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GRANT REPORT

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.

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. 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.

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. The presence of estrogen receptor is the basis for anti-estrogen therapies to treat breast cancer and its presence also indicates good prognosis, longer survival and low recurrence rates. Because of these therapeutic and prognostic reasons, estrogen receptor is detected and quantitated in tumors. Currently its detection and quantification are done using immunohistochemistry procedures in clinical laboratories. However, these procedures require a large amount of sample and cannot detect the changes in the receptor structure. Furthermore, due to increased awareness and improved methods of detection, smaller and smaller tumors are detected in recent times which restricts the amount of tumor tissue available for the immunohistochemisty. This led to a proposal that molecular biological methods may be preferred since they require very small samples. In addition, several prognostic factors could be detected at the same time by these procedures.

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.

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

We have developed the state-of-the art molecular methods for the detection and quantification of estrogen receptor in breast tumors. In particular we have developed molecular procedures to

1) detect and quantify wild type estrogen receptor mRNA, 2) analyzed the alterations in the estrogen receptor mRNA in the breast tumors of African American women 3) devised novel approaches for detecting the estrogen receptor mRNAs which have deletions in one or more exons 4) analyzed several breast cancer cell lines and tumors for the single- and multiple exon deletion transcripts of estrogen receptor and 5) developing methods for the quantification of estrogen receptor exon-deletion variant mRNAs in breast cancer cell lines and tumors. These methodologies are described briefly here.

1 Development of molecular methods to quantitate estrogen receptor mRNA copy numbers. We have devised a method to quantify the mRNA copy numbers of estrogen

receptor by reverse transcription polymerase chain reaction (RT PCR) template competition method. We were the first to develop the quantitative molecular method for the quantification of the receptor mRNA in tumor samples. The procedure is described in detail in the accompanying publication.

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

2. Studying the estrogen receptor profiles in the breast tumors of African American women. After developing the quantification methods for the ER wild type mRNA, we wanted to apply these methods for the quantification of ER in tumor samples. Therefore, we started collecting the tumor samples from Howard university hospital where a large number of breast tumor biopsies are performed. Since the majority of biopsies performed at Howard University hospital are on African American women, it gave us an opportunity to look at any alterations in the ER in these samples. Several reports indicate that the incidence of breast cancer in young African American women is higher and lower in older women compared to Caucasian women. However, the mortality rate with breast cancer is three times as high in African American women compared to women in other populations. The high mortality rate does not appear to be due to differences in socioeconomic status, stage of diagnosis, age, known risk factors or treatment. It has been hypothesized that breast tumors across racial and ethnic groups could have different biologic characters which may account for survival disparities. However, such factors are not identified thus far. There also appears to be differences in tumor biology. The breast tumors from African American women in general are poorly differentiated with lower frequency of the steroid hormone receptors, the estrogen- and progesterone receptor, which are generally associated with well or moderately differentiated tumors and their presence indicate good prognosis. It is not known whether this aggressive tumor phenotype contribute to high mortality with breast cancer. All these studies indicate that the mortality rate is related to biological factors. Preventive and therapeutic strategies can be devised if those factors are identified in the tumors of African American women. Retrospective studies conducted on tumor tissues could be highly valuable in identifying those factors.

To understand the molecular factors which may be responsible for the poorly differentiated aggressive tumors in African American women, we studied the most important prognostic factor, the estrogen receptor in the tumor tissues. In particular, we investigated the alteration in the expression of estrogen receptor gene in the freshly collected breast tumors. Briefly, we studied the estrogen receptor mRNA in 15 immunohistochemically estrogen receptor positive and 5 immunohistochemically negative tumors. We also included five positive tumors from Caucasian women patients. We studied the receptor transcripts by reverse transcription polymerase chain reaction (RT PCR) using a number of primer pairs. Our results indicated that only a third of the tumors have full length receptor transcripts and the majority of tumors had at least three types of modifications. They are 1) truncations in exon 8, which encodes for part of the hormone binding region, 2) base insertions and 3)

absence of naturally occurring exon-7 deletion variant. The significance of these finding and their role in the aggressive tumor types are not clear at this point.

Our results are described in detail in the accompanying manuscript which has been submitted for publication.

3. A novel primer design strategies for the targeted amplification of alternatively spliced sequences by PCR. Estrogen receptor occurs both as unmodified (wild type) and several spliced variants (isoforms) which have the deletions in one or more exons. Several studies have shown that the estrogen receptor isoforms play important functional roles in the estrogen induced signal transduction processes under normal physiological conditions. Recent reports also indicate that the isoforms of estrogen receptor pattern and levels are altered in breast tumors and their content influence the response to anti-estrogen therapies. Because of the importance of isoforms, it is important to develop methods which can precisely detect and quantify their amounts.The currently available methods can only detect the most abundant forms and cannot detect the mRNAs of the low abundance. Therefore, we have developed new strategies to detect even the least abundance isoform by PCR using targeted primers. In particular we designed, tested and identified targeted primers for exon-7 deletion variant, exon-5 deletion variant, exon 2- deletion variant and exons 2-3 deletion variants. The novel primer design strategies are described in detail in the accompanying manuscript.

These results are described in detail in the accompanying manuscript which has been submitted for publication.

4. Analysis of single- and multiple exon deletion variant transcripts of estrogen receptor in breast cancer cell lines and tumors using targeted primers. After developing the new primer design strategies for specific amplification of the mRNAs of isoforms, we used those targeted primers to analyze the variant patterns in breast cancer cell lines and tumor samples. We employed seven breast cancer cell lines, MCF-7, T47D, ZR-75,LCC1, LCC2, LCC9 and MDA-MB-435 and three tumors to test the applicability of the targeted primers. Our results showed that the targeted primers not only amplified the single deletion transcripts, but also multiple deletion isoforms. These transcripts were not detected and characterized until now because of the unavailability of the sensitive methodologies until now. The different cell lines did not differ in the single deletion transcripts. However, they differed in the presence and content of multiple deletion transcripts. Interestingly, we observed only the multiple deletion variant transcripts in the tumor samples.

A manuscript describing this work has been submitted.

5. Quantification of estrogen receptor isoform mRNAs. After establishing the applicability of variant targeted primers for specific amplification of variant transcripts, we decided to apply them in quantification of their transcripts by template competitive PCR

similar to the quantification of wild type estrogen receptor transcripts. We are currently in the final stages of this work and plan to send a manuscript for publication in very near future.

D. CONCLUSIONS. A highly sensitive molecular biological approaches have been developed to detect and quantitate the exact copy numbers of wild type and various single and multiple exon deletion forms 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.

D. ABSTRACTS OR PUBLICATIONS PRODUCED AS A RESULT OF THE GRANT AWARD. The grant award from the Department of Defence has provided support in part for generating the following publications/manuscripts.

1. Title: Quantitation of estrogen receptor mRNA copy numbers in the breast cancer cell lines and tumors.
Authors. **I. Poola,** D.M. Williams, S. Koduri, J. Ramprakash, R.E. Taylor, and W. D. Hankins,
Journal. **Analytical Biochemistry**, 258, 209-215, 1998.

2. Title: Alterations in the estrogen receptor mRNA in the breast tumors of African American women.
Authors: S. Koduri, S. A. W. Fuqua and **I. Poola**
Journal: Revised manuscript **Submitted**

3. Title: A novel approach for the characterization of alternatively spliced estrogen receptors.
Authors. D. M. Williams, S. Koduri, Li, Z, **I. Poola** and W. D. Hankins
Journal. **Submitted**

4. Title. Analysis of single- and multiple exon deletion transcripts of estrogen receptor in breast cancer cell lines and tumors using targeted primers.
Authors. **I. Poola**, S. Chatra, S. Koduri and R. Clark
Journal. Manuscript submitted

5. Title. Quantification of estrogen receptor variant transcripts in breast cancer cell lines and tumors.
Authors. S. Koduri, R. Clark and **I. Poola**
Journal. Manuscript **in preparation.**

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Quantitation of Estrogen Receptor mRNA Copy Numbers in Breast Cancer Cell Lines and Tumors

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Several clinical studies have suggested that the content of estrogen receptor (ER) in breast tumors influences the survival, tumor recurrence, and response to antiestrogen 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 semiquantitation 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 mRNA 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 mRNA copy numbers from various samples, the mRNA 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 7 deletion variant to wild type in the tumor tissues are significantly higher than in the cell lines studied. © 1998 Academic Press

The human estrogen receptor (ER[1]) is a member of the superfamily of nuclear steroid receptors. 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. Domain C, which spans exons 2 and 3, binds DNA and domain E, which spans exons 4–8, contains the hormone binding site (1, 2). The presence of ER in breast tumor cells is considered a good prognosis and the patients who express estrogen receptors in their tumors have an overall longer survival and lower risk of tumor recurrence (3). In addition to its prognostic value, the presence of ER is exploited to treat tumors with antiestrogen therapy (4). Clinical studies have indicated that approximately 50% of patients with ER content >1000 pM/mg protein are found to respond favorably to antiestrogen therapy. Currently, the ER status in breast tumor tissues is determined from rough estimates yielded by microscopically scoring slides subjected to immunohistochemistry techniques. In recent years, due to increased awareness and periodic screening procedures, tumors of small sizes 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 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 quantita-

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[1] Abbreviations used: ER, estrogen receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, reverse-transcription polymerase chain reaction.

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tion 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 mRNA.

In this report, we describe a significantly improved PCR approach with which one can quantitate the exact number of ER mRNA copy numbers of wild-type as well as variant 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, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The significance of our PCR template competition assay, therefore, is twofold: (i) survival prognosis and hormone therapy response may be more readily predictable and (ii) the relationship of ER variants to breast cancer growth can be assessed.

MATERIALS AND METHODS

RNeasy midi kits, Ampli*Taq* 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 (sp act 3000 Ci/mmol, Cat. No. AA0005) was from Amersham. A plasmid containing the full-length ER cDNA gene pIC-ER-F was obtained from ATCC. Trizol reagent for total RNA isolation was from Life Technologies. 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. 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 and Reverse Transcription

Total RNA from breast cancer cell lines was isolated using RNeasy midi kits. This method yielded about 0.8–1.0 mg of total RNA per 10^7 tissue culture cells. Total RNA from powdered breast tumor samples was isolated using Trizol reagent. This method yielded about 30 μg of total RNA per 100 mg of tumor tissue. The isolated RNA was reverse transcribed to cDNA using Moloney murine leukemia virus reverse transcriptase and random hexamers.

PCR

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 antisense primer 5′-TACTTTTGCAAGGAATGCGA-3′ (exon 8, bp 1978–1958) (8). The amplification was carried out in a 12.5-μl reaction volume containing the reverse-transcribed cDNA, 1× PCR buffer, 1× Q solution (Qiagen), 200 μM each of dNTPs, 2 μM each of sense and antisense primers, and 0.6 U of *Taq* polymerase. The PCR conditions were initial denaturation for 5 min at 95°C followed by 94°C for 1 min, annealing for 1 min at 55°C, and extension for 2 min at 72°C for 40 cycles and final extension for 10 min at 72°C. The GAPDH cDNA was amplified using a sense primer, 5′-AAGGCTGAGAACGGGAAGCTTGTCATCAAT-3′ (exon 3, bp 241–270), and an antisense primer, 5′-TTCCCGTCTAGCTCAGGGATGACCTTGCCC-3′ (exon 7, bp 740–711) (9, 10), 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. 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.

