in breast tumors. Once converted into a clinically feasible assay, it could become a highly valuable method to precisely predict the prognosis of the disease and identify patients who are potential candidates for anti-hormone therapy.

Enclosures. 1. A manuscript accepted in Analytical Biochemistry
2. A manuscript which will be sent to Cancer Research very shortly.

ANALYTICAL BIOCHEMISTRY

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November 21, 1997

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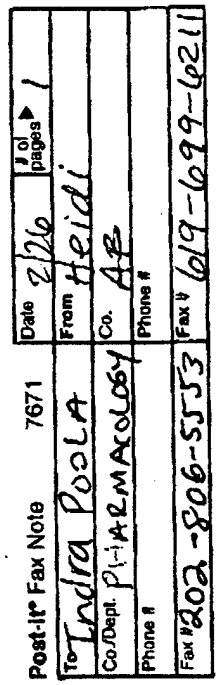

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QUANTITATION OF ESTROGEN RECEPTOR mRNA TRANSCRIPT COPY NUMBERS IN BREAST CANCER CELL LINES AND TUMORS

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Running Title: Quantitation of estrogen receptor copy numbers

ABSTRACT

Several clinical studies have suggested that the content of estrogen receptor (ER) in breast tumors influences the survival, tumor recurrence and response to anti-estrogen therapies. Therefore, the ability to precisely quantitate the ER content in tumor tissues will be of significant benefit to women with breast cancer. Although immunohistochemical and Polymerase Chain Reaction (PCR) methods have been described for the detection and semi-quantitation of ER, none of them precisely quantitate ER copy numbers in tumor samples. In the present report we describe a molecular approach to accurately quantitate ER transcript copy numbers using a reverse transcription- PCR (RT-PCR) template competition method. A competitor template was devised by inserting unrelated nucleic acid sequences into an ER cDNA clone. A template competitive RT-PCR analysis was then performed to determine the number of copies of ER mRNA. As a standard of reference for the ER transcripts from various samples, the copy numbers of a constitutively expressed gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were also quantitated. The ER quantitations were performed in three positive cell lines, MCF-7, T47D and ZR-75 and two positive tumor tissues by this approach. Our results described here show that among the cell lines studied, T47D expresses the highest copy numbers of ER. We also present here that ER as low as 10^3 copies per 10^5 copies of GAPDH can be detected and quantitated in tumor samples by the template competition method. In addition, the molecular approach can simultaneously detect, distinguish and quantitate exon deletion variant copy numbers of ER. The results described in this report indicate that the ratios of exon 7Δ variant to wild type in the tumor tissues are significantly higher than in the cell lines studied.

INTRODUCTION

The human estrogen receptor (ER) is a member of the super-family of nuclear steroid receptors. The molecular cloning of the estrogen receptor has allowed significant advances in our understanding of its structure and mechanism of action (1). The gene for ER is more than 140 Kb in length and contains eight exons. The protein has six functional domains, designated A through F. The domain C, which spans exons 2 and 3, binds DNA and the domain E, which spans exons 4-8, contains the hormone binding site (2). Clinical studies have shown that approximately 60-70% of the breast tumors assayed are ER positive. The presence of ER in tumor cells is considered a good prognosis and indicates that the tumor cells depend on estrogen for growth and survival. The patients who express estrogen receptors in their tumors have an overall longer survival and lower risk of tumor recurrence (3). The loss of the ER in tumor cells is considered to be a bad prognosis. When that happens, breast cancer cells apparently gain the ability to grow even in the absence of the steroid hormones. These tumors appear to be very aggressive, metastasize widely and are resistant to currently available therapies. In addition to its prognostic value, the presence of ER is also exploited to treat tumors with anti-estrogen therapy. It is based upon the breast tumor cell dependency for estrogen for their growth and survival. To treat a tumor with anti-estrogen therapy requires that the tumor cells express ER and bind anti-estrogens specifically (4). Currently, the ER status in breast tumor tissues is determined from rough estimates yielded by microscopically scoring slides subjected to immunohistochemistry techniques. Laboratory studies have shown that about one half of the tumors which have an ER content greater than 10fM/mg of the total

protein are estrogen dependent for growth. Clinical studies, on the other hand, have indicated that approximately 50% of patients with the ER content > 1000 pM/mg protein are found to respond favorably to anti-estrogen therapy. In recent years, due to increased awareness and periodic screening procedures, tumors of small size can be detected. In these cases the limited amount of tumor tissue often restricts ER protein quantitation. Several studies have suggested that molecular biological procedures may be preferred for the ER quantitation since they require much smaller amounts of tumor tissue. This led to the development of PCR based methods for the detection and relative quantitation of ER mRNA in tumor samples (5 - 7) in recent years. However, none of the methods described thus far can estimate the absolute number of copies of the receptor.

In this report, we describe a significantly improved PCR approach with which one can quantitate exact number of copies of wild type as well as variant ER transcripts. As a standard of reference for the ER transcripts, we also describe the quantitation of the mRNA copy number of a constitutively expressed gene, the glyceraldehyde-3-phosphate-dehydrogenase (GAPDH). The significance of our PCR template competition assay, therefore, is threefold: 1) survival prognosis may be easier and more widely applicable 2) patients, likely to respond to hormone therapy can be more readily identified and 3) the relation of ER variants to breast cancer growth can be assessed.

MATERIALS AND METHODS

RNAeasy midi kits, AmpliTaq PCR core kits and QIAquick gel extraction kits were from QIAGEN Inc., Santa Clara, CA. Reverse transcriptase kits were from Applied Biosystems. [α -^{32}P]dCTP (Specific Activity 3000Ci/mMole, Cat# AA0005) was from Amersham. A plasmid containing the full length ER cDNA gene, pIC-ER-F, was obtained from ATCC. The primers for amplifying ER and GAPDH were synthesized at Gibco-BRL Life Technologies. Trizol reagent for total RNA isolation was purchased from Gibco-BRL Life Technologies. Diethyl Pyrocarbonate (DEPC) treated water was from Research Genetics, Al. PCR quality water and Tris- EDTA buffer were from Biofluids, Rockville, Md.

Cell lines and breast tumor samples. The ER positive breast cancer cell lines, MCF-7, T47D and ZR-75 and an ER negative cell line, LCC6 were kind gifts from Dr. Robert Clark, Georgetown University School of Medicine. They were maintained in 90% IMEM without gentamycin and 10% Fetal Bovine Serum (FBS). The ER negative cell lines, HS578T, BT-20. MDA-MB-231 and UACC were obtained from ATCC. Hs 576T was propagated in 90% DMEM and 10% FBS. BT-20 was cultured in MEM with 0.1 mM non-essential amino acids (90%) and 10% FBS. UACC and MDA-MB-231 were grown in Leiboitz's medium (90%) and 10% FBS in the absence of $C0_2$. The breast tumor samples were derived from a breast tumor bank established by one of us (I.P) at Howard University Medical School.

