Identification of ten exon deleted ERβ mRNAs in human ovary, breast, uterus and bone tissues: alternate splicing pattern of estrogen receptor β mRNA is distinct from that of estrogen receptor α

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Abstract Four different human tissues and breast cancer cell lines were screened to identify exon deletion variant transcripts of estrogen receptor β (ERβ) by reverse transcription-polymerase chain reaction using the 'splice targeted primer approach' that amplifies each category of exon deleted variants as a separate gene population. A total of 10 different variant mRNAs that have deletions in various combination of exons were identified by sequence analysis. They were exon 2Δ; exons 2 and 5–6Δ; exon 3Δ; exon 4Δ; exon 5Δ; exons 5 and 2Δ; exon 6Δ; exons 6 and 2Δ; exons 6, 2–3Δ; and exons 5–6Δ. In some cases, deletion of an exon appears to be associated with a mutation of a specific base. Although ERα and ERβ are highly homologous, have identical exon and functional domain organization, exhibit similar ligand-binding profiles and interact with identical DNA response elements, the sequence of exon skipping in ERβ pre-mRNA appears to be distinct from that of ERα mRNA. Furthermore, results described here also suggest that alternate splicing of ERβ mRNA is tissue specific. The presence of a ERβ variant profile together with other ER isoforms in a tissue may have functional implications in binding and response to a particular ligand.

Key words: Exon deletion variant; Splice targeted primer approach; Estrogen receptor α; Alternative splicing; Sequence of exon deletions; Estrogen receptor β; Human ovary; Human breast; Human uterus; Human bone and tissue specific alternate splicing

1. Introduction

The steroid hormone, estrogen, is a key regulator of growth, differentiation and function of a number of tissues including the male and female reproductive tracts, gastrointestinal tract, mammary gland, cardiovascular, skeletal, nervous and immune systems. The biological effects of estrogens are mediated through four different estrogen receptor (ER) pathways: (1) classical ligand-dependent [1], (2) ligand-independent [2], (3) DNA-binding-independent [3] and (4) cell surface non-genomic signaling [4]. It is now well established that the first three pathways are mediated through two structurally related but distinct receptors, the ERα and ERβ, which have functional domain characteristics of the superfamily of nuclear receptors. The ERα and ERβ are highly homologous in DNA- and ligand-binding domains (97 and 60%, respectively) [5], exhibit similar binding profiles to estrogens and interact with identical DNA response elements [6]. Expression patterns of these two receptors suggested that ERα and ERβ genes are differentially expressed in various tissues. The expression of ERα seems to predominate in the female reproductive organs and ERβ predominates in non-reproductive organs and the male reproductive tract [5,7]. In vitro studies have shown that ERβ has overlapping but distinct transcriptional properties compared to ERα [8]. ERβ markedly differs from ERα in activating genes at AP-1 promoter sites. In addition to possessing a constitutive transactivating property in the absence of ligands [9], ERβ seems to inhibit the transcription of genes at AP-1 sites when bound to agonists [10]. These differences in the transactivating properties between the two receptors together with differential expression in different organs are thought to be the basis for tissue specific agonist/antagonistic actions of SERMS (selected estrogen receptor modulators) [11]. However, it is not known whether the expression of specific structurally altered/truncated functionally active ER isoforms also correlate with tissue specific actions of SERMS since most of the estrogen-responsive tissues also express a number of ERs that have deletions in portions of the molecules in addition to the two wild-type ERs. Examination of exon deletion variants of ERα has shown that the deletions could be either a partial or a complete exon or more than one exon in sequence or at distant ends of ER molecule. A total of 20 different exon deletion variant mRNAs have been identified for ERα by reverse transcription-polymerase chain reaction (RT-PCR) using a novel 'splice targeted primer approach' that detects each category of splice variants as a separate gene population [12–13]. They were exon 2Δ; exons 2 and 7Δ; exons 2 and 4Δ; exons 2 and 5Δ; exons 2, 5 and 7Δ; exons 2, 4–5Δ; exons 2, 4–6Δ; exon 3Δ; exons 3 and 7Δ; exons 2–3Δ; exons 2–3 and 7Δ; exons 2–3, 5 and 7Δ; exon 4Δ; exons 4 and 7Δ; exon 5Δ; exon 6Δ; exons 6 and 4Δ; exon 7Δ; exons 7 and 3–4Δ; and exons 7 and 3–5Δ. Fifteen of the 20 identified variants were detected both in normal and neoplastic tissues of the breast [14]. However, not every identified variant was detected in every tissue analyzed suggesting that the expression of variants is not uniform. Among the 15 variants identified, the transcripts that have deletions of exon 3 and exon 5 were elevated in cancer tissues compared to normal tissues. In addition, cancer tissues showed an increased frequency of multiple exon deletion variants. These two ob-

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servations suggested that ERα mRNA undergoes aberrant splicing during malignant transformation [14,15].