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 antisense primers were 5′-CCCGAATTCAAGGCTGAGAACGGGAAGCTT-3′ (exon 3, bp 241–261) and 5′-CCCGGATCCTTCCCGTCTAGCTCAGGGATG-3′ (exon 7, bp 740–720), respectively (10). 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 to 2130) (12) by PCR using primers which had attached *Nco*I restriction enzyme sites to both ends. The sense and antisense primers were 5′-CCCCCATGGCTGGACCGCTACGAAATGCGC-3′ (bp 1920–1940) and 5′-CCCCCATGGCGTTCAACAATGGTCGGG-3′ (bp 2130–2110), re-

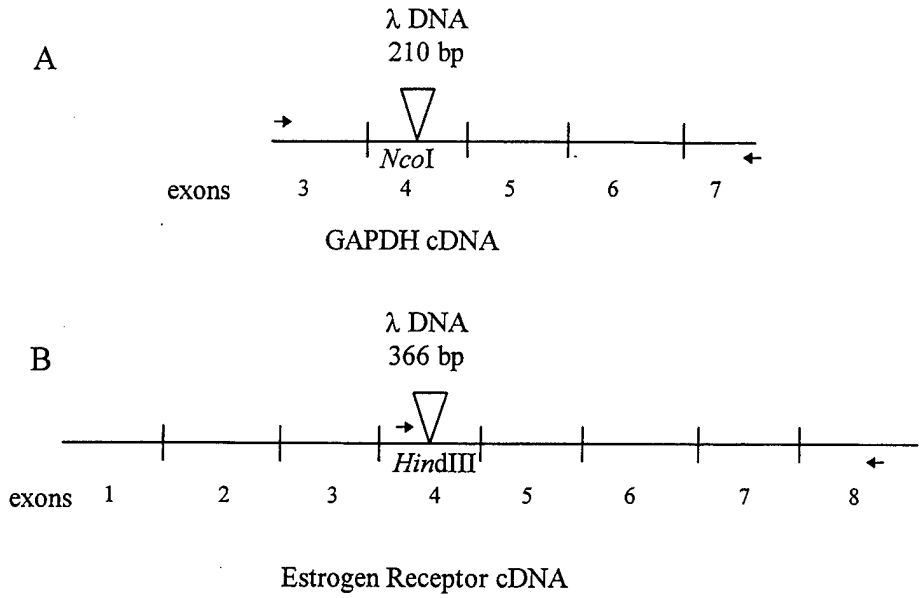

FIG. 1. Design of competitor plasmids for the quantitation of GAPDH and ER copy numbers. (A) pBS-GAPDH-λ. The approximate primer locations are indicated by arrows. A 210-bp λ DNA fragment was inserted into the *Nco*I site in exon 4 of the GAPDH cDNA gene. The resulting GAPDH competitor cDNA generated a 710-bp product by PCR amplification. (B) pSG-ER-λ. The approximate locations of the primers used in exons 4 and 8 are indicated with arrows. A 366-bp λ DNA fragment was inserted at *the Hind*III site in exon 4 of the ER cDNA. The resulting ER competitor cDNA generated a 1233-bp product by PCR amplification.

spectively. 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 Fig. 1A. When pBS-GAPDH-λ was used in a PCR, 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 *Hind*III 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 *Hind*III 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′-GGGAAGCTTAAACCATTCT-TCATAATTCAA-3′ (bp 37101–37121) containing a 5′ *Hind*III linker and the antisense primer 5′-CGCACCAA-CAGGCTCCAAGCC-3′ (bp 37465–37485) which flanked a native *Hind*III site in λ (12). This fragment generated a 366-bp DNA when digested with *Hind*III. The 366-bp fragment generated above was inserted into the *Hind*III site in exon 4 of ER. The competitor thus generated is termed pSG-ER-λ. The schematic representation of the design of the competitor for ER is shown in Fig. 1B. As shown in the figure, the primers used for amplification of ER flank the unique *Hind*III site within exon 4. When this cDNA was used as a competitor template in a PCR 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 numbers 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 and then dividing Avagadro's number by that value as in the following example for a 3500-bp DNA molecule: $(6.023 \times 10^{23}$ molecules/mol)/3500 bp × 660 g/mol/bp = 2.61×10^{17} molecules/g = 2.61×10^{11} molecules/μg. For these experiments the number of copies of ER cDNA and GAPDH cDNA was kept constant at 10^7 while the concentrations of the competitor DNAs ranged from 10^4 to 10^8 copies. 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 as expected or within the range of 1.5–2 times (data not shown), which agrees with the previously

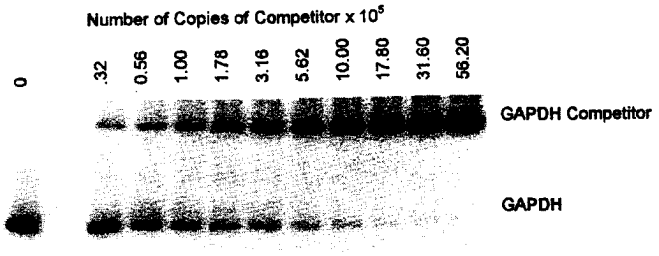

FIG. 2. Template competition assay for GAPDH quantitation in the T47D cell line. PCR amplification with GAPDH specific primers and a constant amount of cDNA prepared by reverse transcription of 50 ng of T47D total RNA was performed in the presence of varying amounts of the competitor pBS-GAPDH-λ. The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

published reports on the template competition method (11). After verifying the feasibility of the 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 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 quantitation of the PCR products, [α-^{32}P]dCTP was included in the PCR at 0.5% of the total reaction volume. The radiolabeled PCR products were electrophoresed in 4.5% acrylamide gels, 90 mM Tris–borate, and 0.2 mM EDTA at a 40-mA constant current for 3.5 h. The gels were dried and autoradiographed. The radioactivity in the individual PCR products was quantitated by scanning the autoradiograms in a laser densitometer (Molecular Dynamics).

RESULTS AND DISCUSSION

Clinical studies have suggested that the content of ER in the tumor tissues influences the prognosis and response to antiestrogen 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 mRNA copy numbers in the tumor tissues by the template competition method. We have also devised a method to quantitate the exact mRNA 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

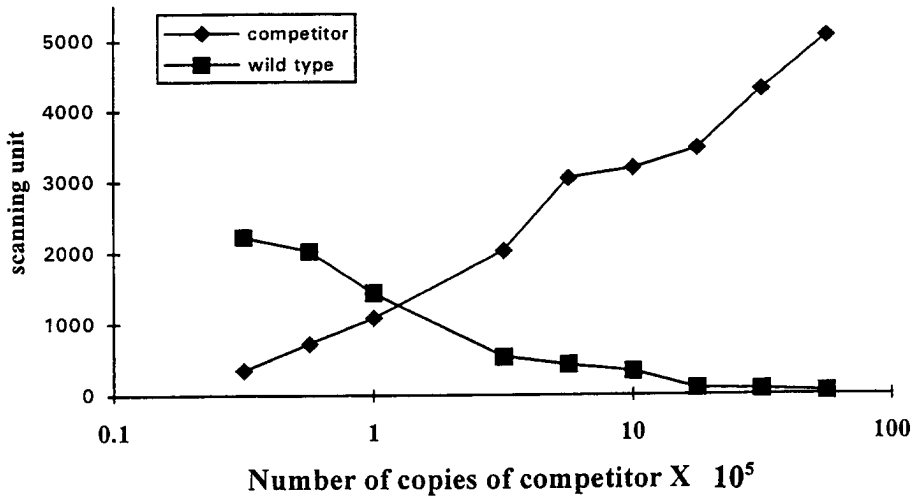

FIG. 3. Absolute quantitation of GAPDH copy numbers in the T47D cell line. The radioactivity in the PCR products of the competition assay was measured by densitometric scanning of the autoradiograms. The normalized scanned units were plotted against the number of copies of the competitor pBS-GAPDH-λ in the PCR. 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 is equal to the number of copies of the competitor at the cross-over point.

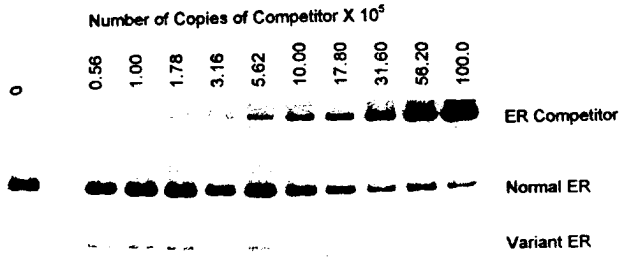

FIG. 4. Template competition assay for ER quantitation in the T47D cell line. PCR amplification with ER specific primers and a constant amount of cDNA prepared by reverse transcription of 250 ng of T47D total RNA was performed in the presence of varying amounts of the competitor pSG-ER-λ. The number of copies of the competitor ranged from 0.58×10^5 (lane 2) to 100×10^5 (lane 11) in quarter-log increments. PCR amplifications were conducted under the conditions described under 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 7Δ variant are seen and designated as normal ER and variant ER.

in exons 2, 3, 2–3, 5, and 7 in both normal mammary and breast tumor tissues is well documented (14–16).

We designed and cloned the competitor templates which enabled us to quantitate the exact copy number of both GAPDH and ER transcripts 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 size of about 700 bp in MCF-7, T47D, and ZR-75 and the two tumors tested. Neither of these products was observed in cell line LCC6 which is 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 the 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 the T47D cell line is presented in Figs. 2 and 3. The amount of competitor in the PCR ranged from 0.32×10^5 copies (Fig. 2, lane 2) to 56.2×10^5 copies (Fig. 2, lane 11). As seen in Fig. 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, first the radioactivity in the normal and the competitor PCR products was scanned densitometrically. Second, since the specific activities of the competitor and normal PCR products are different because of their size difference, the scanning units were normalized to the size of the competitor. Third, the normalized scanning units were plotted against the number of copies of the competitor template in the PCR. A representative graph for GAPDH quantitation in the T47D cell line is shown in Fig. 3. As seen in Fig. 3, the cross-over point at which the competitor and the reverse-transcribed cDNA gave equal

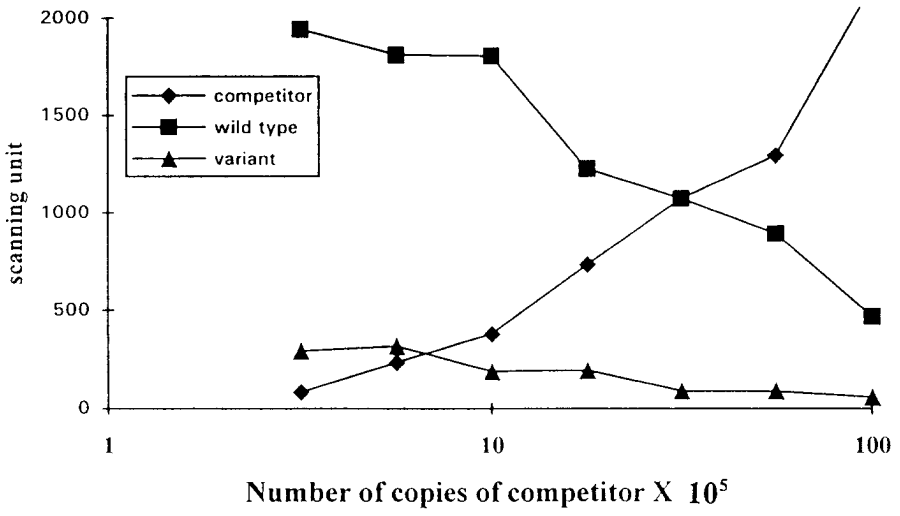

FIG. 5. Absolute quantitation of ER copy numbers in the T47D cell line. The radioactivity in the PCR products of the competition assay was measured by densitometric scanning of the autoradiograms. The normalized scanned units were plotted against the number of copies of the competitor pSG-ER-λ in the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

TABLE 1

No.	Cell type/ tumor	Copies of ER mRNA/ 10^5 copies of GAPDH		Ratio of exon 7Δ variant to wild type
		Wild type	Exon 7Δ variant	
1.	T47D	5.0×10^5	1.0×10^5	0.2
2.	MCF-7	4.8×10^4	1.5×10^4	0.31
3.	ZR-75	1.1×10^4	4.9×10^3	0.44
4.	Tumor 1	2.2×10^4	1.5×10^4	0.68
5.	Tumor 2	$<6.0 \times 10^3$	$<6.0 \times 10^3$	~1.00

amounts of PCR products was 1.25×10^5 copies. Therefore, the number of copies of GAPDH in T47D cDNA prepared from 50 ng of total RNA was 1.25×10^5 copies. The number of copies of GAPDH from MCF-7, ZR-75, tumor 1, and tumor 2 was determined by similar procedures. They were 7.3×10^5, 4.3×10^5, 8.1×10^4, and 3.8×10^4, respectively, per 50 ng of reverse-transcribed total RNA. The values in the tumor samples are about 10 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 the 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 Figs. 4 and 5. The amount of competitor used in the PCR ranged from 0.56×10^5 copies (Fig. 4, lane 2) to 100×10^5 copies (Fig. 4, lane 11). As seen in Fig. 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 were normalized as in the case of GAPDH and the normal and the competitor PCR products were plotted against the number of copies of the competitor template in the PCR. A representative graph for the ER wild-type and exon 7Δ variant quantitation in the T47D cell line is shown in Fig. 5. As seen in Fig. 5, the wild-type ER and competitor templates generated equal amounts of PCR products when the competitor concentration was at 31.6×10^5 copies. The exon 7Δ variant and the competitor products crossed over when the competitor concentration was 6.6×10^5 copies. Thus, the numbers of copies of ER wild-type and exon 7Δ variant in T47D cDNA prepared from 250 ng of reverse-transcribed total RNA were 31.6×10^5 and 6.6×10^5, respectively. The numbers 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 numbers 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 Table 1. 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 Table 1 also show that the ratios of the variant to wild type are highest in ZR-75 (0.44) and lowest in T47D (0.2) among the cell lines studied. The ratios of the exon 7Δ variant to wild type in the tumor tissues are considerably higher (0.68 and ~1 in 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 antiestrogen 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 mRNA 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 establishing an index between the mRNA copy numbers of ER wild- and variant-type transcripts per a given number of copies of GAPDH transcripts in the tumors and response to antiestrogen therapies.