RNA isolation from breast cancer cell lines and tumors: Total RNA from breast cancer cell lines was isolated using RNAeasy midi kits, and the manufacturer's protocol. Briefly, the cells (approximately 5 X 10^7) were lysed with 1.9 ml of lysis buffer, 1.9 ml of 70% ethanol was added and centrifuged at 4°C for 10 min at 5000 g, The supernatant was loaded onto spin columns which were supplied by the manufacturer and centrifuged at 5000 g until all the lysate passed through. The columns were washed with the wash buffer and total RNA was eluted from the columns with DEPC treated water. This method yielded about 0.8 - 1.0 mg of total RNA per 10^7 of tissue culture cells. Although the RNAeasy kits were proved to be excellent for the preparation of total RNA from cell lines, they did not yield RNA from tumor samples. Therefore, an alternative protocol was used for the isolation of total RNA from breast tumor samples using the Trizol reagent and the manufacturer's protocol. Briefly, the tumor tissue was first powdered by pulverizing it in a sterile pestle and mortar in the presence of liquid nitrogen. The tumor powder (50-100 mg) was suspended in 1 ml of Trizol reagent and homogenized with a hand held homogenizer and incubated at room temperature for 5 min to permit the dissociation of nucleoprotein complexes. Then, 0.2 ml of chloroform was added to the above, shaken vigorously and centrifuged at 4°C for 15 min at 12,000 g. Total RNA was precipitated from the supernatant by adding 0.5 ml of isopropanol. The precipitate was washed twice with 75% ethanol, dried briefly and dissolved in DEPC treated water. This method yielded about 30 μg of total RNA per 100 mg of tumor tissue. The concentration of RNA was determined by measuring the optical density at 260 nm.

Reverse Transcription. The isolated RNA was reverse transcribed to cDNA using

Moloney Murine Leukemia Virus (MuLV) reverse transcriptase and random hexamers. Briefly, the standard reaction mixture contained 1 μg of total RNA, 2.5 units of MuLV reverse transcriptase, 1 mM each of dNTPs, 2.5 μM random hexamers, 1 U of RNAse inhibitor, 5 mM $MgCl_2$ and 1 X PCR buffer in a total volume of 20 μl. To reverse transcribe the RNA, the reaction tubes were first left at room temperature for 10 min, followed by incubations at 42^0 C for 15 min, 99^0 C for 5 min and finally 5^0 C for 5 min.

PCR. The Polymerase Chain Reaction was performed in an automatic thermal cycler (MJ Research). For ER cDNA amplification, the sequences between exons 4 and 8 were amplified using the sense primer 5' GCCCGCTCATGATCAAACGC 3' (Exon 4, bp 1112-1132) and an anti-sense primer 5' TACTTTTGCAAGGAATGCGA 3' (Exon 8, bp 1978-1958) (10). The amplification was carried out in a 12.5 μl reaction volume containing the reverse transcribed cDNA, 1 X PCR buffer, 1 X Q solution (Qiagen), 200 μM each of dNTPs, 2 μM each of sense and anti-sense primers and 0.6 U of Taq polymerase. The PCR conditions were initial denaturation for 5 min at 95^0 C followed by 94^0 C for 1 min, annealing for 1 min at 55^0 C, and extension for 2 min at 72^0 C for 40 cycles and final extension for 10 min at 72^0C. The GAPDH cDNA was amplified using a sense primer 5' AAGGCTGAGAACGGGAAGCTTGTCATCAAT 3' (exon 3, bp 241-270) and an anti-sense primer 5' TTCCCGTCTAGCTCAGGGATGACCTTGCCC 3' (exon 7, bp 740 -711) (8, 9) under the same PCR conditions as described for ER.

Design and construction of competitor template DNAs for the quantitation of ER and GAPDH by template competition. In the template competition approach (11), two templates which can be amplified by the same primers are used in the PCR reactions. If two templates are amplified at the same efficiency, the ratio of their 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 two different templates must be distinguishable from each other. For this purpose, a competitor plasmid DNA which contained an extra fragment of DNA internal to the PCR primer location that generates a larger PCR product was prepared. As a standard of reference for the ER copy numbers in different test samples, we also chose to measure the constitutively expressed GAPDH gene. Therefore, we have also designed a competitor DNA for GAPDH. Competitor template DNA for GAPDH. The competitor plasmid for GAPDH was constructed in two steps. First a 500 bp portion of the GAPDH cDNA was amplified by RT-PCR using RNA extracted from the breast cancer cell line, MCF-7. The primers used had an attached upstream *Eco*RI restriction enzyme site and a downstream *Bam*HI restriction enzyme site. The sense and anti-sense primers were 5' CCCGAATTCAAGGCTGAGAACGGGAAGCTT 3' (exon 3, bp 241-261) and 5' CCCGGATCCTTCCCGTCTAGCTCAGGGATG 3' (exon 7, bp 740-720) respectively (9). This fragment was subcloned into the *Eco*RI and *Bam*HI sites of pBluescript II SK+ (Stratagene). The resulting plasmid was designated as pBS-GAPDH. Next a 210 bp fragment of DNA was amplified from bacteriophage λ DNA (from bases 1920-2130) (12) by PCR using primers which had attached *NcoI* restriction enzyme sites to both ends. The sense- and

anti-sense primers were 5' CCCCCATGGCTGGACCGCTACGAAATGCGC 3' (bp 1920-1940) and 5' CCCCCATGGCGTTCAACAATGGTCGGG 3' (bp 2130-2110) respectively. This fragment was then subcloned into the unique *Nco*I site within the 500 bp piece of pBS-GAPDH. The competitor thus generated was termed as pBS-GAPDH- λ. The schematic representation of the competitor design for GAPDH is shown in **Figure 1A**. When pBS-GAPDH-λ was used in a PCR reaction, it generated a fragment 210 bp larger than pBS-GAPDH (not shown). Competitor template DNA for ER. The competitor plasmid for ER was prepared by inserting λ sequences into an unique *Hin*dIII site in exon 4 of ER cDNA. This construction was also performed in two steps. First, since the commercially procured pIC vector in which ER was cloned (13) contains an additional *Hin*dIII site, the ER cDNA gene was subcloned from pIC-ER-F into the *Eco*RI site of the plasmid pSG5 (Stratagene). Second, a fragment of DNA was amplified from bacteriophage λ DNA using the sense primer 5' GGGAAGCTTAAACCATTCTTCATAATTCAA 3' (bp 37101-37101) containing a 5' *Hin*dIII linker and the antisense primer 5'CGCACCAACAGGCTCCAAGCC 3' (bp 37465-37485) which flanked a native *Hin*dIII site in λ (12). This fragment generated a 366 bp DNA when digested with *Hin*dIII. The 366 bp fragment generated above was inserted into the *Hin*dIII site in exon 4 of ER. The competitor thus generated is termed as pSG-ER- λ. The schematic representation of the design of the competitor for ER is shown in the **Figure 1B.** As shown in the figure, the primers used for amplification of ER flank the unique *Hin*dIII site within exon 4. When this cDNA was used as a competitor template in a PCR reaction with the ER primer pair which can amplify exons 4-8, it generated a product that is 366 bp larger than the normal product, and thus distinguishable by gel electrophoresis (data not

shown).