Although the protein products of all spliced variants have not been characterized due to practical limitations of their detection, some of the translated protein products such as the predicted protein product of ERα exon 5Δ has been found naturally in the breast tissues [16]. A few ERβ variant proteins were also described, but their identities were not characterized [17].

In vitro functional analysis of ERα splice variants have shown that exon 5Δ and exon 3Δ proteins translocate to the nucleus and are transcriptionally active. In addition, these two proteins inhibit the wild-type receptor by forming heterodimers and competing for the co-activator, SRC-1e. Exon 5Δ variant protein exhibits constitutive transactivating property in the absence of hormones. Exon 3Δ variant protein binds ligands with the same affinity as the wild-type receptor and has transactivating property at AP-1 sites. Based on these observations, it was proposed that the splice variants act as regulators of gene transcription [18].

The ERβ splice variants are only beginning to be identified and characterized. Three different ERβ variant mRNAs that have deletions in exon 5 or 6 or exons 5–6 have been identified in human breast, uterus and ovary tissues by co-amplification with wild-type receptor sequences [19–21]. Due to several limitations as described [12], co-amplification of alternatively spliced transcripts along with wild-type receptor sequences was not found to be an efficient method for identifying all the splice variant mRNAs. In the current study, we analyzed normal human ovary, breast, uterus and bone tissues and two ERα-positive and two ERα-negative breast cancer cell lines by RT-PCR using the highly sensitive 'splice targeted primer approach' to identify ERβ exon deletion variants. Ten different mRNAs that have deletions in various combinations of exons were detected. Our results also indicate that although both ERα and ERβ are highly homologous, have similar exon and domain organizations and exhibit similar binding profiles to estrogens and interact with identical DNA response elements, they have distinct mRNA alternate splicing patterns. Furthermore, expression of ERβ splice variants appears to be tissue specific.

2. Materials and methods

HotStartTaq PCR core kits, Omniscript reverse transcriptase and MinElute gel extraction kits were from Qiagen, Santa Clara, CA, USA. All the primers used in the current study were synthesized by Gibco-BRL Life Technologies. The pCR®2.1-TOPO cloning vector was obtained from Invitrogen. PCR quality water and Tris–EDTA buffer were from BioWhittaker. Primary cultures of human osteoblasts prepared from trabecular regions of the bone were obtained from Clonetics, normal human ovary and uterus tissues were obtained from Howard University Hospital. They were collected from women undergoing hysterectomy for non-malignant conditions. Total RNA from normal breast tissues, breast cancer cell lines and tumors were available from previous studies [13–15,22,23].

2.1. RNA extraction and cDNA synthesis

Total RNA was extracted from frozen human ovary, uterus and primary osteoblasts using Trizol reagent (Gibco-BRL Life Technologies) as described previously [15,22,23]. RNA integrity was verified by both electrophoresis in 1.5% agarose gels and RT-PCR of the constitutively expressed gene, glyceraldehyde-3 phosphate dehydrogenase (GAPDH). The isolated RNA was reverse transcribed using Omniscript reverse transcriptase as previously described [15,23]. Briefly, the standard mixture contained 1 μg of total RNA, 10 U of RNase inhibitor, 0.5 mM each of dNTPs, 1.0 μM random hexamers and 4 U of Omniscript reverse transcriptase in a total volume of 20 μl. For reverse transcription, tubes were incubated at 37°C for 60 min, followed by 95°C for 5 min and finally rapidly cooled.