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ALTERATIONS IN THE ESTROGEN RECEPTOR mRNA IN THE BREAST TUMORS OF AFRICAN AMERICAN WOMEN

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

ABSTRACT

Several recent reports have shown that the 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 and poorly differentiated with a very low frequency of hormone receptors. To gain an insight into the factors which may be responsible for poorly differentiated aggressive tumors, we investigated the transcript profiles of the estrogen receptor (ER), the most important prognostic factor in breast cancer, in the tumors derived from African American women. We analyzed 15 immunohistochemically ER positive and 6 ER negative malignant tumors for estrogen receptor mRNAs by RT PCR using a number of primer pairs. For comparative purposes, five tumor tissues derived from Caucasian patients were also included. Our results showed that only 5 of the ER positive tumors from African American women patients had full length wild type receptor transcripts and the rest of them had alterations/truncations in exon 8. We also found that the majority of tumors which had alterations/truncations in exon 8 did not express the naturally occurring, most abundant exon 7Δ transcript. Most of the tumors expressed exon-2, exons 2-3, and exon 5 deletion variant transcripts. Unexpectedly, two out of six immunohistochemically ER negative tumors had full length wild type receptor mRNAs and none of the variant transcripts.

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 also seem to differ both by age and race. It was reported that the death rate by breast cancer for African American women under the age 65 has increased, while for women in a similar age group in other population it has declined. Death rates have increased for all women over the age 65 and the increase is reported to be three times as high in African American women as others. Several reports indicate that this 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). However, such factors are not identified thus far. There also appears to be differences in tumor biology in different racial groups. Histochemical studies of the tumors derived from African American women have shown a higher incidence of poorly differentiated tumors and an increased frequency of nuclear atypia, higher mitotic activity, and tumor necrosis (5). Immunohistochemical studies have also shown that the frequency of estrogen- and progesterone receptor expression is significantly lower in the tumors of African American women (6). The presence of these receptors is generally associated with well or moderately

differentiated tumors and considered a good prognosis for longer survival and lower risk of tumor recurrence. The biological factors responsible for the poorly differentiated and aggressive tumors with lower frequency of hormone receptors which may contribute to lower survival in African American women are not known. In the current study, we examined the tumors derived from African American women for alterations in the ER structure which may in part contribute for the aggressive nature of these tumors. We investigated the ER mRNA 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 detectable full length wild type ER mRNAs. The majority of tumors showed alterations/ truncations in exon 8. Another modification observed is that most of the tumors which had exon 8 alterations/truncations did not express the naturally occurring, most abundant exon 7Δ variant mRNA.

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. [α -^{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 from African American women patients were collected from Howard University Hospital and Providence Hospital in Washington D.C. The tumor samples were collected immediately after the surgery and frozen in liquid nitrogen. The ER status of the tumors collected from Howard University hospital and Providence hospital was obtained from the Tumor Registries of the respective hospitals. It was determined immunohistochemically by Oncotech laboratories using the monoclonal antibodies against the NH_2-terminal domain (A/B region) of the receptor.

RNA isolation. Total RNA from African American patient tumors was isolated using Trizol reagent and the manufacturer's protocol. Briefly, the tumors were first ground to a powder using cold, sterile pestle and mortar in the presence of liquid nitrogen. The pulverized 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 to collect the supernatant. 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 integrity of the isolated RNA was verified by electrophoresis in 1.5% agarose gels in Tris-

Acetate EDTA buffer and the concentration was determined by measuring the optical density at 260 nm. Only those RNA samples which demonstrated intact RNA bands as visualized by ethidium bromide staining were chosen for the current study. The integrity of the RNA was further confirmed by amplification of GAPDH. The RNA samples from the breast tumors of Caucasian patients were available in S.A.W.F's laboratory.

Reverse Transcription and PCR. 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 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. For ER cDNA amplification, various primer sequences located in exons 1, 4, 6, 7 and 8 as listed in the text were used. The sequence and locations of all the primers described in the present study are based on the full length ER cDNA sequence published by Green and others (7) The Polymerase Chain Reaction was performed in an automatic thermal cycler (MJ Research) as described previously (8) 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 1 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 72^0 C. The

cDNA prepared from tumor samples were verified for the presence of GAPDH using a sense primer, 5' AAGGCTGAGAACGGGAAGCTTGTCATCAAT 3' (position, exon 3, bp 241-270) and an anti-sense primer, 5' TTCCCGTCTAGCTCAGGGATGACCTTGCCC 3' (position, exon 7, bp 740 -711) (9) under the same PCR conditions as for ER. All the primer pairs were first tested on the cDNA prepared from MCF-7 RNA and included as a positive control in all the PCR amplifications.

Detection and sequence analysis of PCR products. The PCR amplified products were radiolabled in the presence of [α -^{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% Polyacrylamide 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 subjected to autoradiography using Kodak X-Omat AR film and DuPont NEN Lightning Plus intensifying screens at room temperature. In order to confirm the identity of the PCR amplified ER products, they were electrophoresed in agarose gels and purified using QIAquick gel extraction kit. The purified products were cloned into pCR®II-TOPO vector and sequenced by cycle sequencing method (10, 11) on an automated DNA sequencer (carried out at Biopolymer Laboratory, University of Maryland School of Medicine, Baltimore, Md).

RESULTS

The ER mRNA profiles were studied by RT PCR in tumors which were diagnosed for ER status by immunohistochemistry. To detect ER transcripts, we employed primer pairs which could amplify the exons 1-6, 1-4, and 4-8. When primers ER1S (5' CTCCACACCAAAGCATCTGGG 3', position, exon 1, bp 245-265) and ER1A (5' CCAGCAGCATGTCGAAGATC 3', position, exon 6, bp 1520-1501) which amplify exons 1-6 were used, thirteen ER positive and two ER negative tumors from African American patients, and all five Caucasian patient tumors gave an expected PCR product (size ~ 1.275 kb) corresponding to wild type ER. A representative PCR product profile is shown in Figure 1A. Lanes M1 and M2 contain the Gibco-BRL 1kb- and 100 bp molecular weight ladders respectively. Lanes 1-4 contain the PCR products generated using the cDNAs from MCF-7, a representative ER positive Caucasian patient tumor, a representative ER positive African American patient tumor and a representative ER negative African American patient tumor respectively. Two of the ER positive African American women patient tumors did not give any PCR products. We analyzed the exons 4-8 in all the tumors using the primer pair ER2S (5' GCCCGCTCATGATCAAACGC 3', position, exon 4, bp 1113-1132) and ER2A (5' ATACTTTTGCAAGGAATGCGA 3', position, exon 8, bp 1978-1958). They amplified the expected PCR product of size 866 bp corresponding to wild type sequences only in five ER positive African American patient tumors, all five Caucasian patient tumors and two ER negative African American patient tumors which were positive with primers ER1S and ER1A. A representative PCR product profile is shown in Figure 1B. Lane M has the Gibco-BRL 1

kb ladder, lanes 1-3 contain the PCR products amplified with cDNAs from MCF-7, a representative Caucasian patient tumor, and a representative ER positive African American patient tumor respectively. The primers, ER2S and ER2A amplified an additional PCR product of size 682 bp in all the above tumors which by sequence analysis was found to be the exon 7Δ (Figure 1B, lanes 1-3, lower band). The rest of the eight ER positive African American patient tumors which gave positive products for exons 1-6 did not give any product(s) with ER2S and ER2A. A representative tumor sample showing the absence of any product is shown in Figure 1B, lane 4. Two tumors which did not give any products with ER1S and ER1A also did not yield any products with ER2S and ER2A. They are probably false positives and were not analyzed further. Thus it appears that only five out of fifteen ER positive African American patient tumors showed full length wild type sequences. Two of the six ER negative tumors which gave positive products with ER1S and ER1A also gave expected wild type 866 bp product with ER2S and ER2A but did not amplify naturally occurring 682 bp exon 7Δ product. The PCR product from a representative ER negative tumor is shown in Figure 1B, lane 5. Detection of full length wild type sequences only in 5 out of 15 African American women patient tumors which were diagnosed as ER positive by immunohistochemistry was unexpected. We rationalized that the remaining eight ER positive tumors may have alterations/truncations in exon 8. Therefore, we examined them for potential alterations/truncations in exon 8 with an antisense primer, ER3A (5' GCACTTCATGCTGTACAGATGC 3' position, exon 8, bp 1822-1801) in exon 8 upstream of ER2A together with ER2S. The results are shown in Figure 2. Only Two tumors out of eight gave expected wild type and exon 7 deletion products of sizes 710 bp and 526 bp

respectively and five tumors gave only wild type band. Two out these five tumors gave a wild type band which showed slightly lower mobility than the expected wild type product. The profiles of PCR products from representative tumors are presented in Figure 2. Lanes 1-3 contain PCR products generated with cDNAs from MCF-7, a representative Caucasian patient tumor, and a representative African American patient tumor which was positive for full length receptor mRNA respectively as positive controls. Lanes 4- 7 contain representative PCR products generated from eight of the African American patient tumors which were negative with ER2S and ER2A. Lane 4 contains a PCR product representative of the two tumors which gave a slightly higher molecular weight wild type product but did not generate any exon 7Δ product. Lane 5 contains PCR products representative of the two tumors which gave expected wild type and exon 7 deletion products. Lane 7 contains a PCR product generated from a representative of three tumors which gave expected wild type product but did not generate exon 7 deletion product. The gel is overloaded in lane 7 to show the absence of the naturally occurring exon 7Δ variant. One of the eight tumors did not generate any products with ER2S and ER3A (lane 6) suggesting complete deletion of exon 8. To test this possibility, we designed another antisense primer, ER4A (5' GTCCTTCTCTTCCAGAGAC 3', position, exon 7, bp 1651-1633) in exon 7 and tested together with ER2S. The results are shown in Figure 3, lane 4. It amplified an expected 538 bp wild type product and an additional 400 bp product which was identified as exon 5Δ. These results indicated the deletion of complete exon 8 in this tumor. The PCR products from MCF-7, a representative of Caucasian patient tumor and a representative of African American patient tumor which has full length sequences are shown in lanes 1-3 respectively as positive controls. Since the primer pair ER2S and ER4A co-

amplified exon 5Δ along with wild type sequences, they were used to screen all the tumors for the presence of 5Δ. We observed the presence of 5Δ in all the Caucasian patient tumors, and all thirteen ER positive African American patient tumors (data not shown). However, it is absent in two of the ER negative tumors which showed the presence of full length sequences (Figure 3, lane 5). A minor band was visualized which is higher than the exon 5 deletion product but was not characterized because of its very low abundance.

To detect the exon deletion variants in the exons 1-4 region, we designed another set of primers, a sense primer, ER3S, (5' TGCCCTACTACCTGGAGAACG 3', position, exon 1, bp 615-635) downstream of ER1S and an anti-sense primer, ER5A, (5' GGTCAGTAAGCCCATCATCG 3', position, exon 4, bp 1272-1254) in exon 4, and screened all the tumors for the presence of variant transcripts. The primers ER3S and ER5A amplified three PCR products of sizes 658 bp, 487 bp and 370 bp which were identified as the wild type, exon 2Δ and exon 2-3Δ transcripts respectively by sequence analysis. The results from representative tumors are presented in Figure 4. Lanes 1-3 contain PCR products generated with cDNAs from MCF-7, a representative Caucasian patient tumor and a representative African American patient tumor respectively. All Caucasian patient tumors and nine ER positive African American patient tumors showed the presence of both exon 2Δ and exons 2-3Δ transcripts. An additional three ER positive African American patient tumors were positive for exons 2-3Δ deletion variant only. The tumor which had the complete exon 8 deletion did not show the presence of the exon 5-, or exon -2 deletion variants. Two of the ER negative tumors which showed the presence of full length wild type sequences did not

show the presence of either 2Δ or exons 2-3Δ. PCR product from one of the ER negative tumor is shown in Figure 4, lane 4. Thus it appears that the negative tumors which showed the presence of wild type PCR products did not express either exon 5-, exon 7-, exon 2- or exons 2-3 deletion variants.

