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13. ABSTRACT *(Maximum 200 words)*

We have identified in a range of human breast cancer biopsy samples, several known and unknown estrogen receptor (ER) variant mRNAs as well as some ER mRNAs containing inserted nucleotide sequences. The variant ER mRNAs are likely generated by alternative splicing mechanisms, while the inserted ER mRNAs are likely to be generated from a mutated ER allele in the tumor cells. All types of variant ER mRNAs so far identified, could be detected in normal human mammary tissue. Interestingly, the expression of some of these variant ER mRNAs was significantly higher in breast tumors than in normal breast tissue. Moreover, the expression of at least one ER mRNA variant relative to the wild-type ER mRNA was significantly increased in breast tumors with characteristics of poor prognosis and lack of sensitivity to endocrine therapies. Our data suggest that the mechanisms generating ER variant mRNAs exist in normal breast tissue and may be deregulated in breast cancer tissues. Further, the data support and are consistent with our working hypothesis that the expression and/or altered expression of variant/abnormal ER in human breast cancer is one mechanism associated with the development of endocrine resistance and the progression from hormone dependence to independence.

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4. TABLE OF CONTENTS.

5. INTRODUCTION:

The goal of this research is to address the role of variant/abnormal estrogen receptor (ER) expression in the progression of human breast cancers from hormone dependence to independence. The progression of breast cancer from hormone dependence to independence is a clinically significant problem since it limits the effectiveness of the relatively non-toxic hormonal therapies such as antiestrogens and progestins[1]. The hormone-dependent phenotype is characterized by the presence of ER in the breast tumor, but only 50% of receptor positive breast tumors respond to endocrine therapies and of those which initially respond a significant proportion will eventually develop resistance to these therapies. Furthermore, the development of resistance to endocrine therapy occurs despite the continued expression of ER in the tumor, in at least 50% of cases. It is the molecular mechanisms of this form of resistance i.e. the steroid receptor positive/hormone resistant human breast tumors, that this research proposal addresses. Elucidation of these mechanisms will provide information necessary either to prevent the occurrence of hormone resistance, reverse it or develop new treatments for the resistant tumors. As well, novel treatment response markers in human breast tumors are likely to be identified. Although multiple mechanisms are likely to be involved in hormone resistance and progression to hormone independence[1] in human breast cancer, this grant proposal focuses on one possible mechanism: the involvement of variant and/or abnormal forms of ER.

The hypothesis to be tested is that the expression and/or altered expression of variant/abnormal ER in human breast cancer is one mechanism associated with the development of endocrine resistance and the progression from hormone dependence to independence.

The specific aims to address this hypothesis are:

1. To systematically investigate alterations that occur in the ER mRNA in human breast cancers.
2. To characterize structurally and functionally those abnormal ER mRNAs occurring most frequently and determine their involvement in the development of hormone independence and progression in HBC.
3. To develop specific tools to investigate the expression of the corresponding proteins that may be translated from altered or variant ER mRNAs.
4. To assess the biological significance of ER variants in human breast cancer by determining the relationship between the level of expression of ER variants in human breast cancer biopsies and the expression of the normal ER, known estrogen responsive genes, histopathological parameters and other known prognostic factors .

6. BODY:

The first task in the "Statement of Work" was to systematically investigate alterations that occur in the estrogen receptor (ER) mRNA in human breast tumors. Figure 1 (in reference 2, see

appendix 1) shows the approach taken to investigate alterations in the E-domain of the human ER mRNA. Reverse transcription and polymerase chain reaction amplication (RT-PCR) was carried out on RNA isolated from 212 individual human breast tumors. Twenty ul of each PCR reaction were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining. Using these criteria altered sized RT-PCR products could be detected in some human breast biopsy samples. Using primer set 1, a major 483 bp fragment was detected in 86 % of the breast tumors analyzed (Figure 2 in reference 2, see appendix 1). This fragment is the expected RT-PCR product for the normal, wild-type ER mRNA. Fourteen percent of tumors contained little or no detectable ethidium bromide staining bands, and were classified as ER mRNA negative. A faint 344 bp band was detected after ethidium bromide staining in 9% of the tumors analyzed (see 4th and 7th samples in second panel Figure 2, in reference 2, see appendix 1). This band is the size expected for an RT-PCR product derived from a previously identified exon 5 deleted-ER transcript[3]. Southern blotting and hybridization with radiolabelled human ER cDNA indicated that both the 483 bp and the 344 bp PCR products specifically hybridized with human ER cDNA (Figure 3, in reference 2, see appendix 1). Further, Southern blotting and hybridization with radiolabelled human ER cDNA enhanced the frequency with which the exon 5 deleted ER transcript was detected (see Figure 3 in reference 2, see appendix 1), from 9 to 30%. A previously identified exon 7 deleted ER mRNA[4] was also identified in all tumors which expressed the wild-type ER mRNA (Figure 4 in reference 2, see appendix 1).

Interestingly, in several breast tumor samples an RT-PCR product larger than that expected for the wild-type ER mRNA was clearly detected by ethidium bromide staining (Figure 2 in reference 2, see appendix 1). These transcripts were identified as larger than wild-type ER mRNA RT-PCR products in 9.4% of 212 human breast tumors analyzed. The data suggest nucleotide insertions are present in ER mRNA of some breast tumors. Cloning and sequencing of the larger RT-PCR products identified three different types: a complete duplication of exon 6 occurring in 7.5 % of tumors; a complete duplication of both exons 3 and 4 occurring in 1 tumor; and a 69 nucleotide insertion between exons 5 and 6 occurring in 3 tumors . Open reading frame analysis suggested that exon 6 duplicated transcripts encoded a 51.4 kDa ER-like protein truncated just after exon 6 sequences; the exon 3 and 4 duplicated transcript encoded a 83.3 kDa protein containing duplication of ER amino acid residues encoded by exons 3 and 4; the 69 nucleotide insertion was inframe, adding 23 novel amino acid residues between residues 412 and 413 of the normal ER protein to produce a 68.8 kDa protein (Figure 8 in reference 2, see appendix 1). It is unknown if these novel ER-like mRNAs are stably translated *in vivo*. Any resulting protein would be structurally altered, however, possibly resulting in altered function. Experiments are in progress to determine potential function of these proteins.

Our accumulated data suggest that while the generation of the exon deleted ER variant mRNAs and the truncated clone 4 type ER variant mRNA[5] is likely to occur via an alternative splicing mechanism[6] the generation of the inserted and exon-duplicated ER mRNAs is likely to occur due to a mutant ER allele in the breast tumor cells[7].

RT-PCR analysis of other regions of the ER mRNA (Figure 1A in reference 4, see appendix 3) identified previously known ER variant mRNAs deleted in exon 2, exon 3 and a novel exon 2-3 deleted variant[8,9]. Since several different exon deleted ER variant mRNAs are expressed in any one tumor, we now need to consider all exon deleted variants in any one tumor. Therefore, we are developing a competitive RT-PCR approach using primers at each end of the ER coding region, in order to examine the relationship of total ER deleted variants to expression of the normal ER, known estrogen responsive genes, histopathological parameters and other known prognostic factors.

Task 2 in 'Statement of Work' (SOW), was to develop a quantitative RT-PCR approach for measuring selected ER mRNA variants in RNA isolated from microdissected regions of human breast tissue[10]. This approach was necessary to undertake task 5 of the SOW, which is to assess the biological significance of ER variant mRNA in human breast cancer progression. Several different assays were developed[8,10,11,12,13], each with different advantages and limitations. The type of assay used, was dictated by the the question asked and practical issues.

Using assays developed under task 2, we have identified several variant ER mRNA in normal human mammary tissues [8,11,12]. These include the clone 4 ER truncated variant and variants deleted in exon 2, exon 3, exons 2-3, exon 5 or exon 7. The next question addressed was: Is the level of expression of any of these variants different between normal and neoplastic breast tissue? A semi-quantitiative competitive RT-PCR approach was used to determine the relative expression of exon 5- and exon 7- deleted variants to the wild-type ER mRNA in nine normal breast tissues and 19 ER positive breast tumor tissues. The expression of exon 5-deleted ER variant relative to the wild-type ER mRNA was significantly lower ($P < 0.001$) in normal tissue than in tumor tissue (Figure 4 in reference 8, see appendix 3). A similar trend was noted for expression of the exon-7-deleted ER variant mRNA (Figure 4 in reference 8, see appendix 3), but the difference did not achieve statistical significance ($P = 0.476$).

In order to address the question of the level of expression of the truncated clone 4 ER variant in normal and neoplastic tissue, an RT-triple primer-PCR approach was developed[11]. The relative level of expression of clone 4 mRNA to the wild-type ER mRNA was compared in frozen sections of normal human breast tissue (8 samples) and human breast tumors with characteristics of good prognosis (10 samples). The expression of clone 4 variant relative to wild-type ER mRNA was significantly lower ($P = 0.03$) in normal tissue than in tumor tissue (Figure 5 in reference 11, see appendix 6). Previously, using an RNAase protection assay, we had determined that the

expression of clone 4 variant relative to wild-type ER mRNA was significantly higher in those tumors with characteristics of poor prognosis ($P = 0.0004$) and lack of sensitivity to endocrine therapy ($P = 0.011$)[14]. Together these data suggest that the mechanisms generating ER variant mRNAs exist in normal breast tissue and may be deregulated in breast cancer tissues. Further, the data support and are consistent with our working hypothesis that the expression and/or altered expression of variant/abnormal ER in human breast cancer is one mechanism associated with the development of endocrine resistance and the progression from hormone dependence to independence.

Task 3 in the SOW has been initiated. Eukaryotic expression vectors for several of the variant and mutant ER mRNAs have been constructed[2,5]. Transient transfection of the exon duplicated and inserted ER cDNA is being used to determine the subcellular distribution of the resulting proteins; if any of the resulting proteins bind ligand (estrogenic or antiestrogenic), display ligand independent transactivation activity, bind to EREs and inhibit the activity of the wild-type receptor. Clone 4 cDNA under the control of a CMV promoter has been stably transfected into MCF-7 cells together with the neomycin resistance gene. Several colonies resistant to G418 have been isolated and grown up. Analysis for over-expression of the transgene has been initiated. When several independent clones have been identified with overexpression of the transgene, these will be tested, under tissue culture conditions, for their responsiveness to estrogen, monohydroxytamoxifen, ICI 164 384 and progestins.

7. CONCLUSIONS.

Our data suggest that both variant and mutant ER mRNAs exist in human breast cancers. Further, several different types of variant ER mRNAs can be expressed in any one tumor. Therefore, a need exists to develop methods to consider them as a whole in human breast cancer, in order to examine the relationship of total ER variant expression to expression of the normal ER, known estrogen responsive genes, histopathological parameters and other known prognostic factors.

With respect to investigating the biological significance of individual ER variants in human breast cancer, we have shown that several variants can be detected in normal human breast tissue. Moreover at least two of these variants are more highly expressed in breast tumors than in normal mammary tissue. As well we have shown that the expression of one variant ER mRNA (clone 4 truncated variant) relative to wild-type ER mRNA was significantly higher in those tumors with characteristics of poor prognosis and lack of sensitivity to endocrine therapy.

Our data suggest that the mechanisms generating ER variant mRNAs exist in normal breast tissue and may be deregulated in breast cancer tissues. Further, the data support and are consistent with our working hypothesis that the expression and/or altered expression of variant/abnormal ER

in human breast cancer is one mechanism associated with the development of endocrine resistance and the progression from hormone dependence to independence.

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APPENDIX 1

Novel Mutations in the Hormone Binding Domain of the Estrogen Receptor mRNA in Human Breast Cancers *in vivo*.

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INTRODUCTION

The natural history of human breast cancer appears to be a progression from hormone dependence to independence and the development of resistance to endocrine therapies (1). This often occurs despite the continued expression of the estrogen receptor (2). It has been suggested that one mechanism associated with this progression, is the expression, or altered expression of mutant and/or variant forms of the estrogen receptor (ER) protein (3). Support for this hypothesis has been derived from studies over the last decade which have identified a role for mutant or variant steroid hormone receptors in other human diseases (4-6).

Several abnormal or variant forms of ER mRNA species have been recently identified in some breast cancer cell lines and human breast cancer biopsy samples (7-13). These include truncated transcripts and transcripts containing precise exon deletions that potentially could encode ER-like proteins with altered function (7, 9, 10). Moreover, recent studies using gel-shift/antibody supershift and Western blot analyses suggest that ER-like proteins corresponding to some of the variant ER-like transcripts may exist (14 -16). Whether these are the only alterations in the ER to be found in human breast cancers, and the exact frequency of occurrence of abnormal ER mRNAs are important unanswered questions. Such information is essential to understanding the significance of and possible involvement of expression of these abnormal/variant ER molecules in the progression of human breast cancer from hormone dependence to independence.

In undertaking a detailed systematic study of the type and frequency of occurrence of abnormal/variant ER mRNAs in human breast tumors we have now identified novel forms of ER-like mRNAs which contain exon duplications or other inserted sequences.

RESULTS

Figure 1 shows the approach taken to investigate alterations in the E-domain of the human ER mRNA. RT-PCR was carried out on RNA isolated from 212 individual human breast tumors. Twenty ul of each PCR reaction were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining. Using these criteria altered sized RT-PCR

PCR product expected from the wild-type ER mRNA is 668 bp, and this fragment was detected in several tumors. However, an abundant smaller 484 bp fragment was seen in all cases where the wild-type fragment was detected. This fragment corresponded in size to the previously identified exon 7 deleted ER-like transcript (7). Both PCR fragments hybridized with a radiolabelled human ER cDNA following Southern blotting (see panel 3 in Figure 4).

In order to characterize the apparent insertions in the ER mRNA, the larger RT-PCR products were cloned into TA-cloning vectors and their nucleotide sequence determined. The nucleotide sequence of the 616 bp fragment revealed that this transcript contained an exact exon 6 duplication (Figure 5A). Sequencing the 935 bp fragment indicated that the transcript from which it was derived contained exon 4 sequences followed by exon 3 sequences, followed again by exon 4 sequences (Figure 5B). It was suspected that this latter transcript may have a complete duplication of exons 3 and 4. Using the novel exon4/exon3 boundary sequences found in this transcript a lower (antisense) primer was designed to match an upper (sense) primer located in exon 2 of the human ER. If an ER-like transcript duplicated in exons 3 and 4 was present in the RNA isolated from the tumor in which the original 925 bp product had been detected and cloned (Figure 6, panel 1, lane 7), this primer set (Figure 6 primer set 34^2 U and L) would result in a 521 bp RT-PCR product. Using these primers an exon 3 and 4 duplicated ER-like transcript was found at high abundance in only 1 tumor (Figure 6, panel 1, lane 7).

A similar approach (Figure 6, primers 6 x 2 U and L) was used to confirm the exon 6 duplicated ER-like transcript (Figure 6, panel 1, lane 1). Although the expected 443 bp RT-PCR fragment was detected by this approach, other bands were also detected. To further confirm the existence of an exon 6 duplicated ER-like transcript, another set of primers (Figure 6, 6^2 U and L) was designed which would only detect an exon 6 duplicated transcript. These primers would be unable to amplify the wild-type transcript (Figure 6, 6^2 U and L), since the primers would face away from each other on the wild-type transcript. However, if an exon 6 duplicated ER-like mRNA was present a 125 bp fragment would be

predicts another 380 amino acid residues, which contain a complete duplication of the amino acid residues encoded by exons 3 and 4 followed by the normal wild-type ER amino acid residues encoded by exons 5 through to 8 . The unique 69 bp insertion is inframe and codes for 23 novel amino acids inserted between residues 412 and 413 of the normal ER protein. The predicted molecular mass of the protein is 68.8 kDa.

Expression vectors for each of these three different transcripts were constructed and *in vitro* transcription and translation analyses followed by SDS/PAGE showed that the major protein produced from the exon 6 duplicated transcripts was approximately 51 kDa (Figure 9); the major protein from the exon 3/4 duplication was approximately 82 kDa (Figure 9) and the major protein from the unique 69 bp inserted transcript was approximately 69 kDa (Figure 9).

DISCUSSION

There is a large body of molecular data supporting the potential existence of variant and/or abnormal forms of estrogen receptors in some human breast cancer biopsy samples. Several variant ER-like mRNA molecules have been isolated from some human breast cancer biopsy samples (7-10). The most commonly occurring variant ER-like mRNAs fall into two main groups: 1) the truncated ER-like transcripts (10, 17, 18), which consist of various combinations of exons 1, 2 and 3 of the normal ER mRNA followed by sequences that are not found in the wild-type ER mRNA, and 2) the exon-deleted ER-like transcripts (7, 9, 12, 13), in which precise exon deletions have occurred. ER-like transcripts containing deletions of exon 2, exon 3, exon 4, exon 5 and exon 7 have been reported (7, 9, 12, 13, 19, 20). Simultaneous deletions of both 2 and 3 exons have also recently been reported (19, 20). We now report the occurrence of novel ER-like mRNAs which contain either complete exon duplications or novel sequence insertions. This type of alteration seems to occur in at least 9% of the tumors that we have examined.

The exon duplicated ER-like transcripts appear to be novel and have not been reported in human breast cancer biopsy samples previously. Interestingly, the identification

like mRNAs in any of the normal mammary or uterine tissues that we have examined so far (20, unpublished data). It is likely therefore, that the exon-duplicated and inserted ER-like mRNAs are transcribed from an ER gene which has been altered in some human breast cancers. The exon-duplicated and inserted ER-like mRNAs were present however together with an apparently normal wild-type ER mRNA. We have not found any tumor in which only the inserted or exon-duplicated ER mRNA was present. This suggests that only one allele of the ER gene in these tumors is affected, and/or that two populations of tumor cells exist: one in which only the wild-type gene is expressed and one in which the mutated gene is expressed. Interestingly, an MCF-7 sub-line in which an exon 6 and 7 duplicated ER-like transcript is associated with the expression of an 80 kDa ER-immunoreactive protein, also expresses the wild-type 66 kDa protein (21, 22). These cells have been subjected to several rounds of limiting dilution cloning and the cells still express both ER-like proteins. Such data suggest that the cells are not a mixed population of cells i.e. one expressing the 66 kDa protein and the other expressing the 80 kDa protein, but that both ER immunoreactive proteins are expressed together in each cell (21). Further, such data provide support for the translation *in vivo* of these mutant ER transcripts.

If the exon 6 duplicated ER-like mRNA were translated *in vivo* the predicted protein would be identical with the wild-type ER protein up until amino acid residue 457 (24) followed by 5 additional unique amino acids. This predicts for a protein which would contain the A/B and C domains of the wild-type ER but would be truncated in the mid-E domain. Deletion and site-directed mutagenesis data suggest that such a protein would not bind estradiol (25-30). Further, an important dimerization interface and the ligand dependent TAF-2 activity would be missing in the protein predicted from the exon 6 duplicated ER-like mRNA. However, a weaker constitutively active dimerization domain present in the DNA binding domain, as well as the constitutive nuclear localization signal present in exon 4 of the wild-type ER (31) and the ligand independent TAF-1 activity in the A/B domain would still be present (32).

insertion of novel sequence not normally found in the wild-type ER mRNA. It is likely that the mechanism by which these transcripts are generated is different to that involved in generation of the truncated and exon deleted ER transcripts. It is speculated that the expression of these mutant ER-like proteins may alter the ER signal transduction pathway in those tumors which express them, thereby contributing to hormonal progression *in vivo*.

MATERIALS AND METHODS

RNA Isolation: Total RNA was isolated from human breast cancer biopsy samples as previously described (10, 34). The integrity of the RNA was confirmed by denaturing gel electrophoresis as previously described (34).

