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Identification of twenty alternatively spliced estrogen receptor alpha mRNAs in breast cancer cell lines and tumors using splice targeted primer approach

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Abstract

Estrogen receptor (ER) alpha splice variant transcript profiles were analyzed by RT PCR in six ER positive breast cancer cell lines, MCF-7, T47D, ZR-75, LCC1, LCC2 and LCC9, three ER negative cell lines, MDA-MB-435, MDA-MB-235 and LCC6, and three ER positive malignant breast tumors using targeted primers which specifically anneal to the splice junctions of exon 2Δ, exon 3Δ, exons 2–3Δ, exon 4Δ, exon 5Δ, exon 6Δ and exon 7Δ. The partner primers were chosen such that largest possible transcripts were amplified between exons 1 and 8. The results described here show that each splice specific primer amplified not only the single exon deleted transcript but also a number of related transcripts that have deletions in various combinations of exons. The exon 2Δ specific primer amplified five transcripts that have deletions in exon 2, exons 2 and 7, exons 2, 5, and 7, exons 2 and 4–5, and exons 2 and 4–6. The exon 3Δ specific primer amplified two transcripts that have deletions in exon 3, and exons 3 and 7. The exon 2–3Δ specific primer amplified three products that have deletions in exons 2–3, exons 2–3 and 7 and exons 2–3, 5 and 7. The exon 4Δ specific primer amplified two products that have deletions in exon 4, and exons 4 and 7. The exon 5Δ specific primer amplified three transcripts, that have deletions in exon 5, exons 5 and 2, and exons 5, and 2–3. The 6Δ specific primer amplified only one transcript that has a deletion in exon 6. The 7Δ specific primer amplified four transcripts, that have deletions in exon 7, exons 7 and 4, exons 7 and 3–4, and exons 7 and 3–5. None of the above splice specific primers amplified the wild type ER sequences. The six ER positive cell lines differed in the patterns of the variant transcripts and among the three ER negative cell lines analyzed, only MDA-MB-435 showed the presence of exon 2Δ and exon 4Δ transcripts. Analyses in the tumor samples indicated that the above transcripts are extensively modified.

Keywords: ER alpha splice variants; Splice targeted primers; Sequential exon deletions; Breast cancer cell lines and tumors; Distant exon deletions

1. Introduction

The ER alpha mRNA undergoes alternate splicing, generating transcripts containing single, double or multiple exon deletions. The presence of ERα transcripts with deletions in exons 2-, 3-, 2–3, 2–5, 4-, 5-, 6- and 7 has been described in breast cancer cell lines and normal- and malignant breast tissue samples [1–4]. Although the exact function(s) of these splice variants is not established, it has been hypothesized that the splice variant mRNAs may result in proteins that differ in activity. These may differentially modulate the ER signalling pathway in normal tissues. Consequently, changes in the balance of these transcripts could perturb the ER signaling pathway and contrib-

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Nomenclature

ER	estrogen receptor	Exon Δ	exon deletion
PgR	progesterone receptor	AX	anti-sense
GAPDH	glyceraldehyde 3-phosphate dehydrogenase	SX	sense

ute to tumor progression. Several studies suggested that the expression of certain exon deletion transcripts is deregulated during breast tumorigenesis. It was shown that the exon 5 deletion transcript was significantly elevated in ER^- PgR^+ breast tumor tissues [5]. Elevated levels of exon 7 splice transcripts have also been reported in $ER^+/PR^-/pS2$ compared to ER^+/PgR^+ tumors [6]. It has been reported that expression of the exon 3-deleted mRNA is reduced in breast tumor tissue compared with normal tissue [7]. Differential expression of exon 5 and exon 7 deletion transcripts also seem to influence the estrogen responsiveness in breast cancer cell lines [8]. All these reports suggest that expression of some ER variants is altered in human breast tumors and may contribute to tumorigenesis, tumor progression and response to hormones. Therefore, it is important to qualitatively and quantitatively investigate the levels and pattern of ER splice variant expression between normal and neoplastic tissues, and amongst groups of tumors with different characteristics. Yet, there are no specific methods available which can precisely detect and quantify the alternatively spliced ER molecules.

Conventionally, the ER exon deletion variant transcripts are characterized by co-amplification with the wild type sequences using reverse transcription polymerase chain reaction (RT-PCR) approaches which by virtue of specific primer design are focussed on small regions of the known wild type mRNA. However, there are several practical limitations to this approach. Firstly, the threshold of detection — since the wild type transcripts are present in large excess to alternatively spliced molecules, a competitive amplification occurs amongst the wild type and all the alternatively spliced transcripts. Detection of products corresponding to alternatively spliced molecules depends upon the relative expression levels of their mRNA species within the sample. Thus, spliced transcripts expressed at low levels may fall below the threshold of detection. Secondly, this approach cannot distinguish those mRNAs with multiple deletions in distant exons. For example, an ER transcript which has deletions in exons 2 and 7 cannot be distinguished from transcripts having single deletions in exon 2 or exon 7 by this method, and finally transcripts with similar sized deletions cannot be distinguished by gel exclusion chromatography.

To circumvent all the above described limitations, we have developed a new approach to characterize the alternatively spliced molecules. This involves the targeted amplification of the alternatively spliced molecules as separate gene populations without co-amplification of wild type molecules using specific primers designed for the alternative splice junctions [9]. In the current study, we analyzed the ER single, double, and multiple exon deletion variant transcripts in breast cancer cell lines and tumors by RT PCR using the splice targeted primers. We show here that each splice specific primer amplifies not only the single exon deleted transcript but also a number of related transcripts with deletions in various combinations of exons. Our results also show that several alternatively spliced molecules are either missing or extensively modified in tumor samples.