DISCUSSION

We investigated the profiles of ER mRNA by RT PCR in the tumors of African American women which are in general described as poorly differentiated and aggressive. We thought that the variations in the ER structure may, in part, contribute to the above characteristics. The results presented in this report clearly show that only a third of the tumors tested had full length ER mRNAs and a large number of tumors contained mRNAs which appear to have alterations/truncations in the major portion of exon 8. One of the fifteen ER positive tumors had complete deletion of exon 8. Several studies have reported the presence of ER mRNAs which have truncations in exon 2, exon 3 and 6 and were expressed in single breast tumors. In majority of the truncated mRNAs, the transcripts contain entire exon sequences of at least 2 of the 5' ER exon sequences, and then diverge into ER-unrelated sequences (12). The role of one of the truncated variant, namely clone 4 which consists of exons 1 and 2 of the wild type ER mRNA followed by unrelated sequences, has been evaluated and found that its level is significantly elevated in breast tumors compared to normal mammary gland (13). The relative level of this truncated variant was also found to be significantly elevated in breast tumors with characteristics of poor prognosis and endocrine

resistance (14). The nature of truncations we have observed in the Breast tumors of African American women appear to be different from the above reported truncations in that the mRNAs appear to have intact wild type exons 1-7 and part of exon 8. Since the exon-8 sequences encode for a portion of E- and complete F domains which control the ligand binding-, ligand-dependent dimerization, ligand dependent non-acidic transactivating and transcriptional responses to estrogens and anti-estrogens, alterations/truncations in this exon, therefore, will considerably affect the ligand binding properties of the receptor. Another distinguishing characteristic observed in the African American patient tumors studied is that the majority of those which showed the alterations/truncations in exon 8, lacked the presence of the naturally occurring, most abundant exon 7Δ. Although, *in vitro* studies have shown that exon 7Δ has a negative dominant effect on the wild type receptor (15), it was postulated that the variants including the exon 7Δ which are expressed in normal tissues have a role in the estrogen induced signal transduction pathway. The significance of the absence of the exon 7Δ is not known, but it may be relevant that this absence was observed only in tumors which did not demonstrate full length transcripts. Our results also show that two of the truncated tumors which did not express the most abundant exon 7 deletion variant had lower mobility than the expected wild type product on polyacrylamide gels. This lower mobility may be due to base insertions. Due to a very limited amount of RNA from those tumors, the PCR products could not be cloned and sequenced to identify the site and nature of modifications. Since they generated expected size PCR products with primers which amplify exons 1-6, it is possible that the modifications could be either in exon 7 or the untruncated portion of exon 8.

The presence of exon deletion variants between the exons 1-6 was investigated using primers which amplify exons 1-4 and exons 4-7. Our study indicates that majority of tumors express exon-2Δ, exons-2-3Δ and exon-5Δ. The occurrence of the exons 2-3Δ in both normal and cancer tissues has been reported by Leygue and others (15, 16). In a study of 100 tumors, they reported the frequency of this multiple deletion variant in less than 10% of the samples. In our study we found that all but two tumors expressed this variant. Exon 2 deletion variant was detected in 9 out of 15 tumors with primers ER3S and ER5A. Exon 5 deletion variant transcripts were detected in thirteen ER positive African American patient tumors.

Finally, very interesting observations were made with respect to immunohistochemically ER negative tumors. We found that out of six tumors studied two of them showed full length ER mRNA sequences. Unexpectedly, these tumors did not express either exon 7-, exon 2-, exons 2-3- or exon 5Δ transcripts. The physiological significance of these observations are not known. The absence of any of the known variants in these tumors which have wild type sequences suggests two possibilities: i) that the ER mRNAs are not translated in these tissues or ii) the protein amounts are in undetectable levels for immunohistochemistry. It is not known whether the absence of immunohistochemically detectable protein in these tumors is related to the absence of variant transcript molecules. It is possible that the lower frequency of ER positive tumors reported in African American patients may be due to the presence of altered receptors which are not translated to immunoreactive ER protein.

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FIGURE LEGENDS.

Figure. 1. Amplification of ER exons 1-6 and 4-8 in breast tumors. The ER exons were amplified by RT PCR and the products were detected by electrophoresed in 1% agarose gels and ethidium bromide staining.

Panel A contains the PCR products generated with primers ER1S and ER1A, which amplify exons 1-6. Lanes M1 and M2 contain the Gibco-BRL 1kb- and 100 bp molecular weight markers respectively. Lanes 1-4 contain the PCR products generated with cDNAs from MCF-7, a representative from five ER positive Caucasian patient tumors, a representative from thirteen ER positive African American patient tumors and a representative from two ER negative African American patient tumors respectively.

Panel B contains the wild type and exon 7 deletion variant PCR products generated with ER2S and ER2A which amplify exons 4-8. Lane M contains the Gibco-BRL 1 kb ladder, lanes 1-3 contain PCR products generated with cDNAs from MCF-7, a representative from five ER positive Caucasian patient tumors, and a representative from five ER positive African American patient tumors respectively. Lane 4 shows the pattern indicating the absence of any PCR products observed in eight of ER positive African American patient tumors. Lane 5 contains the wild type PCR product from a representative of the two ER negative African American patient tumors.

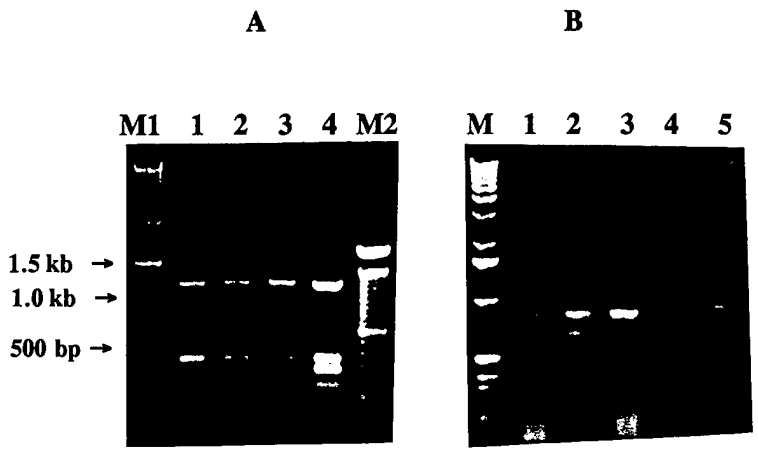
Figure. 2. Testing of ER positive African American patient tumors for possible truncations/alterations in exon 8. All the tumors which did not give any PCR products with

ER2S and ER2A were tested using ER2S and an anti-sense primer, ER3A, upstream of ER2A under the PCR conditions described in methods. The PCRs were conducted in the presence of [α -^{32}P]dCTP and the products were detected by polyacrylamide gel electrophoresis and autoradiography. Lanes 1-3 contain the positive controls amplified using cDNAs from MCF-7, a representative from five ER positive Caucasian patient tumors, and a representative from five ER positive African American patient tumors which contain full length ER sequence respectively. Lanes 4-7 contain representative PCR products from eight tumors which did not amplify with primers ER2S and ER2A. Lane 4 contains a PCR product from a representative of two tumors which generated slightly higher size band than the expected wild type band but not the exon 7Δ band. Lane 5 shows the PCR products from a representative of two tumors which generated expected sized wild type and exon 7Δ bands. Lane 6 shows the absence of any product from one of the above eight tumors and lane 7 shows the wild type PCR product of expected size and the absence of exon 7 deletion product from a representative of three tumors.

Figure. 3. Testing of one of the ER positive tumor for a possible complete deletion of exon 8. One of the ER positive African American patient tumor which was positive for exons 1-6 but did not amplify any product between exons 4-8 was tested with primers which amplify exons 4-7. The amplifications were conducted in the presence of [α -^{32}P]dCTP and the product(s) were analyzed by Polyacrylamide gel electrophoresis and autoradiography. Lanes 1-3 contain PCR products from positive controls amplified with cDNAs from MCF-7, a Caucasian patient tumor and an African American patient tumor which has full length ER

sequence respectively. Lane 4 shows the PCR products amplified from the ER positive African American patient tumor which did not give any PCR products with primers which amplify exons 4-8. Lane 5 contains PCR products from a representative of two ER negative African American patient tumors which were positive for full length ER sequence.

Figure 4. Amplification of exons 1-4 to identify exon deletion variants in this region. To analyze the ER splice variants between exons 1-4, all tumor cDNAs were subjected to PCR amplification in the presence of [α -^{32}P]dCTP and analyzed by polyacrylamide gel electrophoresis and autoradiography. Lanes 1-4 contain the PCR products generated using cDNAs from MCF-7, a representative Caucasian patient tumor, a representative ER positive African American patient tumor and a representative ER negative African American patient tumor respectively.


A
B
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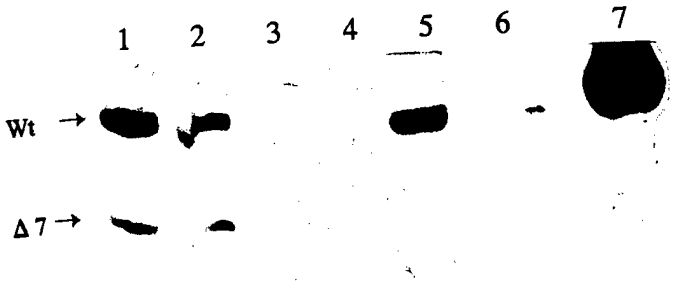
1.0 kb
500 bp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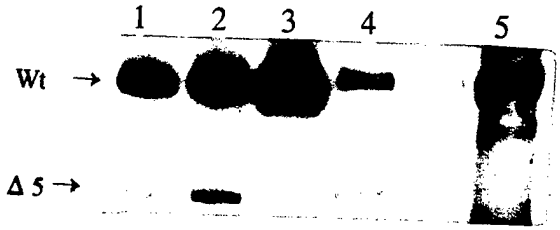
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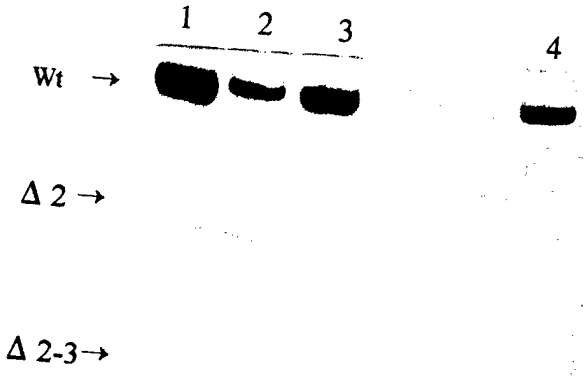
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Δ 2-3→

A NOVEL APPROACH FOR THE CHARACTERIZATION OF ALTERNATIVELY SPLICED ESTROGEN RECEPTORS

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ABSTRACT

Several mRNA molecules undergo alternate splicing giving rise to transcripts which have deletions in one or more exons. In order to define their role in biology and medicine, highly specific detection methods are needed which can characterize them as precisely as possible. They are conventionally detected by amplification of small regions of wild type sequences by RT PCR. However, there are major problems in this approach such as it cannot detect the alternatively spliced mRNAs which are far below the levels of wild type molecules and cannot distinguish those which have multiple deletions in distant exons. In the current study, we describe a novel approach for the amplification of these molecules. Our approach involves targeted amplification of the alternatively spliced molecules as separate gene populations using primers which specifically anneal to the alternatively spliced junctions and do not co-amplify wild type molecules. This approach is described using estrogen receptor (ER) exon 7Δ and exon 5Δ molecules as models. A series of targeted primers were designed which differed in the number of bases in the 3' end unique to the splice junction and total number of overhang bases beyond the junction. The results described here indicate that the targeted primers which have a minimum of 3 out of 4 bases unique to the splice junction in the extreme 3' end are optimal for specific amplification. Our results also indicate that the primers can extend as many as 8 bases beyond the alternate splice junction before amplifying the wild type sequences corresponding to the 3' end of the primer. Based on these parameters, single targeted primers for the splice junctions of ER exon 2Δ and exons 2-3Δ, were designed, tested and found to be very highly specific. We believe that the principles

developed in the current study will have broad applicability to alternatively spliced molecules of a diverse range of genes.

INTRODUCTION

The existence of alternatively spliced mRNAs has been demonstrated for a number of molecules. To mention a few are the estrogen receptor (ER) (1), the human major histocompatibility complex (HLA) class I molecules (2), CD45 (3), and the mineralocorticoid receptor (4). In all these molecules, precise single or multiple exon deletions have been reported. In order to define their role in biology and medicine, new methods are needed which can not only detect and distinguish them from the wild type, but also quantitate the proportion of alternatively spliced molecules in a mixed population. The characterization of alternatively spliced molecules is no where more important than in the ER molecule which mediates the estrogen action in various tissues. A number of alternatively spliced molecules of ER have been reported in cell lines, normal tissues and malignant tissues (5, 6). Because of their presence in normal tissues, they are implicated in the estrogen induced signal transduction pathway. Recent reports also indicate that the levels and patterns of several ER spliced variants are different between normal and neoplastic tissues as well as amongst groups of tumors with different characteristics (6).Yet there are no specific methods available which can precisely detect the alternatively spliced ER molecules. Conventionally, they are detected by RT PCR approaches, which by virtue of specific primer design, are focussed on small regions of the known wild type mRNA. However, there are major problems in this approach, such as: 1) Threshold of detection. Since the wild type molecules are present in large excess to alternatively spliced molecules, competitive amplification occurs amongst the wild type and all the alternatively spliced molecules. Therefore, detection of a particular spliced variant

depends on the relative expression levels of their mRNA species within a sample. Thus, those mRNAs which are expressed at low levels may fall below the threshold of detection. An example of this problem has been observed in our own studies on ER splice variants. When primers which amplify the exons 4-8 of ER cDNA were used, only the most abundant splice variant transcript, the exon 7Δ, was detected but not the less abundant exon 5Δ transcript in any of the breast cancer cell lines examined (7). The exon 5Δ could be, however, detected by amplifying the small region of the wild type between exons 4-6 (8). 2) another disadvantage in detecting the spliced mRNAs by amplifying small regions of wild type has been that the mRNAs which have multiple deletions in distant exons such as the ER molecule with exons 2- and 5 deletions cannot be distinguished by this method. The third disadvantage in the traditional approach of co-amplification with wild type is that the alternatively spliced molecules which have similar sized exon deletions will generate products which cannot be distinguished from each other by size exclusion gel electrophoresis, and therefore, cannot be characterized.