RT-PCR: Total RNA (1 ug per reaction), denatured at 65°C for 3 minutes, was reverse transcribed in a final volume of 50 ul made 1 X with the reaction buffer supplied with the M-MLV reverse transcriptase (5 X: 0.25 M Tris-HCl (pH 8.3), 0.375 M KCl, 15 mM $MgCl_2$, 50 mM DTT), using M-MLV reverse transcriptase (200 units/reaction, Gibco/BRL), 0.01 M DTT, and either an ER specific reverse transcription primer (100 ng/reaction, 5'- GAA CTG AGC AAG CAA ATG AAT GG-3') or random hexamers (final concentration 0.5 uM, Gibco/BRL). The reaction was allowed to proceed for 60 minutes at 37°C and then was terminated by heating at 90°C for 5 minutes. 1 ul of this reaction was amplified by PCR for 30 cycles of 1 min 94°C, 1 min 60°C , and 1 min 72°C . The reaction volume was 50 ul containing 0.01 M Tris-HCl (pH 8.3), 0.05M KCl, 2.5 mM $MgCl_2$, 0.2 mg/ml gelatin, 1 mM dNTPs, 1 ul of each primer (200 ng/ul) and 1 unit of Taq Polymerase (Gibco/BRL). 20 ul of the PCR reactions were electrophoresed in agarose gels (1 - 2% depending on expected sizes of products) and visualized by ethidium bromide staining. The primer sequences are as follows:

a) Set 1: 1 upper (sense) 5' - CAG GGG TGA AGT GGG GTC TGC TG - 3' (priming site in exon 4); 1 lower (antisense) 5' - ATG CGG AAC CGA GAT GAT GTA GC - 3' (priming site in exon 6)

WI). The products were analysed under reducing conditions by SDS/polyacrylamide (7.5 %) gel electrophoresis followed by autoradiography.

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LEGENDS TO FIGURES

FIGURE 1. RT-PCR analysis of the E domain of the human ER mRNA.

Schematic diagram of the approach taken to investigate alterations in the E-domain of the human ER mRNA using RT-PCR. U, upper (sense) PCR primer; L, lower (antisense) PCR primer.

FIGURE 2. RT-PCR analysis using primer set 1 of RNA isolated from human breast tumors.

RT-PCR products obtained using PCR primer set 1. Twenty ul of the resulting PCR products were size separated by electrophoresis on agarose gels. The RT-PCR products were visualized by ethidium bromide staining. M, phi X 174 RF DNA/*Hae* III fragments were used as molecular size markers; the 603 and 872 bp fragments are shown on the left hand side.

Bottom panel shows localization of the PCR primers used in this analysis.

FIGURE 3. Specific hybridization of the human ER cDNA with RT-PCR products from human breast cancer samples.

A Southern blot of 1-5 ul of RT-PCR products from selected human breast tumors shown in Figure 2. The DNA was transferred to nitrocellulose membranes and hybridized with radiolabelled human ER cDNA. Estimated molecular sizes of the specifically hybridizing products are shown on the left hand side of the figure.

Bottom panel shows localization of the PCR primers used in this analysis.

FIGURE 4. RT-PCR products obtained using primer set 4 for the human ER mRNA.

The top two panels show ethidium bromide stained RT-PCR products from several human breast cancer samples using primer set 4. Twenty ul of RT-PCR products were size separated by electrophoresis on agarose gels. M, phi X 174 RF DNA/*Hae* III fragments were used as molecular size markers; the 603 and 872 bp fragment are shown on the left hand side.

The third panel shows a Southern blot of 1-5 ul of RT-PCR products from selected human breast tumors present in the top panels. The DNA was transferred to nitrocellulose membranes and hybridized with radiolabelled human ER cDNA. Estimated molecular sizes of the specifically hybridizing products are shown on the left hand side of the third panel.

Bottom panel shows localization of the PCR primers used in this analysis.

FIGURE 7. Nucleotide sequence of the 69 nucleotide insertion between exon 5 and 6 of the human ER mRNA.

Nucleotide sequence across the novel boundary of the ER-like mRNA which contained a 69 nucleotide insertion precisely between exons 5 and 6 of the wild-type ER mRNA. The 552 bp RT-PCR product was cloned into a TA cloning vector and its nucleotide sequence determined.

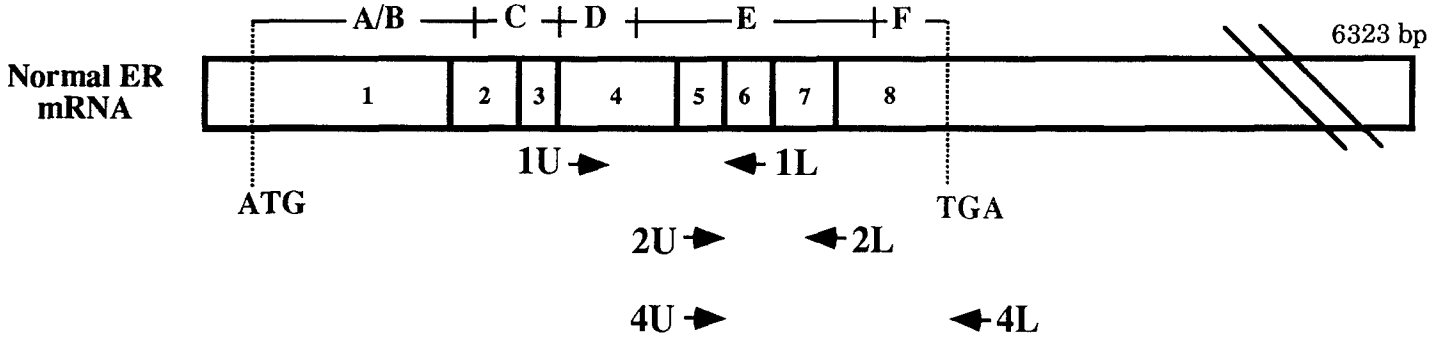
FIGURE 8. Open reading frame analysis of the exon duplicated and inserted ER-like mRNAs.

Schematic representation of the predicted open reading frames for each of the three different larger ER-like mRNAs identified in human breast cancer biopsy samples.

FIGURE 9. *In vitro* expression products of the exon duplicated and inserted ER-like mRNAs.

In vitro transcription and translation analyses of expression vectors for each of the three larger sized ER-like mRNAs. The ^{35}S-labelled proteins were analyzed under reducing conditions by SDS/PAGE(7.5%). An autoradiogram of the results is shown: lane 1, wild-type human ER expression vector obtained from Dr S Tsai (Baylor College of Medicine); lane 2, exon 6 duplicated expression vector; lane 3, exon 3/4 duplicated expression vector; lane 4, 69 nucleotide insertion expression vector; lane 5, expression vector for wild-type ER (HEGO, obtained from Dr P Chambon, INSERM, Strausberg); lane 6, wild-type human ER expression vector obtained from Dr S Tsai (Baylor College of Medicine).

FIGURE 1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C
D
E
F
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Normal ER mRNA
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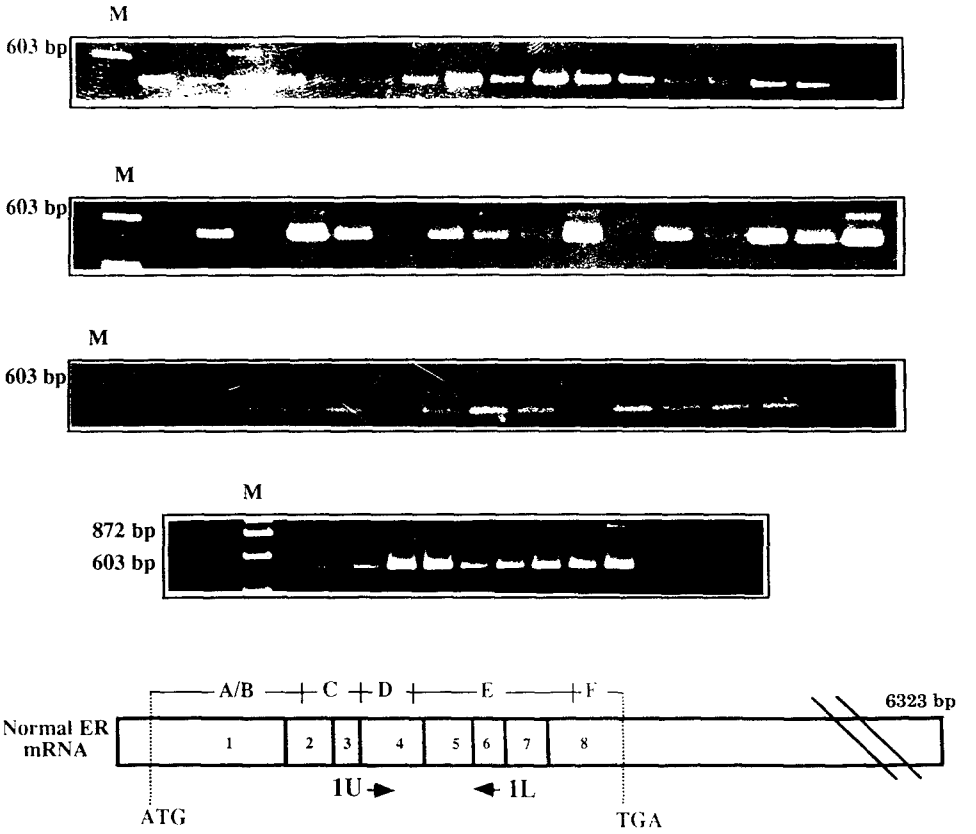
1L
ATG
TGA
2U
2L
4U
4L

Figure 2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M
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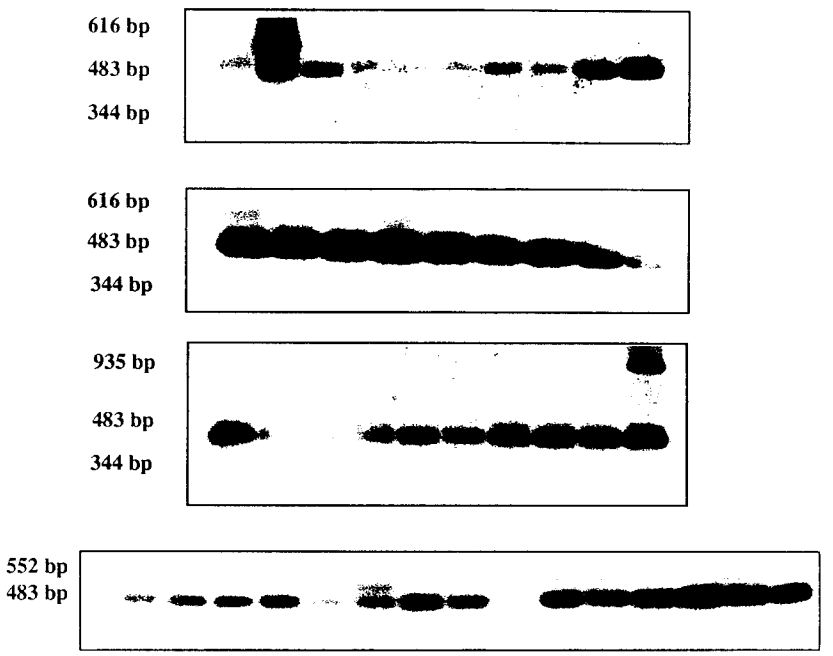
7
8
1U
1L
ATG
TGA

Figure 3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A/B
C
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F
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Normal ER mRNA
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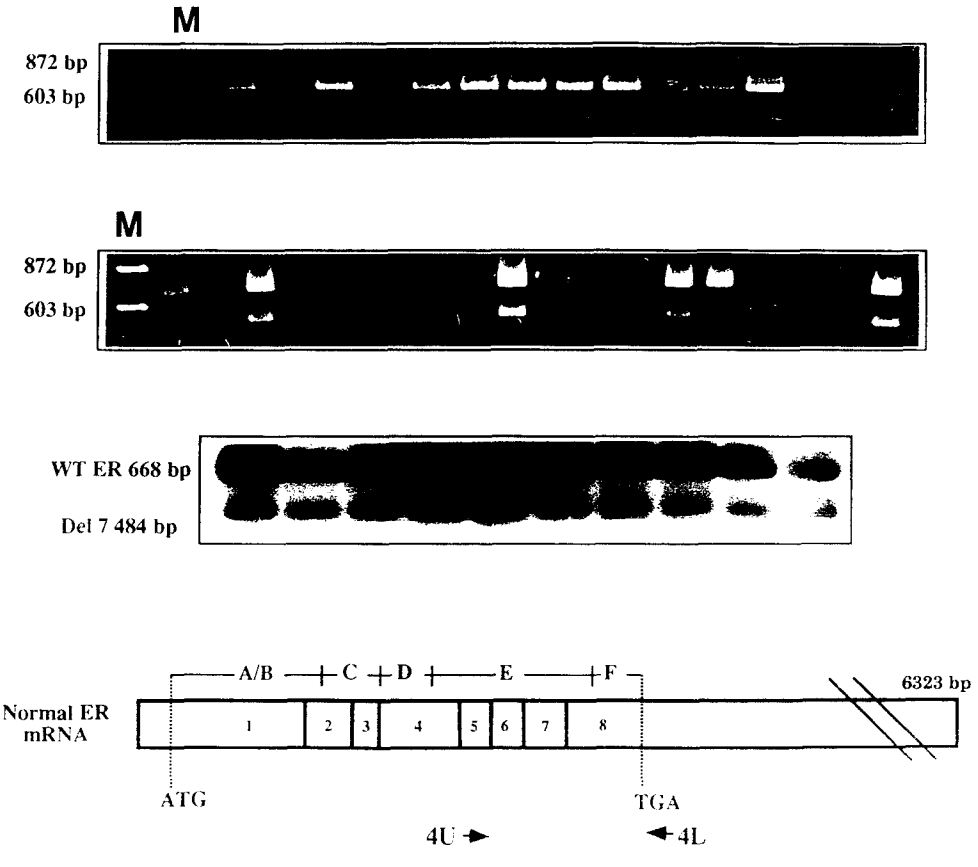
3
4
5
6
7
8
1U
1L
ATG
TGA

Figure 4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603 bp
M
872 bp
603 bp
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Del 7 484 bp
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C
D
E
F
6323 bp
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ATG
TGA
4U
4L

Figure 5

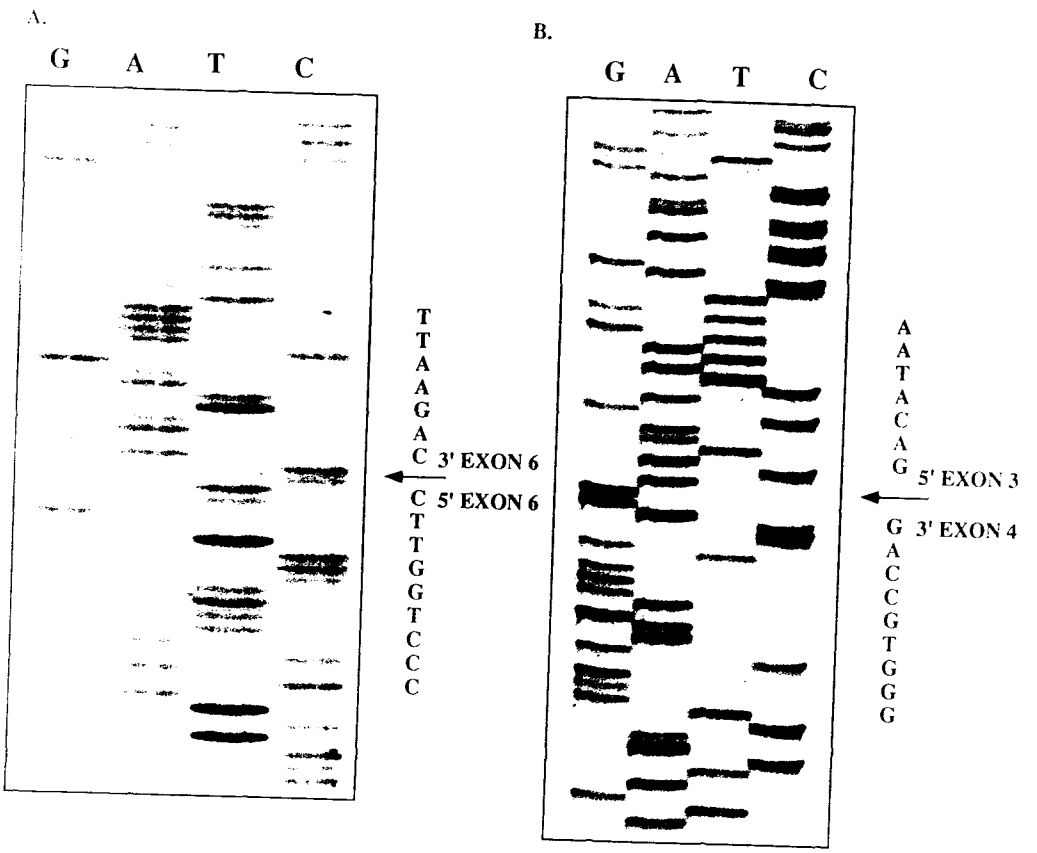
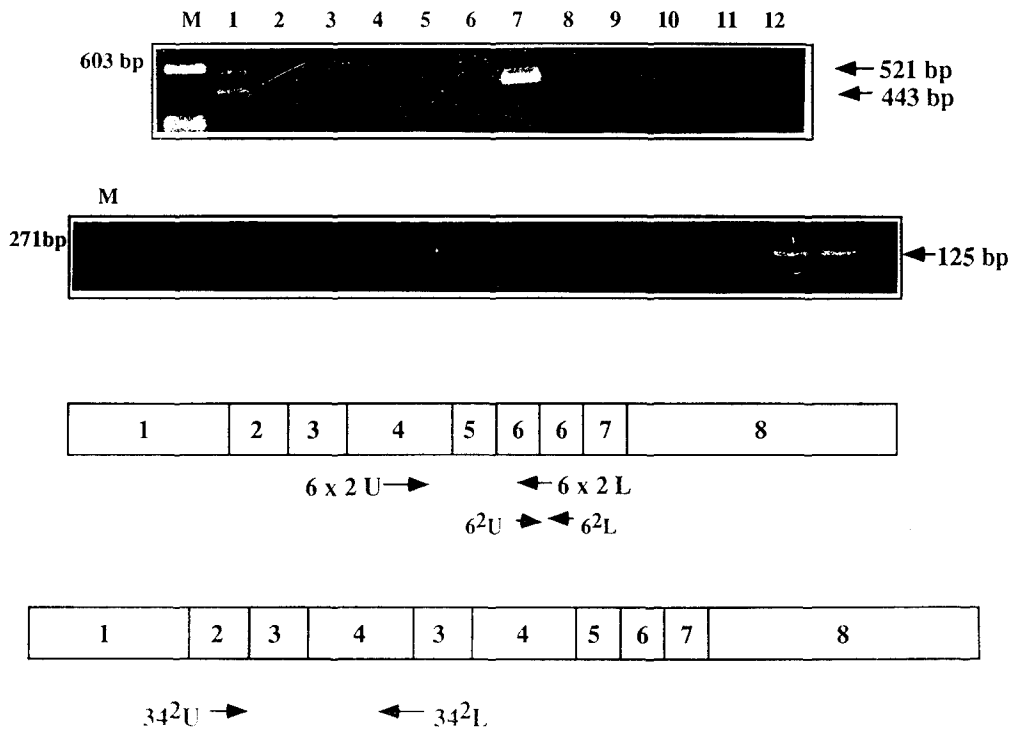

A.
G A T C
T T A A G A C
3' EXON 6
C T T G G T C C C
5' EXON 6
B.
G A T C
A A T A C A G
5' EXON 3
G A C C G T G G G
3' EXON 4

Figure 6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521 bp
443 bp
M
271bp
125 bp
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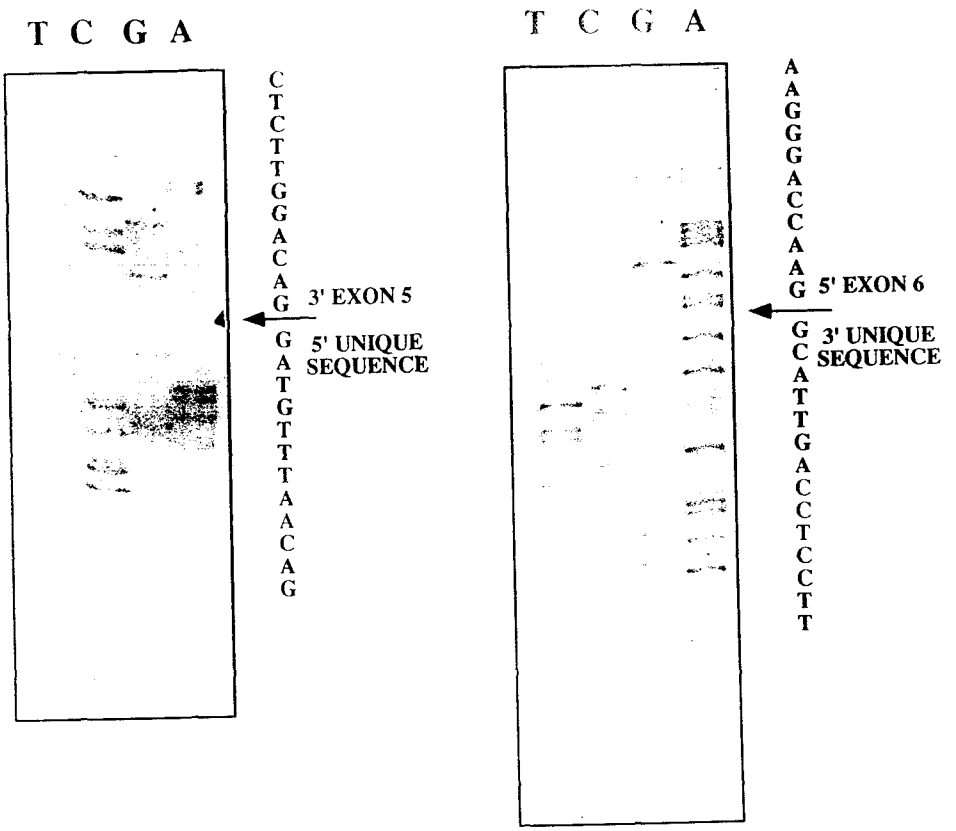
6 x 2 L
6^2U
6^2L
1 2 3 4 3 4 5 6 7 8
34^2U
34^2L

