—**Original**—

Identification of Novel C-Terminally Truncated Estrogen Receptor β Variant Transcripts and Their Distribution in Humans

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Background: The nuclear receptor genes, including estrogen receptor β (ERβ), contain non-conventional internal and terminal exons, and alternative choice of the exons yields multiple mRNA and protein variants with unique structures and functions. However, the genomic structure of the intronic and 3'-downstream regions of the human ERβ gene and the presence of novel ERβ variants with non-conventional sequences have not been re-examined in about 20 years. Therefore, we attempted to re-characterize the structure of the human ERβ gene and identify novel non-conventional exons and distinct splice variants.

Methods: Rapid amplification of cDNA 3'-end and RT-PCR cloning were used to isolate human ERβ mRNA variants from the testis. The identified cDNA sequences were mapped on the human genome assembly. Expression profiles of the variants were assessed by RT-PCR analysis.

Results: We cloned multiple ERβ mRNA variants with novel nucleotide sequences from the testis and identified several alternative splice sites, 3'-elongation of conventional coding exons, and novel terminal exons in the human ERβ gene. The variants encode C-terminally truncated ERβ proteins termed ERβ6, ERβ7, ERβEx. 4_L, and ERβEx. 6_L. Furthermore, we identified exon 7-defective forms of ERβ2/βcx, ERβ4, ERβ6, and ERβ7. Subsequently, we noted distinct expression patterns of the variants in human peripheral organs and brain subregions.

Conclusion: This study clarified complicated genomic organization and splicing patterns of the human ERβ gene that contribute to the distinct heterogeneity of human ERβ mRNAs and proteins.

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Key words: alternative splicing, ESR2, estrogen receptor β, splice variants

Introduction

Pleiotropic hormones, estrogens, have powerful effects on diverse physiological events in reproductive and non-reproductive organs[1] and are involved in pathophysiological processes such as breast cancer, ischemic stroke, and Alzheimer disease[2–4]. Estrogen signaling is mediated mainly via activation of nuclear estrogen receptors (ERs) and estrogen receptor α and β (gene symbols: *ESR1* and *ESR2*, respectively).

The ER genes encode ligand-induced nuclear transcription factors that contain distinct functional domains: the N-terminal transactivation function, DNA-binding, hinge, and ligand-binding C-terminal transactivation function domains[5,6]. The genes consist of several 5'-untranslated exons and eight conventional coding exons[7–10]. Furthermore, the gene transcripts are subject to complicated alternative splicing[11]. Since the discovery of the full-length ERβ (ERβ1), in 1996[12,13], various exon-skipping ERβ variants have been identified[14–18]. In addition, the human ERβ gene contains multiple non-conventional terminal exons, and alternative choice of the terminal exons generate mRNAs encoding C-terminally truncated ERβ variants (ERβ2/ERβcx, ERβ3, ERβ4, and ERβ5)[19,20]. Although the variants themselves lack transcriptional transactivation abilities, they were reported to heterodimerize with full-length ERα and/or ERβ proteins and to modulate trans-

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Table 1 Oligonucleotide primers used for 3'-RACE and RT-PCR experiments