To circumvent all the above described problems, we have developed a new approach to characterize the alternatively spliced molecules. Our approach involves the targeted amplification of the alternatively spliced molecules as separate gene populations with out co-amplification of wild type molecules. We demonstrate our approach by describing the targeted amplification of alternatively spliced ER molecules. We believe that the principles developed in the current study will have broad applicability to splice variants of a diverse range of genes. We also feel that the new approach will find applications to study changes in the

splice variants in cancer and other diseases.

MATERIALS AND METHODS

AmpliTaq PCR core kits and QIAquick gel extraction kits were from QIAGEN Inc., Santa Clara, CA. All the primers used in the current study were synthesized at Gibco-BRL Life Technologies. The pCR®II-TOPO cloning vector was from Invitrogen. PCR quality water and Tris-EDTA buffer were from Biofluids, Rockville, Md. The plasmid, pIC-ER-F, containing the full length ER cDNA was obtained from ATCC, Rockville, Md.

Construction of plasmids containing alternatively spliced ER variant sequences. The construction of ER plasmids containing the exon 7-, 5-, 2-, and 2-3 deletion sequences was accomplished as follows: First, their respective nucleotide sequences were co-amplified with wild type sequences using the cDNA prepared from the breast cancer cell line, MCF-7 and the PCR conditions described previously (6). The products corresponding to alternatively spliced variants were isolated by electrophoresis in 1% agarose gels and extraction using QIAquick gel extraction kits. The identity of the gel extracted products was verified by sequence analysis (carried out at the Biopolymer Laboratory, University of Maryland School of Medicine, Baltimore, Md). Next, the isolated variant sequences were cloned into pCR®II-TOPO vector following the manufacturer's protocol. To construct the ER exon 7 deletion splice variant plasmid, the nucleotide sequences were co-amplified with wild type sequences between the exons 4 and 8 using the sense primer, ER1S 5' GCCCGCTCATGATCAAACGC

3' (position, exon 4, bp 1113-1132) and an anti-sense primer, ER1A, 5' TACTTTTGCAAGGAATGCGA 3' (position, exon 8, bp 1977-1958). The sequence and locations of all the primers described here are based on the full length ER cDNA sequence, which is about 1.8 kb long and is divided into eight exons, published by Green and others (9). The primers ER1S and ER1A generated two PCR products of sizes 865 bp and 688 bp, which were identified by sequence analysis as the wild type and exon 7 deletion variant respectively. The 688 bp exon 7 deletion product was cloned into the pCR®II-TOPO vector. The resulting plasmid was termed as pCR®II-TOPO-7Δ. The exon 5 deletion splice variant nucleotide sequences (300 bp) was generated by co-amplification with wild type sequences (439 bp) between the exons 4-6, using the sense primer, ER2S, 5'GGAGACATGAGAGCTGCCAAC3' (position, exon 4, bp 1082-1102) and an anti-sense primer, ER2A, 5' CCAGCAGCATGTCGAAGATC 3' (position, exon 6, bp 1520-1501). The resulting plasmid was termed as pCR®II-TOPO-5Δ. The exon 2 deletion variant (468 bp) and exons 2-3 deletion variant (350 bp) sequences were generated by co-amplification with the wild type sequences (659 bp) between the exons 1-4, using the sense primer, ER3S, 5' TGCCCTACTACCTGGAGAACG 3' (position, exon 1, bp 615-635), and an anti-sense primer, ER3A, 5' GGTCAGTAAGCCCATCATCG 3' (position, exon 4, bp 1273-1254). The resulting exon 2- and exons 2-3 splice variant plasmids were termed as pCR®II-TOPO-2Δ and pCR®II-TOPO-2-3Δ respectively.

PCR. The targeted primers listed in the Table were tested separately on two different templates, 1) pIC-ER-F and 2) pCR®II-TOPO-7Δ or pCR®II-TOPO-5Δ depending upon

which set of primers were tested. The PCRs were carried out in a 25 µl reaction volume containing the test plasmid, 1 X Qiagen PCR buffer, 1 X Qiagen Q solution, 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 either 61^0 C or 68^0 C as indicated in the text, and extension for 2 min at 72^0 C for 30 cycles plus a final extension for 10 min at 72^0 C.

RESULTS AND DISCUSSION

Our goal was to design an approach which can specifically amplify a particular category of alternatively spliced molecules from a pool of other types of alternatively spliced- and wild type genes. The approach described here involves the targeted amplification of the spliced molecules using primers which anneal to the alternatively spliced junctions. To achieve this, we designed several primers for the splice junctions of ER exon 7Δ and exon 5Δ, tested, and identified the primer requirements and conditions which are necessary for the targeted amplification of these molecules. Specifically, we tested 1) the number of extreme 3' bases in the primer, that were required to be unique for the targeted amplification of the alternatively spliced molecules and 2) the number of bases that the primer could extend beyond the splice junction and still maintain specific amplification of the target sequences and 3) the influence of annealing temperature for the specific amplifications. We tested a series of primers which differed in the number of bases in the 3' end that are unique to the splice junction and number of overhang bases beyond the junction for ER exon 5Δ- and exon 7Δ.

As illustrated in the Table, we designed a series of antisense primers, ER AX6/8-1 (positions, exon 8, bp 1804 to 1786 and exon 6, bp 1601-1600) through ER AX6/8-7 (positions, exon 8, bp 1798-1786, and exon 6, bp 1601-1594) which have a varying number of overhanging bases at the 6/8 splice junction (Table, panel A). These primers were then tested together with the sense primer, ER1S, at an annealing temperature of 61° C. If the targeted primers specifically recognize only the 6/8 splice junction, we would expect them to generate a PCR product ranging from 508 bp for ER AX6/8-1 to 502 bp for ER AX6/8-7 with pCR®II-TOPO-7Δ and no product with pIC-ER-F. On the other hand, if the exon 8 portion of the primers anneal and amplify the wild type sequences in pIC-ER-F, we would expect to observe a PCR product from 692 bp with ER AX6/8-1 to 686 bp with ER AX6/8-7. Similarly, if the exon 6 portions of the primers recognize and amplify exon 6 of the wild type sequences in pIC-ER-F, we would expect to observe PCR products as described for the pCR®II-TOPO-7Δ. Because of these reasons, it is critical to examine the primers for their specific amplification of alternatively spliced junctions. The results observed with pCR®II-TOPO-7Δ and pIC-ER-F are shown in Figure 1. Lane M contains the Gibco-BRL 1 kb ladder molecular weight markers. Lanes 1-7 contain the PCR products amplified with the targeted primers ER AX6/8-1 through ER AX6/8-7 respectively using pCR®II-TOPO-7Δ as the template. As seen in lanes 1-7, all the targeted primers generated the expected ~500 bp product with pCR®II-TOPO-7Δ. However, when the template was pIC-ER-F, the targeted primers ER AX6/8-1 through ER AX6/8-3 which have fewer than three bases unique to the splice junction in the extreme 3' end, generated an approximately 690 bp PCR product (Figure 1, lanes 8-10) indicating the annealing of these primers to exon 8 of the wild type ER

sequences. On the other hand, the primers ER AX6/8-4 through ER AX6/8-7, all of which have a minimum of three or more bases unique to the splice junction in the extreme 3' end, did not generate any PCR products with the full length wild type plasmid, pIC-ER-F (Figure 1, lanes 11-14). These results indicated the absence of amplification by their annealing to either exons 7/8 or 6/7 junctions in the wild type. Based on these results, it can be interpreted that the ER 7Δ targeted primers which have a minimum of 3 out of 4 unique bases at the extreme 3' end will specifically amplify the spliced junction without amplifying the flanking exons. In addition, it appears that as many as eight bases past the exon 6/8 splice junction are permitted without amplification of the wild type sequences.

Next, we designed anti-sense primers, ER AX4/6-1 (positions, exon 6, bp 1484-1468 and exon 4, bp 1328-1325) through ER AX4/6-7 (positions, exon 6, bp 1478-1468 and exon 4, bp 1328-1319) (Table, panel B) to test for the specific amplification of ER exon 5 deletion variant. As seen in panel B of the Table, both exons 4/6 and 5/6 junctions have the same initial three bases, CTG. Therefore, we designed primers further beyond the splice junction to target the unique sequences and tested together with the sense primer, ER1S, under the PCR conditions described above and at an annealing temperature of 61^0 C. If the targeted primers are specific for the 4/6 junction, we would expect to observe PCR products ranging from 233 bp with ER AX4/6-1 to 227 bp with ER AX4/6-7 when amplifying pCR®II-TOPO-5Δ and no product with pIC-ER-F. Alternatively, if the targeted primers anneal and amplify exon 6 or exon 4 of the wild type junctions in pIC-ER-F, we would expect to observe PCR products of sizes approximately 370 bp or 230 bp respectively. The PCR results with these primers are

shown in Figure 2A. Lanes 1-7 contain the PCR products amplified with the targeted primers ER AX4/6-1 through ER AX4/6-7 respectively with pCR®II-TOPO-5Δ as the template. As seen in lanes 1-7, all of the targeted primers generated the expected ~230 bp product with pCR®II-TOPO-5Δ. However, when the template was pIC-ER-F, primers AX4/6-1 through AX4/6-3 generated an approximately 370 bp PCR product indicating the annealing of 5' end of these primers to exon 6 and amplification of the wild type ER sequences (Figure 2A, lanes 8-10). These observations are similar to the results obtained with the ER exon 7 splice variant targeted primers, ER AX6/8-1 through ER AX6/8-3, which have fewer than three unique bases in the extreme 3' end and resulted in annealing of their 5' end portion to exon 8 and amplification of wild type junction.

The targeted primers, AX4/6-4 through AX4/6-7, generated an ~230 bp band with the wild type plasmid indicating that the 3' end portion of these primers recognized and amplified exon 4 of the wild type sequences (Figure 2A, lanes 11-14). The primer ER AX4/6-3 generated both 370 and 231 bp products indicating that it amplified from both exons 4- and 6 of the wild type sequences (Figure 2A, lane 10). These results with ER AX4/6-4 through ER AX4/6-7 contrasted the observations made with the exon 6/8 splice junction targeted primers, ER AX6/8-4 through ER AX 6/8-7, in that they are not specific to the target splice junctions. This is probably due to the increased number of total overhang bases in the exon 4/6 splice junction targeted primers and their higher GC content. Since none of the targeted primers were completely specific at the annealing temperature of 61^0 C, we subsequently tested them at an annealing temperature of 68^0 C. The results are shown in Figure 2B. The primers,

ER AX4/6-2 through ER AX4/6-7 maintained their ability to amplify the targeted sequences at 68^0 C (Figure 2B, lanes 2-7) with pCR®II-TOPO-5Δ. The PCR results with pIC-ER-F are shown in Figure 2B, lanes 8-14. The primer ER AX4/6-1 did not recognize either pCR®II-TOPO-5Δ or the pIC-ER-F (lanes 1 and 8 respectively). The primers, ER AX4/6-2 through ER AX4/6-5 did not generate any PCR products with pIC-ER-F (Figure 2B, lanes 9-12) indicating their specificity to 4/6 junction at an annealing temperature of 68^0 C. The primers, AX4/6-6 and AX4/6-7 amplified a minor product of size ~230 bp (Figure 2B, lanes 13-14), indicating amplification of sequences in exon 4 of the wild type. Thus it appears that in addition to the number of unique overhang bases in the extreme 3' end of the primer, the total number of overhang bases in the 3' end and the annealing temperatures influence the specific amplification of the targeted sequences. The above described results with targeted primers for ER exon 5 splice variant provide evidence for our earlier hypothesis that a minimum of three out of four bases unique to the splice junction in the extreme 3' end of the primer are required to specifically amplify the alternate splice junction. It also appears that in order to design such a primer, the overhang sequences can extend up to eight bases past the splice junction without annealing and amplification of the wild type sequences.