Figure 7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3' EXON 5
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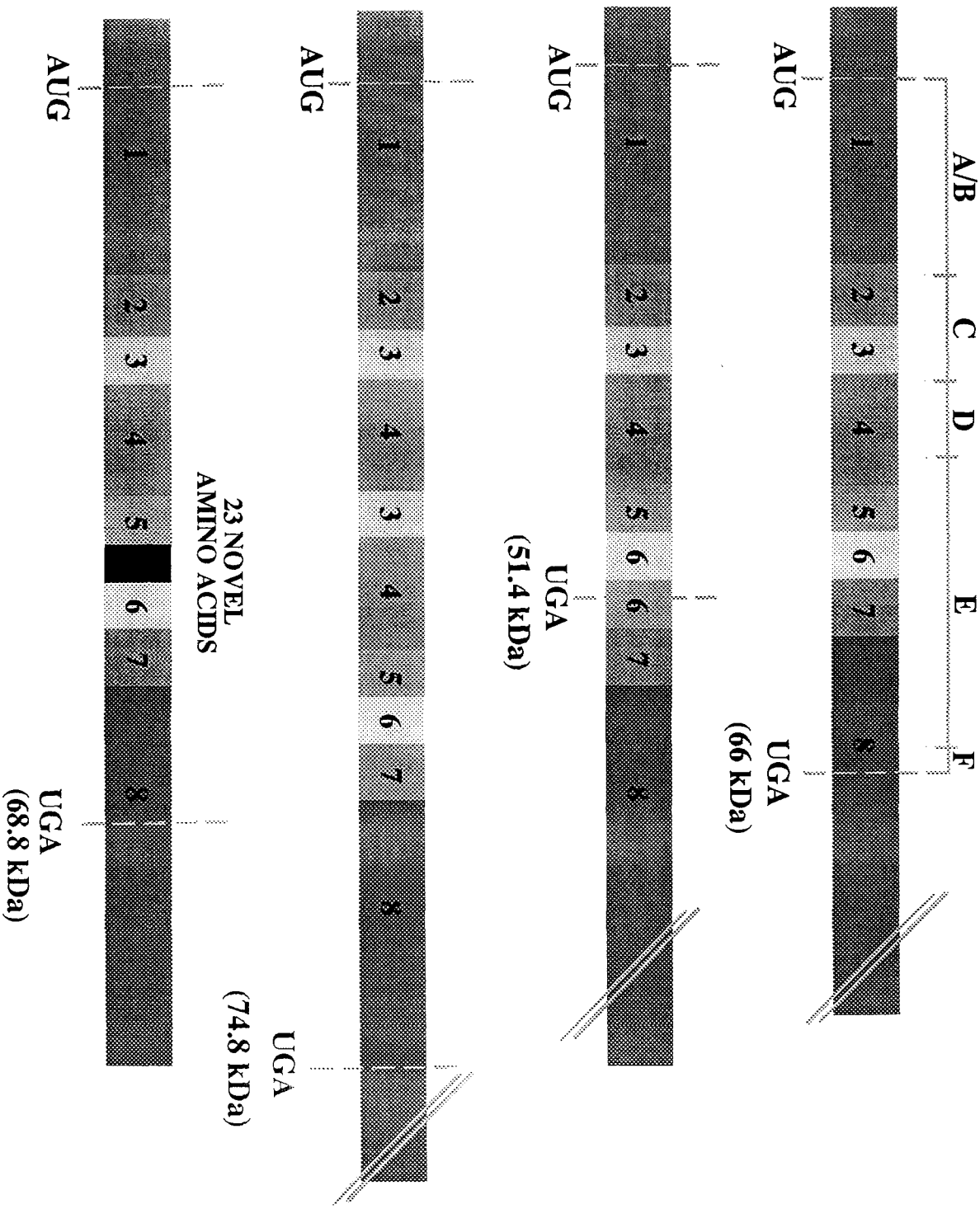
3' UNIQUE SEQUENCE

Figure 8


AUG
A/B
C
D
E
F
UGA
(66 kDa)
AUG
UGA
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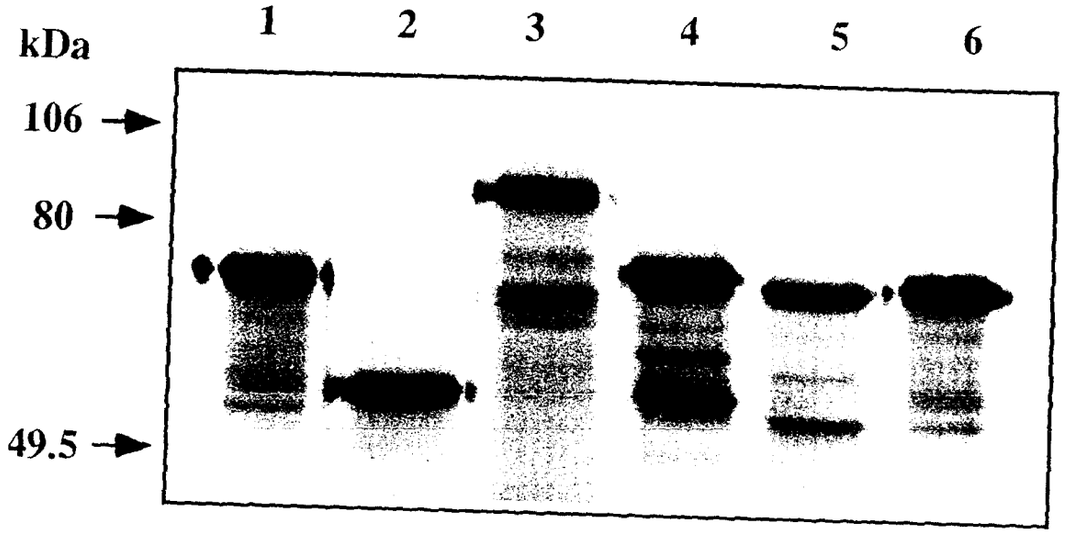
AUG
23 NOVEL
AMINO ACIDS
UGA
(74.8 kDa)
AUG
UGA
(68.8 kDa)

Figure 9



APPENDIX 2

Unpublished Data

A Point Mutation in the Human Estrogen Receptor Gene is associated with the Expression of an Abnormal Estrogen Receptor mRNA containing a 69 Novel Nucleotide Insertion.

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Summary

A novel ER-like mRNA containing a 69 nucleotide insertion precisely between exon 5 and 6 sequences was previously identified in human breast cancer biopsy samples. Data are presented which suggest that the 69 nucleotide sequence is normally present in intron 5 of the human estrogen receptor gene. The region corresponding to and surrounding this 69 nucleotide sequence was cloned and the nucleotide sequence determined. Cloning and sequencing of the corresponding region in genomic DNA isolated from a breast tumor expressing the 69 nucleotide inserted ER mRNA, revealed an A--->G point mutation immediately 3' to the 69 nucleotide sequence. This point mutation resulted in the generation of a consensus splice donor site. A consensus splice acceptor site sequence is normally present immediately 5' to the 69 nucleotide sequence. These data are consistent with the 69 nucleotide sequence being recognized as an exon by the splicing machinery, and resulting in processing of a mature ER mRNA containing the 69 nucleotide insert.

Introduction

We have previously identified in approximately 9% of human breast tumors estrogen receptor (ER)-like mRNAs, which contained inserted sequences [1]. Two types of inserted sequences were identified: one in which complete duplications of normal ER exons were found and one in which 69 novel nucleotides had been inserted between the exons 5 and 6 sequences of the normal ER mRNA. Other altered ER-like mRNAs have also been found in human breast tumors [2]. However, these were mostly truncated ER-like mRNAs [3] or exon deleted ER-like mRNA [4, 5], both of which were most likely generated by some alternative splicing mechanism [6]. It was difficult, however, to suggest an alternative splicing mechanism for either the exon duplicated transcripts or the 69 nucleotide inserted transcript. More likely these transcripts were generated from a mutated ER allele present in some human breast tumors. In this study we present evidence which supports the presence of a mutated ER allele in a breast tumor from which a 69 nucleotide inserted ER-like mRNA was identified and cloned.

Materials and Methods

RNA Isolation: Total RNA was isolated from human breast cancer biopsy samples as previously described [7,8]. The integrity of the RNA was confirmed by denaturing gel electrophoresis [8].

DNA Isolation and Southern Blot Analysis. Southern blot analysis and isolation of genomic DNA from human tumors was as previously described [7]. DNA from bacteriophage clones containing genomic fragments of the human estrogen receptor gene (GHER : gift from Dr P Chambon) [9] was isolated using a plate lysis method [10]. Southern blotting of bacteriophage plaque lifts was carried out according to standard methods [11].

PCR: The thermal profile used was 30 cycles of 1 min at 94^0C, 1 min at 60^0C , and 1 min at 72^0C . The reaction volume was 50 ul containing 0.01 M Tris-HCl (pH 8.3), 0.05 M KCl, 2.5 mM $MgCl_2$, 0.2 mg/ml gelatin, 0.2 mM dNTPs, 1 ul of each primer (200 ng/ul) and 1 unit of Taq DNA Polymerase (Gibco/BRL). 20 ul of the PCR reactions were electrophoresed in agarose gels (1 - 2% depending on expected sizes of products) and visualized by ethidium bromide staining. The primer sequences used are as follows:

Set A: Upper (sense) 5'-TTT GCT CCT AAC TTG CTC TTG - 3' (priming site in exon 5).

Lower (antisense) 5' - CGT AAC TGG AGG AAG TGG - 3' (priming site in novel 69 nucleotides).

Set B: Upper (sense) 5'- TGC CAG TAG CAA CCT CCA CTT - 3' (priming site in novel 69 nucleotides).

Lower (antisense) 5' - CGG AAC CGA GAT GAT GTA GCC - 3' (priming site in exon 6).

Set C: Upper (sense) 5'- TGC CAG TAG CAA CCT CCA CTT - 3' (priming site in novel 69 nucleotides).

Lower (antisense) 5' - CGT AAC TGG AGG AAG TGG - 3' (priming site in novel 69 nucleotides).

Set D: Upper (sense) 5' - CCC AGT CTC AGG TAG GTC TTT - 3' (priming site in intron 5, 5' to novel 69 nucleotides).

Lower (antisense) 5' - GAG TTG GGA AAG CAT AGA GTG - 3' (priming site in intron 5, 3' to novel 69 nucleotides).

Preparation of Radiolabelled Probes. The human ER cDNA (OR-8) [12] was labelled with ^{32}P by nick-translation as described previously [7]. A specific probe for the novel 69 nucleotide sequence was prepared by PCR amplification using primer set C (see above). This primer set generates a 64 bp PCR product which was separated from free nucleotides and primers by low melting point agarose gel electrophoresis (NuSieve GTG Agarose; FMC, Rockland, ME). This product was labelled with ^{32}P-dCTP (0.33 uM final concentration) using 1 PCR cycle under the conditions described above except that the other nucleotides were at a final concentration of 0.33 uM each. The labelled fragment was separated from unincorporated radionucleotides on a Sephadex G-10 column.

Long PCR. This was accomplished using a Perkin Elmer GeneAmp XL Kit (Roche Molecular Systems Inc, Branchburg, NJ) according to the manufacturers instructions.

Cloning and Sequencing: PCR products were isolated from low melting point agarose gels (NuSieve GTG, FMC Bioproducts, Rockland, ME) and ligated into the TA cloning vector, pCR™II using the TA Cloning™Kit (Invitrogen, San Diego, CA). The inserts were sequenced using the T_7 Sequencing Kit (Pharmacia, Baie d'Urfe, Quebec). The region surrounding the 69 nucleotide sequence present in DNA obtained from a tumor expressing the abnormal ER-like transcript was cloned using primer set D.

RESULTS.

We had previously identified 3 human breast tumors which expressed a novel ER mRNA which contained a 69 nucleotide insert precisely between exons 5 and 6 of the wild-type ER mRNA [1]. Due to the precise insertion it was hypothesized that the 69 nucleotide

sequence was normally present in intron 5 of the human estrogen receptor gene and that a mutation in the ER gene of those tumors expressing the abnormal ER mRNA resulted in the 69 nucleotide sequence being recognized as an exon and thus being processed into the mature mRNA. This hypothesis predicts that the 69 nucleotide sequence is present in intron 5 of the normal human ER gene. To address this hypothesis two experiments were undertaken.

Firstly, genomic DNA from T-47D and MCF-7 human breast cancer cells, which do not express the 69 nucleotide inserted abnormal ER mRNA, was isolated and subjected to PCR analysis using specific primers for the 69 nucleotide sequence matched with primers either to sequences in wild-type exon 5 (primer set A) or wild-type exon 6 (primer set B). No PCR products were obtained with primer set B (upper primer specific for the novel 69 nucleotide sequence and lower primer specific for exon 6). However, with primer set A (upper specific for exon 5 and lower specific for the 69 nucleotide sequence), a PCR product of approximately 2.5 kbp was obtained in DNA isolated from both these cell lines (Figure 1).

Secondly, several genomic clones spanning the regions around exon 5 and exon 6 of the human ER (gift from Dr P Chambon) [9] were subjected to Southern blotting of bacteriophage plaques. Duplicate lifts were made. One filter was hybridized with radiolabelled hER cDNA while the duplicate lift was hybridized with a radiolabelled 64 bp probe specific for the novel 69 nucleotide sequence (primer set C). Figure 2 shows the results of this experiment. Human genomic ER clones GHER 9, 10 and 11 all hybridized with the hER cDNA, but only GHER 10 hybridized with the 64 bp probe specific for the novel 69 nucleotide inserted sequence. When DNA isolated from GHER 10 was subjected to long PCR using the primer set A (upper specific for exon 5 and lower specific for the novel 69 sequence), a PCR product of approximately 2.5 kbp was obtained which appeared to be identical in size to that obtained from DNA isolated from the breast cancer cell lines (Figure 1).

Digestion of DNA isolated from GHER 10 with Eco RI yielded four bands. Southern blotting and hybridization with the 64 bp radiolabelled probe specific for the novel 69 nucleotide sequence identified only one band of approximately 3 kbp (data not shown). This band was subcloned into the plasmid Bluescript SK- (Stratagene, La Jolla, CA) and primers specific (sense and antisense) for the novel 69 nucleotide sequence were used individually as sequencing primers. The nucleotide sequence obtained from this analysis confirmed the presence of the novel 69 nucleotide sequence within the clone. As well the sequence of approximately 170 nucleotides either side of the novel 69 nucleotide sequence was also obtained (Figure 3). As shown in Figure 3, the sequence immediately 5' to that of the novel 69 nucleotide sequence, is AG. This is a potential splice acceptor site to partner the splice donor site at the end of exon 5. The sequence immediately 3' of the novel 69 nucleotide sequence is AT. We reasoned that the simplest explanation for the 69 nucleotides being recognized as an exon in the tumor cells expressing the abnormal ER-like transcript, would be a mutation which generated a new splice donor site at the end of the 69 nucleotides which could partner the splice acceptor site preceeding exon 6.

Therefore, DNA was isolated from the human breast tumor biopsy sample originally found to express the 69 nucleotide inserted ER-like mRNA. The region surrounding the novel 69 nucleotide sequence was selected and PCR amplified using primers designed from the known normal sequence around this area (Figure 3, primer set D). These primers amplified the expected 252 bp fragment in DNA isolated from non-expressing tumors as well as from the abnormal ER-like mRNA expressing tumor. The 252 bp fragment from the expressing tumor was subcloned and sequenced. Two independent PCRs led to clones some of which when sequenced contained an A--->G mutation immediately 3' to the novel 69 nucleotide sequence (Figure 4). This mutation generates a new splice donor site to partner the splice acceptor site preceeding exon 6. Such a mutation would be consistent with the novel 69 nucleotide sequence being recognized as

an exon and being processed into the mature ER-like mRNA. It should be noted that clones containing the wild-type sequences were also obtained.

DISCUSSION.

Several variant ER-like transcripts have been characterized in human breast cancers [2]. They fall into two main categories: the precise exon deleted transcripts [4, 5] and the truncated transcripts [3, 7] both of which are likely to be generated by an alternative splicing mechanism [6]. More recently we have identified an ER-like transcript which was larger than the wild-type ER mRNA, and indeed cloning and sequencing of this transcript identified a precise insertion of 69 nucleotides between exon 5 and exon 6 sequences [1]. It seemed more likely that this novel transcript was generated from a mutant ER gene, rather than some alternative splicing mechanism. The data presented in this manuscript identify a point mutation in the ER gene present in DNA isolated from the tumor originally found to be expressing high levels of the novel 69 nucleotide inserted ER-like mRNA [1].

The 69 nucleotide sequence was found to be present in DNA obtained from cells containing only the wild-type estrogen receptor gene, at least, as defined by the lack of expression of any detectable abnormal inserted ER-like mRNA. The sequence was further mapped to intron 5 of the human estrogen receptor gene [9]. Sequencing of the region surrounding the 69 nucleotide sequence in the wild-type gene, identified a consensus splice acceptor site immediately 5' to the 69 bp sequence, but not a donor splice site immediately 3' to the 69 bp sequence. When the same region was characterized in DNA isolated from the tumor expressing the abnormal transcript, an A--->G transition was found immediately 3' to the 69 nucleotide sequence which generated a new consensus splice donor site. Indeed the presence of the 69 nucleotide inserted ER-like mRNA in this tumor is consistent with the 69 nucleotides being surrounded with appropriate splicing signals and being recognized as an exon and therefore processed into mature mRNA.

Although wild-type clones were also obtained from the DNA of the tumor expressing the abnormal ER-like mRNA, the A--->G mutation is unlikely to be a PCR

induced error, since the identical mutation was obtained from clones generated from two independent PCR reactions. The mixture of wild-type and mutant is more likely to reflect the presence of both the wild type allele and the mutant allele in the tumor sample. Further, such data are consistent with the original RT-PCR data in which both the wild-type RT-PCR product and the abnormal RT-PCR product were observed in the same RNA extract [1]. It is unclear whether this represents heterozygosity for the mutant allele or alternatively heterogenous cell populations within the tumor sample.

In conclusion, our data support the generation of abnormal ER-like mRNA from mutations occurring in the estrogen receptor gene in some human breast cancers.

ACKNOWLEDGEMENTS

This work was supported in part by the Canadian Breast Cancer Research Initiative and the U.S.Army Breast Cancer Research Program. LCM is an MRC (Canada) Scientist. We thank Dr Pierre Chambon for his generous gifts of the GHER clones and the OR-8 cDNA.

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LEGENDS TO FIGURES

FIGURE 1

The top panel shows the results of long PCR using primer set A of DNA isolated from GHER 10 bacteriophage containing part of the human estrogen receptor gene [9], and genomic DNA isolated from T47D and MCF-7 human breast cancer cells. Primer set A produced a similar approximately 2.5 kbp fragment as visualized by ethidium bromide staining, from all three DNA samples. Markers are Hind III restriction fragments of lambda phage DNA. The bottom panel shows a schematic representation of the abnormal ER-like transcript with the novel 69 nucleotide insertion (cross-hatched box). The arrows show the approximate positions of primer set A (long PCR) and primer set C (specific probe).