Competition assay. The ability of the competition assay to accurately measure ER and GAPDH transcript copy numbers was first confirmed by performing the assay with known amounts of both the competitor templates and the cloned normal GAPDH and ER cDNA genes. The number of copies of normal and competitor DNA molecules were determined by multiplying the average mass of a base pair (660 daltons) by the number of base pairs then dividing Avagadro's number by that value as in the following example for a 3500 bp DNA molecule: (6.023 X 10^{23} molecules/mole)/3500 bp X 660 g/mole/bp = 2.61 X 10^{17} molecules/g = 2.61 X 10^{11} molecules/ μg. For these experiments the number of copies of ER cDNA and GAPDH cDNA were kept constant at 10^7 while the concentrations of the competitor DNAs ranged from 10^4 to 10^8 copies. The GAPDH competition was performed in the presence of the competitor which ranged from 10^4 copies to 10^8 copies by half log dilutions. ER competition assay was performed in the presence of the competitor which ranged between 10^5 to 10^8 in quarter log dilutions. In both ER and GAPDH competition assays, we expect the normal and the competitor plasmid DNAs to cross over at equimolar concentrations (10^7 copies). It was found that the amounts of competitors needed to generate equal ratios of the normal and the competitor PCR products for GAPDH and ER were 10^7 and $10^{7.25}$ respectively. They crossed over at expected or with in the range of 1.5 - 2 times, which agrees with the previously published reports on template competition method (11) (data not shown). After verifying the feasibility of template competition method, we went on to measure the copy numbers of both ER and GAPDH in breast cancer cell lines and tumor

samples. To determine the GAPDH and ER transcript copy numbers, the template competitive PCR assays were conducted with reverse transcribed cDNA prepared from a known amount of RNA and in the presence of various amounts of competitor template DNAs. To determine ER copy numbers, the cDNA prepared from 250 ng of total RNA was kept constant and the competitor concentration was increased from 10^4 to 10^8 copies in quarter log dilutions. To determine the number of copies of GAPDH, the cDNA prepared from 50 ng of total RNA was kept constant and the competitor concentration was increased from 10^4 to 10^8 in half log dilutions. The PCRs were performed as described before.

Detection and quantitation of PCR products. For initial screening experiments, the PCR products were electrophoresed in 1% agarose gels and visualized by ethidium bromide staining. For quantitation of the PCR products, [α -^{32}P]dCTP was included in the PCR reaction at 0.5% of the total reaction volume. The radiolabelled PCR products were electrophoresed in a Bio-Rad vertical slab gel apparatus in 4.5% acrylamide gels, 90 mM Tris-Borate and 0.2 mM EDTA at a 40 mA constant current for 3.5 hrs. The gels were dried in a Savant gel dryer and autoradiographed using Kodak X-Omat AR film and DuPont NEN Lightning Plus intensifying screens at room temperature. The radioactivity in the individual PCR products was quantitated by scaning the autoradiograms in a laser densitometer (Molecular Dynamics).

Sequence analysis: In order to confirm the identity of the PCR amplified ER products, they were electrophoresed in agarose gels and purified using the QIAquick gel extraction kit and sequenced by cycle sequencing method on an automated DNA sequencer

using the ER sense primer (14, 15).

RESULTS AND DISCUSSION

Clinical studies have suggested that the content of ER in the tumor tissues influences the prognosis and response to anti-estrogen therapy in breast cancer (3, 4). Therefore, the ability to precisely quantitate the ER content in the tumor tissues will be of significant benefit to women with breast cancer. Although immunohistochemical and several PCR based methods have been described, none of them quantify the exact copy numbers of ER in tumor tissues. In the present study we describe a molecular approach to precisely quantitate the ER transcripts in the tumor tissues by template competition method. We have also devised a method to quantitate the exact copy numbers of a constitutively expressed gene, GAPDH, so that the ER copies from various samples can be expressed with reference to the copy numbers of GAPDH. In addition, we show that the template competition method described here can simultaneously quantitate the copy numbers of ER exon Δ variants. The presence of the variant ERs which have deletions in exons 2, 3, 2-3, 5, and 7 in both normal mammary-, and breast tumor tissues is well documented (16-18).

We designed and cloned the competitor templates which enabled us to quantitate the exact copy number of both GAPDH and ER transcipts by template competition. For ER quantitations, we used the primers which amplified sequences between exons 4 and 8. The primers were first tested on the cDNA prepared by reverse transcription of total RNA from

various breast cancer cell lines. We observed the expected 865 bp PCR product plus an additional product of size about 700 bp in MCF-7, T47D and ZR-75 and the two tumors tested. Neither of these products were observed in the cell lines, MDA-MB-231, HS578T, LCC6, UACC and BT-20 which are typed as ER negative. Restriction enzyme digestion of the 700 bp fragment suggested that it was the previously characterized exon 7Δ variant and subsequent sequence analysis confirmed its identity (data not shown). After verifying the identity of 700 bp fragment, we performed the quantitations of GAPDH and ER copy numbers in the ER positive breast cancer cell lines and tumor tissues.

A typical quantitation assay performed for GAPDH using the reverse transcribed cDNA prepared from T47D cell line is presented in Figures 2 and 3. The amount of competitor in the PCR reaction ranged from 0.32 x 10^5 copies (Figure 2, lane 2) to 56.2 x 10^5 copies (Figure 2, lane 11). As seen in Figure 2, the GAPDH PCR product from the T47D cell line (lower band) decreased in the presence of increasing amounts of the competitor plasmid pBS-GAPDH-λ (upper band). To determine the exact copy numbers of GAPDH, the desitometric scans of the radioactivity in the normal and the competitor PCR products were plotted against the number of copies of the competitor template in the PCR reaction. A representative graph for GAPDH quantitation in the T47D cell line is shown in Figure 3. As seen in Figure 3, the cross over point at which the competitor and the reverse transcribed cDNA gave equal amounts of PCR products was 1 X 10^5 copies. Therefore, the number of copies of GAPDH in T47D cDNA prepared from 50 ng of total RNA was 1 X 10^5 copies. The number of copies of GAPDH from MCF-7, ZR-75, tumor 1 and tumor 2 were determined

by similar procedures. They were 5.62 X 10^5, 3.16 X 10^5, 6.00 X 10^4, and 3.16 X 10^4 respectively per 50 ng of reverse transcribed total RNA. The values in the tumor samples are about ten times lower than the tissue culture cell lines for the same amount of reverse transcribed total RNA. The reasons for the lower values in tumor samples are not clear. This observation, however, validates the importance of normalizing the ER values to GAPDH in tumor samples instead of relying only on RNA concentration.

A typical quantitation assay for the ER wild type and exon 7Δ variant copy numbers using the reverse transcribed cDNA prepared from the T47D cell line is presented in Figures 4 and 5. The amount of competitor used in the PCR reactions ranged from 0.56 x 10^5 copies (Figure 4, lane 2) to 100 x 10^5 copies (Figure 4, lane 11). As seen in Figure 4, the ER wild type and exon 7Δ variant products (middle and lower bands respectively) from the T47D cell line decreased in the presence of increasing amounts of the competitor plasmid, pSG-ER-λ (upper band). To determine the exact copy numbers of ER wild type and the exon 7Δ variant, the densitometric scans of the radioactivity in the normal and the competitor PCR products were plotted against the number of copies of the competitor template in the PCR reaction. A representative graph for the ER wild type and exon 7Δ variant quantitation in the T47D cell line is shown in Figure 5. As seen in Figure 5, the wild type ER and competitor templates generated equal amounts of PCR products when the competitor concentration was at 20 X 10^5 copies. The exon 7Δ variant and the competitor products crossed over when the competitor concentration was 4.5 X 10^5 copies. Thus, the number of copies of ER wild type and exon 7Δ variant in T47D cDNA prepared from 250 ng of reverse transcribed total RNA were