Keeping these parameters in mind, we next designed the targeted primers, ER SX1/3 (positions, exon 1, bp 669-684, and exon 3, bp 876-880) and ER SX1/4 (positions, exon 1, bp 670-684 and exon 4, bp 993-998), for the exons 1/3 and 1/4 splice junctions respectively. The primer design is illustrated in Figure 3. As seen in Figure 3, the first and third bases in the overhang of the 1/3 splice junction of the sense strand are identical to the first and third bases

of the 1/2 junction. However the bases in positions 2, 4 and 5 are unique. Thus in a total of 5 overhang bases of the 1/3 splice junction, 3 out of the 4 extreme 3' bases are unique. Similarly, we designed a primer targeted to the 1/4 junction of the sense strand of the ER exon 2-3 deletion cDNA. As seen in Figure 3, in positions 1, 2 and 5 of the overhang bases in the splice junction of exons 1/4 junction are identical to those in the overhang of the 1/2 junction. However, bases in position 3, 4 and 6 are unique. Thus in a total of 6 overhang bases of the 1/4 splice junction, 3 out of the 4 extreme 3' bases are unique. Each of these primers satisfy the requirements of our target primer design based on the results described above. We next individually tested these two sense primers using the anti-sense primer, ER3A, at an annealing temperature of 61^0 C. The obtained results are shown in Figure 4. Lane M has the 123 bp molecular weight ladder. Lanes 1-3 contain the PCR products generated with the targeted primer, ER SX1/3, and the anti-sense primer ER3A using no DNA, pCR®II-TOPO-2Δ and pIC-ER-F as templates respectively. Similarly, lanes 4-6 contain the PCR products with the targeted primer, ER SX1/4, and the ER3A using no DNA, pCR®II-TOPO-2-3Δ and pIC-ER-F respectively as templates. As seen in the Figure 4, the targeted primers designed for the exon 2Δ and exons 2-3Δ molecules amplified only the targeted splice junctions and generated the expected 414 bp and 296 bp products (lanes 2 and 5 respectively) but did not generate any products with the ER wild type sequences (lanes 3 and 6 respectively). Thus the targeted primers which meet the requirement of atleast three out of four unique bases in the extreme 3' end exclusively amplify the alternate sequences. These results obtained with the primers ER SX1/3 and ER SX1/4 provide further support for our earlier hypothesis.

The current study illustrates the selective amplification of alternatively spliced molecules as individual genes with splice specific primers. Since the targeted primers specifically anneal to the alternatively spliced junctions, this method could be applied to detect molecules which are present in very minute amounts and cannot be detected by conventional methods. The current approach also permits the detection of mRNAs with multiple deletions in distant exons by using selected partner primers. We believe that the principals derived for the specific amplifications of ER splice variants in this study could be broadly applied for the targeted amplification of the alternatively spliced molecules of any gene. The specific amplification approach will highly facilitate in assessing the functional roles of the splice variants in biology and medicine and in monitoring the changes in their expression levels in cancer and other diseases.

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FIGURE LEGENDS

Figure 1. Testing of targeted primers for the ER exons 6/8 splice junction with the templates pCR®II-TOPO-7Δ and pIC-ER-F. The primers ER AX6/8-1 through ER AX6/8-7 were tested together with ER1S using the above two plasmids under PCR conditions described in the Materials and Methods. Lane M contains the 1 kb molecular weight ladder. Lanes 1-7 contain the PCR products amplified with the targeted primers, ER AX6/8-1 through ER AX6/8-7, respectively using pCR®II-TOPO-7Δ as the template. Lanes 8-14 contain the PCR products amplified with the targeted primers ER AX6/8-1 though ER AX6/8-7 respectively using pIC-ER-F as the template.

Figure 2. Testing of targeted primers for the ER exons 4/6 splice junction using the templates pCR®II-TOPO-5Δ and pIC-ER-F. The primers ER AX4/6-1 through ER AX4/6-7 were tested together with ER 1S using the above two plasmids under PCR conditions described in the Materials and Methods. Panels A and B show the PCR products obtained at the annealing temperatures of 61° C and 68° C respectively. In each of these panels, lanes 1-7 contain the PCR products amplified with the primers ER AX4/6-1 through ER AX4/6-7 respectively with pCR®II-TOPO-5Δ as the template; lanes 8-14 contain the PCR products amplified with the primers ER AX4/6-1 though ER AX4/6-7 respectively with pIC-ER-F as the template; and in panel B, lane M contains the Gibco-BRL Life Technologies 1 kb molecular weight ladder.

Figure 4. Testing of targeted primers for the ER exons 1/3 and ER exons 1/4 splice junctions with pCR®II-TOPO-2Δ and pCR®II-TOPO-2-3Δ respectively and pIC-ER-F. The targeted primers ER SX1/3 and ER SX1/4 were tested together with ER3A using their respective cloned plasmids and pIC-ER-F under PCR conditions described in the Materials and Methods. Lane M contains the 123 bp molecular weight ladder. Lanes 1-3 contain the PCR products amplified with ER SX1/3 and ER3A and using no DNA, pCR®II-TOPO-2Δ and pIC-ER-F respectively as the templates. Lanes 4-6 contain the PCR products amplified with the targeted primer, ER SX1/4, and ER 3A using no DNA, pCR®II-TOPO-2-3Δ and pIC-ER-F respectively as templates.

Figure 1

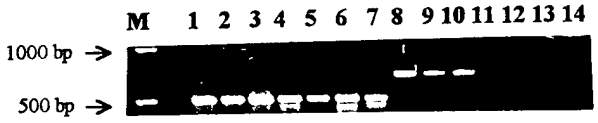

Figure 2

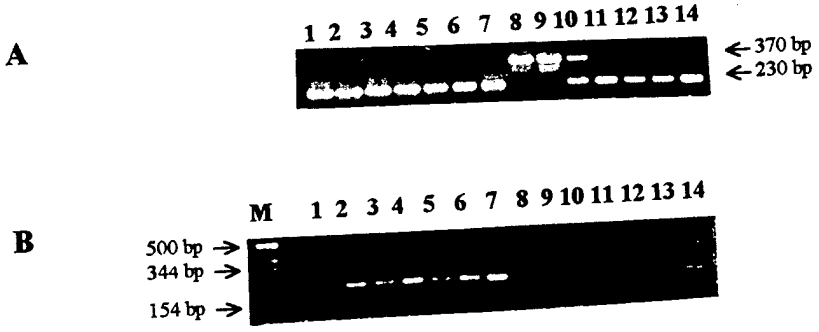
A
1 2 3 4 5 6 7 8 9 10 11 12 13 14
370 bp
230 bp
B
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14
500 bp
344 bp
154 bp

Figure 3

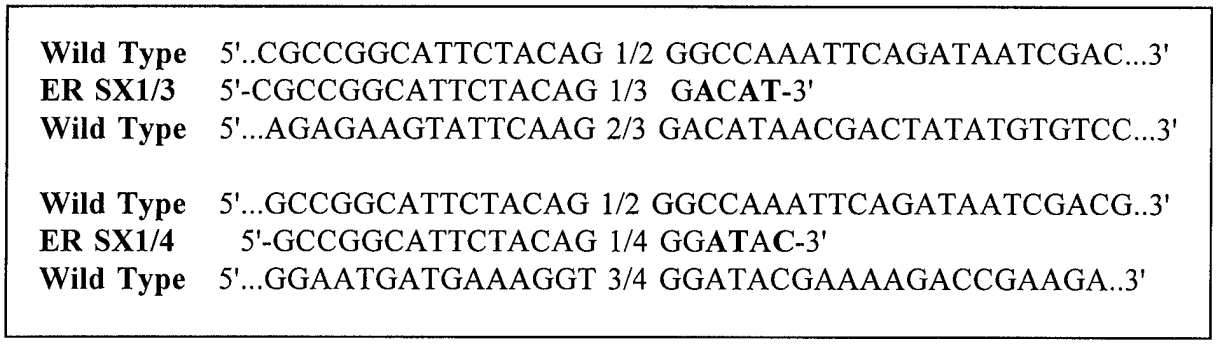

Figure 3. Targeted primer design for ER exons- 1/3 and 1/4 splice junctions.

Figure 4

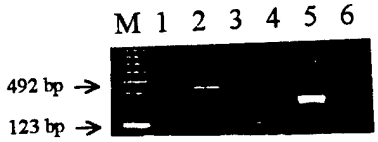

Table. Design of antisense primers for targeting ER splice variants, ER 7Δ and ER 5Δ

A. PRIMERS FOR EXON 7Δ

		Number of 3' overhang bases		Target Specific	
	Exon 8 Exon 6	**Total**	**Unique in the extreme 3' end**	**(At annel. Temp)**	
				(61^0 C)	**(68^0 C)**
ER AX6/8-1	5' ATGCTCCATGCCTTTGTTA//C**A** 3'	2	1/1	NO[1]	N/T
ER AX6/8-2	5' TGCTCCATGCCTTTGTTA//C**AG** 3'	3	2/2	NO[1]	N/T
ER AX6/8-3	5' GCTCCATGCCTTTGTTA//C**AG**A 3'	4	2/3	NO[1]	N/T
ER AX6/8-4	5' CTCCATGCCTTTGTTA//C**AGAA** 3'	5	3/4	YES	N/T
ER AX6/8-5	5' TCCATGCCTTTGTTA//C**AGAAT** 3'	6	4/5	YES	N/T
ER AX6/8-6	5' CCATGCCTTTGTTA//C**AGAATT** 3'	7	4/6	YES	N/T
ER AX6/8-7	5' CATGCCTTTGTTA//C**AGAATTA** 3'	8	5/7	YES	N/T
Wild Type	5.....TGCCTTTGTTA//CTCATGTG. 3'				
	Exon 8 Exon 7				

B. PRIMERS FOR EXON 5Δ

	Exon 6 Exon 4				
ER AX4/6-1	5' CACATTTTCCCTGGTTC//CTG**G** 3'	4	1/1	NO[1]	YES
ER AX4/6-2	5' ACATTTTCCCTGGTTC//CTG**GC** 3'	5	1/2	NO[1]	YES
ER AX4/6-3	5' CATTTTCCCTGGTTC//CTG**GCA** 3'	6	2/3	NO[1,2]	YES
ER AX4/6-4	5' ATTTTCCCTGGTTC//CTG**GCAC** 3'	7	3/4	NO[2]	YES
ER AX4/6-5	5' TTTTCCCTGGTTC//CTG**GCACC** 3'	8	4/5	NO[2]	YES
ER AX4/6-6	5' TTTCCCTGGTTC//CTG**GCACCC** 3'	9	5/6	NO[2]	NO[2]
ER AX4/6-7	5' TTCCCTGGTTC//CTG**GCACCCT** 3'	10	6/7	NO[2]	NO[2]
Wild Type	5'.....CCCTGGTTC//CTGTCCAAGA..3'				
	Exon 6 Exon 5				

N/T. Not tested

[1] Amplifies the wild type exon at the 5' end of the primer.

[2]Amplifies the wild type exon at the 3' end of the primer.

ANALYSIS OF SINGLE- AND MULTIPLE EXON DELETION TRANSCRIPTS OF ESTROGEN RECEPTOR IN BREAST CANCER CELL LINES AND TUMORS BY A NOVEL APPROACH

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Running Title: Analysis of ER splice variants by a novel approach.

ABSTRACT

Estrogen receptor (ER) splice variant transcript profiles were analyzed by RT PCR in six ER positive breast cancer cell lines, MCF-7, T47D, ZR-75, LCC1, LCC2, LCC9, one ER negative cell line, MDA-MB-435, and three ER positive breast tumors using targeted primers which specifically anneal to alternatively spliced junctions. Analyses were conducted to detect exon 2Δ and exons 2-3Δ, exon 5Δ and exon 7Δ containing transcripts. For all the specific primers except 5Δ, the partner primers were chosen such that the transcripts were amplified between exons 1 and 8. The results described here show that each splice specific primer amplified not only the single exon deleted transcript but also a number of related cDNAs which have deletions in various combinations of exons. A total of fourteen different transcripts were amplified by the above four primers out of which eleven had double- or multiple exon deletions. The exon 2Δ specific primer amplified five transcripts which have deletions in exon 2, exons 2 and 7, exons 2, 5, and 7, exon 2 and exons 4-5, and exon 2 and exons 4-6. The exon 2-3Δ specific primer amplified three products which have deletions in exons 2-3, exons 2-3 and 7 and exons 2-3, 5 and 7. The exon 5Δ specific primer amplified three transcripts which have deletions in exon 5, exons 5 and 2, and exons 5, 7 and 2-3. The 7Δ specific primer amplified four transcripts which have deletions in exon 7, exons 7 and 4, exon 7 and exons 3-4, and exon 7 and exons 3-5. None of the splice specific primers amplified the wild type ER sequences. The six ER positive cell lines differed in the levels and patterns of the variant transcripts and the ER negative cell line expressed only the exon 2Δ transcripts. Unlike the cell lines which expressed both single- and multiple exon deletion

products, the tumor samples amplified predominantly multiple exon deletion transcripts which also appear to be modified for base pair deletions/insertions.