FIGURE 2

Top panels: Bacteriophage containing genomic clones of the regions around exons 5 and 6 of the human estrogen receptor (GHER 9, GHER 10, GHER 11) [9] were subjected to plaque lifts and Southern blotting, using either a human estrogen receptor cDNA (OR-8) [12] or a probe specific for the novel 69 nucleotide insertion (novel sequence probe, see figure 1). The bottom panel shows a schematic representation of the human estrogen receptor cDNA, human estrogen receptor gene and the bacteriophage clones containing various regions of the human estrogen receptor gene [9].

FIGURE 3

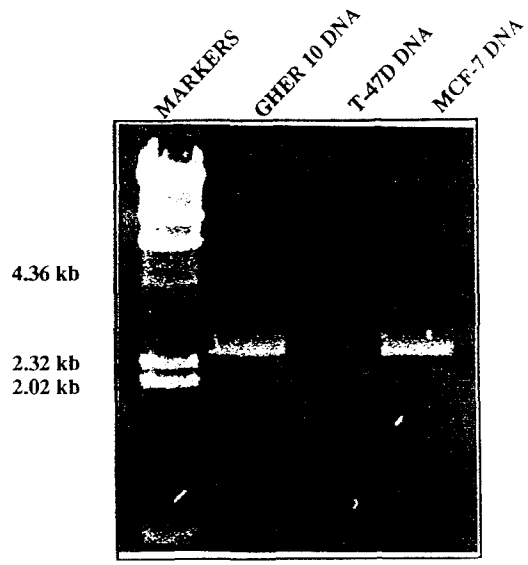
Wild-type nucleotide sequence of the region surrounding the 69 nucleotide sequence, which was subcloned from DNA isolated from GHER 10 bacteriophage. The 69 nucleotide sequence is in italics and underlined. The AG immediately upstream of the 69 sequence is bold-faced, as is the AT immediately downstream of the 69 nucleotide sequence. The two bold-faced 21 nucleotide sequences upstream and downstream of the 69 nucleotide sequence are the sequences used to generate PCR primers (primer set D)

which were then used to clone the corresponding region from DNA of a breast tumor expressing the 69 nucleotide inserted ER mRNA .

FIGURE 4

Nucleotide sequence of wild-type and mutant clones obtained from the PCR analysis (primer set D) of DNA from a breast tumor expressing the 69 nucleotide inserted ER mRNA. The arrows identify the relevant nucleotides in each clone. The asterisk identifies the G point mutation in the mutant clone. Panel A shows representative sequencing gels of both wild-type and mutant clones, and panel B shows the mutant nucleotide sequence, with the 69 nucleotides in italics and underlined and the G mutation bold-faced.

Figure 1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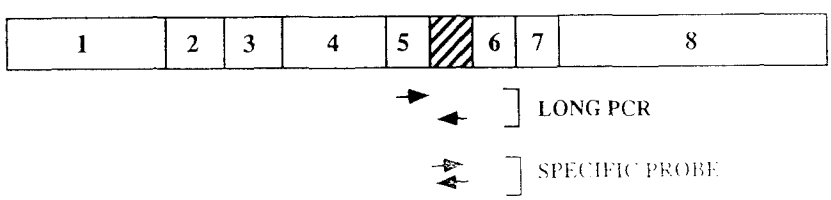
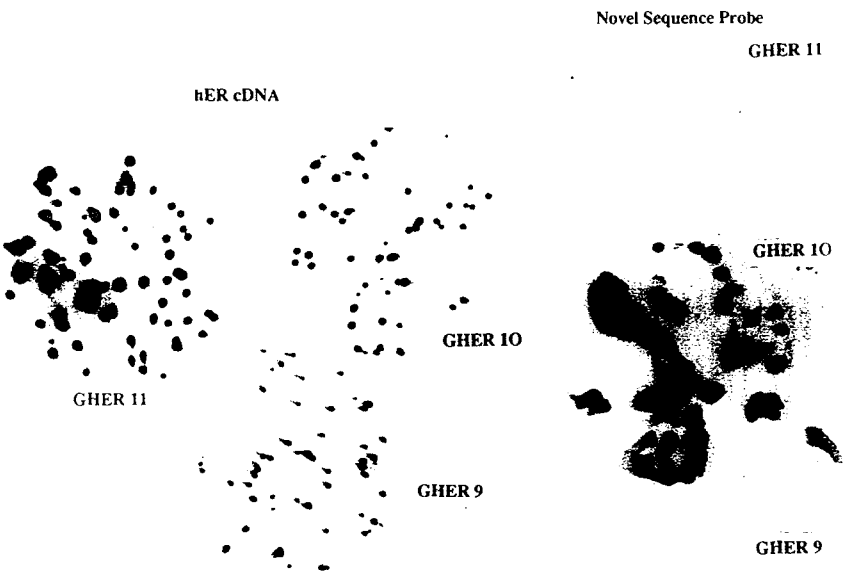

1
2
3
4
5
6
7
8
LONG PCR
SPECIFIC PROBE

Figure 2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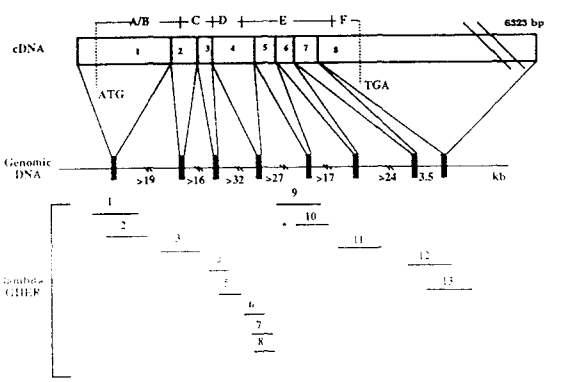
hER cDNA
GHER 10
GHER 11
GHER 9
GHER 10
GHER 9


A/B
C
D
E
F
6323 bp
cDNA
ATG
TGA
Genomic DNA
>19
>16
>32
>27
>17
>24
3.5
kb

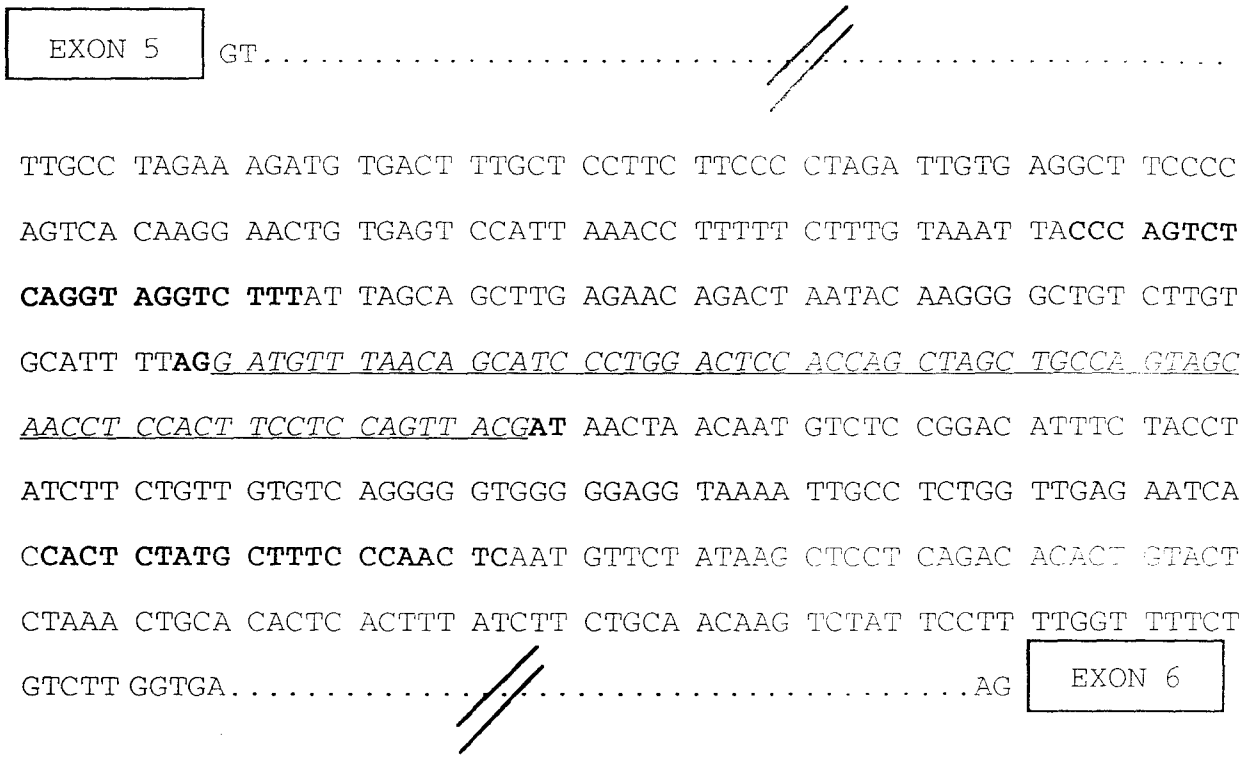
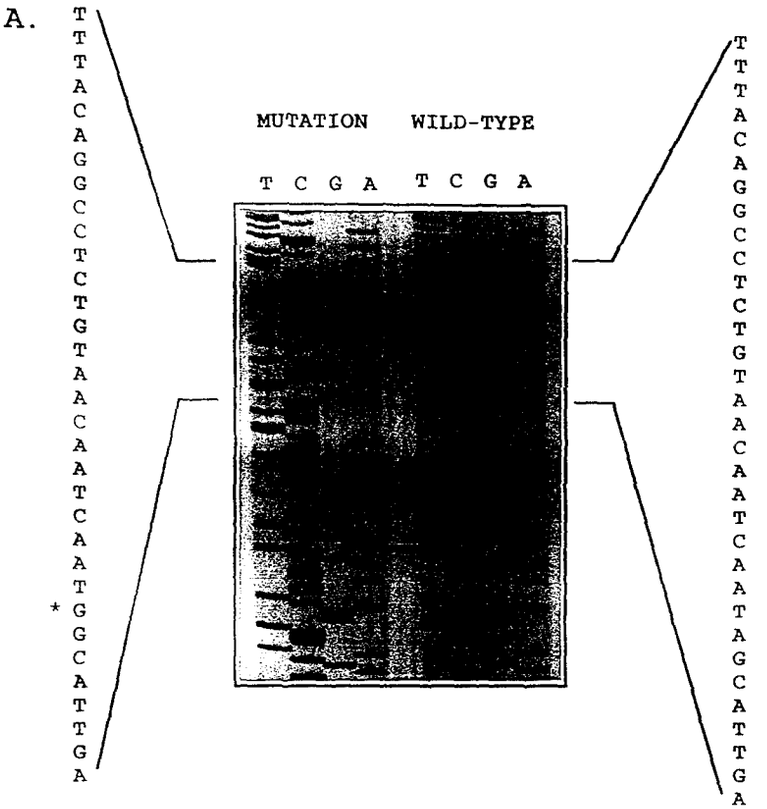

EXON 5
GT
1 TTGCC TAGAA AGATG TGACT TTGCT CCTTC TTCCC CTAGA TTGTG AGGCT TCCCC
56 AGTCA CAAGG AACTG TGAGT CCATT AAACC TTTTT CTTTG TAAAT TACCC AGTCT
111 CAGGT AGGTC TTTAT TAGCA GCTTG AGAAC AGACT AATAC AAGGG GCTGT CTTGT
166 GCATT TTAGG ATGTT TAACA GCATC CCTGG ACTCC ACCAG CTAGC TGCCA GTAGC
221 AACCT CCACT TCCTC CAGTT ACGAT AACTA ACAAT GTCTC CGGAC ATTTC TACCT
276 ATCTT CTGTT GTGTC AGGGG GTGGG GGAGG TAAAA TTGCC TCTGG TTGAG AATCA
331 CCACT CTATG CTTTC CCAAC TCAAT GTTCT ATAAG CTCCT CAGAC ACACT GTACT
386 CTAAA CTGCA CACTC ACTTT ATCTT CTGCA ACAAG TCTAT TCCTT TTGGT TTTCT
441 GTCTT GGTGA AG
EXON 6

Figure 4

A.



B.

AT

TAGCA GCTTG AGAAC AGACT AATAC AAGGG GCTGT CTTGT

GCATT TTAGG ATGTT TAACA GCATC CCTGG ACTCC ACCAG

CTAGC TGCCA TTATT AACCT CCACT TCCTC CAGTT ACGGT

AATTA ACAAT [illegible] ATTTC TACCT ATCTT CT

APPENDIX 3

ESTROGEN RECEPTOR VARIANTS IN NORMAL HUMAN MAMMARY TISSUE.

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Key words:

Estrogen receptor variants, normal human breast, breast cancer, differential splicing.

Abstract

Background: Several estrogen receptor (ER) variant mRNAs have been identified previously in human breast cancer biopsy samples and cell lines. In vitro assays of function of polypeptides encoded by some of these variant mRNAs have led to the speculation that ER variants may be involved in progression from hormone dependence to independence in breast cancer.

Purpose: We set out to establish whether ER variant mRNAs are expressed in normal human breast tissues and, if so, to compare levels of expression of these variants between normal and neoplastic human breast tissues.

Methods: Four human breast tissue samples from reduction mammoplasties and 5 breast tissue samples adjacent to breast tumors were analyzed. The tissues were confirmed to be normal (i.e., not malignant) by histopathologic analysis. RNA was extracted immediately from adjacent frozen sections. Human breast tumor specimens originally obtained from 19 patients were acquired from a tumor bank and processed in the same way as the normal tissue samples. The RNAs were then reverse transcribed and subsequently amplified using polymerase chain reaction (RT-PCR). PCR primer sets were designed to detect several different exon-deleted ER variants and a truncated ER variant (called clone 4). A semi-quantitative PCR-based method was used to determine the relative expression of exon 5- and exon 7-deleted variants to wild-type ER mRNA in the nine normal breast tissues and 19 ER positive breast tumor tissues.

Results: ER variant mRNAs corresponding to the clone 4 ER truncated variant and to variants deleted in either exon 2, exon 3, exons 2-3, exon 5 or exon 7 were detected in all normal samples. The results were confirmed by restriction enzyme analyses and sequencing of the PCR products. The expression of exon 5-deleted ER variant relative to the wild-type ER mRNA was significantly lower ($P<.001$) in normal tissue than in tumor tissue. A similar trend was noted for expression of the exon 7-deleted ER variant mRNA; however, the difference did not achieve statistical significance ($P = .476$).

Conclusion: Several ER variant mRNAs are present in normal human breast tissue, but the level of expression of some of these variants may be lower in normal tissue than in tumor tissue.

Implication: These data suggest that the mechanisms generating ER variant mRNAs exist in normal breast tissue and may be deregulated in breast cancer tissues. Further investigation of the role of altered variant ER expression in development and progression in human breast cancer appears warranted.

Introduction

The estrogen receptor (ER), which belongs to the superfamily of steroid/thyroid/retinoic acid receptors (1), is an important regulator of growth and differentiation of the mammary gland. The receptor can be divided into several structural and functional domains (A-F) (2). In the absence of hormone, the receptor is thought to be associated with a protein complex including heat-shock proteins such as hsp90 (3). Upon binding of ligand, the ER dissociates from this complex, dimerizes, and binds to specific sequences (ERE) located in the 5' flanking region of ER-responsive genes. Such interactions alter the transcription of estrogen responsive genes. Region E of the receptor is implicated in ligand binding, dimerization and trans-activating functions (TAF-2). The DNA-binding domain is located in the C region and another cell and promoter specific trans-activating function (TAF-1) lies within the A/B regions. Nuclear localization and dimerization functions reside in the E and in the C domains.

Several ER variant mRNAs have been identified previously in human breast cancer biopsies and cell lines (4, 5, 6, 7). While it is unclear if these mRNAs are translated in vivo, some of the predicted ER-like proteins, lacking some domains, exhibit altered functions or may interfer with wild-type (WT) ER function when recombinantly expressed. Exon 3- and exon 7-deleted variants were shown to act as dominant negative regulators of WT ER (8, 9). In contrast, exon 5-deleted ER has ligand independent trans-activating activity in a yeast expression system (10). It has been shown that the relative levels of some of these ER variants were increased during tumor progression. Exon 5-deleted ER variant mRNA expression was found higher in estrogen receptor negative/progesterone receptor positive (ER-/PR+) than estrogen receptor positive/progesterone receptor positive (ER+/PR+) tumors (6, 7). Further, exon 7-deleted variant mRNA levels were shown to be higher in ER+/PR- than ER+/PR+ tumors (9). Further data from our own laboratory, suggest that elevated expression of another ER variant, called clone 4 truncated

ER variant (11), is correlated with parameters of poor prognosis and endocrine insensitivity (12). It has thus been speculated that these ER variants may be involved in progression from hormone dependence to independence in breast cancer (13). However, the expression of ER variants in normal human breast tissue remains unknown. A study was recently published showing the detection of several exon-deleted ER mRNAs in a single normal breast tissue sample (14). The authors did not exclude the eventual development of breast cancer in the individual from whom the sample was derived. Moreover, no data were provided addressing relative expression of ER variants between normal and tumorous breast tissues. It was therefore important to establish definitively the existence of these ER variants in multiple normal breast tissue samples as well as to determine their relative level of expression between normal and tumorous breast tissues.

The aim of this study was to determine if multiple ER variant mRNAs can be detected in normal human breast tissue and where possible to compare the level of expression in normal tissue with that observed in tumors tissues.

Materials and methods

Human breast tissues and cell lines.

Normal breast tissues were obtained from reduction mammoplasty surgical specimens collected at the Necker Hospital, France (4 case patients) and from normal tissues adjacent to tumors in mastectomy specimens obtained through the Manitoba Breast Tumor Bank (5 case patients). Human breast tumor specimens were also obtained from the Manitoba Breast Tumor Bank (19 case patients). In all cases the specimens had been rapidly frozen at -70°C as soon as possible after surgical removal. Subsequently, a portion of the frozen tissue from each case (normal and tumor) was processed to create formalin-fixed and paraffin embedded tissue blocks that are matched and orientated relative to the frozen tissue. These blocks provide tissue for high quality histological sections for pathological interpretation and assessment. The presence of normal ducts and lobules was confirmed in all normal tissue specimens, as well as the absence of any atypical lesion. The nineteen primary invasive ductal carcinoma used were selected from the Tumor

Bank database were all associated with high ER levels (105 to 284 fmol/mg protein). Within this group ten tumors were progesterone receptor positive (PR+), four were PR negative (PR-) and five were borderline positive (<15 fmol/mg protein) as determined by ligand binding assay. Specific frozen tissue blocks were chosen in each case on the basis of several further criteria as assessed in histological sections. These criteria included: a cellular content of greater than 30% invasive tumor cells with minimal normal lobular or ductal epithelial components, good histological preservation and absence of necrosis. In all tumor cases, grading was performed using the Nottingham grading system (15) and additional clinical and staging information (e.g. patient age, tumor size, nodal status) was obtained from the Tumor Bank database. The age distribution of patients associated with the normal samples was similar to that of the tumor group (mean: 70.2 years old, standard deviation 13 years). For reduction mammoplasties women were younger (mean: 20 years, standard deviation: 3 years).

Ishikawa cells, an endometrial adenocarcinoma cell line initially established by Dr. Iwasaki (Tsukuba, Japan), were provided by Dr. E. Gurpide (Mount Sinai School of medicine, New-York). The breast cancer cell line T-47D-5 was kindly provided by Dr. RL Sutherland (Garvan Institute for Medical Research, Sydney, Australia). These cells are known to express different ER variant mRNAs (16, 17 and unpublished data) and have therefore been used as positive controls. Cos-1 cells (American Type Culture Collection, Rockville, Maryland) do not express ER mRNA and were used as a negative control in our experiments. Cells were grown, harvested and cell pellets stored at -70°C, as previously described (18).

Extraction of mRNA and reverse transcription (RT).

Total RNA was extracted from histologically defined regions within 20 µm frozen normal and tumor cryostat sections using a small scale RNA extraction protocol (Trireagent, MRCI, Cincinnati) according to the manufacturers instructions. The yield was quantitated by spectrophotometer in a 50 µl microcuvette. The average yield of total RNA per 20 µm section was 4 $\mu g/cm^2$ for tumor and 0.6 $\mu g/cm^2$ for normal tissues (± 20% variation with cellularity) and Optical Density $^{260/280}$ >1.8.