2.2. PCR

PCRs were performed in an automatic thermal cycler (MJ Research) as previously described [24] in a total volume of 12.5 μl containing the cDNA, reverse transcribed from 125 ng of total RNA, 1×PCR buffer, 1×Q solution, 200 μM each of dNTPs, 2μM each of sense and anti-sense primers and 0.6 U of HotStartTaq polymerase. GAPDH was amplified in parallel for only 25 cycles with cDNA reverse transcribed from 50 ng of total RNA. The PCR conditions were initial denaturation for 1 min at 95°C, followed by 94°C for 1 min, annealing for 1 min at the specified temperature depending on the primer pair used, extension for 2 min at 72°C for 40 cycles and final extension for 15 min at 72°C. The annealing temperature for amplification of all the transcripts described below is 55°C except for exons 5–6Δ transcripts. To amplify these transcripts, annealing temperature was 61°C. The wild-type ERα sequences were amplified using a sense primer, ERαS, 5′-TGCCCTACTACCTGGAGAACG-3′ (position, exon 1, bp 615–635) and an anti-sense primer, ERαA, 5′-GTCCTTCTCTTCCAGAGAC-3′ (position, exon 7, bp 1651–1633). ERβ wild-type sequences were amplified using a sense primer, ERβS, 5′-CGCTAGAACACACCTTACCTG-3′ (position, exon 1, bp 433–453) and an anti-sense primer, ERβ1A, 5′-CTGTGACCAGAGGGTACAT-3′ (position, exon 7, bp 1344–1326). The 'splice targeted primers' for amplifying ERβ exon 2Δ, exon 3Δ, exon 4Δ, exon 5Δ, exon 6Δ, exons 5–6Δ, and exon 7Δ transcripts were ERβ SX1/3, 5′-ACCTTACCTGTAAACAG1/3GACA-3′ (positions, exon 1, bp 444–460 and exon 3, bp 634–637), ERβSX2/4, 5′-TAAAAGAAGCATTCAAG2/4GCTC-3′ (positions, exon 2, bp 617–633 and exon 4, bp 751–754), ERβSX 3/5, 5′-GAATGGTGAAGTGTG3/5GCTTTG-3′ (positions, exon 3, bp 736–750 and exon 5, bp 1051–1056), ERβAX 6/4, 5′-GCATTTCCCCTCATCC6/4CGGGA-3′ (positions, exon 6, bp 1190–1205 and exon 4, bp 1046–1050), ERβAX 7/5, 5′-GACCAGAGGGTACATAC7/5CTGT-3′ (positions, exon 7, bp 1340–1324 and exon 5, bp 1189–1186), ERβAX 7/4, 5′-CCAGAGGGTACATAC7/4CGGGAA-3′ (positions, exon 7, bp 1340–1324 and exon 4, bp 1045–1050), and ERβAX8/6, 5′-GTTCCATGCCCTTGTTA8/6TGGA-3′ (positions, exon 6, bp 1320–1323 and exon 8, bp 1505–1521), respectively. The partner primer for 2Δ, 3Δ and 4Δ was ERβ2A, 5′-TTGAGCAGATGTTCCATGCCC-3′ (position, exon 8, bp 1511–1531). For 5Δ, 6Δ, 5–6Δ and 7Δ, the partner primer was ERβS. The design, specificity, and applicability of splice targeted primers in amplifying and quantifying alternatively spliced ER mRNAs as separate gene populations in cell lines and tissues have been well established [12–15,23]. The GAPDH was amplified using a sense primer, 5′-AAGGCTGAGAACGGGAAGCTTGTCATCAAT-3′ (position, exon 3, bp 241–270) and an anti-sense primer, 5′-TTCCCGTCTAGCTCAGGGATGACCTTGCCC-3′ (position, exon 7, bp 740–711) [25]. The sequence and locations of ER primers are based on the full-length cDNA sequences [26–27].

2.3. Detection and identification of PCR products

The PCR amplified products (6.5 μl each) were separated by electrophoresis in 1% agarose gels in Tris–acetic acid–EDTA buffer and detected by ethidium bromide staining. To detect the PCR products of GAPDH, 1 μl was used. The identities of the PCR amplified ER products were determined by cloning the gel purified products into pCR®2.1-TOPO vector and sequence analysis as previously described [13–15].

3. Results and discussion

Alternate splicing of pre-mRNAs is thought to be one of the cellular mechanisms for generating a functionally diverse pool of gene products derived from a single gene. It is also recognized as a process that contributes to the complexity and diversity of human proteome given the presence of only 30 000 to 40 000 structural genes [28–29], just twice as many as for a worm or a fly. Alternate splicing is also recognized as an important mechanism for regulating the wild-type proteins.