Purpose	Gene	Exon (s)	Direction	Oligonucleotide sequence (5′ to 3′)
3′-RACE	Universal		Reverse	5′-GCTGTCAACGATACGCTACGTAACGGCATGACAGTG (T)$_{18}$-3′
			Reverse	5′-GCTGTCAACGATACGCTACGTAACG-3′
			Reverse	5′-CGCTACGTAACGGCATGACAGTG-3′
	ESR2	4	Forward	5′-AGAGATGTGGGTACCGCCTTGTG-3′
		4	Forward	5′-TGATCAGCCGCCCCAGTGC-3′
		5	Forward	5′-GGTGTTAATGATGGGGCTGATGTG-3′
		6	Forward	5′-GGATGAGGGGAAATGCGTAGAAGG-3′
RT-PCR	*ESR2*	6	Forward	5′-GATGAGGGGAAATGCGTAGA-3′
		8	Reverse	5′-TTCAGCCTGTGACCTCTGTG-3′
		6	Forward	5′-GATGAGGGGAAATGCGTAGA-3′
		β2	Reverse	5′-TTCTTTAGGCCACCGAGTTG-3′
		4, 5	Forward	5′-AAGAAGATTCCCGGCTTTGT-3′
		β3	Reverse	5′-TGGCTTCCCTCAGCATAAAC-3′
		4, 5	Forward	5′-AAGAAGATTCCCGGCTTTGT-3′
		β4	Reverse	5′-CAAATCTTTCATTGCCCACA-3′
		4, 5	Forward	5′-AAGAAGATTCCCGGCTTTGT-3′
		β5	Reverse	5′-CACATAATCCCATCCCAAGC-3′
		4, 5	Forward	5′-AAGAAGATTCCCGGCTTTGT-3′
		β6	Reverse	5′-TGCACTGGATACCAGGACTTT-3′
		4, 5	Forward	5′-AAGAAGATTCCCGGCTTTGT-3′
		β7	Reverse	5′-AGGAAGGGAAAGCAGGTCTC-3′
		3	Forward	5′-ACGAAGTGGGAATGGTGAAG-3′
		4_L	Reverse	5′-GCACAGCTCATGGACCTCTA-3′
		4, 5	Forward	5′-AAGAAGATTCCCGGCTTTGT-3′
		6_L	Reverse	5′-CGAAGTCCAAAAGGAAACCA-3′
	GAPDH	1	Forward	5′-TTCGACAGTCAGCCGCATCTTCTTTTG-3′
		5	Reverse	5′-CGCCAGCATCGCCCCACTTG-3′

activation of full-length variants[20–23]. Several studies have reported that these C-terminally truncated ERβ variants are associated with diseases such as breast cancer, lung cancer, prostate cancer, brain tumor, and cerebral apoplexy[24–31].

Recently, multiple C-terminally truncated ERα variants have been characterized in humans, mice, and rats. They were generated by alternative choice of novel internal and terminal exons between conventional coding exons and exhibited distinct transcriptional transactivation in transfected cells[32–35]. These results suggest that there are novel C-terminally truncated ERβ variants generated from novel intronic exons with unique structures and functions in humans. However, the genomic structure of the 3′-region of the human ERβ gene and the presence of novel C-terminally truncated ERβ variants have not been re-examined for about 20 years. Therefore, we decided to re-characterize the structure of the human ERβ gene and identify other human C-terminally truncated ERβ splice variants.

Materials and Methods

Rapid Amplification of cDNA 3′-End (3′-RACE) and RT-PCR

Human total RNAs were purchased from Thermo Fisher Scientific (Waltham, MA, USA) and TaKaRa-Clontech (Shiga, Japan). Information on the total RNAs is presented in our previous report[33]. 3′-RACE was performed as described in our previous studies[32–36]. Human testis total RNA (TaKaRa-Clontech) was reverse-transcribed by using an adaptor-oligo(dT) primer. Human ERβ 3′-end fragments were amplified by nested PCR using LA Taq polymerase (TaKaRa-Clontech). The cDNAs for RT-PCR were synthesized as described elsewhere[37]. The cDNAs (25 ng/tube) were amplified in three steps by using Blend Taq polymerase (Toyobo, Osaka, Japan), as described in our previous studies[38,39]. Oligonucleotide primers used in the 3′-RACE and RT-PCR experiments were purchased from Nihon Gene Research Laboratories (Sendai, Japan) and are shown in **Table 1**. Electrophoresis in agarose gels was used to separate the 3′-RACE and RT-PCR products. The products were stained with ethidium bromide, and the gel images were

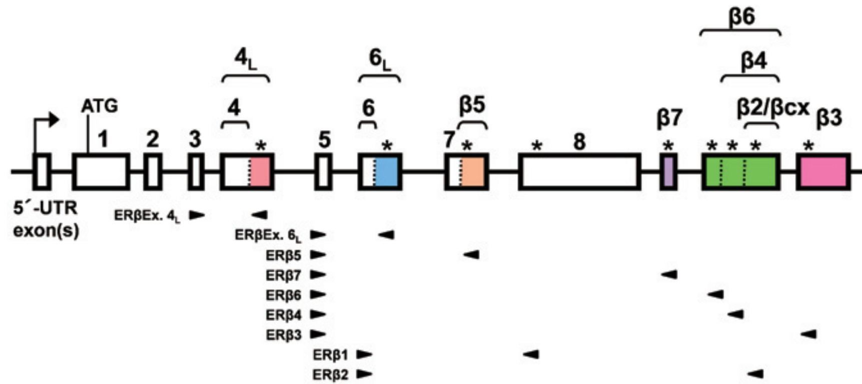

Fig. 1 Human ERβ gene structure

The genomic organization of the human ERβ gene is shown schematically. The gene is mapped to 14q23.2-q23.3 in human chromosome 14. The open and filled boxes indicate conventional exons and non-conventional terminal sequences, respectively. The bent arrow, dotted lines, and asterisks symbolize a transcriptional start site, alternative splicing sites, and stop codons, respectively. The inward-facing arrowheads show the locations of primer pairs used for RT-PCR.