RT was performed in a final volume of 15 µl. RNA (600 ng) was reverse transcribed in the presence of 1 mM deoxyadenosine-5'-triphosphate (dATP), 1 mM deoxycytidine-5'-triphosphate (dCTP),

1 mM deoxyguanosine-5'-triphosphate (dGTP), 1 mM deoxythymidine-5'-triphosphate (dTTP), 5 mM dithiothreitol (GIBCO-BRL), 1 unit/µl ribonuclease inhibitor (Promega), 20 µM random primers, 50 mM Tris-HCl (pH 7.5), 75 mM KCl, 3 mM $MgCl_2$, and 5 units/µl Moloney Murine Leukemia Virus reverse transcriptase (GIBCO-BRL) for 10 minutes at 22°C and 1 hour at 37°C. After 5 minutes at 95°C, 1 µl of the reaction mixture was taken for subsequent amplification using the Polymerase Chain Reaction (PCR).

Primers and PCR conditions

Four sets of primers were used in this study (Fig.1). The primer set which detected exon 5-deleted ER mRNA was called D5 set, consisted of D5U primer ($^{5'}$-CAGGGGTGAAGTGGGGTCTGCTG-$^{3'}$; sense; located in WT ER exon 4; 1060-1082) and D5L primer ($^{5'}$-ATGCGGAACCGAGATGATGTAGC-$^{3'}$; antisense; located in WT ER exon 6; 1520-1542). This primer set allows amplification of 483 bp and 344 bp fragments corresponding to WT ER and exon 5-deleted ER variants, respectively. The primer set designed to detect exon 7-deleted ER mRNA was called D7 primer set and consisted of D7U primer ($^{5'}$-TCCTGATGATTGGTCTCGTCTGG-$^{3'}$; sense; located in WT ER exon 5; 1389-1411) and D7L primer ($^{5'}$-CAGGGATTATCTGAACCGTGTGG-$^{3'}$; antisense; located in WT ER exon 8; 2035-2057). The primer set allows amplification of 668 bp, 534 bp, 484 bp and 350 bp fragments corresponding to WT ER, exon 6-deleted, exon 7-deleted and exon 6-7-deleted ER variants, respectively. The primer set which detects exon 2-, exon 3- and exon 2-3-deleted ER mRNAs was called D2/3 primer set and consisted of D2/3U primer ($^{5'}$-TGCCCTACTACCTGGAGAA-$^{3'}$; sense; located in WT ER exon 1; 615-633) and D2/3L primer ($^{5'}$-TGTTCTTCTTAGAGCGTTTGA-$^{3'}$; antisense; located in WT ER exon 4; 1125-1145). This primer set allows amplification of 531 bp, 414 bp, 340 bp and 222 bp fragments corresponding to WT ER, exon 3-deleted, exon 2-deleted and exon 2-3-deleted ER variants, respectively. Primers designed to specifically detect clone 4 truncated ER mRNA consisted of Clone 4 U primer ($^{5'}$-TGCCCTACTACCTGGAGAA-$^{3'}$; sense; located in WT ER exon 1; 623-641) and Clone 4 L primer ($^{5'}$-GGCTCTGTTCTGTTCCATT-$^{3'}$; antisense; 941-959). This set allows amplification of a 337 bp fragment corresponding specifically to clone 4 truncated ER variant mRNA. Positions given correspond to

published sequences of ER cDNA (19) and clone 4 cDNA (11) for exon-deleted-primer sets and clone 4 primer set, respectively.

PCR amplifications were performed using 1 µl of RT mixture in a final volume of 50 µl, in the presence of 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM dATP, 0.2 mM dTTP, 0.2 mM dGTP, 0.2 mM dCTP, 4 ng/µl of each primer and 1 unit of Taq DNA polymerase (GIBCO-BRL). Each PCR consisted of 40 cycles (1 minute at 60°C, 30 seconds at 72°C and 30 seconds at 94 °C) using a Thermocycler (Perkin Elmer). PCR products were then separated on 2% agarose gels before staining with ethidium bromide (15 µg/ml).

Identification of PCR products

PCR products were identified by restriction enzyme analysis and sequencing. Bands corresponding to the clone 4 and exon 7-deleted ER variants were excised from gels after staining with ethidium bromide. The corresponding DNA was extracted with phenol-chloroform-isoamyl alcohol (25:24:1) and precipitated with 70% ethanol in presence of 100 mM CH_3COONa. PCR amplified DNA product corresponding to Clone 4 was digested for 18 hours at 37°C with Taq1 alone or Taq1 plus Kpn1 (5 unit each /µg of DNA). The PCR product corresponding to exon 7-deleted ER variant was digested under similar conditions with Pst1. Digestion products were separated on 2% agarose gels and their sizes determined by reference to size markers (ΦX174 RF DNA/Hae3 fragments, GIBCO-BRL). In parallel, approximatively 50 ng of DNA were sequenced using Clone 4 and D7 primer sets and dsDNA cycle sequencing system (GIBCO-BRL) according to the manufacturers instructions.

For exon 5-, exon 2-, exon 3- and exon 2-3-deleted ER variants, the PCR products were labelled with dCTP [α-^{32}P] (see below). One µl of PCR product corresponding to exon 5-deleted ER variant was digested with Hind3 as described above. Similarly, PCR products corresponding to exon 2-, exon 3- and exon 2-3-deleted ER variants were digested with Taq1 alone or Taq1 plus Hha1. Digestion products were separated on 6% polyacrylamide gels containing 7M urea (PAGE). Following electrophoresis, the gels were dried and exposed to Kodak XAR Film at -70°C with an intensifying screen and the size of digestion products determined by reference to size markers. In parallel, slices of gel corresponding to each labelled

PCR product were excised from the dried gel and rehydrated overnight in 100 µl of sterile water. For each sample, five different PCR reactions were performed as described above using 1 µl of this solution previously boiled for 10 minutes. PCR products corresponding to each set of 5 reactions were pooled, purified (Wizard PCR preps kit, Promega), cloned using Invitrogen TA Cloning kit and sequenced as previously described (11).

Labelling of PCR products

In order to label PCR products, a standard PCR reaction was carried out in 10 µl supplemented with 10 nM of dCTP [α-^{32}P] (ICN Pharmaceuticals, Inc, Irvine, California). 2 µl of the reaction were denaturated in 80% formamide buffer and subjected to PAGE. Following electrophoresis, the gels were dried and autoradiographed for 6-18 hours.

Quantification and statistical analysis

The method used to quantitate exon-deleted variant mRNAs relative to WT ER mRNA is a modification of a method described by Daffada et al. (20, 21). These authors showed that co-amplification of WT ER and exon 5-deleted variant generates 2 bands whose ratio was constant with varying cycle number. This assay provides a semi-quantitative RT-PCR whose internal control is the WT ER mRNA co-amplified and in which relative expression of variant mRNA can be determined for individual samples. In our study, quantification of signals was carried out after excision of the band corresponding to variant and WT mRNA, followed by addition of 5 ml scintillant (ICN Pharmaceuticals, Inc, Irvine, California) and counted in a scintillation counter (Beckman). The exon-deleted signal was expressed as a percentage of the WT ER signal. Preliminary studies showed that for each sample, the ratio of exon 5-deleted/WT ER signal and exon 7-deleted/WT ER signal remained constant (± 20%) and independent of the number of PCR cycles or initial cDNA input quantities. It should be noted that the percentage obtained reflects the relative ratio of variant to WT ER RT-PCR product and does not provide absolute initial mRNA levels.

For each sample, at least 3 independent assays were performed and the mean determined. The ratio of exon 5-deleted/WT ER signal and exon 7-deleted/WT ER signal measured during these assays never varied by more than 20%. The statistical significance of differences in the relative levels of expression of

exon 5- and exon 7-deleted ER mRNAs in normal breast versus breast tumor tissue was determined using the Mann-Whitney rank-sum test (two-sided).

Results

Detection of ER variants in normal breast tissues

Total RNA from nine normal breast tissue specimens from nine different women was analyzed by RT-PCR using the oligonucleotide primer pairs described in the Methods section and depicted in Fig.1. Primers were designed to allow the detection of different ER variants previously observed in breast cancer tissues or cell lines : exon 3- (8), exon 5- (6) and exon 7- (9) deleted ER variants and clone 4 ER truncated variant (11). These variants were detected using D2/3, D5, D7 and clone 4 primers set, respectively.

In the first series of experiments, PCR products were stained with ethidium bromide after separation on 2% agarose gel (Fig.2).

Using the D7 primer set (Fig.2A), 2 bands which corresponded in size to WT ER (668 bp) and to exon 7-deleted ER variant (Del 7, 484 bp) were obtained. These bands comigrated with those observed in the positive controls: T-47D-5 breast and Ishikawa uterine cancer cell lines. In order to confirm the identity of exon 7 deleted ER variant, the lower band was purified and digested with different restriction enzymes (data not shown). Nucleotide sequence obtained by cycle sequencing revealed a perfect boundary between exon 6 and exon 8 ER WT sequences (data not shown). These data definitively confirmed the identity of the exon 7 deleted ER PCR product obtained.

Using the clone 4 primer set, a band migrating with the expected size of 337 bp was obtained (Fig.2B). This band comigrated with that seen in the positive controls. Identity of this band was confirmed by enzymatic digestion and cycle sequencing (not shown).

Using the D2/3 or D5 primer sets followed by ethidium bromide staining, no exon 3- and exon 5-deleted ER variant mRNAs were detected in normal tissues (data not shown). A more sensitive technique consisting of incorporation of labelled nucleotide into the PCR reaction followed by separation of PCR products on 6% denaturating polyacrylamide, was subsequently used.

This technique together with the D5 primer set, detected 2 bands corresponding in size to the WT ER mRNA (483 bp) and exon 5-deleted variant (344 bp) in all normal breast tissue samples (Fig.3A). Identity of the PCR products was confirmed following restriction enzyme digestion and sequencing (data not shown).

The D2/3 primer set and labelled PCR reactions resulted in the detection of 4 different PCR products in normal breast tissue samples. These products corresponded in size to WT ER (531 bp), exon 3 deleted (414 bp), exon 2 deleted (340 bp), and exon 2-3 deleted (222 bp) ER variant mRNAs (Fig.3C). Identity of these bands was confirmed with restriction enzyme digestion analysis and sequencing (data not shown).

Comparison of exon-deleted ER variant expression in normal and tumor tissues

The relative level of exon-deleted variant mRNA expression was compared in 9 normal breast tissues and 19 ER+ breast cancer tissues. Expression relative to the WT ER mRNA was measured in each sample by incorporating a labelled nucleotide into the PCR products, which were then separated by PAGE (Fig.3). Our preliminary studies confirmed the previous observation that amplification of WT and deleted variant transcripts occurs with similar efficiency (20, 21) and therefore the assay could be used to determine the relative levels of variant mRNA in individual samples. For exon 5- and exon 7-deleted variants, it was possible to express the signal measured as a percentage of the signal provided by the WT ER mRNA.

Because there was a substantial age difference between the reduction mammoplasty patients and the normal breast taken from the tumour patients, it was important to determine if the exon 5- and exon 7-deleted ER variant expression relative to WT ER were identical. No statistically significant difference was observed between these two subgroups of normal patients (data not shown).

The level of exon 5-deleted variant mRNA relative to the WT ER mRNA was found to be significantly less ($P<.001$) in normal (median = 21%) than in neoplastic breast tissues (median = 35%).

Although a similar trend was observed for the exon 7-deleted variant between normal (median 88%) and breast cancer tissues (median = 107%), the difference failed to reach statistical significance (P = .476).

While expression of exon 2-, exon 3- and exon 2-3-deleted variants was reproducibly observed in normal tissues, their relative expression changed from experiment to experiment, suggesting that the efficiency of RT-PCR varied when determination of relative expression of three different transcripts was attempted simultaneously. Figure 3C shows an experiment performed where the exon 2-3-deleted variant was not detected in normal sample N7. This could be explained by similarly low equivalent transcript levels of these variant mRNAs in normal tissues: the amplification occuring randomly on one variant rather than another. It was therefore not possible to quantify relative expression of these variants in normal tissues compared with that seen in cancer tissues.

Discussion

Using RT-PCR, it was possible to observe in each normal tissue studied 5 different exon-deleted ER variant mRNAs and 1 truncated ER variant mRNA. During the preparation of this manuscript a paper describing the detection of exon 2-, exon 3- and exon 7-deleted variant ER mRNAs in one normal human breast tissue sample was published (14). Our data confirm these observations and significantly add information concerning the expression of these variants in multiple normal human breast tissue samples. Further, our studies have detected the expression of an exon 5-deleted ER transcript in multiple normal human tissue samples as well as identifying a previously unknown deletion variant exon 2-3-deleted, in normal and breast cancer tissues. These 2 variants were not observed in the study of Pfeffer et al. (14). Our ability to detect these variants in normal tissues is probably due to our use of a highly sensitive technique. The detection of ER variants in each of 9 different normal tissue samples strongly suggests that the mechanisms generating these variants already exist in normal breast tissue. The primer sets we have used could potentially detect exon 6- and exons 6-7-deleted ER variants, but these were not observed. This

suggests that the mechanisms used to generate deletion and truncated ER variants display some specificity, and that generation of ER variants may have some role in normal ER regulation and/or function.

Many of these variants have been suggested to be involved in progression from estrogen-dependence to independence in breast cancer (7, 22, 23, 24). However, acquisition of hormone independence often occurs late in tumorigenesis and so, it was of interest to compare the expression of these ER variants between normal and cancer tissues with characteristics of good prognostic (ie ER+), to gain further insight into their function and possible involvement in early tumorigenesis.

In order to have a representative group of ER+ breast tumors, selection was made such that approximatively half was ER+/PR+ and the other half was ER+/PR-. While no significant difference in exon 5-deleted ER variant expression was observed between the two groups, the tumor group as a whole had significantly higher levels of exon 5-deleted variant expression relative to WT transcript than in normal breast tissues. Similarly, using a new PCR-based quantitative method, we have recently demonstrated that clone 4 ER variant expression was increased in breast tumors compared to normal breast tissue (Leygue ER, Murphy LC, Watson PW: submitted data). Taken together, these data suggest that the exon-5 deleted variant as well as clone 4 ER variant may have some role in early steps of tumorigenesis.

The absence of statistically significant differences between normal and breast tumor tissues with respect to the exon 7-deleted variant expression suggests that this variant may have a role in normal breast tissue that is not altered due to tumorigenesis. A similar finding, that altered expression of exon 5-deleted ER variant mRNA may occur in association with tumor progression while the same changes do not necessarily occur in the exon 7-deleted variant has also been found in breast cancer cell lines that have developed tamoxifen resistance in vitro (25). This absence of difference may also be due to the small number of samples studied. Additional studies of larger numbers of normal breast and tumor breast tissue may be useful in clarifying this issue.

In conclusion, we have demonstated that a range of ER variant mRNAs can be consistently detected in multiple independent samples of normal human breast tissues. Furthermore, by comparison between normal and neoplastic tissues we have shown that the relative level of expression of specific

variants in normal tissue can be altered and higher in tumor tissues. These data suggest that the mechanism(s) generating ER variant mRNAs already exist(s) in normal breast tissue and may be deregulated in breast cancer tissues; it is our speculation that such deregulation may contribute to progression in human breast cancer.

Acknowledgements

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Figures legend

Figure 1

A Schematic representation of wild-type estrogen receptor (WT ER) cDNA and primers used to detect exon-deleted ER variants: ER cDNA contains 8 different exons coding for a protein divided into structural and functional domains (A-F). Region A/B of the receptor is implicated in trans-activating function (TAF-1). The DNA-binding domain is located in the C region. Region E is implicated in hormone binding and another trans-activating function (TAF-2). D5 primer set allows amplification of 483 bp and 344 bp fragments corresponding to WT ER and exon 5-deleted ER variants, respectively. D7 primer set allows amplification of 668 bp, 534 bp, 484 bp and 350 bp fragments corresponding to WT ER, exon 6-deleted, exon 7-deleted and exon 6-7-deleted ER variants, respectively. D2/3 primer set allows amplification of 531 bp, 414 bp,340 bp and 222 bp fragments corresponding to WT ER, exon 3-deleted, exon 2-deleted and exon 2-3-deleted ER variants, respectively.

B Schematic representation of clone 4 ER variant cDNA and primers used to detect this variant: Clone 4 cDNA contains the first 2 exons of ER cDNA followed by sequences with similarity to Line-1 sequences (17). Clone 4 primer set allows amplification of 337 bp fragment corresponding specifically to clone 4 truncated ER variant mRNA.

Figure 2

Detection of exon 7-deleted and clone 4 ER variants in normal human breast: Total RNA from normal human breast tissue samples (Normal 1-4), T-47D-5 and Ishikawa cancer cell lines was reverse transcribed and PCR amplified as described in material and methods using the D7 primers set (**A**) and clone 4 primers set (**B**). PCR products were separated on 2 % agarose before staining with ethidium bromide.

Figure 3

Comparison of exon -deleted ER variants expression between normal and tumor breast tissues: Total RNA from normal human breast tissue samples (N1-N2), and from breast tumors (T1-T4) was

reverse transcribed and labelled PCR amplification was performed as described in material and methods using D5 primer set (A), D7 primer set (**B**) and D2/3 primer set (**C**). PCR products were separated on 6% polyacrylamide gel containing 7M urea. Following electrophoresis, the gel was dried and autoradiographed for 6-18 hours.

Figure 4

Comparison of exon 5- (del5) and exon 7-deleted (del7) ER variant expression between normal and tumor breast tissues: Total RNA from 9 normal human breast tissue samples and 19 breast tumors was analyzed using D5 primer set and D7 primer set as described in Fig.3. Quantification of signals was carried out after excision of the band corresponding to variant and WT mRNA, as described in material and methods. The exon-deleted signal was expressed as a percentage of the WT ER signal. For each sample, at least 3 independent assays were performed and the mean determined (circles). Differences in exon 5- and exon 7-deleted ER relative expression were then compared using the Mann-Whitney rank sum test (two-sided). Bar represents median of each group. NS : non significant.