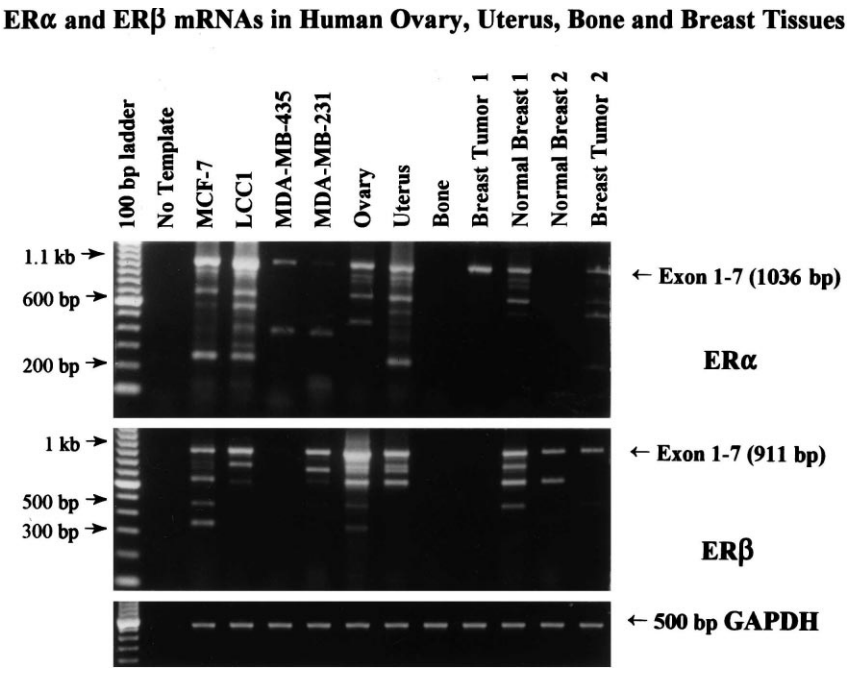

Fig. 1. Expression of ERα and ERβ wild-type mRNA in ovary, uterus, breast and bone tissues and breast cancer cell lines. The cDNAs from the tissues and cell lines were amplified for ERα and ERβ wild-type (exon 1–7) by RT-PCR as described in Section 2. 6 μl of the PCR amplified products were analyzed by agarose gel electrophoresis and detected by ethidium bromide staining. The house keeping gene, GAPDH gene, was amplified in parallel.

For example, two splice variants of ERα, the exon 3Δ and 5Δ have been shown to modulate the wild-type receptor [18] in addition to possessing their own functional activities. The wild-type bullfrog glucocorticoid receptor-3 (bfGnRHR-3) activity has also been shown to be inhibited by alternatively spliced variant proteins in a dose-dependent manner [30]. In this case, splice variant proteins did not show any transcriptional activity. A recent analysis of human expressed tag databases showed that 38% of human primary transcripts are alternatively spliced and there are approximately 2.75 splice forms per differentially spliced transcript [31].

Although the presence of alternatively spliced variants for several genes has been known for some time, little is known about the factors that regulate this process. In particular it is not known whether the sequence of exon skipping in a pre-mRNA is specific to the gene or the type of tissue. In the current study we have examined the alternate splicing pattern of ERβ mRNA and compared it with the closely related ERα mRNA. To detect all the possible ERβ splice variant transcripts, we analyzed four different estrogen-responsive human tissues, breast, ovary, uterus and bone tissues and four different breast cancer cell lines by RT-PCR using a highly specific 'splice targeted primer approach'. For comparative purposes, we also amplified wild-type ERα and ERβ mRNAs.

The expression of ERα and ERβ wild-type (exon 1–7), 10 exon deletion variants of ERβ in ovary, uterus, bone and breast tissues, two ERα-positive breast cancer cell lines (MCF-7 and LCC1) and two ERα-negative breast cancer cell lines (MDA-MB-435 and MDA-MB-231) are shown in Figs. 1 and 2, respectively, and in Table 1. Ten exon deletion variants of Erβ were identified. They were exon 2Δ, exons 2 and 5–6Δ, exon 3Δ, exon 4Δ, exon 5Δ, exons 5 and 2Δ, exon 6Δ, exons 6 and 2Δ, exons 6 and 2–3Δ, and exons 5 and 6Δ (Fig. 3). Among all the tissues and cell lines examined only ovary showed the presence of nine of the 10 identified transcripts (Fig. 3, Table 1). Although the wild-type receptors were not detected in bone tissue (osteoblasts) presumably due to low level of expression, two exon deletion variants