20 X 10^5 and 4.5 X 10^5 respectively. The number of copies of the wild type and the exon 7Δ variant in MCF-7, ZR-75 and tumor tissues were determined by similar procedures. The number of copies of both the wild type and exon 7Δ variant in all the cell lines and tumor tissues studied were normalized to 10^5 copies of GAPDH. The results are presented in the Table. The numbers were found to be repeatable in multiple experiments. Our results indicate that the T47D cell line expresses the highest amounts of ER transcripts among the cell lines studied. The results presented in the Table also show that the ratios of the variant to wild type is highest in ZR-75 (0.36) and lowest in T47D (0.225) among the cell lines studied. The ratios of the exon 7Δ variant to wild type in the tumor tissues is considerably higher (0.69 and ~ 1 in the tumors 1 and 2 respectively) than in the cell lines studied. The significance of this higher variant to wild type ratio in tumor tissues is not known. It was previously shown that the exon 7Δ variant forms a heterodimer with the wild type protein and exerts a dominant negative effect due to constitutive DNA binding activity in the absence of the hormone. It was also suggested that the ratio of variant to wild type influences the response to anti-estrogen therapies (19). However it is not known whether the increase in the copy numbers of this variant is responsible for the genesis and progression of breast cancers.

The results presented in this report clearly establish the feasibility of determining the exact copy numbers of the ER wild type and variants in tumor tissues. Since the ER competitor we have prepared has the insert in exon 4, it can be used with any set of primer pairs which encompasses exon 4. The quantitation procedures described here will highly facilitate in establishing an index between the copy numbers of ER wild- and variant type

transcripts per a given number of copies of GAPDH transcripts in the tumors and response to anti-estrogen therapies.

ACKNOWLEDGEMENTS

This work was supported in part by grants from the National Cancer Institute (CA 71150), the Susan G. Komen breast cancer foundation (to I. P) and a grant from the Department of Defense (DAMD 17-94-J-4495) (to I. P and R. E. T). The technical assistance of Ms. Chung Ling Go and Dr. Zang Li is acknowledged. Dr. Robert Clark is gratefully acknowledged for his gifts of breast cancer cell lines.

ABBREVIATIONS USED

ER, Estrogen receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, Reverse Transcription Polymerase Chain Reaction. FBS, Fetal bovine serum; DEPC, Diethyl pyrocarbonate.

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FIGURE LEGENDS

Figure 1. Design of competitor plasmids for the quantitation of GAPDH and ER copy numbers. A. pBS-GAPDH-λ The approximate primer locations are indicated by arrows. A 210 bp λ DNA fragment was inserted into the *Nco*I site in exon 4 of the GAPDH cDNA gene. The resulting GAPDH competitor cDNA generated a 710 bp product by PCR amplification. B. pSG-ER-λ The approximate location of the primers used in exons 4 and 8 are indicated with arrows. A 366 bp λ DNA fragment was inserted at *the Hin*dIII site in exon 4 of the ER cDNA. The resulting ER competitor cDNA generated a 1201 bp product by PCR amplification.

Figure 2. Template competition assay for GAPDH quantitation in the T47D cell line. PCR amplification with GAPDH specific primers and a constant amount of cDNA prepared by reverse transcription of 50 ng of T47D total RNA was performed in the presence of varying amounts of the competitor, pBS-GAPDH- λ. The number of copies of the competitor ranged from 0.32 X 10^5 (lane 2) to 56.2 X 10^5 (lane 11) in half-log increments. The PCR amplifications were conducted under the conditions described in the materials and methods. The amplified products were electrophoresed in 4.5% acrylamide gels and detected by autoradiography.

Figure 3. Absolute quantitation of GAPDH copy numbers in the T47D cell line. The radioactivity in the PCR products of the competition assay was measured by densitometric scanning of the autoradiograms. The scanned units were plotted against the number of copies of the competitor, pBS-GAPDH- λ, in the PCR reaction. The cross over point is shown at which the GAPDH products generated from both the competitor and T47D

cDNA are equal. The number of copies of GAPDH transcripts are equal to the number of copies of the competitor at the cross over point.

Figure 4. Template competition assay for ER quantitation in the T47D cell line. PCR amplification with ER specific primers and a constant amount of cDNA prepared by reverse transcription of 250 ng of T47D total RNA, was performed in the presence of varying amounts of the competitor, pSG-ER- λ. The number of copies of the competitor ranged from 0.58 X 10^5 (lane 2) to 100 X 10^5 (lane 11) in quarter-log increments. PCR amplifications were conducted under the conditions described in the materials and methods. The amplified products were electrophoresed in 4.5% acrylamide gels and detected by autoradiography. Two ER products from cDNA derived from the T47D cell line corresponding to wild type and exon 7 Δ variant are seen and designated as Normal ER and Variant ER.

Figure 5. Absolute quantitation of ER copy numbers in the T47D cell line. The radioactivity in the PCR products of the competition assay was measured by densitometric scanning of the autoradiograms. The scanned units were plotted against the number of copies of the competitor, pSG-ER- λ, in the PCR reaction. The cross over points are shown at which the specific ER wild type and exon 7 Δ variant PCR products generated from T47D cDNA and competitor DNA are equal. The number of copies of ER wild type and variant transcripts are equal to the number of copies of the competitor at the respective cross over points.

FIGURES.