Figure 1

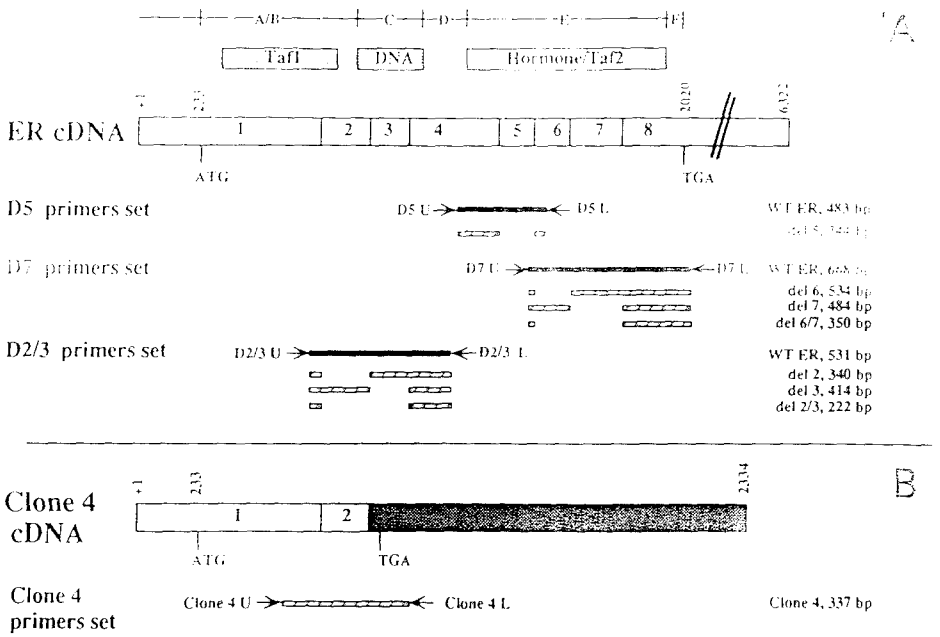


Figure 2

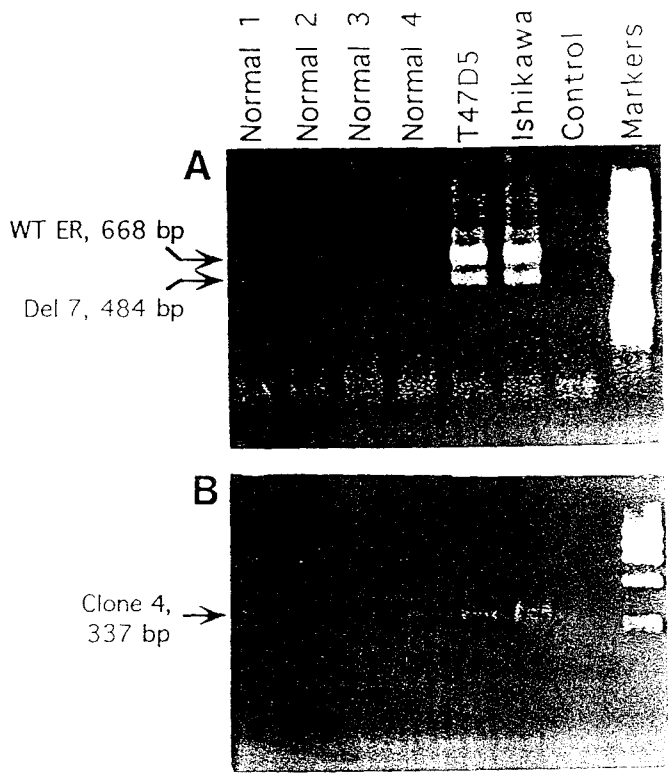


Figure 3

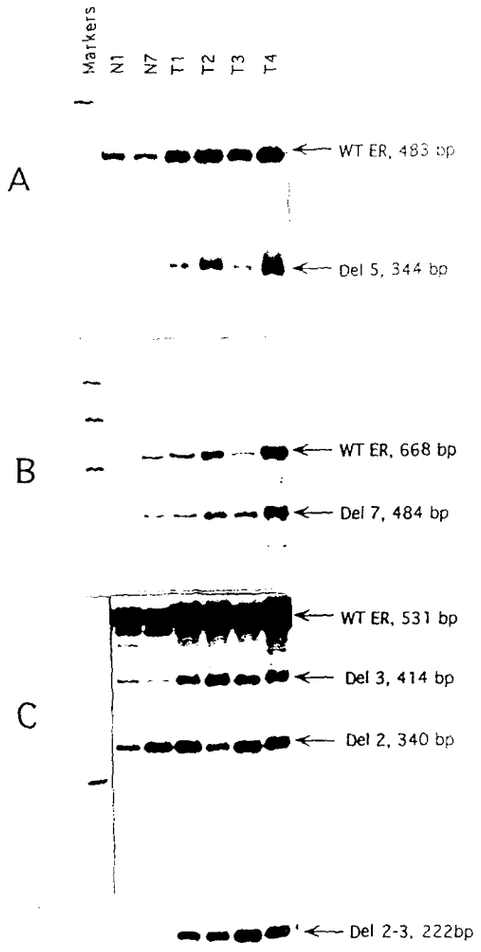
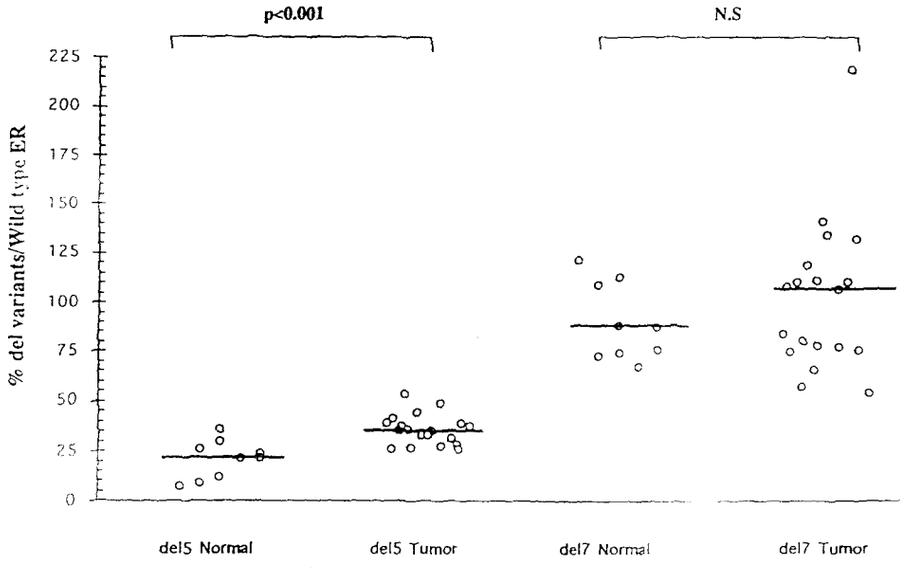


Figure 4



APPENDIX 4

Unpublished Data

EXPRESSION OF EXON-2-3-DELETED ESTROGEN RECEPTOR VARIANT mRNA IN HUMAN BREAST TUMORS.

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Running title:

Del-2-3-ER mRNA in breast tumors

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Summary

The presence of a new estrogen receptor (ER) variant mRNA deleted in exon 2-3 (del-2-3-ER), recently identified in normal human breast tissue, was investigated by reverse transcription and polymerase chain reaction in nineteen ER positive (ER+) breast tumors differing in size, grade, progesterone receptor level and axillary nodal status. Del-2-3-ER was detected in all samples, suggesting that this variant is uniformly expressed in ER+ breast tissue.

Key words

Breast cancer, Differential splicing, Estrogen receptor, mRNA variant, PCR.

Introduction

Several ER variant mRNAs have now been identified in human breast cancer biopsies and cell lines [1, 2, 3, 4]. Some of the predicted ER-like proteins, encoded by these variants and lacking some ER functional domains, have been shown to exhibit altered functions including interference with wild-type ER (WT-ER) function when recombinantly expressed [3, 5]. The presence of some of these ER variant mRNAs in normal breast tissue has recently been reported by several groups [6, 7, 8]. A new ER variant mRNA, deleted in exon 2-3 (del-2-3-ER) and not previously observed in breast tumor samples, was detected in normal breast samples [7]. In order to determine if this variant was also expressed in breast cancer tissue, its expression was investigated using reverse transcription followed by polymerase chain reaction (RT-PCR) in a set of ER+ primary invasive ductal carcinomas with various characteristics of progesterone receptor (PR) level, size, grade and axillary nodal status.

Materials and methods

Human breast tissues and cell lines.

Nineteen primary invasive ductal carcinomas (T1-T19) and two normal tissue samples adjacent to tumor in mastectomy specimens (N1-N2) were selected from the Manitoba Breast Tumor Bank. Tumors were all associated with high ER levels but had various characteristics of PR level, as measured by ligand binding assay, size, grade and axillary nodal status (Table 1). The two normal tissue samples known to express a high (N1) and an undetectable (N2) level of del-2-3-ER mRNA were used as a positive and a negative control, respectively.

Extraction of mRNA and reverse transcription (RT).

Total RNA were extracted from histologically defined regions within 20 µm frozen cryostat sections using Trireagent protocol (Trireagent, MRCI, Cincinnati). RT was performed in a final volume of 15 µl as described previously [7]. 1 µl of the reaction mixture was taken for subsequent amplification using the Polymerase Chain Reaction (PCR).

PCR conditions and detection of PCR products

The D2/3 primer set consisted of D2/3U primer ($5'$-TGCCCTACTACCTGGAGAA-$3'$; located in WT ER exon 1; 615-633) and D2/3L primer ($5'$-TGTTCTTCTTAGAGCGTTTGA-$3'$; located in WT ER exon 4; 1125-1145). The positions given correspond to published sequences of ER cDNA [9].

PCR amplifications were performed in the presence of 10 nM of dCTP [α-^{32}P] (ICN Pharmaceuticals, California), as previously described [7]. PCR products were separated on 6% polyacrylamide gels containing 7M urea (PAGE). Following electrophoresis, the gels were dried and exposed to Kodak XAR Film at -70°C. The identity of the PCR products was confirmed after subcloning using Invitrogen TA Cloning kit and sequencing as previously described [7, 10].

The method used to evaluate exon-deleted variant mRNA expression relative to WT-ER mRNA has been already described [7, 8, 11, 12]. Co-amplification of WT-ER and exon-deleted variants generates bands whose ratio was constant and related to their initial relative expression. This assay provides a semi-quantitative RT-PCR whose internal control is the WT-ER mRNA co-amplified and in which relative expression of variant mRNA can be evaluated for individual samples.

Results

Nineteen different breast tumors whose characteristics are summarized in Table 1 were first analyzed to investigate the presence of del-2-3-ER mRNA. After reverse transcription of total RNA, PCR amplification was performed using primers able to anneal with ER-exon 1 and ER-exon 4 sequences. This amplification resulted in the detection of 4 different PCR products in each breast tumor sample (T1-T19) and in the normal breast sample (N1) known to express del-2-3-ER mRNA (Fig.1A). Only three PCR products were detected in N2, known to contain undetectable levels of del-2-3-ER mRNA. These products corresponded in size to WT-ER, exon 3-deleted, exon 2-deleted, and del-2-3-ER variant mRNAs. Sequencing of del-2-3-ER PCR product revealed a perfect match between sequences corresponding to exon 1 and exon 4 (Fig.1B).

For each sample, the relative expression of del-2-3-ER mRNA to wild-type mRNA, evaluated by comparison of signals of the corresponding amplified bands, was found constant within the set of tumors studied (data not shown).

Discussion

Several ER variant mRNAs previously characterized in breast tumor tissues, were recently detected in normal breast tissue [6, 7, 8]. Some of these variants, such as exon 3-deleted, exon 5-deleted or exon 7-deleted, were shown, using *in vitro* systems, to encode proteins which were able to either interfer with wild-type ER signal transduction or display ligand independent activity [3, 5]. In contrast, some of them, such as exon 2-deleted, encoded a short truncated ER protein, with little effect on this signaling pathway [13]. The role of these variants *in vivo* as well as their biological significance in normal human breast remains to be determined. Recently, a new exon-deleted ER variant, del-2-3-ER, was detected in several normal human breast tissue samples. This variant is expected to encode a truncated 169 amino-acid ER-like protein whose function is unknown. Since this ER variant had not been previously described in breast tumors it was important to determine if it could be detected in ER+ breast tumor tissues.

Del-2-3-ER mRNA was detected in all ER+ breast tumors studied. Use of different primer set is therefore probably the reason why this novel ER variant was not detected in previous studies [6, 8].

Several studies have now been published where exon-deleted ER variant mRNA expression relative to wild-type was evaluated by comparison of signals measured after PCR [7, 8, 11, 12]. Using such a semi-quantitative method to evaluate the relative expression of del-2-3-ER mRNA, no differences were found within the set of tumors studied. This suggests that expression of this ER variant is not significantly altered in ER+ tumors with various characteristics of size, grade, PR status or axillary node status. This result remains however to be confirmed by more reliable quantitative methods.

In conclusion, del-2-3-ER mRNA, already detected in normal breast tissue, was observed in all ER+ breast tumors studied. The biological significance of this ubiquitous ER variant mRNA as well as the possible function of the encoded protein remain to be determined.

Acknowledgements

This work was supported in part by grants from the Canadian Breast Cancer Research Initiative (CBCRI) and the U.S. Army Medical Research and Material Command (USAMRMC). The Manitoba Breast Tumor Bank is supported by funding from the National Cancer Institute of Canada (NCIC) and the "Terry Fox Foundation". PHW is a Medical Research Council of Canada (MRC) Clinician-Scientist, LCM is an MRC Scientist, EL is a recipient of a University of Manitoba Faculty of Medicine Postdoctoral Fellowship.

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Table 1

Tumor	ER (fmol/mg prot)	PR (fmol/mg prot)	Grade	Tumor size (cm)	Axillary nodal status
T1	163	147	4	2.2	nd[a]
T2	105	136	7	5.6	- [b]
T3	110	6	6	2.5	-
T4	140	13	7	4	+ [c]
T5	279	10	8	3	+
T6	199	165	5	1.5	-
T7	108	145	7	3	+
T8	386	12	8	2.2	nd
T9	169	14	9	2.2	+
T10	151	0	5	1.5	-
T11	142	164	6	3	-
T12	284	190	7	1.6	-
T13	279	12	7	1.5	nd
T14	127	105	7	4	+
T15	144	12	8	2.5	+
T16	249	177	6	3.2	+
T17	143	134	5	3	-
T18	164	5	6	1.6	-
T19	177	190	6	1.5	-

[a] not determined, [b] axillary node negative tumor, [c] axillary node positive tumor

Tables legend

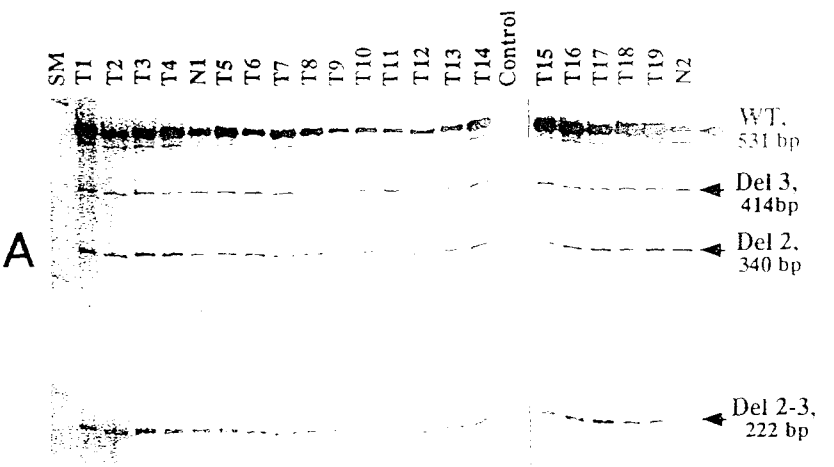
Table 1. Characteristics of the nineteen tumors investigated for the presence of del-2-3-ER mRNA.

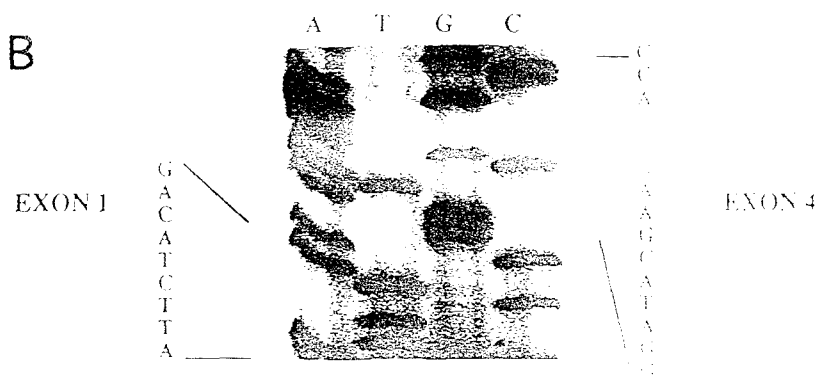
Figures legend

Figure 1

A Total RNA from nineteen breast tumors (**T1-T19**) was reverse transcribed. PCR amplification was performed in the presence of dCTP [α-^{32}P] using D2/3 primer set. PCR products were separated on PAGE and detected as described in Material and methods. D2/3 primer set allows amplification of 531 bp, 414 bp, 340 bp and 222 bp fragments corresponding to WT ER, exon 3-deleted, exon 2-deleted and exons 2-3-deleted ER variants, respectively. **SM**: molecular size marker; **N1**: positive control; **Control**: no cDNA added during PCR amplification; **N2**: negative control.

B Band corresponding to exons 2-3-deleted was excised from the gel, reamplified, subcloned and sequenced. Sequence shown corresponds to the observed junction between exon 1 and exon 4 sequences.


SM
T1
T2
T3
T4
N1
T5
T6
T7
T8
T9
T10
T11
T12
T13
T14
Control
T15
T16
T17
T18
T19
N2
WT,
531 bp
Del 3,
414bp
Del 2,
340 bp
Del 2-3,
222 bp
A


B
A
T
G
C
EXON 1
G
A
C
A
T
C
T
T
A
EXON 4

APPENDIX 5

Unpublished Data

MICRODISSECTION/RT-PCR ANALYSIS OF GENE EXPRESSION IN FROZEN TISSUE SECTIONS

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Keywords: mRNA expression, RT-PCR, microdissection, Breast cancer

Any attempt to translate laboratory hypotheses concerning gene expression to the human disease should recognise the problems in interpretation that are posed by tumor composition and tumor heterogeneity. For example, in solid tumors such as breast cancer, the gross tumor specimen may include normal, in-situ and invasive tumor components as well as stroma and inflammatory elements. Heterogeneity of cell phenotype and gene expression within the invasive component is also well documented. The interpretation of molecular studies is therefore critically dependent on the ability to apply sensitive assays to human tumor tissue that is of the highest quality with respect to pathological definition and cellular preservation.

The assessment of gene alteration at the level of mRNA expression is important for our understanding of tumor progression. Alteration and quantitation of level of mRNA expression can be assessed by a variety of techniques. Some such as Northern Blot, Slot Blot, and RNase protection assay are widely used in the laboratory but are not sensitive enough to allow the study of small samples that are usually available for research. Alternatively, in-situ hybridisation allows the assessment of individual cell expression. However sensitivity, accurate quantitation of signal, and information concerning alteration of mRNA structure can be a significant limitation in some situations. Reverse-Transcription-Polymerase-Chain-Reaction (RT-PCR) assay offers a sensitive alternative that can allow accurate measurement of both structure and level of mRNA based on very small samples. We and others have previously demonstrated the feasibility of extracting DNA from microdissected regions within archival formalin fixed and paraffin embedded tissue sections to assess alterations in gene

structure by PCR (3,8-9). Several groups have also reported RNA extraction from paraffin sections (1, 4-7). However in some instances this required specialised approaches to tissue fixation (4) and in many cases, and in our own experience, the amplification of only relatively stable and abundant RNA species such as 'housekeeping genes' can be reliably demonstrated. (2,5). Alternatively RNA can be extracted from frozen tissues, however this has the limitation of suboptimal histological detail which can make it difficult to assess parameters such as tumor grade or to make the distinction between subtle lesions such as ductal hyperplasia and carcinoma-in-situ in breast tissues. In this report we describe an approach that we have now developed to facilitate microdissection of small pathologically defined regions from frozen tumor sections to provide mRNA for the analysis of gene expression by RT-PCR.

Fresh tissue samples from breast cancer cases are obtained through a standardised and timed collection protocol instituted by the National Cancer Institute of Canada - Manitoba Breast Tumor Bank. Portions of these tissues (typically 0.5 cm^3) are then rapidly bissected, orientated on the external and cut surfaces with different coloured dyes (black india ink, alcian blue and mercurachrome), and each of the two halves placed in either 10% neutrral-buffered formalin or snap frozen in liquid nitrogen within a cryovial. The formalin fixed blocks are then processed routlinely to produce matching and 'mirror image' formalin-fixed-paraffin-embedded and frozen tissue blocks. Thin (5 μm) hematoxylin and eosin (H&E) stained sections are then prepared from the paraffin blocks to allow interpretation of the detailed histology and tumor composition by light microscopy. The corresponding frozen blocks can then be sectioned in a cryostat to provide thin 5 to 20 μm sections when required, in which the distinction of tumor grade, mitotic rate, in-situ tumor vs florid ductal hyperplasia and other

subtle features can be determined by direct comparison with the adjacent high quality paraffin section.

High quality total-RNA is extractable from 20 μm frozen tumor sections using a small scale extraction protocol (Tri-Reagent, Molecular Research Center Inc., Cincinnati) to provide an average yield of 4 $\mu g/cm^2$/20 μm tumor section (consistently $OD^{260/280}$ >1.8 as quantitated by spectrophotometer in a 50 μl microcuvette). But this yield varies (+/- 50%) in direct proportion to the tumor cellularity. Nevertheless, for a typical frozen tumor section measuring 0.25 cm^2 , we obtain 1μg of total RNA which is sufficient to be used as a substrate for multiple RT-PCR assays. We have used this approach to reliably amplify a range of genes such as estrogen receptor, pS2 and CD44. We have also examined the effect of re-use and storage on the yield and quality of RNA that can be extracted from frozen tissue sections. GAPDH and estrogen receptor gene expression was assessed by RT-PCR using RNA extracted from frozen tissue sections, obtained from four different tumor cases. These had previously been sectioned for successful RT-PCR analysis and then re-frozen from 1½ to 2½ years previously. As shown in figure 1 we have found no significant loss of RT-PCR signal in RNA extracted from previously sectioned frozen blocks by comparison with the original RNA extracted, if the tissue blocks are stored at -70^{0}C and carefully handled during processing. By comparison, and as illustrated by the tumor in lane 6, degradation of stored RNA samples can occur.