captured with an ASTEC Gel Scene System (ASTEC, Fukuoka, Japan).

Cloning and DNA Sequencing

The electrophoresed amplicons of different sizes were extracted from agarose gels with a Wizard SV Gel and PCR Clean-up System (Promega, Madison, WI, USA) and separately cloned into pGEM-T-Easy vectors (Promega). After the sizes of the cloned products were estimated by direct colony PCR, the clones with differently sized products were selected and DNA-sequenced with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems). In the RT-PCR experiments, at least three separate clones from each band were DNA-sequenced.

Results

Identification of Novel ERβ mRNA Variants

Unlike the rodent ERβ genes, the human ERβ gene is abundantly expressed in the testis[8,10,19]. Therefore, we used 3′-RACE to clone human ERβ variant mRNAs, including novel nucleotide sequences, in the testis. The 3′-RACE fragments were amplified by nested PCR by using forward gene-specific and reverse universal primers and cloned into pGEM-T-Easy vectors. We sequenced the amplicons and identified multiple clones containing novel nucleotide sequences. We then mapped them on the human genome assembly (GRCh38/hg38 human assembly) with the BLAT alignment program[40] and identified several alternative splice sites, 3′-elongation of conventional coding exons, and novel terminal exons. **Figure 1** shows the genomic structure of the human ERβ gene schematically. We labelled exons containing novel nucleotide sequences as exons 4_L, 6_L, β6, and β7 and named the variants containing the respective sequences as ERβEx. 4_L, ERβEx. 6_L, ERβ6, and ERβ7. Their nucleotide sequences are shown in detail in **Figure 2**. Exons β2, β4, and β6 were generated from alternative choice of splice acceptor sites. Exons 4_L and 6_L were produced by 3′-elongation of exons 4 and 6, respectively. Exon β7 is a novel non-conventional terminal exon located downstream of exon 8 and contains a putative polyadenylation signal (AATAAA).

Expression and Splicing Patterns of Novel ERβ mRNA Variants in the Testis

Expression and splicing profiles of the C-terminally truncated ERβ variant mRNAs in the testis were analyzed by using RT-PCR with forward primers designed in conventional coding exons and reverse primers in novel terminal sequences (**Fig. 3**). The locations of the primer pairs are shown in **Figure 1**. We confirmed expression and splicing profiles of the novel variants in the testis and further observed the presence of exon 7-defective forms in the ERβ2/βcx, ERβ4, ERβ6, and ERβ7 amplicons (named as ERβ2δ7, ERβ4δ7, ERβ6δ7, and ERβ7 δ7, respectively).

The open reading frames of the variants were confirmed by RT-PCR cloning and DNA sequencing analysis. The mRNA and potentially encoded protein structures of the ERβ variants are shown in **Figure 4**. The nucleotide

(A) Exon 4_L

```
GCTCCCGGAG AGAGAGATGT GGGTACCGCC TTGTGCGGAG ACAGAGAAGT GCCGACGAGC
AGCTGCACTG TGCCGGCAAG GCCAAGAGAA GTGGCGGCCA CGCGCCCCGA GTGCGGGAGC
TGCTGCTGGA CGCCCTGAGC CCCGAGCAGC TAGTGCTCAC CCTCCTGGAG GCTGAGCCGC
CCCATGTGCT GATCAGCCGC CCCAGTGCGC CCTTCACCGA GGCCTCCATG ATGATGTCCC
TGACCAAGTT GGCCGACAAG GAGTTGGTAC ACATGATCAG CTGGGCCAAG AAGATTCCCG
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GTAGGGCTTT CTGGCTATCA GTTTTCCATG TACTTGTAGA AAGGCCGGCC GCTAATATTT
AAGGGGCAAG AGTACAAAGT AGAGGTCCAT GAGCTGTGCC TAGATATTTA ACAGGTCCTC
AGCTGGATTT GTAACTTTTA AGTGCAATAT GTTCCTTCCT TCTGTCTTGG CATACCTACC
TTCAACAAGG CCGTGTTCTG ATTTAG
```