2. Materials and methods

AmpliTaq PCR core kits and QIAquick gel extraction kits were obtained from QIAGEN, Santa Clara, CA. All the primers used in the current study were synthesized by Gibco-BRL Life Technologies. Reverse transcriptase kits were purchased from Applied Biosystems. The pCR®2.1-TOPO cloning vector was obtained from Invitrogen. PCR quality water and Tris-EDTA buffer were from Biofluids, Rockville, MD. The total RNA samples from breast cancer cell lines and tumors were prepared using Trizol reagent (Gibco-BRL). The integrity of all the RNA preparations was confirmed by electrophoresis and ethidium bromide staining and amplification of the constitutively expressed gene, glyceraldehyde-3 phosphate dehydrogenase (GAPDH). The ER status of all the tumors used in the current study was determined immunohistochemically by Oncotech laboratories using monoclonal antibodies against the NH_2 terminal (A/B region) of the receptor. The six tumors used were ER positive by the above immunohistochemical method.

2.1. Targeted primers for the amplification of single, double and multiple exon deletion variant cDNAs of ER

We have previously shown that the primers targeted at the alternate splice junctions that have a minimum of three out of four unique bases at the extreme 3′ end

will specifically amplify the spliced junction without amplifying the flanking wild type exons and in order to design such a primer, the overhang sequences can extend up to eight bases past the splice junction [9]. The splice specific primers used in the current study were designed based on these principles. The splice specific primers used for amplifying 2Δ, 3Δ, 2–3Δ, 4Δ, 5Δ, 6Δ, and 7Δ were ER SX1/3, 5′ CGCCGGCATTCTACAG 1/3 GACAT 3′ (positions, exon 1, bp 669–684, and exon 3, bp 876–880), ER SX2/4, 5′ AAGAGAAGTATTCAAG 2/4 GGATA 3′ (positions, exon 2, bp 860–875 and exon 4, bp 993–997), ER SX1/4, 5′ GCCGGCATTCTACAG 1/4 GGATAC 3′ (positions, exon 1, bp 670–684 and exon 4, bp 993–998), ER SX3/5, 5′ GTGGGAATGATGAAAGGTG 3/5 GCTTT 3′ (positions, exon 3, bp 974–992 and exon 5, bp 1329–1333), ER AX4/6, 5′ ATTTTCCCTGGTTC 6/4 CTGGCAC 3′ (positions, exon 6, bp 1481–1468 and exon 4, bp 1328–1322), ER AX 5/7, 5′ CAGAAATGTGTACACTC 7/5 CTGT 3′ (positions, exon 7, bp 1618–1603 and exon 5, bp 1468–1465) and ER AX6/8, 5′ CTCCATGCCTTTGTTA 8/6 CAGAA 3′ (positions, exon 8, bp 1801–1786 and exon 6, bp 1601–1597), respectively. The partner primer for 2Δ, 3Δ, 2–3Δ, and 4Δ splice specific primers was ERA, 5′ GCACTTCATGCTGTACAGATGC 3′ (position, exon 8, bp 1822–1801) and for 5Δ, 6Δ, and 7Δ primers was ERS, 5′ TGCCCTACTACCTGGAGAACG 3′ (position, exon 1, bp 615–635). The sequence and locations of all the primers described here are based on the full length ER cDNA sequence published by Green et al. [10].

2.2. Reverse transcription and PCR

The total RNA was reverse transcribed to cDNA using Maloney Murine Leukemia Virus reverse transcriptase and random hexamers. Briefly, the standard reaction mixture contained 1 μg of total RNA, 2.5 units of MuLV reverse transcriptase, 1 mM each of dNTPs, 2.5 μM random hexamers, 20 U of RNAse inhibitor, 5 mM $MgCl_2$ and 1 × PCR buffer in a total volume of 20 μl. To reverse transcribe the RNA, the reaction tubes were first left at room temperature for 10 min, followed by incubations at 42°C for 15 min, 99°C for 5 min and finally 5°C for 5 min. The polymerase chain reactions were performed in an automatic thermal cycler (MJ Research) as described previously [11] in a 25 μl reaction volume containing the cDNA reverse transcribed from 250 ng of total RNA, 1 × PCR buffer, 1 × Q solution, 200 μM each of dNTPs, 2 μM each of sense and anti-sense primers and 0.6 U of Taq polymerase. The GAPDH was amplified using a sense primer, 5′ AAGGCTGAGAACGGGAAGCTTGTCATCAAT 3′ (position, exon 3, bp 241–270), an anti- sense primer, 5′ TTCCCGTCTAGCTCAGGGATGACCTTGCCC 3′ (position, exon 7, bp 740–711) [12] and cDNAs prepared from reverse transcription of 25 ng of total RNA. To amplify the exon deletion variant cDNAs in the tumor samples, PCRs were performed using cDNAs prepared from reverse transcription of 500–750 ng of total RNA. The PCR conditions were initial denaturation for 1 min at 95°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 10 min at 72°C. The annealing temperature for 2Δ, 2–3Δ, 4Δ and 6Δ specific primers was at 61°C, for 3Δ and 7Δ primers at 55°C and for 5Δ specific primer at 65°C.

2.3. Detection and sequence analysis of PCR products

To detect the PCR amplified ER splice variant products from cell lines, an aliquot (4–7 μl) was electrophoresed in 1% agarose gels in Tris-acetate EDTA buffer and detected by ethidium bromide staining. To detect the PCR products of GAPDH, 1 μl was electrophoresed and the ER splice variant products amplified from tumor samples, 12–25 μl of the products were analyzed on the gel. To determine the identity of the PCR amplified ER splice variant products, they were electrophoresed in 1.2% agarose gels and purified individually using the QIAquick gel extraction kit. The purified products were cloned into pCR®2.1-TOPO vector and sequenced by cycle sequencing method on an automated DNA sequencer (carried out at the Biopolymer Laboratory, University of Maryland School of Medicine, Baltimore, MD).