In order to be able to assess gene expression in a defined region within a tissue section we have found that it is possible to microdissect specific tumor components from within H&E stained frozen sections for RNA extraction and RT-PCR analysis. Multiple

individual 20 μm frozen sections are cut from the frozen tumor block in Leica cryostat at -30^{0}C and each then mounted onto a glass slide that has been previously coated with 2% agarose (Boehringer Mannheim). We have found that frozen sections directly mounted onto glasss slides dry out rapidly during dissection and are difficult to dissect. Slides coated by 2% agarose, to a depth of approximately 1mm, can be prepared by pouring molten agarose in autoclaved ddH_2O onto slides. These are then stored at 4^{0}C for up to 1 hour prior to use to prevent dessication. Mounted sections are then immersed in a Harris's Hematoxylin solution (Mallinckodt) for 1 to 2 seconds, rinsed in water for ten seconds, immersed into Eosin Y in 95% ethanol (Mallinckodt) for 1 to 2 seconds, rinsed again in water for 10 seconds and then placed under a dissection microscope (Leica, wild M3C). Sections can then be oriented and the histological details confirmed with reference to a paraffin H&E stained section from the adjacent and matching paraffin block. Using a scalpel blade and fine needle, specific tumor components can be dissected under a 20X objective and lifted off the agarose to be placed into a pre-cooled microfuge tube and stored on ice. Histologically defined areas less than 1 to 2 mm^2 can be readily microdissected. We have found that the brief H&E stain provides essential cellular discrimination without significantly affecting the ability to perform successful RT-PCR (as shown in figure 2).

After completion of dissection and a brief centrifugation to pellet the dissected material, 10 ul of ddH_20 is added and the material is snap frozen by immersion in liquid nitrogen to disrupt tissue architecture. RNA can then be extracted and used as a substrate for RT-PCR assay.

To demonstrate the feasibilty of this approach we selected an estrogen receptor positive tumor where a well defined invasive tumor component could be identified adjacent to a region of lymphocyte rich stroma. Following microdissection, 100 ng total RNA was used for reverse transcription in a volume of 20 ul of RT mix (1X RT buffer and 200 U MMLV RTase (BRL); 0.5 mM each dGTP, dATP, dTTP, dCTP; 1 uM BSA; 0.01 M DTT; 1.25 mM oligo d(T) primer; 5% DMSO) and incubated for 60 minutes at 37^0C.

PCR amplification of GAPDH and Estrogen Receptor cDNA was then performed in an MJ Research PTC-100 thermal cycler. Each PCR reaction was performed in a 50 ul volume utilizing 2 ul of the completed RT reaction containing cDNA; 1X PCR buffer, 2 mM $MgCl^2$, 1.1 U taq polymerase (Promega); 200mM each dGTP, dATP. dTTP, dCTP; DMSO and 0.5 mM PCR primers. The 40 cycle PCR protocol consisted of a 5 minute preheating step at 94^0C; 45 seconds denaturing at 93^0C, 45 seconds annealing at 56^0C and a 90 sec 75^0C extension step. The final cycle consisted of a 7 minute 72^0C extension step. After thermal cycling was completed, 1.5 ul of gel loading buffer was added to 15 ul of the PCR reaction and samples were electrophoresed on a 2% agarose gel. PCR products were visualized by subsequent Ethidium Bromide staining and photography under UV light. The primer sequences used were as follows; $GAPDH^{943}$ 5' ACC CAC TCC TCC ACC TTT G 3', $GAPDH^{1102}$ 5' CTC TTG TGC TCT TGC TGG G 3', ER^{675} 5' TGC CCT ACT ACC TGG AGA A 3', ER^{860} 5' TGG TAG CCT GAA GCA TAG TC 3'.

In the experiment shown in figure 1, expression of the GAPDH 'housekeeping' gene is detected in the entire unstained frozen tumor section, in the frozen tumor section following brief H&E staining, and in both microdissected tumor and stroma compartments. Similarly expression of the estrogen receptor is seen in the entire tumor section and in the region of invasive carcinoma but as expected is absent in the immediately adjacent lymphocyte rich stroma.

In conclusion, we have described an approach involving a specific protocol for tissue processing that allows for the extraction of mRNA from single histologically defined tumor sections. We have also shown that it is feasible to microdissect small areas from within H&E stained frozen tumor sections and to extract RNA that is suitable for RT-PCR analysis of specific components within tumors.

ACKNOWLEDGEMENTS

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FIGURE LEGENDS

Figure 1. RT-PCR analysis of estrogen receptor and GAPDH expression within frozen sections from re-sectioned tumor blocks. RNA was extracted from frozen sections obtained from tumor blocks that had been sectioned and successfully used for RNA extraction and RT-PCR up to 2½ years previously. In all four tumors the expression of estrogen receptor and GAPDH can be readily detected in the re-used blocks (lanes 1 to 4) and this is similar to the expression seen in the corresponding samples of RNA stored at -70^{0}C since the original extraction. (lanes 5 to 8). Note that the integrity of one stored RNA sample (lane 6) has been lost.

Figure 2. Microdissection/RT-PCR analysis of estrogen receptor and GAPDH expression within a breast tumor section. The upper panel (A) shows the original H&E stained frozen section from a breast tumor with separate regions consisting of invasive tumor and stroma (top left); the adjacent serial section mounted on agarose after microdissection (top right); the detail of the histology of the lymphocyte rich stroma and tumor (bottom left) and the microdissected tumor region (bottom right, scale bar = 0.2 cms). The lower panel (B) shows the results of RT-PCR analysis of ER and GAPDH expression from the entire frozen tumor section (lane 1), the entire H&E stained frozen tumor section (lane 2), the microdissected region of invasive tumor (lane 3), the microdissected region of stroma (lane 4), and the RT-PCR negative control (RNA-).

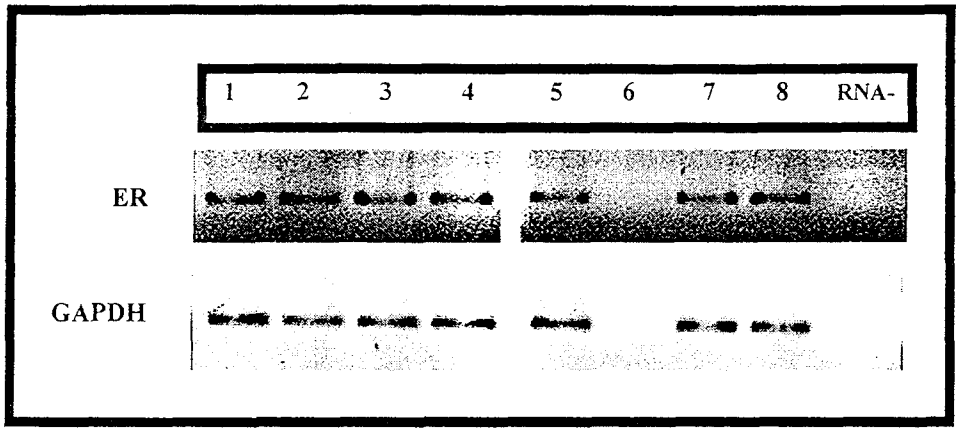

Figure 1. RT-PCR analysis of estrogen receptor and GAPDH expression within frozen sections from re-sectioned tumor blocks.

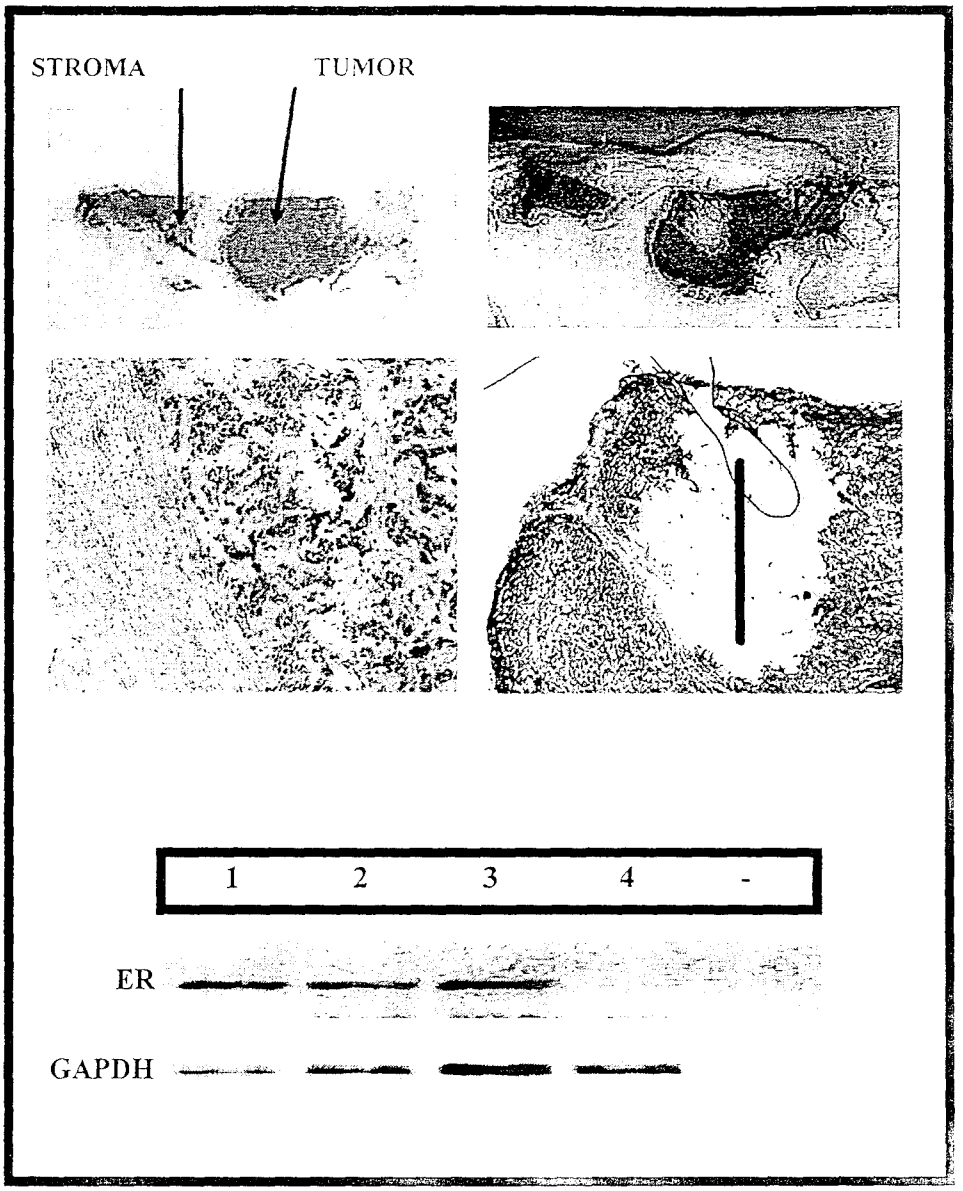

Figure 2 Microdissection/RT-PCR analysis of estrogen receptor and GAPDH expression within a breast tumor section.

APPENDIX 6

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TRIPLE PRIMER-POLYMERASE CHAIN REACTION: A NEW WAY TO QUANTIFY TRUNCATED mRNAs EXPRESSION.

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Short running title: Triple primer-PCR

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Key words:

Estrogen receptor variants, normal human breast, breast cancer, quantitative PCR.

Abstract

The most practical method to quantify mRNA expression within small tumor samples is reverse transcription (RT) followed by quantitative polymerase chain reaction (PCR). Competitive RT-PCR allows absolute quantitation by reference to synthetic RNA standards but is time consuming and requires multiple manipulations which limit its usefulness as a screening assay. We describe here a new approach to quantify truncated type mRNAs relative to the wild-type transcripts in small amounts of tissue. This technique, called RT-Triple Primer-PCR, consists of co-amplification of wild type and truncated cDNAs using three primers in the PCR. To validate this approach, a truncated estrogen receptor variant (clone 4) was quantified relative to the wild-type estrogen receptor using plasmid preparations. The ratio of triple primer-PCR products obtained was directly related to the initial ratio of input cDNAs. RT-Triple primer-PCR was then used to compare the relative expression of clone 4 mRNA to the wild-type estrogen receptor mRNA in frozen sections of normal human breast tissue and human breast tumors with characteristics of good prognosis. The statisticallly significant difference ($p = 0.03$) observed between normal (median = 82.5%) and tumor (median = 107.5%) tissues suggests that elevated expression of the clone 4 variant is associated with early steps of tumorigenesis

This technique provides a useful alternative to competitive or quantitative RT-PCR for the quantification of truncated mRNA within small amounts of biological material.

Introduction

One manifestation of altered gene expression in numerous human pathological diseases is the production of truncated or modified mRNAs which may be translated into modified proteins that act abnormally. Such mRNAs are involved in diseases as different as Glanzmann thrombasthenia,[1] the most common inherited disorder of platelets, Aspartylblucosaminuria,[2] an inherited lysosomal storage disorder, Duchenne and Becker muscular dystrophies[3] or cancer progression.[4,5] Several estrogen receptor (ER) variant mRNAs have also been identified in human breast cancer biopsies.[6,7,8,9] The knowledge of the relative proportion of these modified mRNAs to the wild-type mRNA can provide a useful tool in diagnosis, prognosis or survey of the disease. The accurate quantification of such mRNAs is often difficult and the more commonly used quantitative techniques such Northern blot or RNase protection assay are not sensitive enough to allow assessment of expression within small pathologically defined regions of tissue or small cell numbers. Reverse transcription associated with polymerase chain reaction (RT-PCR) is often the only method that can be used to qualitatively or quantitatively determine mRNAs when sample size and/or abundance are limiting factors.[10,11,12] Comparative RT-PCR, which involves assessment by reference to a "housekeeping" gene is often used but suffers from the assumption that the reference point is stable.[13] An alternative is competitive RT-PCR, which involves the co-amplification of a synthetic standartd that is distinguishable from the cDNA to be quantified by the presence of an introduced restriction site or by differences in length.[14,15] This approach is based on the principle that the two targets will compete for the annealing of the two primers and so an equivalent signal after amplification means that the control DNA and the cDNA to be quantified are present in the same proportion. Techniques based on this approach have been successfully used by many laboratories. Theoretically this approach will allow absolute quantitation but this can be at the cost of multiple manipulations, and larger quantities of RNA and therefore precious tissue samples. For these reasons we concluded that comparative and competitive RT-PCR techniques were not optimal for rapidly quantitating the relative expression of truncated mRNAs to wild-type mRNA in multiple small breast tissue samples. We therefore tested a new approach called

RT-Triple Primer-PCR (RT-TP-PCR) to assess estrogen receptor (ER) variant expression in microdissected human breast specimens.

To validate this assay, we chose to measure the relative expression of a truncated ER mRNA variant (clone 4) to the wild-type ER (WT-ER) mRNA. This truncated ER mRNA variant was initially characterized by sequencing a 2333-base pair cDNA isolated from a human breast tumor cDNA library,[16] and was shown to present significantly elevated expression relative to the WT-ER transcript in tumors with parameters of poor prognosis and endocrine insensitivity.[17] As shown in Figure 1, clone 4 cDNA consists of sequences identical to exon 1 and 2 of the human ER, followed by sequences which are unrelated to those found in human ER cDNA. To perform TP-PCR, three primers are used. The upstream primer (E2U) recognizes both the truncated variant and the wild-type cDNAs. The 2 downstream primers (E3L, C4L) are specific for the WT cDNA and ER-clone 4 cDNA, respectively. Since the upstream primer can anneal to both cDNAs, TP-PCR leads to a competitive amplification of truncated and wild-type cDNA, the final ratio between the co-amplified products being related to the initial input cDNA ratio.

After validating this technique, it was used to compare the relative expression of clone 4 mRNA to WT-ER mRNA in normal human breast tissue and human breast tumors which displayed characteristics of good prognosis.

Materials and methods

Human breast tissues and cell lines.

Normal breast tissues were obtained from reduction mammoplasty specimens collected at the laboratory of F. Kuttenn, Necker Hospital, France (4 cases) and at the Manitoba Breast Tumor Bank (4 cases). Human breast tumor specimens were obtained from the Manitoba Breast Tumor Bank (10 cases). All specimens had been rapidly frozen at -70°C as soon as possible after surgical removal. A portion of the frozen tissue from each case (normal and tumor) was processed to create formalin-fixed and paraffin embedded tissue blocks, matched and orientated relative to the frozen tissue. This allows high quality histological sections to be assessed and pathological interpretation of the corresponding frozen sections

from the immediately adjacent frozen tissue block. The presence of normal ducts and lobules was confirmed in all normal tissue specimens, as well as the absence of any atypical lesion. The ten primary ductal carcinomas were selected from the Tumor Bank on the basis of a set of several parameters that are indicative of a good prognosis. All tumors were well differentiated (Nottingham grade 4 or 5), ER and progesterone receptor (PR) positive as determined by ligand binding assay (ER>3 fmol/mg protein, PR>15 fmol/mg protein), and axillary node negative. Specific frozen tissue blocks were chosen in each case on the basis of several further criteria as assessed in histological sections. These tissue criteria included: a cellular content of greater than 30% invasive tumor cells with minimal normal lobular or ductal epithelial components, good histological preservation and absence of necrosis.

Ishikawa cells, an endometrial adenocarcinoma cell line initially established by Dr. Iwasaki (Tsukuba, Japan), were provided by Dr. E. Gurpide (Mount Sinai School of Medicine, New York). These cells are known to express different ER variant mRNAs (unpublished data and submitted data) and have therefore been used as positive controls. Cos-1 cells (American Type Culture Collection, Rockville, Maryland) do not express ER mRNA and were used as a negative control in our experiments. Cells were grown, harvested and cell pellets stored at -70°C, as previously described.[18]

Plasmids

The pHEGO plasmid contains the previously cloned and sequenced WT-ER cDNA and was kindly provided by P. Chambon.[19] Clone 4 plasmid contains the previously cloned and sequenced clone 4 truncated ER variant cDNA.[16] These 2 plasmids were mixed in order to obtain solutions where the clone 4/WT-ER cDNA proportions range from 1/1000 to 1/1, while maintaining a total constant plasmid concentration of 0.1 ng/µl.

Extraction of mRNA and reverse transcription (RT).

Total RNA were extracted from histologically defined regions within 20 µm cryostat sections of frozen normal and tumor tissue using a small scale RNA extraction protocol (Trireagent, MRCI, Cincinnati) according to the manufacturers instructions. The yield was quantitated by spectrophotometer in

a 50 µl microcuvette. The average yield of total RNA per 20 µm section was 4 µg/cm^2 for tumor and 0.6 µg/cm^2 for normal tissues (± 20% variation with cellularity) with minimal contamination by DNA (Optical Density $^{260/280}$ >1.8).

RT was performed in a final volume of 15 µl. RNA (600 ng) was reverse transcribed in the presence of 1 mM deoxyadenosine-5'-triphosphate (dATP), 1 mM deoxycytidine-5'-triphosphate (dCTP), 1 mM deoxyguanosine-5'-triphosphate (dGTP), 1mM deoxythymidine-5'-triphosphate (dTTP), 5 mM dithiothreitol (GIBCO-BRL), 1 unit/µl ribonuclease inhibitor (Promega), 20 µM random primers, 50 mM Tris-HCl (pH 7.5), 75 mM KCl, 3 mM $MgCl_2$, and 5 units/µl Moloney Murine Leukemia Virus reverse transcriptase (GIBCO-BRL) for 10 minutes at 22°C and 1 hour at 37°C. After 5 minutes at 95°C, 1µl of the reaction mixture was taken for subsequent amplification using the Polymerase Chain Reaction (PCR).

Primers and TP-PCR conditions

Three primers were used in this study (Figure 1). E2U ($^{5'}$-AGGGTGGCAGAGAAAGAT-$^{3'}$, sense, located in WT-ER exon 2; 708-725) and E3L ($^{5'}$-TCATCATTCCCACTTCGT-$^{3'}$, antisense, located in WT-ER exon 3; 969-986) allowed amplification of a 281 bp fragment corresponding to WT-ER mRNA. E2U and C4L ($^{5'}$-GGCTCTGTTCTGTTCCATT-$^{3'}$, antisense; 941-959) allowed amplification of a 249 bp fragment corresponding specifically to clone 4 truncated ER variant mRNA. Positions given correspond to published sequences of ER cDNA[20] for E2U and E3L and of clone 4 cDNA[16] for C4L primer.