INTRODUCTION

The estrogen receptor (ER) mRNA undergoes alternate splicing giving rise to transcripts containing precise single or multiple exon deletions. The presence of ER transcripts which have deletions in exons 2-, 3-, 2-3, 2-5, 4-, 5-, 6- and 7 has been described in breast cancer cell lines, normal- and malignant breast tissue samples (1-4). Although the exact function(s) of these splice variants is not established, it has been hypothesized that the splice variant mRNAs may result in quite different proteins that differ in activity and may modulate differentially the ER signalling pathway in the normal tissues. Consequently, changes in the balance of these transcripts could perturb the ER signaling pathway and contribute to tumor progression. Several studies suggested that the expression of some exon deletion transcripts is deregulated during breast tumorigenesis. It was recently shown that the exon 5 deletion transcript was significantly elevated in breast tumor tissues compared with normal breast tissue (5, 6). Elevated levels of exon 7 splice transcripts have also been reported in ER+/PR-/pS2- compared to ER+/PR+ tumors (4, 7). It has also been reported that the exon 3- deleted mRNA is reduced in breast tumor tissue compared with normal tissue (8). All these reports suggest that expression of some ER variants is altered in human breast tumors and may contribute to tumorigenesis and tumor progression. Therefore it is highly important to investigate qualitatively and quantitatively the levels and the pattern of ER splice variant expression between normal and neoplastic tissues as well as amongst groups of tumors with different characteristics. Yet there are no specific methods available which can precisely detect the alternatively spliced ER molecules and quantify them.

Conventionally, the ER exon deletion variant transcripts are characterized by co-amplification with the wild type sequences using reverse transcription polymerase chain reaction (RT-PCR) approaches which by virtue of specific primer design are focussed on small regions of the known wild type mRNA. However, there are several practical limitations to this approach such as: 1) threshold of detection- since the wild type transcripts are present in large excess to alternatively spliced molecules, a competitive amplification occurs amongst the wild type and all the alternatively spliced transcripts. Therefore, detection of bands corresponding to alternatively spliced molecules depends upon the relative expression levels of their mRNA species with in the sample. Thus, the spliced transcripts which are expressed at low levels may fall below the threshold of detection. An example of this problem has been observed in our own studies on ER splice variants. When primers which amplify the exons 4-8 of ER cDNA were used, only the most abundant splice variant transcript, the exon 7Δ, was detected but not the less abundant exon 5Δ transcript in any of the breast cancer cell lines examined (9). The exon 5Δ could be, however, detected by amplifying a small region of the wild type between exons 4-6 (10). 2) another disadvantage has been that it cannot distinguish the mRNAs which have multiple deletions in distant exons. For example, an ER transcript which has deletions in exons 2- and 7 cannot be distinguished from transcripts having single deletions in exon 2 or exon 7 by this method. The third disadvantage is that the alternatively spliced molecules which have similar sized exon deletions will generate products which cannot be distinguished from each other by size exclusion gel electrophoresis, and therefore, cannot be characterized.

To circumvent all the above described limitations, we have developed a new approach to characterize the alternatively spliced molecules of ER. Our approach involves the targeted amplification of the alternatively spliced molecules of ER as separate gene populations with out co-amplification of wild type molecules using targeted primers designed for the splice junctions (11). In particular, we designed a number of targeted primers for the splice junctions of ER exons 5Δ-, 7Δ-, 2Δ- and exons 2-3Δ and tested their specific amplification properties using plasmids containing wild type sequences and alternatively spliced sequences. We have identified the targeted primers which exclusively amplify the above splice junctions without amplifying the wild type sequences. In the current study, we have analyzed the ER single- and multiple exon deletion variant transcripts in breast cancer cell lines and tumors by RT PCR using the splice targeted primers. We show here that the splice specific primers amplify both single and several multiple deletion transcripts. We have identified fourteen different ER splice variant transcripts using primers which are specific for the junctions of exon 2Δ, exons 2-3Δ, exon 5Δ and exon 7Δ. Our results also show that the tumor samples analyzed predominantly expressed multiple exon deletion variants where as the breast cancer cell lines expressed both single and multiple exon deletion variant transcripts.

MATERIALS AND METHODS

AmpliTaq PCR core kits and QIAquick gel extraction kits were from QIAGEN Inc., Santa Clara, CA. All the primers used in the current study were synthesized at Gibco-BRL Life Technologies. Reverse transcriptase kits were from Applied Biosystems. The pCR®II-TOPO cloning vector was from Invitrogen. PCR quality water and Tris-EDTA buffer were from Biofluids, Rockville, Md.

Total RNA from breast cancer cell lines and tumor samples. The total RNA samples isolated from the breast cancer cell lines, MCF-7, T47D, ZR-75, LCC1, LCC2, LCC9 and MDA-MB-435 were available from previous studies (12) in R. C's laboratory. The total RNA samples isolated from breast tumor samples were available from previous studies (13) in I. P's laboratory.

Reverse Transcription and PCR. The total RNA was reverse transcribed to cDNA using Maloney 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. The Polymerase Chain Reactions were performed in an automatic thermal cycler (MJ Research) as described

previously (9) in a 25 µl reaction volume containing the cDNA reverse transcribed from 250 ng of total RNA, 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. To amplify the exon deletion variant cDNAs in the tumor samples, PCRs were performed using cDNAs prepared from reverse transcription of 500-750 ng of total RNA. The PCR conditions were initial denaturation for 1 min at 95^0 C followed by 94^0 C for 1 min, annealing for 1 min either at 55^0 C, or 61^0 C or 65^0 C depending on the primer pair used, extension for 2 min at 72^0 C for 40 cycles and final extension for 10 min at 72^0 C.

Targeted primers for the amplification of single- and multiple exon deletion variant cDNAs of ER. In the current study we used the primers which are targeted at the splice junctions of exon 2Δ, exons 2-3Δ, exon 5Δ, and exon 7Δ. They were previously identified and shown not to co-amplify the wild type sequences (11). For amplifying cDNAs which have exon-7 deletions, an anti-sense primer targeted at the splice junction of exons 6/8, ER AX6/8, 5' CTCCATGCCTTTGTTA//CAGAA 3' (positions, exon 8, bp 1801 to 1786 and exon 6, bp 1601-1597), was used together with a sense primer ERS, 5' TGCCCTACTACCTGGAGAACG 3' (position, exon 1, bp 615-635) at an annealing temperature of 55^0 C. For amplifying 5Δ transcripts, an anti-sense primer targeted at the splice junction of exon 4/6, ER AX4/6, 5' ATTTTCCCTGGTTC//CTGGCAC 3' (positions, exon 6, bp 1481-1468 and exon 4, bp 1328-1322), was used with ERS at an annealing temperature of 65^0 C. For amplifying ER exon 2Δ containing transcripts, a sense primer targeted at the splice junction of exons 1/3, ER SX1/3, 5' CGCCGGCATTCTACAG//GACAT

3' (positions, exon 1, bp 669-684, and exon 3, bp 876-880), was used together with an anti-sense primer, ERA , 5' GCACTTCATGCTGTACAGATGC 3' (position, exon 8, bp 1822-1801) at an annealing temperature of 61^0 C. For amplifying ER exons 2-3Δ containing cDNAs, a sense primer targeted at the splice junction of exons 1/4, ER SX1/4, 5' GCCGGCATTCTACAG//GGATAC 3' (positions, exon 1, bp 670-684 and exon 4, bp 993-998), was used with the anti-sense primer, ERA at an annealing temperature of 61^0 C. The sequence and locations of all the primers described here are based on the full length ER cDNA sequence published by Green and others (14).

Detection and sequence analysis of PCR products. In order to detect the PCR amplified products, an aliquot was electrophoresed in 1% agarose gels in Tris-acetate EDTA buffer and detected by ethidium bromide staining. To determine the identity of the PCR amplified ER products, they were electrophoresed in 1.2% agarose gels and purified individually using the QIAquick gel extraction kit. The purified products were cloned into pCR®II-TOPO vector and sequenced by cycle sequencing method on an automated DNA sequencer (carried out at Biopolymer Laboratory, University of Maryland School of Medicine, Baltimore, MD).

RESULTS

We analyzed the ER single- and multiple exon deletion transcripts by RT PCR using primers targeted at the splice junctions of exon 2Δ, exons 2-3Δ, exon 5Δ and exon 7Δ. The partner primers were chosen such that the transcripts were amplified from exons 1-8. This permitted the amplification of not only the single exon deletion transcripts but also those with multiple deletions in distant exons. The analyses were carried out in six ER positive breast cancer cell lines, MCF-7, T47D, ZR-75, LCC1, LCC2, and LCC9 and one ER negative cell line, MDA-MB-435. Three ER positive breast tumor samples were also included to test the applicability of splice targeted primers in analyzing the above transcripts.

Analysis of exon 2Δ transcripts. The exon 2Δ transcript profiles in seven cell lines and three tumors are shown in Figure 1A. The lanes M1 and M2 contain Gibco-BRL 1 kb- and 100 bp ladders respectively. As seen in the figure, the ER positive cell lines, MCF-7, ZR-75, LCC1, LCC2, LCC9 amplified three bands of sizes of about 960 bp, 780 bp and 640 bp. The cell line T47D did not amplify the 960 band but amplified two products which are higher than 960 bp. All the six ER positive cell lines amplified several minor bands ranging from 480-330 bp. Unexpectedly, the ER negative cell line, MDA-MB-435, also amplified 960-, 640- and 480 bp bands and three additional bands which showed lower mobility than 960 bp band. Tumor 3 did not amplify any product. Tumor 2 amplified minor bands at 640- and 480 bp and tumor 1 amplified only the 480 bp as minor band. To determine the identify of the above products, the PCR products from LCC1 cell line were purified from agarose gels,

individually cloned into pCR®II-TOPO and sequenced. The identities of these products are illustrated in Figure 1B. The 960-, 780-, 640-, 480- and 330 bp products were identified as ER transcripts which have deletions in exon 2, exons 2 and 7, exons 2, 5, and 7, exon 2 and exons 4-5, and exon 2 and exons 4-6 respectively. It was also found that the 330 bp band had 20 bps missing in exon 7.

Analysis of exons 2-3Δ transcripts. The PCR product profiles of exon 2-3Δ transcripts in seven cell lines and three tumors are shown in Figure 2A. The lanes M1 and M2 contain Gibco-BRL 1 kb- and 100 bp ladder standards respectively. As seen in Figure 2A, all the six ER positive cell lines amplified three products which have approximate sizes of 840 bp, 660 bp and 520 bp. Two minor bands in between 840 bp and 660 bp bands are also seen. The ER negative cell line, MDA-MB-435, generated a minor product which is slightly bigger than the 840 bp product. The amounts and ratios of these three products in the six cell lines appear to be different. The cell line MCF-7 generated equal amounts of 840 bp and 660 bp bands and a minor 520 bp band. The cell line T47D generated equal amounts of all the three products. The cell lines ZR-75, LCC1, LCC2 and LCC9 generated more amounts of 840- and 660 bp products than MCF-7 and T47D. To determine the identity of 840-, 660- and 520 bp products, the PCR products from LCC1 were individually cloned into pCR®II-TOPO and sequenced. The identities of these products are illustrated in Figure 2B. The 840-, 660-, and 520 bp products were identified as ER transcripts which have deletions in exons 2-3, exons 2-3 and 7, and exons 2-3, 5 and 7 respectively. Tumor 1 generated three bands of which two of them corresponded to exons 2-3Δ, and exons 2-3Δ and 7Δ. The third band

showed slightly higher mobility than the exons 2-3Δ, 5Δ and 7Δ product. Tumor 2 amplified two bands of approximate sizes 700 bp and 550 bp which are slightly higher than the exons 2-3Δ and 7Δ, and exons 2-3Δ, 5Δ and 7Δ products. The third tumor generated only the exons 2-3Δ and exon 7Δ product.

Analysis of exon 5Δ splice variant cDNAs. The profiles of exon 5Δ transcripts in seven cell lines and three tumors are shown in Figure 3A. The lanes M1 and M2 contain Gibco-BRL 1 kb- and 100 bp ladders respectively. As seen in the figure, all the ER positive breast cancer cell lines except MCF-7 amplified one major product and two minor products of approximate sizes, 730 bp, 540 bp and 420 bp respectively. The MCF-7 and ER negative cell line, MDA-MB-435, did not generate any products. To determine the identify of 730-, 540- and 420 bp products, the PCR products from ZR-75 were individually cloned into pCR®II-TOPO and sequenced. The 730-, 540- and 420 bp products were identified as ER transcripts which have deletions in exon 5, exons 5 and 2, and exons 5 and 2-3 respectively (Figure 3B). The three tumor samples analyzed gave very distinct products. The tumor 1 amplified all the three exon 5Δ, exons 5Δ and 2Δ, and exons 5Δ and 2-3Δ products and an additional product in between exon 5Δ and exons 5Δ and 2Δ products. Tumor 2 amplified one product in between exon 5Δ and exons 5Δ and 2Δ products similar to tumor 1 and two products of approximate sizes 500 bp and 350 bp. Tumor 3 amplified only the 500 bp and 350 bp products. Neither tumor 2 or 3 amplified the major single deletion product.