3. Results

We analyzed the ER single, double, and multiple exon deletion transcripts by RT PCR using primers targeted at the splice junctions of exon 2Δ, exon 3Δ, exons 2–3Δ, exon 4Δ, exon 5Δ, exon 6Δ and exon 7Δ. The partner primers were chosen such that the largest possible transcripts were amplified between the exons 1 and 8. This permitted the amplification of not only the single exon deletion transcripts but also those with multiple deletions in distant exons. The PCR analyses were carried out in six ER positive breast cancer cell lines, MCF-7, T47D, ZR-75, LCC1, LCC2, and LCC9 and three ER negative cell lines, MDA-MB-435, MDA-MB-235 and LCC6. Three ER positive breast tumor samples were also included to test the applicability of splice targeted primer approach in analyzing the above transcripts in clinical samples. The results described here on the analysis of various alternatively spliced ER transcripts were repeated in 20 experimen-

tal trials with cell lines and three trials with tumor samples.

3.1. Analysis of exon 2Δ transcripts

The exon 2Δ transcript profiles in seven cell lines and three tumors are shown in Fig. 1. The lanes M1 and M2 contain Gibco-BRL 1 kb and 100 bp ladders, respectively. The ER positive cell lines, MCF-7, ZR-75, LCC1, LCC2, and LCC9, amplified three major bands of sizes about 960, 780, and 640 bp. The cell line T47D did not amplify the 960 band, instead it amplified two products which are higher than 960 bp. All six ER positive cell lines amplified several minor bands ranging from 480–330 bp. Unexpectedly, one of the three ER negative cell lines tested, MDA-MB-435, also amplified 960, 640 and 480 bp bands and three additional bands that showed lower mobility than the 960 bp band. Tumor 3 did not amplify any product. Tumor 2 amplified minor bands at 640 and 480 bp and tumor 1 amplified only the 480 bp one as a minor band. To determine the identify of the above products, the PCR products from LCC1 cells were cloned and sequenced. The 960, 780, 640, 480 and 330 bp products were identified as ER transcripts with deletions in exon 2, exons 2 and 7, exons 2, 5, and 7, exons 2 and 4–5, and exons 2 and 4–6, respectively (Fig. 1B). It was also found that the exons 2Δ and 4–-6Δ product had 20 bps missing in exon 7. Fig. 1(A) also shows the expression levels of GAPDH in the above cell lines and tumors and no template control.

3.2. Analysis of exon 3Δ transcripts

The exon 3Δ transcript profiles in seven cell lines and three tumors are shown in Fig. 2. Lanes M1 contain Gibco-BRL 100 bp ladders. The ER positive cell lines, MCF-7, T47D, ZR-75, LCC1, LCC2 and LCC9, amplified two products of sizes about 845 and 661 bp. The ER negative cell lines and two of the tumors in the study did not amplify these two bands. Only one of the three tumors (Tumor 5) amplified the 845 bp but not 661 bp product. To determine the identity of the above products, the PCR products from LCC1 cell line were cloned and sequenced. The 845 and 661 bp products were identified as ER transcripts that have deletions in exon 3, and exons 3 and 7, respectively (Fig. 2B). Fig. 2(A) also shows the expression levels of GAPDH in the above cell lines and tumors and no template control.

3.3. Analysis of exons 2–3Δ transcripts

The PCR product profiles of exon 2–3Δ transcripts

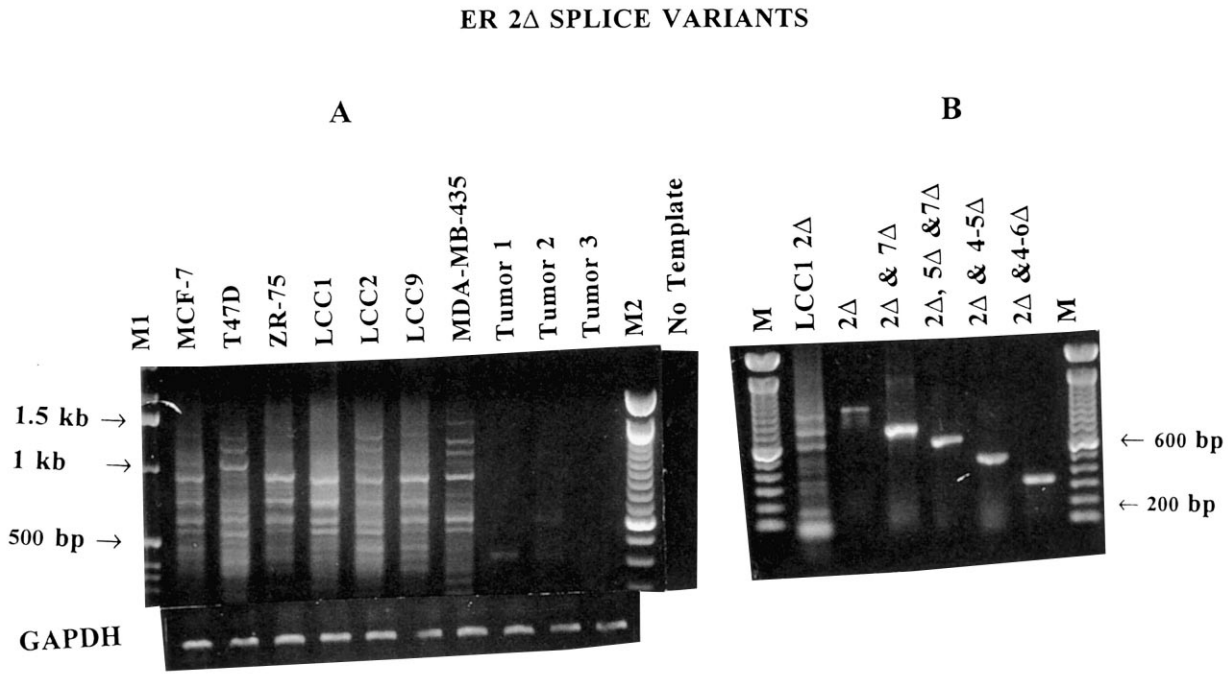

Fig. 1. Analysis of ER exon 2Δ transcript profiles in breast cancer cell lines and tumors by RT PCR using 2Δ specific primer. The ER exon 2Δ transcripts were analyzed using the specific sense primer, ER SX1/3, and an anti-sense primer ERA. To determine the identity of various PCR products, the products from LCC1 were cloned and sequenced. Panel A shows the PCR products amplified from breast cancer cell lines, MCF-7, T47D, ZR-75, LCC1, LCC2, LCC9 and MDA-MB-435, and the tumors 1, 2, and 3. Lanes M1 and M2 contain the Gibco-BRL 1 kb and 100 bp ladders, respectively. The GAPDH profile in all the above samples and no template control are also shown. Panel B illustrates the identity of the PCR products as determined by sequence analysis. Lanes M have 100 bp ladders.