Table 1
Expression of ERα wild-type, ERβ wild-type and ERβ exon deletion variants in human ovary, uterus, bone and breast tissues and cell lines

Tissue/cell line	ERα wild-type	ERβ wild-type	ERβ exon 2Δ	ERβ exon 3Δ	ERβ exon 4Δ	ERβ exon 5Δ	ERβ exons 5 and 2Δ	ERβ exon 6Δ	ERβ exons 6 and 2Δ	ERβ exons 6, 2–3Δ	ERβ exons 5–6Δ	ERβ exons 5–6, 2Δ
MCF-7	+	+	−	−	−	+	−	−	−	−	+	−
LCC1	+	+	+	−	−	+	−	−	+	−	+	+
MDA-MB-435	+	−	−	−	−	−	−	−	−	−	+	−
MDA-MB-231	−	+	+	−	−	+	+	−	−	−	−	−
Ovary	+	+	+	+	+	+	+	+	+	−	+	+
Uterus	+	+	−	−	−	+	−	+	−	+	+	+
Bone	−	−	−	−	−	−	+	−	−	−	−	+
Breast tumor 1	+	−	+	−	−	−	−	−	−	−	−	−
Normal breast 1	+	+	−	−	−	+	−	+	−	−	+	+
Normal breast 2	−	+	−	−	−	−	−	+	−	−	+	−
Breast tumor 2	+	+	−	−	−	−	−	+	−	−	−	−

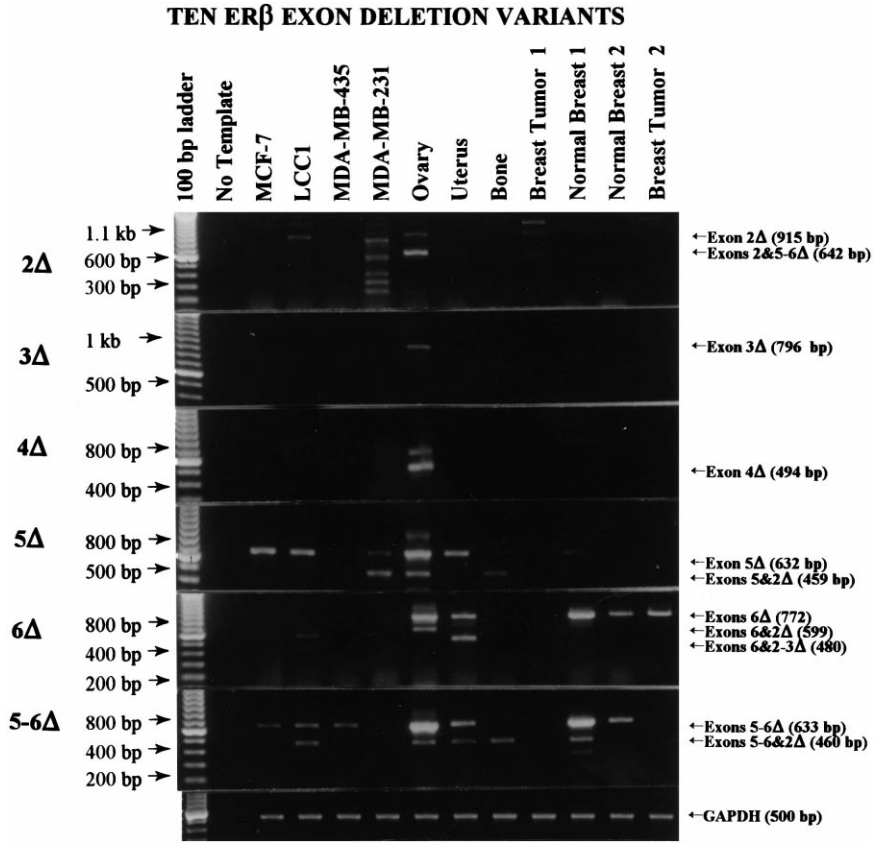

Fig. 2. Expression of ERβ exon deletion variants in ovary, uterus, breast and bone tissues and breast cancer cell lines. Exon deletion variant mRNAs were amplified by RT-PCR using splice targeted primers. Partner primers were either in exon 1 or 8 depending upon the targeted primer used. This permitted the amplification of transcripts with multiple exon deletions in distant exons. PCR amplified products were identified by cloning and sequence analysis as described in Section 2. Expression of 10 identified exon deletion variants in various tissues and cell lines are shown. GAPDH was amplified in parallel.