Analysis of exon 7Δ splice variant cDNAs. The profiles of exon 7Δ cDNAs in seven

cell lines and three tumors are shown in Figure 4A. The lanes M1 and M2 contain Gibco-BRL 1 kb- and 100 bp ladders respectively. As seen in figure 4A, all the six ER positive breast cancer cell lines generated a major 1 kb band and a minor band of size approximately 665 bp. The cell line LCC2 generated additional two minor bands of sizes 560 bp and 410 bp. The cell line LCC1 also generated 560 bp minor band and LCC9 generated the 410 bp minor band. To determine the identities of 1kb-, 665-, 560- and 410 bp products, the PCR products from LCC1 were cloned and sequenced. They were identified (Figure 4B) as ER transcripts which have deletions in exon 7, exons 7 and 4, exon 7 and exons 3-4, and exon 7 and exons 3-5 respectively. The three tumor samples analyzed gave very distinct products. The tumor 1 amplified all the above four products similar to LCC1 cell line. However, the exons 7Δ and 4Δ product is seen as a major band and the single deletion 1000 bp product as a minor band. Tumor 2 gave similar profile as tumor 1 and the tumor 3 did not amplify any product. Tumor 3 was previously shown not to have any exon 7Δ transcript when analyzed by co-amplification with wild type sequences between exons 4-8 (13).

DISCUSSION

In the current study we applied a novel approach to specifically amplify a particular category of alternatively spliced ER molecules from a pool of other types of alternatively spliced- and wild type ER genes using primers which anneal to the spliced junctions. We used the primers targeted at the splice junctions of exon 2Δ, exons 2-3Δ, exon 5Δ and exon 7Δ. The results described here show that each splice specific primer amplified not only the single

exon deleted transcript but also a number of related cDNAs which have deletions in various combinations of exons. None of the splice specific primers amplified the wild type ER sequences. The four specific primers amplified a total of fourteen different transcripts out of which eleven of them had double or multiple exon deletions. Majority of the multiple deletion transcripts described here were not reported until now. Our results also show that eight of the fourteen transcripts identified contained exon 7Δ suggesting that it is the most frequently deleted exon. Examination of the products amplified by exon 2Δ and exons 2-3Δ specific primers indicated a similar trend in that the second and the third products contained exon 7Δ, and exons 5Δ and 7Δ respectively (Figures 1B and 2B). These results also suggest that deletion of exon 5 is preceded by exon 7 deletion. Interestingly, the exon 7Δ specific primer did not recognize any of the above four products, instead, it preferentially amplified exon 4Δ containing transcripts (Figure 4B). The factor(s) influencing this preferential amplification appears to be other than the size and amounts of the transcripts since the exon 2Δ and exons 2-3Δ specific primers amplified products are comparable to those amplified with exon 7Δ specific primer in these properties. The results presented in Figures 1-4 also reveled that none of the ten multiple exon deletion transcripts contained single exon 3Δ, instead it is always associated with either exon 2 or exon 4 deletion (Figures 2B and 4B). The only transcript which is recognized by two different specific primers is the one containing exons 2-3Δ and 5Δ (Figures 2B and 3B).

The exon deletion transcript analysis in tumor samples showed very interesting findings. In the cell lines, the most abundant product each specific primer amplified is the

single deletion product and the second most abundant product is the double exon deleted transcript in the case of exon 2Δ, exon 5Δ and exon 7Δ and in the case of exons 2-3Δ specific primer they are triple- and quadruple exon deleted transcripts. However, different primers gave different results in tumor samples. When three tumors were analyzed with exon 7Δ specific primer, two tumors showed the presence of four transcripts similar to cell lines. However, the ratio of each transcript appears to be different compared to the cell lines. In the case of exon 2Δ transcripts, only two tumors showed the presence of minor bands and none of them amplified the single- or double deletion products. When analyzed for the exons 2-3Δ containing transcripts, only one of the tumors generated 2-3Δ product and the other two amplified only the multiple deletion products which also appear to have other modifications such as base pair insertions/deletions. Similar observations were made when analyzed for exon 5Δ containing transcripts. These results suggest that the patterns and levels of ER variants undergo extensive alterations in tumor tissues. They also suggest a progressive loss of exons.

The results presented in the current study clearly demonstrate the efficacy of the targeted primers designed at alternate splice junctions for the analysis of various ER splice variants in both cell lines and clinical samples. We believe that the new approach described here will highly facilitate in 1) delineating the functional roles of ER exon deletion variants in estrogen induced signal transduction processes, 2) analyzing the changes in the profiles of splice variants in the tumor tissues compared to normal tissues, 3) evaluating their role in tumor progression and loss of hormone dependency, 4) predicting prognosis and response to

anti-hormone therapy and finally 5) developing tissue specific synthetic estrogens and anti-estrogens.

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FIGURE LEGENDS

Figure 1. Analysis of ER exon 2Δ transcript profiles in breast cancer cell lines and tumors by RT PCR using a 2Δ specific primer. The ER exon 2Δ transcripts were analyzed using the specific sense primer, ER SX1/3, and an anti-sense primer ERA in exon 8 under the experimental conditions described in materials and methods. To determine the identity of various PCR products, the products amplified from the cell line, LCC1, were isolated from agarose gels, cloned into pCR®II-TOPO vector and sequenced.

Panel A shows the PCR products amplified from breast cancer cell lines, MCF-7, T47D, ZR-75, LCC1, LCC2, LCC9 and MDA-MB-435 and the tumors 1, 2, and 3. Lanes M1 and M2 contain the Gibco-BRL 1 kb- and 100 bp ladders respectively.

Panel B illustrates the identity of the PCR products as determined by sequence analysis. Lanes M have 100 bp ladders.

Figure 2. Analysis of exons 2-3Δ transcript profiles in breast cancer cell lines and tumors by RT PCR using 2-3Δ specific primer. The exon 2-3Δ transcripts were analyzed using the specific sense primer, ER SX1/4, and an anti-sense primer ERA under the experimental conditions described in materials and methods. To determine the identity of various PCR products, the products amplified from the cell line, LCC1, were isolated from the agarose gels, cloned into pCR®II-TOPO vector and sequenced. In both A and B panels, lanes M1 and M2 contain the Gibco-BRL 1 kb- and 100 bp ladders respectively.

Panel A shows the PCR products generated from the breast cancer cell lines, MCF-7,

T47D, ZR-75, LCC1, LCC2, LCC9 and MDA-MB-435 and the tumors 1, 2, and 3.

Panel B illustrates the identity of the PCR products as determined by sequence analysis.

Figure 3. Analysis of exon 5Δ transcript profiles in breast cancer cell lines and tumors by RT PCR using 5Δ specific primer. The exon 5Δ transcripts were analyzed using the specific anti-sense primer, ER AX4/6, and a sense primer ERS in exon 1 under the experimental conditions described in materials and methods. To determine the identity of various PCR products, the products amplified from the cell line, ZR-75, were isolated from agarose gels, cloned into pCR®II-TOPO vector and sequenced.

Panel A shows the PCR products generated from breast cancer cell lines, MCF-7, T47D, ZR-75, LCC1, LCC2, LCC9 and MDA-MB-435 and the tumors 1, 2, and 3. Lanes M1 and M2 contain the GiBco-BRL 1 kb- and 100 bp ladders respectively.

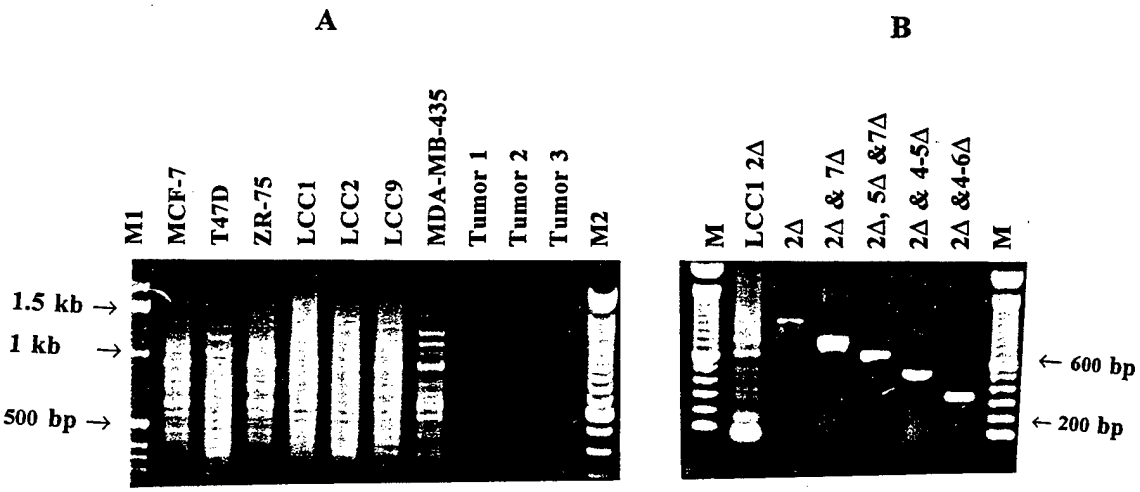
Panel B illustrates the identity of the PCR products as determined by sequence analysis. Lane M has the Gibco-BRL 100 bp ladder.

Figure 4. Analysis of exon 7Δ transcript profiles in breast cancer cell lines and tumors by RT PCR using 7Δ specific primer. The exon 7Δ transcripts were analyzed using the specific anti-sense primer, ER AX6/8, and a sense primer ERS under the experimental conditions described in materials and methods. To determine the identity of various PCR products, the products amplified from the cell line, LCC1, were isolated from agarose gels, cloned into pCR®II-TOPO vector and sequenced.

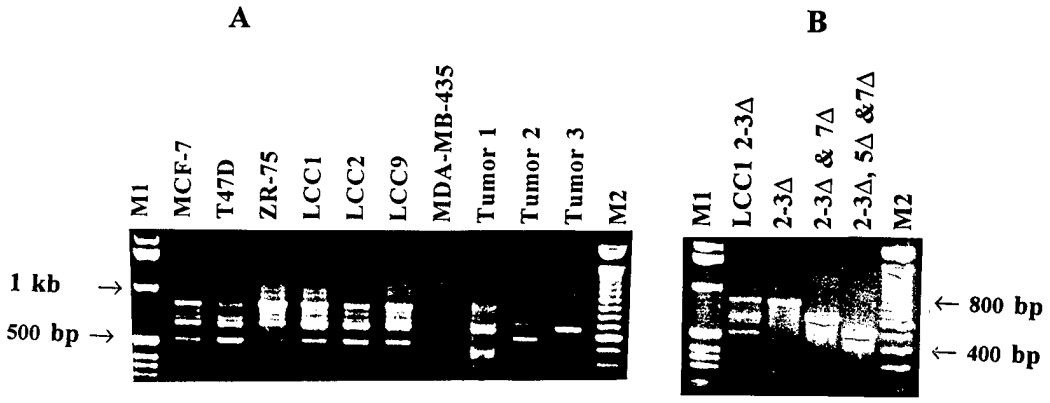
Panel A shows the PCR products generated from breast cancer cell lines, MCF-7, T47D, ZR-75, LCC1, LCC2, LCC9 and MDA-MB-435 and the tumors 1, 2, and 3. Lanes M1 and M2 contain the Gibco-BRL 1 kb- and 100 bp ladders respectively.

Panel B illustrates the identity of the PCR products as determined by sequence analysis. Lane M has the Gibco-BRL 100 bp ladder.

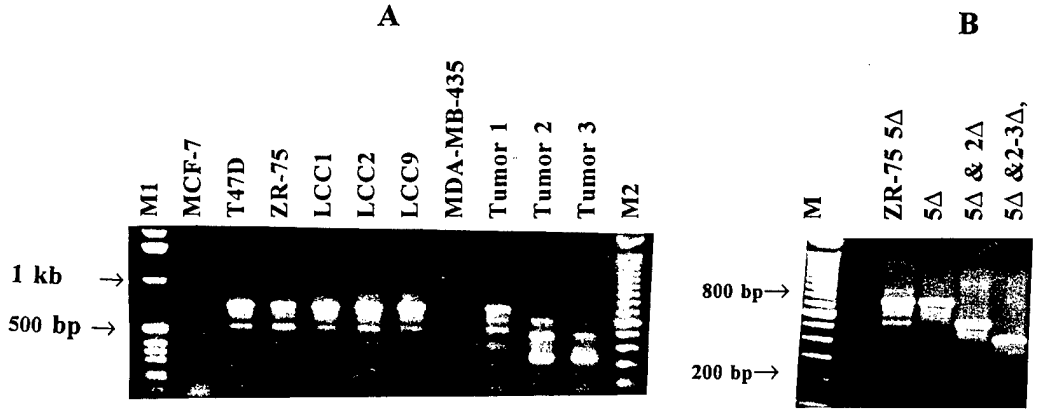
ER 2Δ SPLICE VARIANTS



ER 2-3Δ SPLICE VARIANTS



ER 5Δ SPLICE VARIANTS



ER 7Δ SPLICE VARIANTS