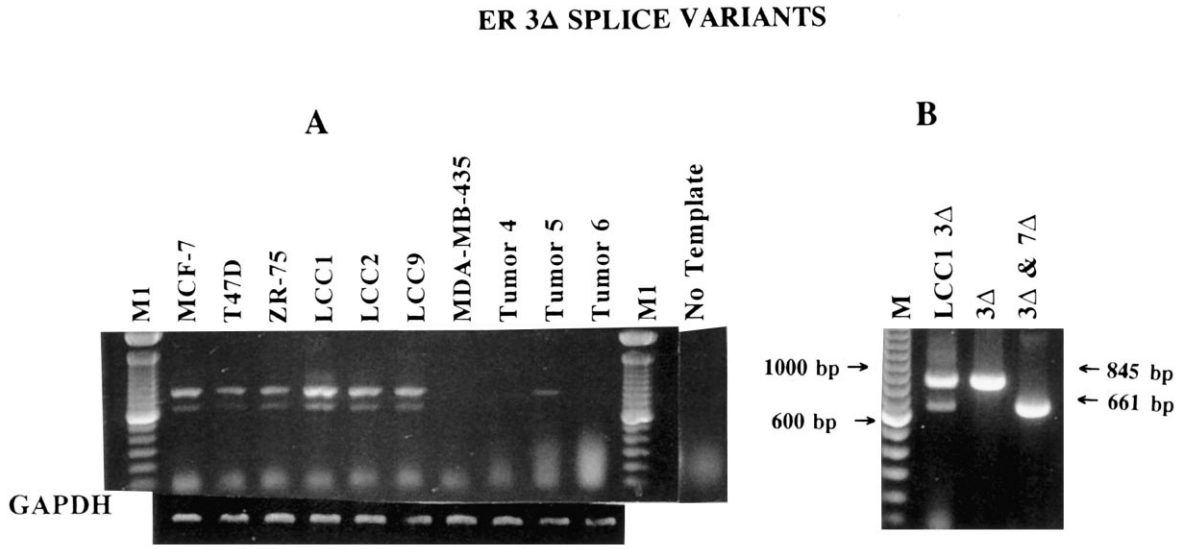

Fig. 2. Analysis of ER exon 3Δ transcript profiles in breast cancer cell lines and tumors by RT PCR using 3Δ specific primer. The ER exon 3Δ transcripts were analyzed using ER SX2/4 and ERA. To determine the identity of various PCR products, the products from LCC1 were cloned and sequenced. Panel A shows the PCR products amplified from breast cancer cell lines, MCF-7, T47D, ZR-75, LCC1, LCC2, LCC9 and MDA-MB-435 and the tumors 4, 5, and 6. Lanes M1 contain the Gibco-BRL 100 bp ladders. The GAPDH profile in all the above samples and no template control are also shown. Panel B illustrates the identity of the PCR products as determined by sequence analysis. Lane M has the 100 bp ladder.

in seven cell lines and three tumors are shown in Fig. 3(A). The lanes M1 and M2 contain Gibco-BRL 1 kb and 100 bp ladders, respectively. All the six ER positive cell lines amplified three products with approximate sizes of 840, 660 and 520 bp. Two minor bands between 840 and 660 bp are also seen. One of the three ER negative cell line, MDA-MB-435, generated a minor product slightly bigger than the 840 bp product. To determine the identities of 840, 660 and 520 bp products, the PCR products from LCC1 were cloned and sequenced. The 840, 660, and 520 bp products were identified as ER transcripts with deletions