Figure 1

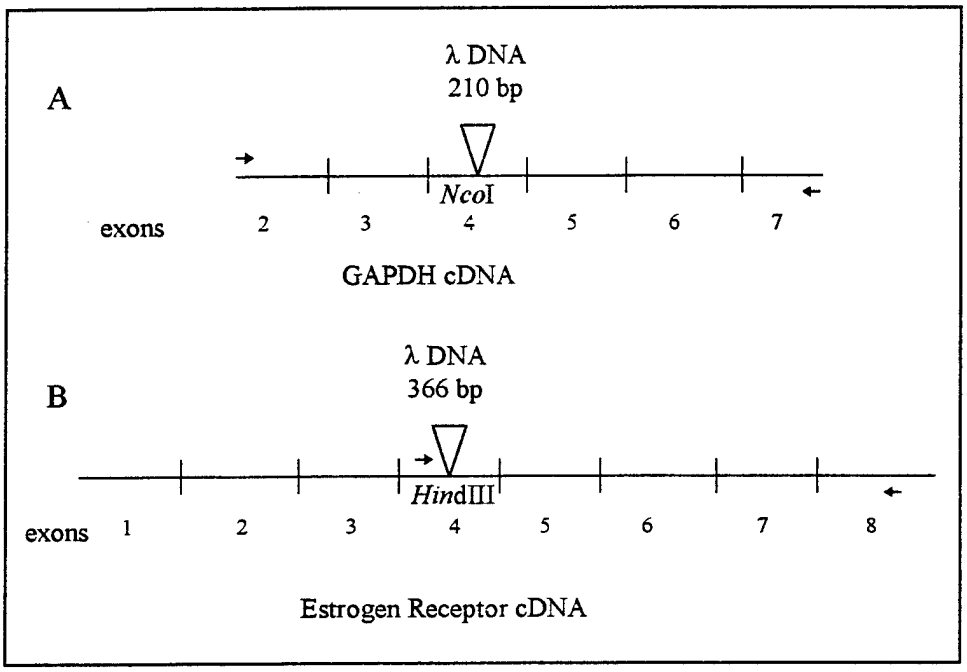


Figure 2

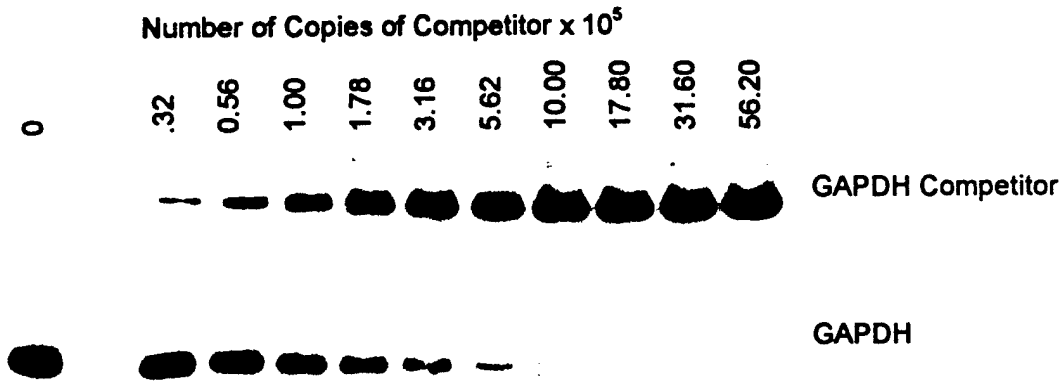

Number of Copies of Competitor x 10^5
0
.32
0.56
1.00
1.78
3.16
5.62
10.00
17.80
31.60
56.20
GAPDH Competitor
GAPDH

Figure 3

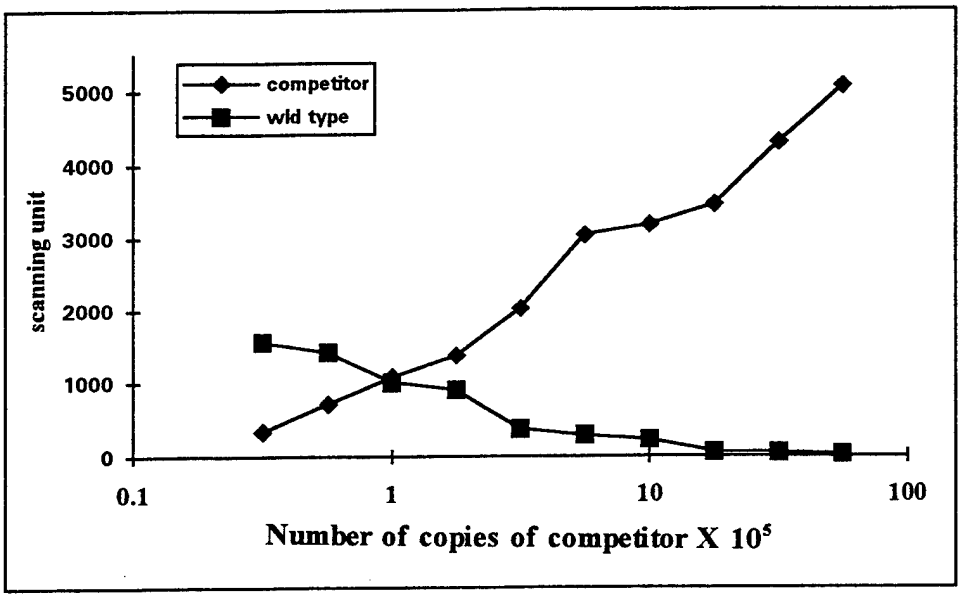

competitor
wld type
5000
4000
3000
2000
1000
0
scanning unit
0.1
1
10
100
Number of copies of competitor X 10⁵

Figure 4

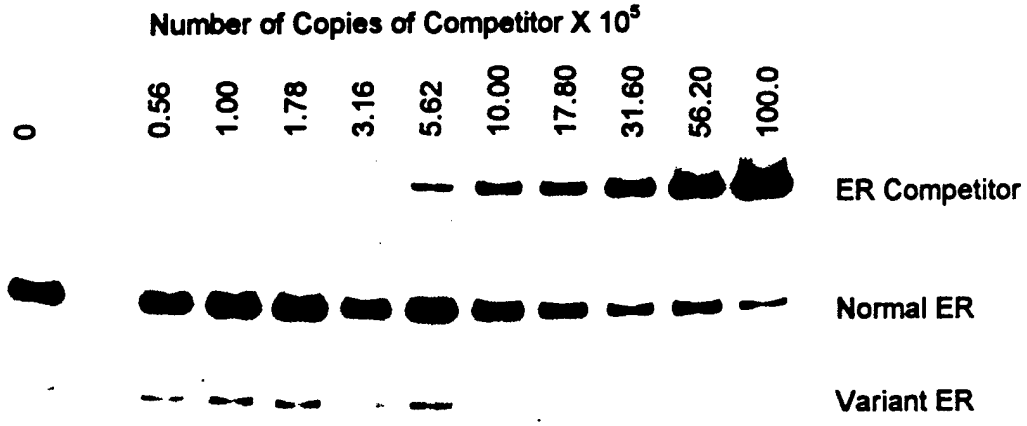
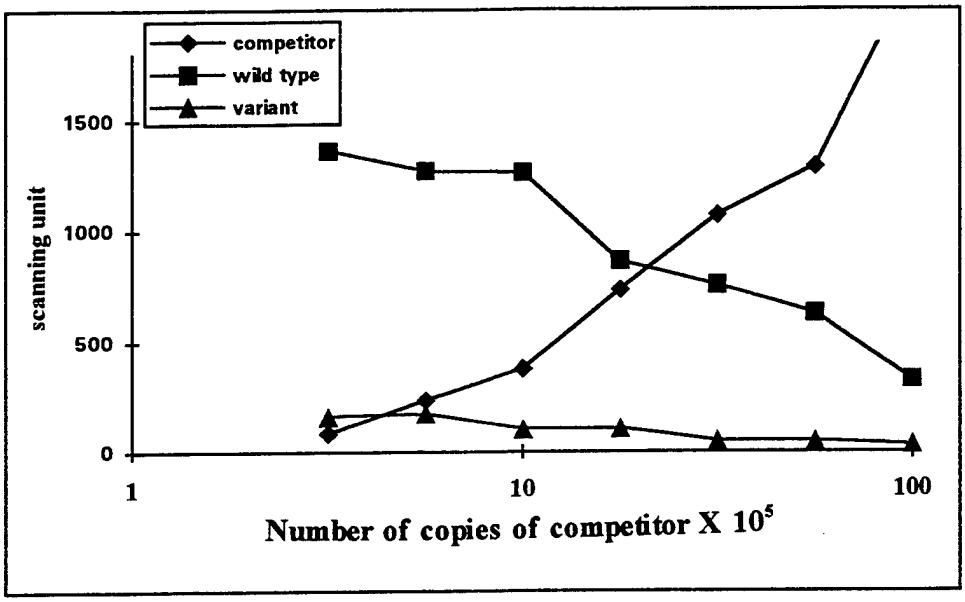