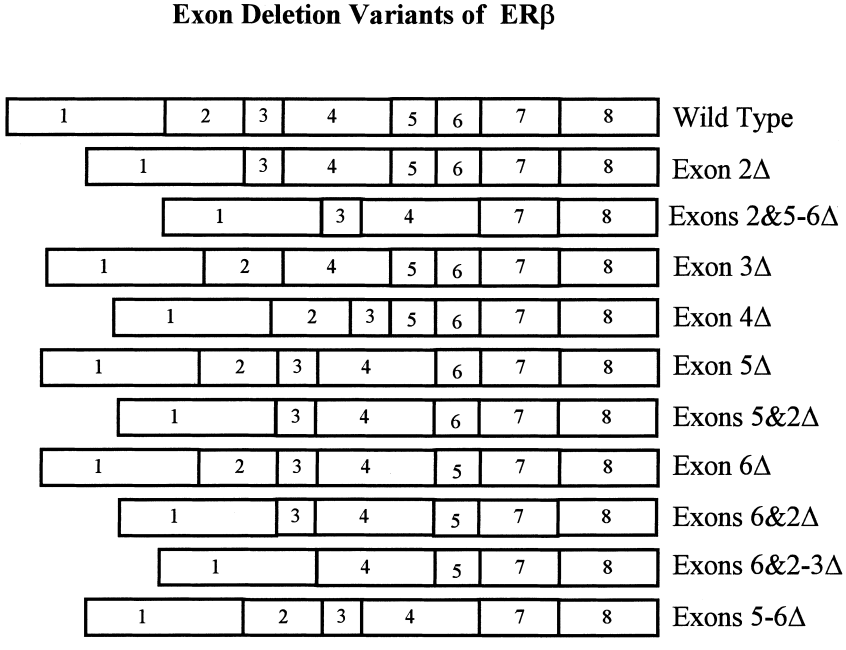

Fig. 3. Schematic diagram of 10 human ERβ exon deletion variants. The wild-type and 10 exon deletion variant mRNAs identified are represented.

Table 2
Summary of base changes in ERβ exon deleted transcripts

Variant	Base position	Base in wild-type	Base in variant
Exon 2Δ	1087	T	C
	1201	A	G
	1237	T	C
	1315	T	C
	1427	G	-
Exon 3Δ	1347	T	C
Exon 4Δ	1078	A	–
Exons 5 and 2Δ	989	A	–
	1035	**C**	**–**
Exons 5–6Δ	1000	A	–
	1035	**C**	**–**
Exons 5–6, 2Δ	**1035**	**C**	**–**
Exon 6Δ	539	G	–
	542	G	AA
	551	G	A
	717	**G**	**A**
Exons 6 and 2Δ	**717**	**G**	**A**
	1169–1170	–	T
	1172	T	–

were detected. The ERα-negative cell line, MDA-MB-435, although it did not show the presence of any ERβ wild-type, was positive for the presence of exons 5–6Δ transcript. Interestingly, breast tumor 1 and normal breast tissues 2, each showed the expression of only one type of ER isoform transcripts. ERβ exon 7 deletion variants, which are the most abundant among the ERα mRNAs (13–14), are not detected in any of the tissues or cell lines analyzed. The results shown in Figs. 1 and 2 and Table 1 also suggest that expression of ERβ exon deleted variants is tissue specific.

Sequence analyses of both strands of the above variants also revealed several base changes in various transcripts as summarized in Table 2. We observed that deletion of a second exon in exon 5Δ transcript is associated with a deletion mutation (position, exon 4, bp 1035). This deletion mutation was observed in exons 5–6Δ, exons 5 and 2Δ and exons 5–6, and 2Δ transcripts (Table 2). We also observed an association between exon 6 deletion and substitution of base A in the place of G (position, exon 3, bp 717). This substitution mutation was observed in exon 6Δ and exon 6 and 2Δ transcripts. The functional significance of these mutations is not known.