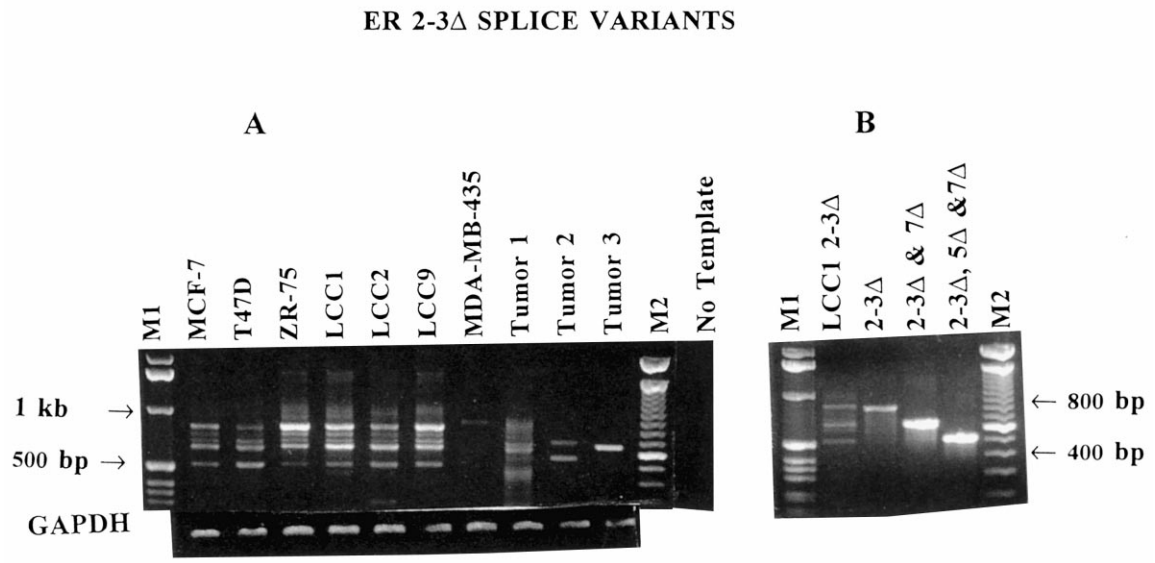

Fig. 3. Analysis of exons 2–3Δ transcript profiles in breast cancer cell lines and tumors by RT PCR using 2–3Δ specific primer. The exon 2–3Δ transcripts were analyzed using ER SX1/4 and ERA. To determine the identity of various PCR products, the products from LCC1 were cloned and sequenced. In both A and B panels, lanes M1 and M2 contain the Gibco-BRL 1 kb and 100 bp ladders, respectively. Panel A shows the PCR products generated from the breast cancer cell lines, MCF-7, T47D, ZR-75, LCC1, LCC2, LCC9 and MDA-MB-435 and the tumors 1, 2, and 3. The GAPDH profile in all the above samples and no template control are also shown. Panel B illustrates the identity of the PCR products as determined by sequence analysis.

in exons 2–3, exons 2–3 and 7, and exons 2–3, 5 and 7, respectively (Fig. 3B). Tumor 1 generated three bands of which two corresponded to exons 2–3Δ, and exons 2–3Δ and 7Δ. The third band showed slightly higher mobility than the exons 2–3Δ, 5Δ and 7Δ product. Tumor 2 amplified two bands of approximate sizes 700 and 550 bp, which are slightly higher than the exons 2–3Δ and 7Δ, and exons 2–3Δ, 5Δ and 7Δ products. The third tumor generated only the exons 2–3Δ and 7Δ product. Fig. 3(A) also shows the expression levels of GAPDH in the above cell lines and tumors and no template control.

3.4. Analysis of exon 4Δ transcripts

The exon 4Δ transcript profiles in seven cell lines and three tumors are shown in Fig. 4. The lane M1 contains Gibco-BRL 100 bp ladder. The ER positive cell lines, MCF-7, T47D, ZR-75, LCC1, LCC2 and LCC9, amplified two products of sizes about 512, and 328 bp. One of the three ER negative cell lines, MDA-MB-435, also amplified faint bands of 512 and 328 bp. All three of the tumors tested amplified these two products. To identify the above products, the PCR products from LCC9 cell line were cloned and sequenced. The 512, and 328 bp products were identified as ER transcripts with deletions in exon 4, and exons 4 and 7, respectively (Fig. 4B). Fig. 4(A) also shows the expression levels of GAPDH in the above cell lines and tumors and no template control.

3.5. Analysis of exon 5Δ transcripts

The profiles of exon 5Δ transcripts in seven cell lines and three tumors are shown in Fig. 5. The lanes M1 and M2 contain Gibco-BRL 1 kb and 100 bp ladders, respectively. All the ER positive breast cancer cell lines except MCF-7 amplified one major product and two minor products of approximate sizes, 730, 540 and 420 bp, respectively. The MCF-7 and all the three ER negative cell lines did not generate any products. To determine the identity of 730, 540 and 420 bp products, the PCR products from ZR-75 were cloned and sequenced. The 730-, 540- and 420 bp products were identified as ER transcripts having deletions in exon 5, exons 5 and 2, and exons 5 and 2–3, respectively (Fig. 5B). The three tumor samples analyzed gave very distinct products. Tumor 1 amplified all the above three products and an additional product between exon 5Δ and the exons 5Δ and 2Δ products. Tumor 2 amplified one product between exon 5Δ and exons 5Δ and 2Δ products similar to tumor 1 and two products of approximate sizes 500 and 350 bp. Tumor 3 amplified only the 500 and 350 bp products. Neither tumor 2 nor 3 amplified the major single deletion product. Fig. 5(A) also shows the expression levels of GAPDH in the above cell lines and tumors and no template control.

3.6. Analysis of exon 6Δ transcripts

The profiles of exon 6Δ transcripts in seven cell lines and three tumors are shown in Fig. 6. The lanes M1 and M2 contain Gibco-BRL 1 kb and 100 bp ladders,

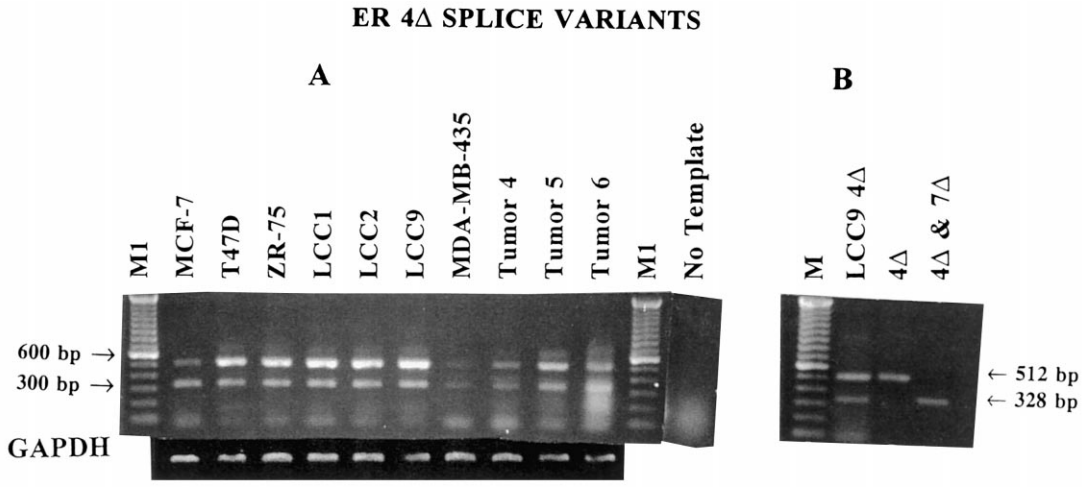

Fig. 4. Analysis of ER exon 4Δ transcript profiles in breast cancer cell lines and tumors by RT PCR using 4Δ specific primer. The ER exon 4Δ transcripts were analyzed using ER SX3/5 and ERA. To determine the identity of various PCR products, the products from LCC9 were cloned and sequenced. Panel A shows the PCR products amplified from breast cancer cell lines, MCF-7, T47D, ZR-75, LCC1, LCC2, LCC9 and MDA-MB-435 and the tumors 4, 5, and 6. Lanes M1 contain the Gibco-BRL 100 bp ladders. The GAPDH profile in all the above samples and no template control are also shown. Panel B illustrates the identity of the PCR products as determined by sequence analysis. Lane M has the 100 bp ladder.