Number of Copies of Competitor X 10⁵
0
0.56
1.00
1.78
3.16
5.62
10.00
17.80
31.60
56.20
100.0
ER Competitor
Normal ER
Variant ER

Figure 5


competitor
wild type
variant
1500
1000
500
0
scanning unit
1
10
100
Number of copies of competitor X 10^5

TABLE

#	Cell Type/ tumor	Copies of ER/10^5 copies of GAPDH		Ratio of Exon 7Δ variant to wild type
		Wild type	Exon 7Δ variant	
1.	T47D	4.0×10^5	9.0×10^4	0.225
2.	MCF-7	3.6×10^4	1.1×10^4	0.3
3.	ZR-75	1.1×10^4	4.0×10^3	0.36
4.	Tumor 1	2.3×10^4	1.6×10^4	0.69
5.	Tumor 2	$<6.0 \times 10^3$	$<6.0 \times 10^3$	~1.00

PROFILES OF EXON- DELETION AND TRUNCATED VARIANTS OF ESTROGEN RECEPTOR IN BREAST TUMORS OF AFRICAN AMERICAN WOMEN

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Key words: Estrogen Receptor, Exon deletions and truncations, Variants, Breast tumors and African American women

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Running Title: Estrogen receptor variants in breast tumors of African American women

ABSTRACT

Several recent reports have shown that mortality rate with breast cancer is about three times as high in African American women as other populations. In addition, the available data also indicate that the tumors are very aggressive, poorly differentiated with very low frequency of hormone receptors. To gain an insight into the factors which may be responsible for poorly differentiated tumors, we investigated the profiles of estrogen receptor (ER), the most important prognostic factor in breast cancer. We analyzed 15 ER positive and 6 ER negative malignant tumors for wild type and variant receptor mRNAs by RT PCR. Our results showed that only 33% of the tumors had full length wild type receptors, 28% of them had truncations in both exons 1 and 8, and 10 had truncations in exon 8 only. We also found that the most predominantly expressed variants are the exon 2 and exons 2-3 deleted molecules. We also detected exon 5 deletion variant and exon 7 deletion variant mRNAs in 80% of the tumors. Our results also indicate that the ratios of variants to wild types are significantly high in all the tumors studied.

INTRODUCTION

Recent reports indicate that the experience with breast cancer varies in different populations. The incidence of breast cancer is reported to be slightly lower in African American women compared to other women (1). However, the incidence seems to differ in different age groups. It is higher in young and lower in middle aged and older African American women compared to women of similar age groups in other populations (2). Trends in survival rate with the disease also seem to differ both by age and race. It was reported that death rate for African American women under the age 65 has increased while for women in similar age group in other population has declined. Death rates in women age over 65 has increased for all the women and the increase seems to be three times as high in African American women as others. Several reports indicate that the high mortality rate is not due to differences in socioeconomic status, stage of diagnosis, known risk factors or methods of treatment (3). It has been hypothesized that breast tumors across racial and ethnic groups could have different biologic characteristics which may account for survival disparities (4).

There appears to be differences also in tumor biology in different racial groups. Histochemical studies of the tumors derived from African American women have found a higher incidence of poorly differentiated tumors and an increased frequency of nuclear atypia, higher mitotic activity, and tumor necrosis (5). Immunohistochemical studies have shown that the frequency of estrogen- and progesterone receptor expression, which are generally associated among well or moderately differentiated tumors, is significantly lower in the tumors of African American women (6). The presence of these two receptors is generally

considered a good prognosis for longer survival and lower risk of tumor recurrence. A number of studies on the estrogen receptor mRNA expression have shown that it undergoes alternate splicing resulting in several alternatively spliced species in addition to wild type. The presence of both wild type and alternatively spliced exon deleted variant mRNAs were reported in normal and tumor tissues (7). It has been suggested that the relative ratios of certain variants to the wild type are significantly changed in tumors compared to normal tissues (8). Interestingly, preferential detection of some deleted variants was found to be associated with known prognostic markers in breast cancer. For example, deletions in exons 3-7 were associated with higher grade tumors (9).

To understand whether the poorly differentiated tumors observed in African American women are associated with the expression of certain variant ER molecules, we investigated ER mRNA expression by RT PCR using a number of primer pairs. Our results described here show that only a fraction of the tumors which were diagnosed as ER positive by immunohistochemistry had wild type ER mRNAs, a majority of them had truncations in exon 1 and 8 and the predominantly expressed deletion variants were exon 2-, and exons 2-3 D molecules.

MATERIALS AND METHODS

AmpliTaq PCR core kits and QIAquick gel extraction kits were from QIAGEN Inc., Santa Clara, CA. Reverse transcriptase kits were from Applied Biosystems. [a -^{32}P]dCTP (Specific Activity 3000Ci/mMole, Cat# AA0005) was from Amersham. The primers for

amplifying ER and glyceraldehyde-3 phosphate dehydrogenase (GAPDH) were synthesized at Gibco-BRL Life Technologies. Trizol reagent for total RNA isolation was purchased from Gibco-BRL Life Technologies. Diethyl Pyrocarbonate (DEPC) treated water was from Research Genetics, Al. PCR quality water and Tris-EDTA buffer were from Biofluids, Rockville, Md.

Breast tumor samples. The breast tumor samples were collected from Howard University hospital. All the tumors used in this study were derived exclusively from African American women. They were collected immediately after the surgery and zap frozen in liquid nitrogen. The ER status of the tumors was obtained from the Tumor Registry, Howard University Hospital. It was determined for Howard University hospital by Oncotech laboratories.

RNA isolation. Total RNA from breast tumor samples was isolated using Trizol reagent and the manufacturer's protocol. Briefly, the tumor tissue was first powdered by pulverizing in a sterile pestle and mortar in the presence of liquid nitrogen. The tumor powder (50-100 mg) was suspended in 1 ml of Trizol reagent and homogenized with a hand held homogenizer and incubated at room temperature for 5 min to permit the dissociation of nucleoprotein complexes. Then, 0.2 ml of chloroform was added to the above, shaken vigorously and centrifuged at 4°C for 15 min at 12,000 g. Total RNA was precipitated from the supernatant by adding 0.5 ml of isopropanol. The precipitate was washed twice with 75% ethanol, dried briefly and dissolved in DEPC treated water. This method yielded about 30 mg

of total RNA per 100 mg of tumor tissue. The integrity of the isolated RNA was verified by 1.5% agarose gel electrophoresis in Tris- acetate EDTA buffer and the concentration was determined by measuring the optical density at 260 nm.

Reverse Transcription. The isolated RNA was reverse transcribed to cDNA using Maloney Murine Leukemia Virus (MuLV) reverse transcriptase and random hexamers. Briefly, the standard reaction mixture contained 1 mg of total RNA, 2.5 units of MuLV reverse transcriptase, 1 mM each of dNTPs, 2.5 mM random hexamers, 1 U of RNAse inhibitor, 5 mM $MgCl_2$ and 1 X PCR buffer in a total volume of 20 ml. To reverse transcribe the RNA, the reaction tubes were first left at room temperature for 10 min, followed by incubations at 42^0 C for 15 min, 99^0 C for 5 min and finally 5^0 C for 5 min.