Examination of the sequence of exon deletions in ERβ mRNA suggests that it is distinct from that of ERα mRNA. Our data on ERα splice variants indicated that exon 2 was the most commonly deleted exon since it was detected in every normal breast tissue analyzed, and 10 of the 20 exon deleted variants had the deletion of exon 2 [13–14]. Our studies also suggested that initial deletion of an exon in a transcript is mostly followed by the deletion of exon 7, and the third most common exon to be deleted after the deletion of exon 7 is exon 5. After the deletion of exon 2 in a transcript, if the second deletion is initiated at exon 4, the deletions seem to proceed up to exon 5 or 6. Initial deletion of exon 7 in a transcript seem to always follow the deletion of either exon 3 or exon 4 and proceed up to exon 5. Exon deletions of ERβ mRNA appear to be not as extensive as ERα since only 10 mRNAs were detected while 20 ERα were identified. Deletion of exon 6 which is very rare in ERα mRNA [13–14] appears to be one of the most frequently deleted exon of ERβ mRNA. Exon 2 appears to be as frequently deleted as exon 6 in an ERβ mRNA. Detection of exons 5 and 2Δ, exons 6 and 2Δ, exons 6 and 2–3Δ and exons 5–6 and 2Δ mRNAs suggests that deletion of exon 2 is associated with the deletion of exon 5 and/or 6. However, it is not clear whether the deletion of exon 2 in an ERβ mRNA precedes or follows the deletion of exon 5 or 6 or 5–6.

Deletion of exon 2, 5 or 6 in ERβ mRNA causes a frame shift mutation resulting in premature termination of translation, thereby generating a diverse class of C-terminally truncated receptor forms. Deletion of either exon 3 or 4 does not disrupt the mRNA reading frame, but produces receptor proteins with internal deletions. The proteins translated from exon 2Δ and 4Δ lack nuclear localization signals and remain in cytoplasm. The proteins translated from exon 3 deletion results in an in-frame loss of 117 nucleotides that encode 39 amino acid (aa) in the carboxyl-terminal half of the DNA-binding domain (DBD), including the second zinc finger. The exon 5 and 6 deleted splice variant proteins are unlikely to bind ligands because of truncation in the ligand-binding region. The exon 5–6 deleted splice variant is in frame but deleted in 91 aa which are within the hormone-binding domains and has glycine at aa residue 409 instead of serine. A putative protein encoded by exon 5–6 deleted variant will have lower affinity for ligands. However, it will retain the AF-2 site required for binding with co-activators and co-repressors. The multiple exon deleted transcripts, exons 5 and 2Δ, exons 6 and 2Δ, exons 6, 2–3Δ, and exons 2,5–6Δ are likely to generate proteins that are defective in either ligand binding or DNA binding or both.

There has been a debate for a long time whether the ER splice variant mRNAs are translated into proteins and the truncated ERs are functionally active. Recent reports show that both ERα and ERβ splice variant mRNAs are translated into proteins [16–17] and ERα exon 5Δ and 3Δ proteins are functionally active in ligand binding and transactivation [18] as described earlier. Since portions of the variant molecules retain the structural characteristics of intact receptor, these proteins may directly or indirectly participate in ligand binding and transactivating properties by protein–protein interactions. The ERβ exon 3Δ and 5Δ proteins, by analogy to ERα variants, could translocate into the nucleus and may modulate the wild-type receptor. Exon 6Δ and exons 5–6Δ proteins, although lacking portions of ligand-binding domain, have the nuclear localization signal and, therefore, may translocate to the nucleus, bind EREs and modulate the transactivation property of the wild-type receptor similar to ERα exon 5Δ protein. In addition, exons 5–6 deletion variant proteins may be involved in binding to co-repressors and co-activators and regulate the wild-type receptor. Proteins translated from other single and multiple exon deletion variant transcripts that lack nuclear localization signal may remain in the cytoplasm. The functional properties of cytosol localized splice variant ER proteins are not known. It remains to be established whether the cytosol localized splice variant proteins are involved in cross talk between the growth factor receptor mediated intracellular kinase pathways and ligand independent transactivation of nuclear ERs.

The results presented in Figs. 1 and 2 also show considerable differences in the expression profiles of ER isoforms in various estrogen-responsive tissues. It remains to be established whether differential expression of types and relative levels of various ER isoforms are the basis for tissue specific agonist/antagonistic actions of SERMS.

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