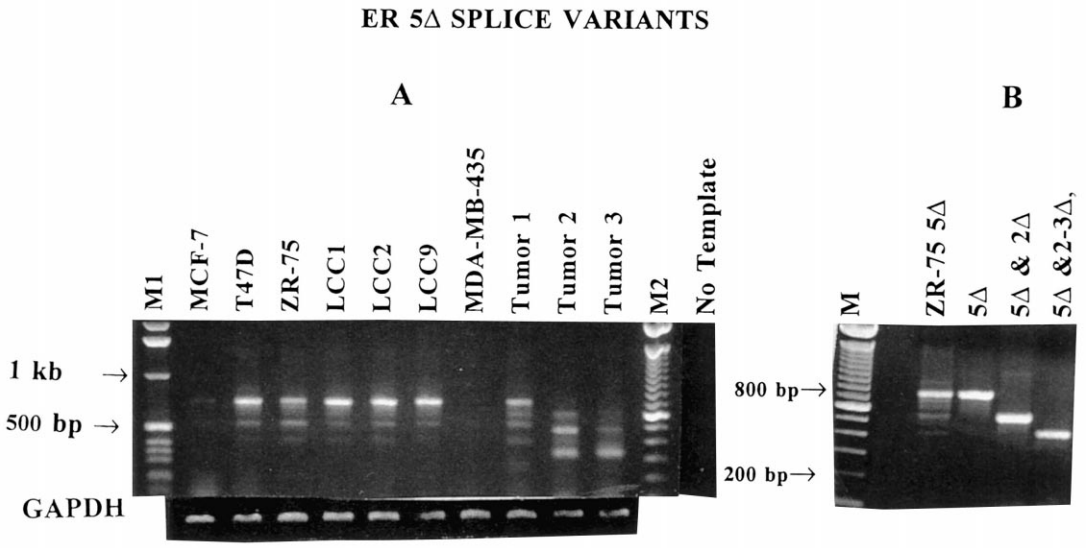

Fig. 5. Analysis of exon 5Δ transcript profiles in breast cancer cell lines and tumors by RT PCR using 5Δ specific primer. The exon 5Δ transcripts were analyzed using ER AX4/6 and a sense primer ERS. To determine the identity of various PCR products, the products from ZR-75 were cloned and sequenced. Panel A shows the PCR products amplified from breast cancer cell lines, MCF-7, T47D, ZR-75, LCC1, LCC2, LCC9 and MDA-MB-435 and the tumors 1, 2, and 3. Lanes M1 and M2 contain the GiBco-BRL 1 kb and 100 bp ladders, respectively. The GAPDH profile in all the above samples and no template control are also shown. Panel B illustrates the identity of the PCR products as determined by sequence analysis. Lane M has the Gibco-BRL 100 bp ladder.

respectively. All ER positive breast cancer cell lines amplified one major product of approximate size 866 bp. It was identified as the transcript that has a deletion in exon 6 (Fig. 6B). None of the ER negative cell lines amplified any product. We could not detect any double or multiple deletion transcripts with 6Δ primer. The three tumors analyzed did not amplify any products (Fig. 6A). Fig. 6(A) also shows the expression levels of GAPDH in the above cell lines and tumors and no template control.

3.7. Analysis of exon 7Δ transcripts

The profiles of exon 7Δ cDNAs in seven cell lines and three tumors are shown in Fig. 7. The lanes M1 and M2 contain Gibco-BRL 1 kb and 100 bp ladders,

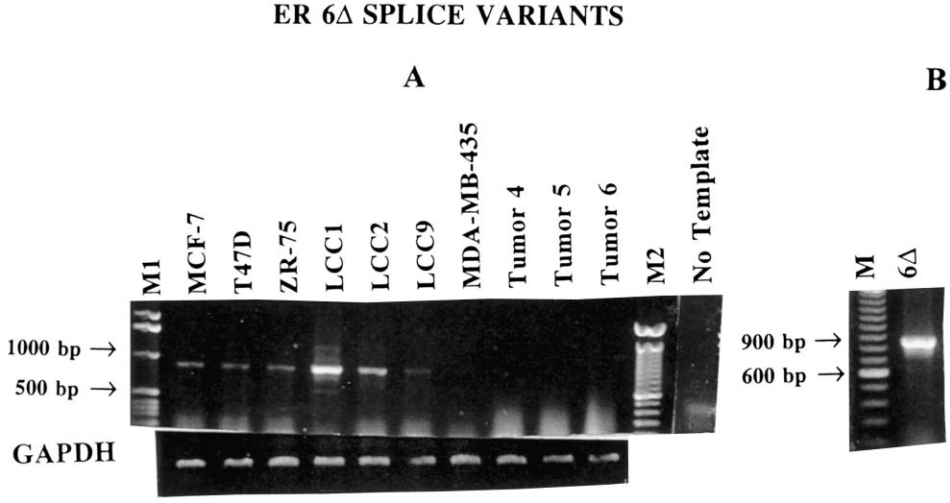

Fig. 6. Analysis of exon 6Δ transcript profiles in breast cancer cell lines and tumors by RT PCR using 6Δ specific primer. The exon 6Δ transcripts were analyzed using ER AX5/7 and ERS. To determine the identity of various PCR products, the products from LCC1 were cloned and sequenced. Panel A shows the PCR products generated from the breast cancer cell lines, MCF-7, T47D, ZR-75, LCC1, LCC2, LCC9 and MDA-MB-435, and the tumors 4, 5, and 6. Lanes M1 and M2 contain the Gibco-BRL 1 kb and 100 bp ladders, respectively. The GAPDH profile in all the above samples and no template control are also shown. Panel B illustrates the identity of the PCR product as determined by sequence analysis. Lane M has the Gibco-BRL 100 bp ladder.