PCR. The Polymerase Chain Reaction was performed in an automatic thermal cycler (MJ Research). For ER cDNA amplification, various primer sequences located in exons 1, 4, 6, 7 and 8 (10) as listed in the Table were used. The positions of the primers are also shown schematically in Figure 1. The amplification was carried out in a 12.5 ml reaction volume containing the reverse transcribed cDNA, 1 X PCR buffer, 1 X Q solution (Qiagen), 200 mM each of dNTPs, 2 mM each of sense and anti-sense primers and 0.6 U of Taq polymerase. The PCR conditions for amplifying ER mRNA were as described before (11). The cDNA prepared from tumor samples were first verified for the presence of constitutively expressed gene, GAPDH. The GAPDH cDNA was amplified using a sense primer 5' AAGGCTGAGAACGGGAAGCTTGTCATCAAT 3' (exon 3, bp 241-270) and an anti-sense

primer 5' TTCCCGTCTAGCTCAGGGATGACCTTGCCC3' (exon 7, bp 740 -711) (12) under the same PCR conditions as for ER. All the primer pairs were first tested on cDNA prepared from MCF-7 RNA and included as a control in all the PCR amplifications.

Detection of PCR products. The PCRs were conducted in the presence of [a -^{32}P]dCTP at 0.5% of the total reaction volume. The radiolabelled PCR products were electrophoresed in a Bio-Rad vertical slab gel apparatus in 4.5% acrylamide gels, 90 mM Tris-Borate and 0.2 mM EDTA at a 40 mA constant current for 3.5 hrs. The gels were dried in a Savant gel dryer and autoradiographed using Kodak X-Omat AR film and DuPont NEN Lightning Plus intensifying screens at room temperature. The radioactivity in the individual PCR products was quantitated by scaning the autoradiograms in a laser densitometer (Molecular Dynamics).

Sequence analysis. In order to confirm the identity of the PCR amplified ER products, they were electrophoresed in agarose gels and purified using the QIAquick gel extraction kit and sequenced by cycle sequencing method (13, 14) on an automated DNA sequencer.

RESULTS AND DISCUSSION

Several reports on breast cancer in different racial groups have shown that African American women bear poorly differentiated, aggressive tumors and experience higher death rates compared to other populations. Recent studies also indicate that the frequency of ER

expession in the tumors found in African American women is significantly lower. It has been hypothesized that breast tumors across racial and ethnic groups could have different biologic characteristics which may account for survival disparities. However, such factors have not been identified thus far.

To get insights into the factors which may contribute for the above, we investigated the pattern and levels of ER variant expression. We thought that the variations in the ER structure may contribute, in part, for the poorly differentiated tumors. The ER mRNA profiles were studied by RT PCR in the tumors which were diagnosed for ER status by Oncotech Laboratories. We employed primer pairs which could amplify the exons 1-8, 1-4, 4-6 and 4-8. When primers ER1S and ER1A (Table 1) which amplify exons 1-8 were used, we detected expected PCR product of size 1.75 kb only in 33% of the tumors (data not shown). It was unexpected that only a small percentage of ER positive tumors gave wild type mRNAs. We rationalized that ER mRNAs in other tumors are probably altered in the primer annealing regions in either exon 1 or 8 or both. To investigate this possibility, those tumors which did not amplify with ER1S and ER1A were tested with primer pairs which can amplify exons 1-4 (ER1S and ER2A) and 4-8 (ER2S and ER1A). We rationalized that those which have truncations in exon 8 will amplify for exons 1-4 and those which have alterations in exon 1 will amplify at least for exons 4-8. We could amplify an expected PCR product (size ~1 kb) (Figure 2, lane 2, upper band) with ER1S and ER2A but could not amplify any product with ER2S and ER1A in 19% of the tumors, suggesting that they possibly have alterations/ truncations in exon 8. An additional product of size 720 bp was also detected with ER1S and

ER2A which by sequence analysis was identified as exon 2-3 deletion variant (Figure 2, lane 2, lower band). The rest of the tumors were tested for possible alterations in both exons 1 and 8. For this we designed a sense primer (ER3S) down stream of ER1S and an antisense primer (ER3A) upstream of ER1A. When we used ER3S and ER2A, 28% of the tumors gave an expected PCR product of size about 630 bp (Figure 2, lane 4, upper band) suggesting that they have alterations/truncations upstream of the primer ER3S in exon 1. The primer pair ER3S and ER2A also generated two additional PCR products of sizes 440 bp, and 330 bp (Figure 2, lane 4 middle and lower bands respectively) which by sequence analysis were identified as exon 2, and exon 2-3 deletion variants respectively. These products were either minor or not visible with primer pairs ER1S and ER1A or ER1S and ER2A. It is probably because of the competion of the more abundant templates for the minor templates. Similarly, primer pair ER2S and ER3A gave an expected PCR product of size 721 bp and an additional product of size 521 bp (Figure 2, lane 6, upper and lower bands respectively) in 50% of those which have alterations/truncations in exon 1, suggesting that they also have truncations downstream of primer ER3A. The 521 bp product was identified as exon 7 deletion variant. The remaining 50% of the tumros which have alterations/truncations in exon 1 were tested with primers ER2S and an antisense primer in exon 7 (ER4A). All of them gave an expected PCR product of size 570 and an additional product of size 430 bp which by sequence analysis was identified as exon 5 deletion variant (Figure 2, lane 8), suggesting that in these tumors exon 8 was completely deleted. The exon 5 deletion variant was not detected with primers ER2S and ER1A or ER2S and ER3A. The remaining 19% of the tumors were negative for ER mRNAs with all the primers tested. We tested the negative tumors for the presence of

exons 4-6 sequences since they were reported to be present in the ER negative tumors using the primers ER4S and ER5A. We found that 50% of the negative primers showed the presence of exon 5 deletion sequences in addition to expected wild type exons 4-6 sequences (Figure 2, lane 10, lower and upper bands respectively).

Since the exon 2 and exons 2-3 deleted products could be detected with the ER3S and ER2A primer pair, it was used to screen all the tumors for the presence of these deletion variants. We detected the exon 2 deletion variant in 62% of the tumros and exons 2-3 deletion variant in 57% of the tumors. The exon 7 deletion variant and exon 5 deletion variant which are also found in the normal human mammary tissues were detected in 62% of the tumors. The results described above are summarized in Table 2. Interestingly, as seen in the Table, two out of six ER negative tumors tested had full length receptor mRNAs.

The results presented in this report clearly show that only a third of the tumors tested have wild type mRNA and a significant number of tumors contain mRNAs which have truncations in either exon 8 or in both exons 1 and 8. The occurance of exons 2-3 deletion variant in both normal and cancer tissues has been reported by Leygue and others (9, 15). However, the frequency of this multiple deletion variant is reported to be only ..% of tumors. No data is available with regard to potential function of this protein in human breast cells. Exon 2 deletion variant has been reportedA possible correlation between the presence of truncations/deletions in exon 1 and 8 observed in African American women to poorly differentiated tumors needs to be evaluated. It is possible that the lower frequency of ER

positive tumors reported in African American women is due to the absence of wild type receptors and the presence of altered receptors which are not reactive with the antibodies used in immunohistochemical detection in the clinical laboratories.