PCR amplifications were performed using 1 µl of RT mixture or plasmid solution in a final volume of 10 µl, in the presence of 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM dATP, 0.2 mM dTTP, 0.2 mM dGTP, 0.2 mM dCTP, 4 ng/µl of each primer (E2U, E3L and C4L), 1 unit of Taq DNA polymerase (GIBCO-BRL) and 10 nM of dCTP [α-^{32}P] (ICN Pharmaceuticals Inc, Irvine, California). Each PCR consisted of 40 cycles (1 minute at 60°C, 1 minute at 72°C and 1 minute at 94 °C) using a Thermocycler (Perkin Elmer). 3 µl of the reaction was then denaturated in 80% formamide buffer and subjected to electrophoresis on 6% polyacrylamide gels containing 7M urea (PAGE). Following

electrophoresis, the gels were dried and exposed to Kodak XAR Film at -70°C with two intensifying screens for 2 hours.

Quantification and statistical analysis

Autoradiographs were analyzed with a densitometry system based on a CCD camera (DAGE 72) and MCID M4 software (Imaging Research Inc). The signal corresponding to clone 4 was expressed as a percentage of the corresponding wild-type ER signal.

For each sample, at least 3 independent assays were performed and the mean determined. Relative expression obtained from the 8 normal breast samples was then compared to that found in the 10 tumor tissue samples using the Mann-Whitney rank sum test.

Results

Co-amplification of wild-type ER and clone 4 truncated ER variant cDNAs using triple primer-PCR (TP-PCR).

The ability of WT- ER and clone 4 cDNAs to be amplified simultaneously in a PCR reaction using 3 primers (TP-PCR) was initially determined. Total RNA from 2 normal breast tissue samples was analyzed by RT-PCR using E2U-E2L, E2U-C4L or E2U-E3L-C4L primers (Figure 2). Using E2U and E3L, a band of 281 bp, corresponding to WT-ER cDNA was obtained. E2U and C4L primers allowed amplification of a 249 bp band, corresponding to clone 4 cDNA. When the 3 primers were present during the PCR reaction, both bands were obtained.

Ratio of the clone 4 signal relative to wild-type signal is constant and proportional to the initial wild-type/clone 4 cDNA ratio.

The maintenance of a constant ratio of clone 4/WT-ER signals after TP-PCR was examined under varying PCR conditions. To address this issue, RNA from Ishikawa cells, known to express clone 4 truncated ER mRNA (unpublished data and submitted data) was reverse transcribed and amplified using E2U-E3L-C4L primers for a varying number of cycles ranging from 20 to 45. Quantification of signals showed that the ratio clone 4/WT signals did not vary by more than 20% (data not shown). Similarly,

using a constant number of PCR cycles (40 cycles), variation of input cDNA ranging from 50 ng to 0.1 ng did not affect this ratio by more than 20% (data not shown).

The relationship between the input ratio of clone 4/WT-ER cDNA and the clone 4/WT-ER signal ratio after TP-PCR was determined next. pHEGO and clone 4 plasmids, containing WT-ER cDNA and clone 4 cDNA, respectively, were mixed in varying proportion, ranging from 1000/1 to 1/1. TP-PCR was performed on these samples. Autoradiography showed that the intensities of the 2 PCR products are directly related to the initial ratio of cDNAs added (Figure 3A). Quantification of signals revealed a linear relationship between the final percentage of clone 4 and the log of the initial WT/clone 4 ratio (Figure 3B).

Comparison of clone 4 truncated ER variant mRNA expression in normal and tumor tissues with characteristics of good-prognosis.

The relative level of clone 4 variant ER mRNA expression was then measured by RT-TP-PCR in 8 normal breast tissues and 10 breast tumors with characteristics of good prognosis (Figure 4 and 5). Using the Mann-Whitney rank sum test, the relative expression of clone 4 truncated variant ER mRNA to WT-ER mRNA was found to be significantly ($p = 0.03$) lower in normal breast (median = 82.5%) versus neoplastic breast tissues (median = 107.5%).

Discussion

In this manuscript, we provide strong evidence that triple primer-PCR (TP-PCR) is a reliable quantitative technique to determine relative expression of truncated transcripts: the percentage of signals measured after TP-PCR being directly correlated to the initial input ratio. This new PCR based quantification provides several advantages over existing techniques. There is no need to synthetize controls, the internal control for both reverse transcription and PCR amplification being provided by wild-type mRNA. Moreover, serial dilutions are not necessary and the handling of one tube only decreases the risk of contamination and degradation as well as decreasing variability/error associated with multiple pipetings. This technique is optimal for the study of multiple tissue samples where the quantity of material is limited. During the preparation of this manuscript, a similar approach was used to co-amplify wild-type

glucocorticoid receptor and a truncated variant in myeloma patients.[21] However, in this study, the technique was used to compare relative expression and the quantitative nature of this PCR approach was not tested.

TP-PCR was applied to assess the clone 4 truncated ER variant mRNA expression in normal and tumor human breast tissue. Several ER variants have previously been identified in breast cancer biopsies and cell lines.[6,7,8] Some of the ER-like proteins encoded by these variant mRNAs, lack some ER functional domains, and have been shown to exhibit altered functions or interfer with wild-type ER function.[8,22,23] Therefore, it has been speculated that these ER variants may be involved in progression from hormone dependence to independence in breast cancer.[24] Many of these variant ER mRNAs have now also been detected in normal breast tissue (submitted data).[25] This suggests that variant ER-like proteins may play a role in the normal ER signalling pathway and that any change in relative proportion of these variants could therefore lead to deregulation of this pathway which may contribute to tumorigenesis. Some support for such an hypothesis had been obtained previously when we used an RT-PCR approach to show that the relative expression of exon-5 deleted ER mRNA to wild-type transcript was significantly higher in tumorous breast tissue compared to normal breast tissue (submitted data). Furthermore, we had previously shown, using an RNase protection assay that clone 4 truncated mRNA was significantly elevated relative to WT-ER mRNA in those breast tumors which had characteristics of poor prognosis and hormone independence.[17] Using RT-TP-PCR, we report here that the expression of clone 4 truncated ER mRNA is significantly lower in normal tissues compared to human breast tumors with characteristics of good prognosis (ER+, PR+, node negative). This result together with our previous data strongly support the hypothesis that deregulation of ER variant mRNA expression occurs at relatively early steps in human breast tumorigenesis, and may indeed have a role in this process.

TP-PCR can be adapted to the study of numerous biological problems involving variant mRNA containing unique sequences linked to the sequences shared with wild-type transcripts. Apoptosis involving several partners, including bcl-2, bcl-x and bax, provides an example where relative expression

of such variant mRNAs could be explored using TP-PCR.[26,27] Baxβ mRNA effectively contains unspliced sequences absent from baxα mRNA. The short form of the orphan receptor FTZ-F1 that was recently found to regulate the wild-type protein activity[28] is coded by a variant mRNA whose expression could also be explored by this technique. TP-PCR can also be adapted to DNA studies. Quantification of translocated DNA regions relatively to the wild-type DNA could allow the estimation of an abnormal cell population relatively to the normal one in leukemia patients. TP-PCR can therefore provide a useful tool in diagnosis, prognosis or survey of numerous diseases.

Acknowledgements

This work was supported in part by grants from the Canadian Breast Cancer Research Initiative (CBCRI) and the U.S. Army Medical Research and Development Command (USAMRDC). The Manitoba Breast Tumor Bank is supported by funding from the National Cancer Institute of Canada (NCIC) and the "Terry Fox Foundation". PHW is a Medical Research Council of Canada (MRC) Clinician-Scientist, LCM is an MRC Scientist, EL is a recipient of a University of Manitoba Faculty of Medicine Postdoctoral Fellowship. The laboratory of Pr. F.Kuttenn is gratefully aknowledged from providing us with normal breast tissue samples.

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Figures legend

Figure 1: Schematic representation of Triple Primer-Polymerase Chain Reaction (TP-PCR)

Three primers are used simultaneously during the PCR. The upper primer (E2U) is able to recognize both wild-type estrogen receptor (ER) cDNA and truncated clone 4 cDNA. The lower primers (E3L and C4L) are specific for each cDNA. Competitive amplification of a 281 bp and a 249 bp fragment occurs giving a final PCR-product ratio related to the initial input cDNAs ratio.

Figure 2: Amplification of normal breast tissue cDNA using 2 or 3 primers in PCR

RNA from 2 normal breast tissue samples (N1 and N2) was reverse transcribed and amplified by PCR using E2U-E3L, E2U-E3L-C4L or E2U-C4L primers. PCR products were separated by PAGE and analyzed as described in Material and Methods. Upper and lower arrows show wild-type (281 bp) and clone 4 (249 bp) corresponding signals, respectively.

Figure 3: Validation of TP-PCR technique using different plasmid preparations

A Different plasmid preparations (0.1 ng) were amplified by TP-PCR: plasmid containing wild type ER cDNA alone (0/1), a mix of plasmids containing WT and truncated cDNA in varying ratio: clone 4/wild type ranging from 1/1000 to 1/1, or plasmid containing clone 4 alone (1/0). PCR products were separated by PAGE and analyzed as described in Material and Methods.

B A mix of plasmids containing WT-ER and truncated clone 4 ER variant in varying proportion were analyzed by TP-PCR as described above. The percentage of clone 4 signal relative to the wild-type signal is expressed as a function of the log of WT/clone 4 input cDNA ratio.

Figure 4: Measurement of the relative expression of clone 4 variant ER mRNA in normal and tumor human breast tissues

RNA extracted from normal (N1-N4) or tumorous (T6-T10) breast tissues was analyzed by RT-TP-PCR. PCR products were analyzed as described above. Negative controls consisted of RNAs from Cos-1 cells analyzed simultaneously (Cos cells), or no added cDNA in TP-PCR reaction (control). Upper and lower arrows show wild-type and clone 4 corresponding signals, respectively.

Figure 5: Quantitative comparison of the relative expression of clone 4 variant ER mRNA in normal human breast tissue and human breast tumors with characteristics of good prognosis

RNAs extracted from 8 normal breast tissue samples and 10 tumors with characteristics of good prognosis were analyzed by RT-TP-PCR as described in Material and Methods. For each sample, at least 3 separate experiments were performed. Bars represent medians.

Figure 1

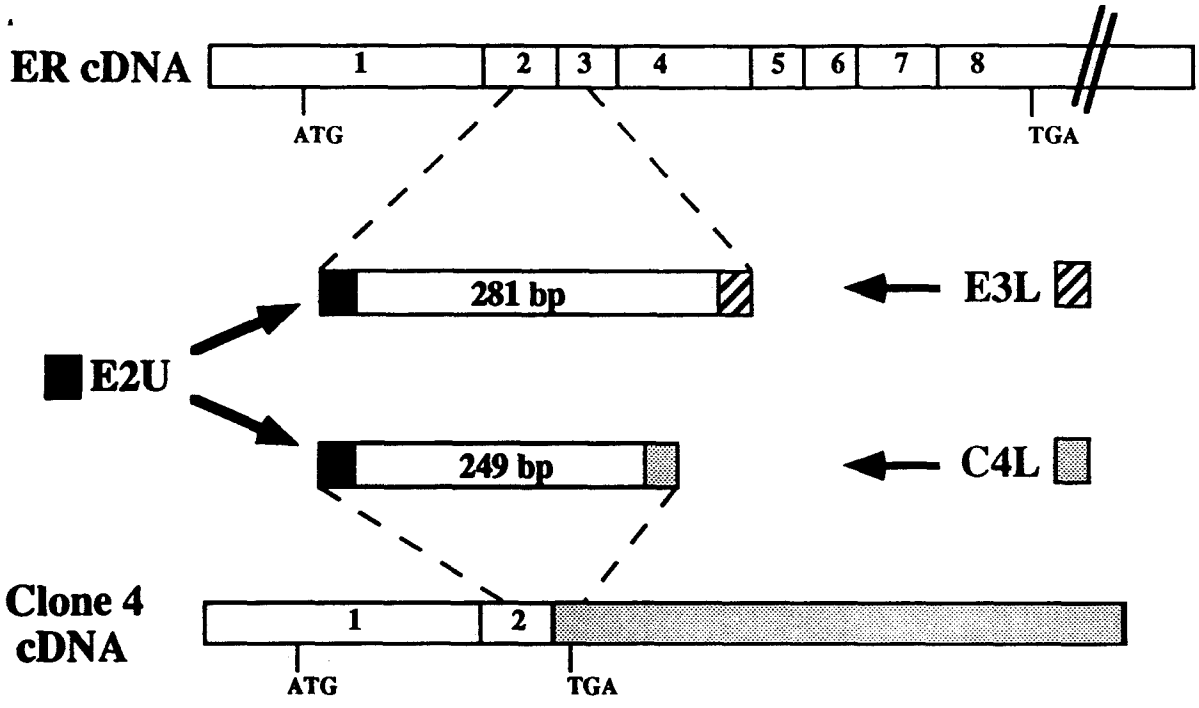


Figure 2

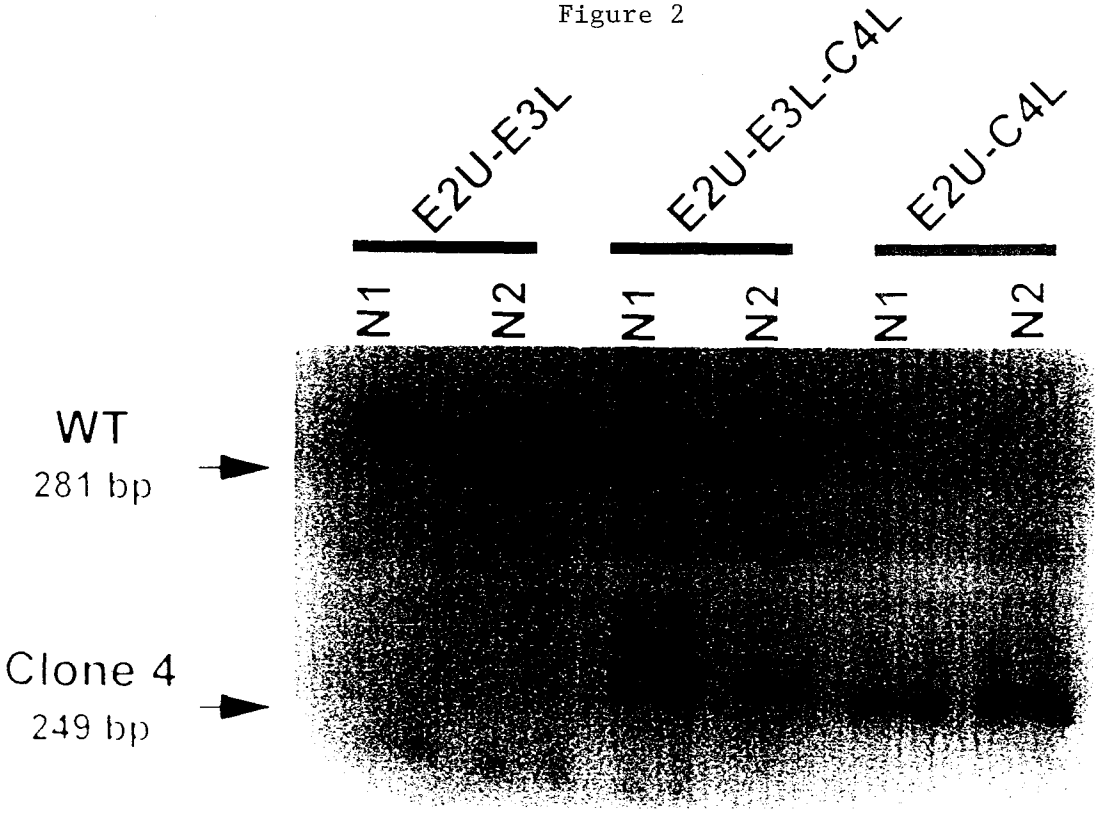


Figure 5

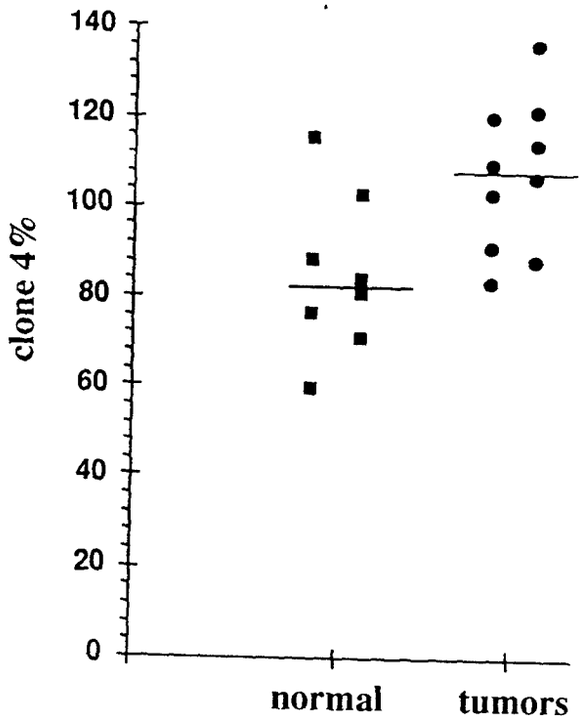


Figure 3

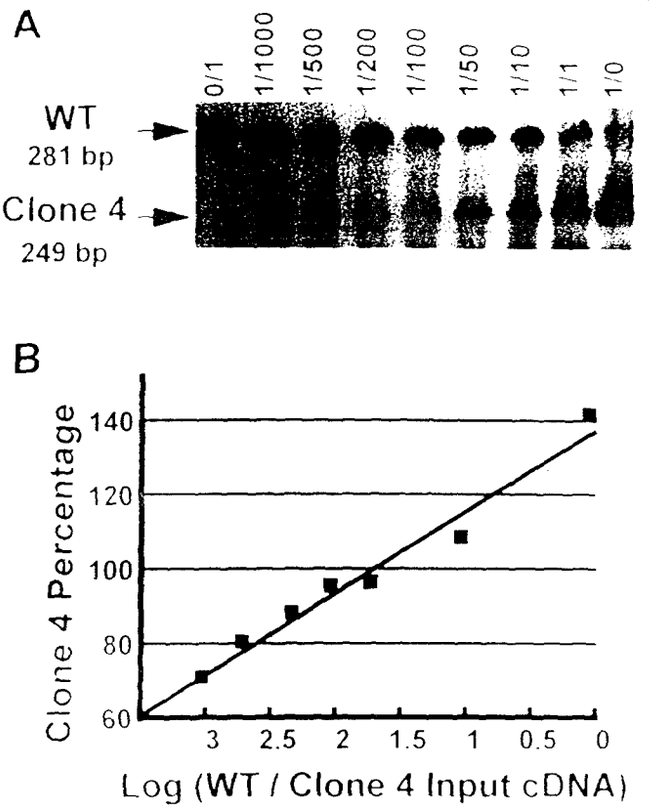
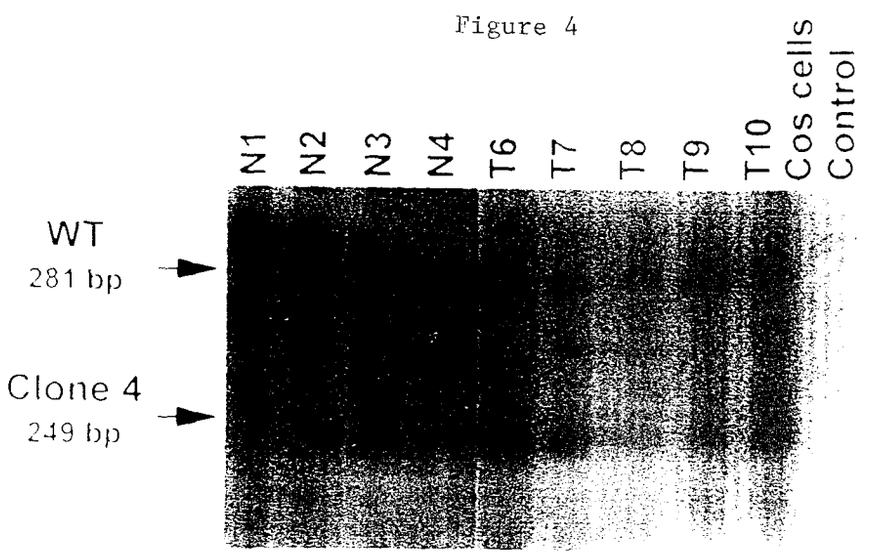


Figure 4



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