respectively. All the six ER positive breast cancer cell lines generated a major 1 kb band and a minor band of approximately 665 bp. The cell line LCC2 generated an additional two minor bands of sizes 560 and 410 bp. The cell line LCC1 also generated 560 bp minor band and LCC9 generated the 410 bp minor band. In all these cell lines, several closely spaced minor bands were visualized between 1 kb and 665 bp products. To determine the identities of 1 kb, 665, 560 and 410 bp products, the PCR products from LCC1 were cloned and sequenced. They were identified as ER transcripts with deletions in exon 7, exons 7 and 4, exons 7 and 3–4, and exons 7 and 3–5, respectively (Fig. 7B). The three tumor samples analyzed gave very distinct products. Tumor 1 amplified all the above four products, similar to LCC1 cell line. However, the exons 7Δ and 4Δ product is seen as a major band and the single deletion 1000 bp product as a minor band. Tumor 2 gave a similar profile to tumor 1, and tumor 3 did not amplify any product. Tumor 3 was previously shown not to have any exon 7Δ transcript when analyzed by co-amplification with wild type sequences between exon 4–8 [13]. Fig. 7(A) also shows the expression levels of GAPDH in the above cell lines and tumors and no template control.

4. Discussion

In the current study we applied a novel approach to specifically amplify a particular category of alternatively spliced ER molecules, from a pool of other alternatively spliced and wild type ER genes, using primers which anneal to the spliced junctions. We used primers targeted at the splice junctions of exon 2Δ, exon 3Δ, exons 2–3Δ, exon 4Δ, exon 5Δ, exon 6Δ and exon 7Δ transcripts. The results described above on the identities of various transcripts, amplified by the seven splice specific primers, are summarized in Table 1. Each splice specific primer amplified not only the single exon deleted transcript but also a number of related cDNAs that have deletions in various combinations of exons. None of the splice specific primers amplified the wild type ER sequences. The seven specific primers amplified a total of 20 transcripts, of which 14 had double or multiple exon deletions. Although single, a few double, and multiple deletion variants have been described, most of the double and multiple deletion transcripts described here were not previously reported.

Our results show that 10 of the 20 transcripts identified have exon 7 deletion, suggesting that this is the most frequently deleted exon. Examination of the products amplified by exon 2Δ, exon 3Δ, and exon 4Δ specific primers indicated a trend in the deletion of exons. In all these cases, the double deletion transcript identified had the deletion of exon 7 (Figs. 1B, 2B and 4B). A similar trend was seen for the exons 2–3Δ primer amplified products (Fig. 3B). These results suggest that initial deletion of a particular exon is mostly followed by the deletion of exon 7. Interestingly, the exon 7Δ specific primer recognized only one of the double deletion products, the exons 7Δ and 4Δ (Fig. 7B). This preferential amplification may be due

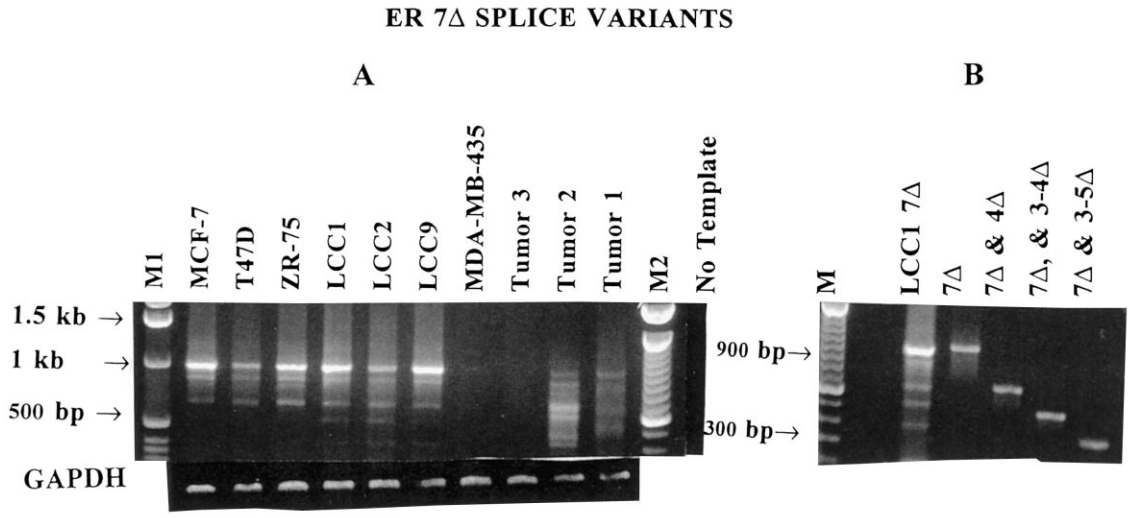

Fig. 7. Analysis of exon 7Δ transcript profiles in breast cancer cell lines and tumors by RT PCR using 7Δ specific primer. The exon 7Δ transcripts were analyzed using ER AX6/8 and ERS. To determine the identity of various PCR products, the products from LCC1 were cloned and sequenced. Panel A shows the PCR products generated from the breast cancer cell lines, MCF-7, T47D, ZR-75, LCC1, LCC2, LCC9 and MDA-MB-435 and the tumors 1, 2, and 3. Lanes M1 and M2 contain the Gibco-BRL 1 kb and 100 bp ladders, respectively. The GAPDH profile in all the above samples and no template control are also shown. Panel B illustrates the identity of the PCR products as determined by sequence analysis. Lane M has the Gibco-BRL 100 bp ladder.

to competition among various transcripts. The detection of double deletion transcripts, the exons 5Δ and 7Δ, and exons 6Δ and 7Δ, was not possible in our studies because of the 5Δ and 6Δ specific primers design. The data presented here also show that the third largest cDNA amplified by 2Δ and exons 2–3Δ specific primers had the deletion of exon 5, suggesting that the third most common exon to be deleted in a transcript after the deletion of exon 7 is the exon 5. These observations also indicate that alternative splicing of the ER transcript takes place in a sequential manner, rather than at random. The 3Δ targeted primer did not amplify the triple deletion transcript, which lacked exons 3, 5, and 7 in our studies, probably due to its low abundance. The 4Δ primer did not amplify because of its unique design.