ACKNOWLEDGEMENTS

This work was supported in part by grants from the Susan G. Komen breast cancer foundation and the Department of Defence (DAMD 17-94-J-4495) awarded to I.P. The authors are thankful to Drs, Robert DeWitty, Lassale Laffale, E.B Chung, and Robert E. Taylor for their help in collection of fresh tumors. Thanks are also to Dr. Vivian Pinn, director of NIWH, NIH for her support and encouragement. Mrs. Arubala Reddy and Ms. Tamica Cooper are acknowledged for their assistance in tumor collection.

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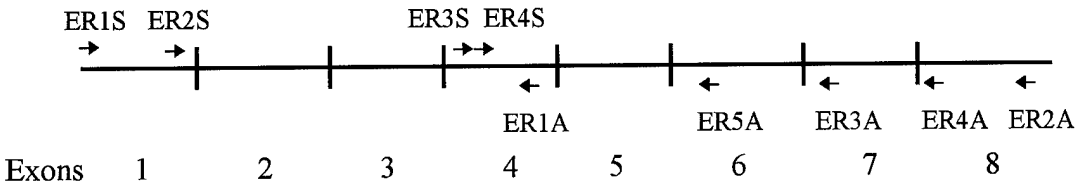
FIGURE !



Estrogen Receptor cDNA

FIGURE 2

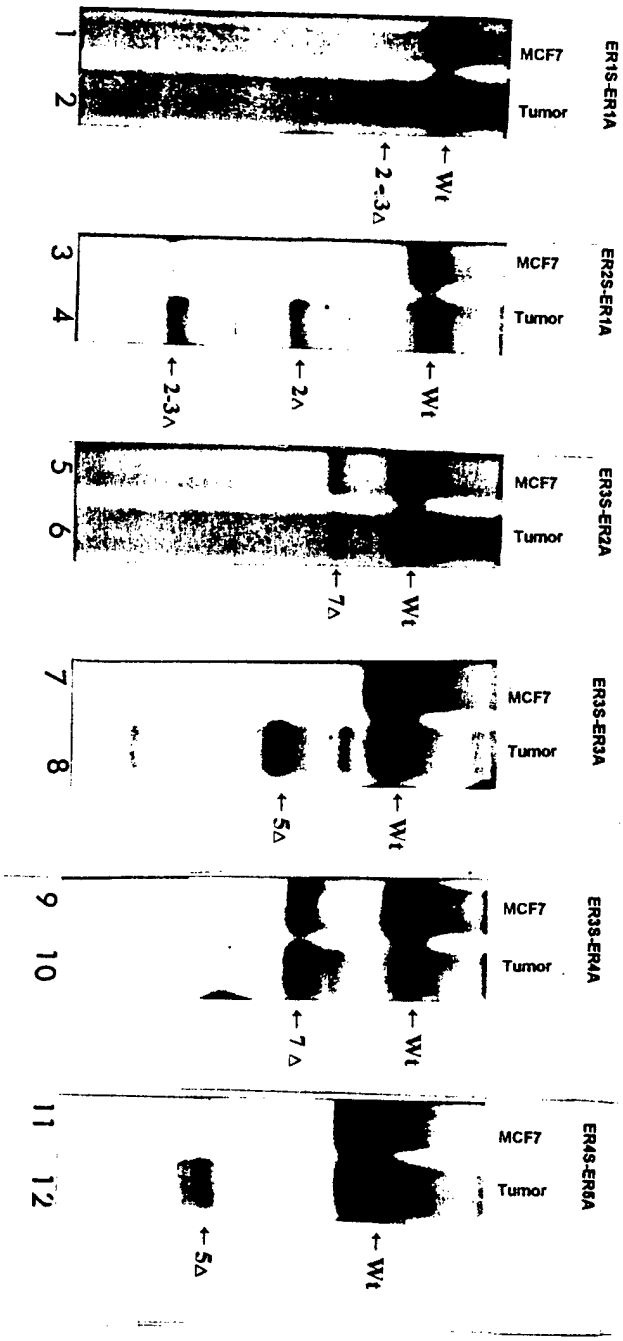

1
2
MCF7
Tumor
ER1S-ER1A
← 2 -3Δ
← Wt
3
4
MCF7
Tumor
ER2S-ER1A
← 2-3Δ
← 2Δ
← Wt
5
6
MCF7
Tumor
ER3S-ER2A
← 7Δ
← Wt
7
8
MCF7
Tumor
ER3S-ER3A
← 5Δ
← Wt
9
10
MCF7
Tumor
ER3S-ER4A
← 7Δ
← Wt
11
12
MCF7
Tumor
ER4S-ER6A
← 5Δ
← Wt

TABLE 1. ER PRIMER SEQUENCES

Primer pairs used	**Location** (10)
1. ER1S 5' ATGACCATGACCCTCCACACC 3'	Exon 1 Position, 233-254
2. ER1A 5' ATACTTTTGCAAGGAATGCGA 3'	Exon 8 Position, 1978-1958
3. ER1S 5' ATGACCATGACCCTCCACACC 3'	Exon 1 Position, 233-254
4. ER2A 5' GGTCAGTAAGCCCATCATCGAAGCTT 3'	Exon 4 Position, 1253-1228
5. ER2S 5'GCCCGCTCATGATCAAACGC 3'	Exon 4 Position, 1081-1101
6. ER1A 5' ATACTTTTGCAAGGAATGCGA 3'	Exon 8 Position, 1978-1958
7. ER3S 5' TGCCCTACTACCTGGAGAACG 3'	Exon 1 Position, 615-635
8. ER2A 5'GGTCAGTAAGCCCATCATCGAAGCTT 3'	Exon 4 Position, 1253-1228
9. ER2S 5'GCCCGCTCATGATCAAACGC 3'	Exon 4 Position, 1081-1101
10.ER3A 5' GCACTTCATGCTGTACAGATGC 3'	Exon 8 Position, 1822-1801
11.ER2S 5'GCCCGCTCATGATCAAACGC 3'	Exon 4 Position, 1081-1101
12. ER4A 5'GTCCTTCTCTTCCAGAGAC 3'	Exon 7 Position, 1652-1634
13. ER4S 5'GCCCGCTCATGATCAAACGC 3'	Exon 4 Position, 1111-1130
14. ER5A 5' CCAGCAGCATGTCGAAGATC 3'	Exon 6 Position, 1519-1501

TABLE 2. PROFILES OF ER VARIANTS IN THE TUMORS STUDIED

	Full length		Deletions			Truncations	
Tumor #	Wt	7Δ	5Δ	2Δ	2-3Δ	Exon 1	Exon 8
			(ER positive tumors)				
1.	-	+	+	-	+	+	+
2.	-	-	-	-	+	+	+
3.	+	+	+	+	+	-	-
4.	+	+	+	+	+	-	-
5.	+	+	+	+	+	-	-
6.	-	+	+	-	-	+	+
7.	-	+	+	+	+	+	+
8.	-	+	+	+	+	+	+
9.	+	+	+	+	+	-	-
10.	-	-	-	-	-	-	-
11.	-	+	+	+	+	-	+
12.	-	+	-	-	+	-	+
13.	-	+	+	-	-	+	+
14.	+	+	+	+	+	-	-
15.	-	+	+	+	+	-	+
			(ER negative tumors)				
16.	-	-	-	+	-	-	+
17.	-	-	-	+	-	-	-
18.	-	-	+	+	-	-	-
19.	+	-	-	-	-	-	-
20.	+	-	-	+	-	-	-
21.	-	-	-	-	-	-	-