Among the seven targeted primers tested, only 2Δ and 7Δ primers amplified the transcripts with deletions in consecutive exons (Figs. 1B and 7B, respectively and the Table). The profile of these transcripts suggests that 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. Similarly, after exon 7 deletion, if the second deletion is initiated at exon 3, the deletions seem to proceed up to exon 4 or 5. Examination of the other multiple deletion transcripts indicated that none of those had single exon 3Δ, instead, the deletion of exon 3 appears to be associated with either exon 2 or exon 4 deletion (Fig. 3B and 7B, respectively).

The results presented in Fig. (1)–(7) show some differences between estrogen dependent and independent ER positive cell lines in the patterns of variant transcripts. The LCC1, LCC2, and LCC9 are estrogen independent cell lines derived from the estrogen-sensitive parent cell line, MCF-7, after exposure to steroidal (ICI 182, 780)- or non-steroidal (Tamoxifen) anti-estrogens [14], [15]. These three cell lines did not show any differences in variant expression, suggesting that no ER remodeling is associated with either acquired Tamoxifen [14] or Tamoxifen and ICI 182,780 crossresistance [15]. In contrast, there seems to be some differences in ER variant expression associated with acquired estrogen-independence in these cells. For example, all three of the estrogen-independent cells contain the exons 7Δ, and 3–4Δ and exons 7Δ, and 3–5Δ transcripts. These are absent in the parental MCF-7 cells, and in the T47D and ZR-75 cells. Loss of exon 7 might be expected to affect ligand binding as might deletion of exon 5 and possibly exon 4. The entire hinge region would be lost in the 3–4Δ and 3–5Δ containing transcripts. Elimination of the ligand binding domain and part of the hinge region can produce transcriptionally active protein [16], overexpression of which could contribute to estrogen independence. While expression of the exons 7Δ, and 3–4Δ and exons 7Δ, and 3–5Δ transcripts is associated with acquired estrogen-independence, their function and whether significant amounts of these proteins are made, remain unclear. Another major difference observed is the absence of exon 5Δ, exons 5Δ and 2Δ and exons 5Δ, and 2–3Δ transcripts in the parental MCF-7 cells (Fig. 5A). It is possible that, these cells are estrogen dependent, in part, because of the absence of 5Δ transcript, which was reported to possess ligand independent transcriptional property. However, absence of 5Δ transcript alone may not determine the estrogen dependency because this transcript is detected in both T47D and ZR-75. It is possible that several splice variants, and their relative amounts to the wild type alpha receptor and the amounts of beta receptor in a given cell may influence estrogen dependency rather than a single transcript.

The exon deletion transcript analysis in tumor samples showed very interesting findings. In the cell lines, the most abundant product each specific primer amplified was the single deletion product and the second most abundant product was the double exon deleted transcript in the case of exon 2Δ, exon 3Δ, exon 4Δ, exon 5Δ and exon 7Δ. In the case of exons 2–3Δ specific primer, they are double and triple exon deleted transcripts. However, different primers gave different results in tumor samples. When three tumors were analyzed with exon 7Δ specific primer, two tumors showed the presence of four transcripts similar to the cell lines. However, the ratio of each transcript appears to be different compared to the cell lines. In the case of exon 2Δ transcripts, only two tumors

Table 1
Identities of twenty ER alpha spliced variants amplified by seven targeted primers

No.	Splice specific primer	cDNAs amplified
1.	ER SX1/3	2Δ, 2Δ & 7Δ, 2Δ, 5Δ & 7Δ, 2Δ & 4–5Δ and 2Δ & 4–6Δ
2.	ER SX2/4	3Δ and 3Δ & 7Δ
3.	ER SX1/4	2–3Δ, 2–3Δ & 7Δ and 2–3Δ, 5Δ & 7Δ
4.	ER SX3/5	4Δ and 4Δ & 7Δ
5.	ER AX4/6	5Δ, 5Δ & 2Δ, and 5Δ & 2–3Δ
6.	ER AX5/7	6Δ
7.	ER AX6/8	7Δ, 7Δ & 4Δ, 7Δ & 3–4Δ, 7Δ & 3–5Δ

showed the presence of minor bands and none of them amplified the single or double deletion products. When analyzed for the exons 2–3Δ containing transcripts, only one of the tumors generated 2–3Δ product, and the other two amplified the multiple deletion products, that appear to have other modifications, such as base pair insertions/deletions (Fig. 3A). Similar observations were made when analyzed for exon 5Δ transcripts (Fig. 5A). In summary, 5Δ and 2–3Δ transcripts are altered for base pair deletions and alterations, 2Δ, 3Δ and 6Δ transcripts are mostly absent, 7Δ transcript ratios are altered and 4Δ transcripts are unchanged in the tumor samples. These results suggest that the patterns and levels of ER variants undergo extensive alterations in tumor tissues.

The results presented in the current study clearly demonstrate the efficacy of the novel approach for analyzing the ER splice variant transcripts in the cell lines and tissue samples using targeted primers designed at alternate splice junctions. We believe that the new approach described here will be useful in: (1) delineating the functional roles of ER exon deletion variants in estrogen induced signal transduction processes, (2) analyzing the changes in the profiles of splice variants in the tumor tissues compared to normal tissues, (3) evaluating their role in tumorigenesis, tumor progression and loss of hormone dependency, (4) predicting prognosis and response to anti-hormone therapy, and finally (5) developing tissue specific synthetic estrogens and anti-estrogens.

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