(B) Exon 6_L

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GGATGAGGGG AAATGCGTAG AAGGAATTCT GGAAATCTTT GACATGCTCC TGGCAACTAC
TTCAAGGTTT CGAGAGTTAA AACTCCAACA CAAAGAATAT CTCTGTGTCA AGGCCATGAT
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CCTGCTCAAT TCCAGTAAGT AATCACACAG CTGGGCCATG TTTTATCGGG GAGAGATGCT
GTTTCTACAA CTAGCGTGAT ATTAAGAAGA ATGTTGAACT TCTATTTTAT TTGAAAGGGT
AAAATGGTTT CCTTTTGGAC TTCGTTTTTA TTTTGATAGC GATTTAAACT GTAGGTAACT
TTTGGTAACT TGGACATAAA TTACTCATTA AGTGAATGAC TGGCAATCAA TTTAAAAGTA
GCTCAAGCCA CTTGCTG
```

(C) Exons β2, β4, and β6

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GTCCTGTGTA TACAAGTAAA ATGCAGCTCA CAAAAGTCCT GGTATCCAGT GCATCGATTA
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TTTGGATAGA TTTTCTGTAA TCATTCTGAG TTTGATTAGA ATTATATCCT TTACAGATGG
GGAGAAAAGC AATTCATTCA TTTGAAGTTA TCTTAGTGCC AAGAGTCATG TGAAAATGTC
CCTTGCATGT GGGCAATGAA AGATTTGCAG ACGATATAAA ACCCAGACTA CCTCATAAAA
GAGTTTTGGG AATACACTGA GCTTTGAGTG AAAGAAGCTG CAGTGGCCTC CCTGGAGATG
GGGAGCAAAC CAGCTTAAAG GCCCTTATCC TGAGGAAGAG ACAAAAATTG ACATGCACAA
TATTAAGCTT TGAAATGCAG ACCACACTTC CTTTCACTGC AACTTTGACT TGTCCCGCAT
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CTCTACTTAA GGGCAGAAAA GGCCTCTCAA ACACTCACCT CATTTGGAAT GAAGATGGAG
ACTCTTTTGC CTGAAGCAAC GATGGAGCAG TGACCCTCTA ATCAACTCGG TGGCCTAAAG
AAAAATCTTG GGTAACATTT TCACTTCAGT TTCCCTCTGG GATCATTGTA ATCCATGAAA
AAAATAATTT TAAAGAAAGA GTTAAAAT
```

(D) Exon β7

```
GCTTTACATT TGGGCCTTGT AGAAATGAAT GTTTGCTGCT CTGTGAAAGC AGATTTTGAG
ACCTGCTTTC CCTTCCTCCA GGGAGTGTTT TCCTTACTGT GTCCCTTTAA TGTCTATGGC
ACTGTCGTAG AGAGTTTAAC ATGATATAAA TAAAGTGTTT CATTATTTTG GCTTT
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Fig. 2 Nucleotide sequences of novel non-conventional exons

Nucleotide sequences of exons 4_L (A), 6_L (B), β6 (C), and β7 (D) are detailed. The AG-GT splicing boundaries are underlined. The black and white arrowheads indicate alternative splicing acceptor and donor sites, respectively. The putative polyadenylation signal in exon β7 is boxed.

sequences of human C-terminally truncated ERβ variants were registered to the DDBJ/EMBL/GenBank database. The accession numbers are LC122965 for ERβ2δ7, LC 122966 for ERβ4δ7, LC122967 for ERβ6, LC122968 for ERβ6δ7, LC122969 for ERβ7, LC122970 for ERβ7δ7, LC 122971 for ERβEx. 4_L, and LC122972 for ERβEx. 6_L. The potentially encoded proteins contained the N-terminal transactivation, DNA-binding, and hinge domains but lacked the 1/3-2/3 C-terminal parts of the ligand-binding domain. Only ERβ1 mRNA encoded the complete ligand-binding/C-terminal transactivation domain.

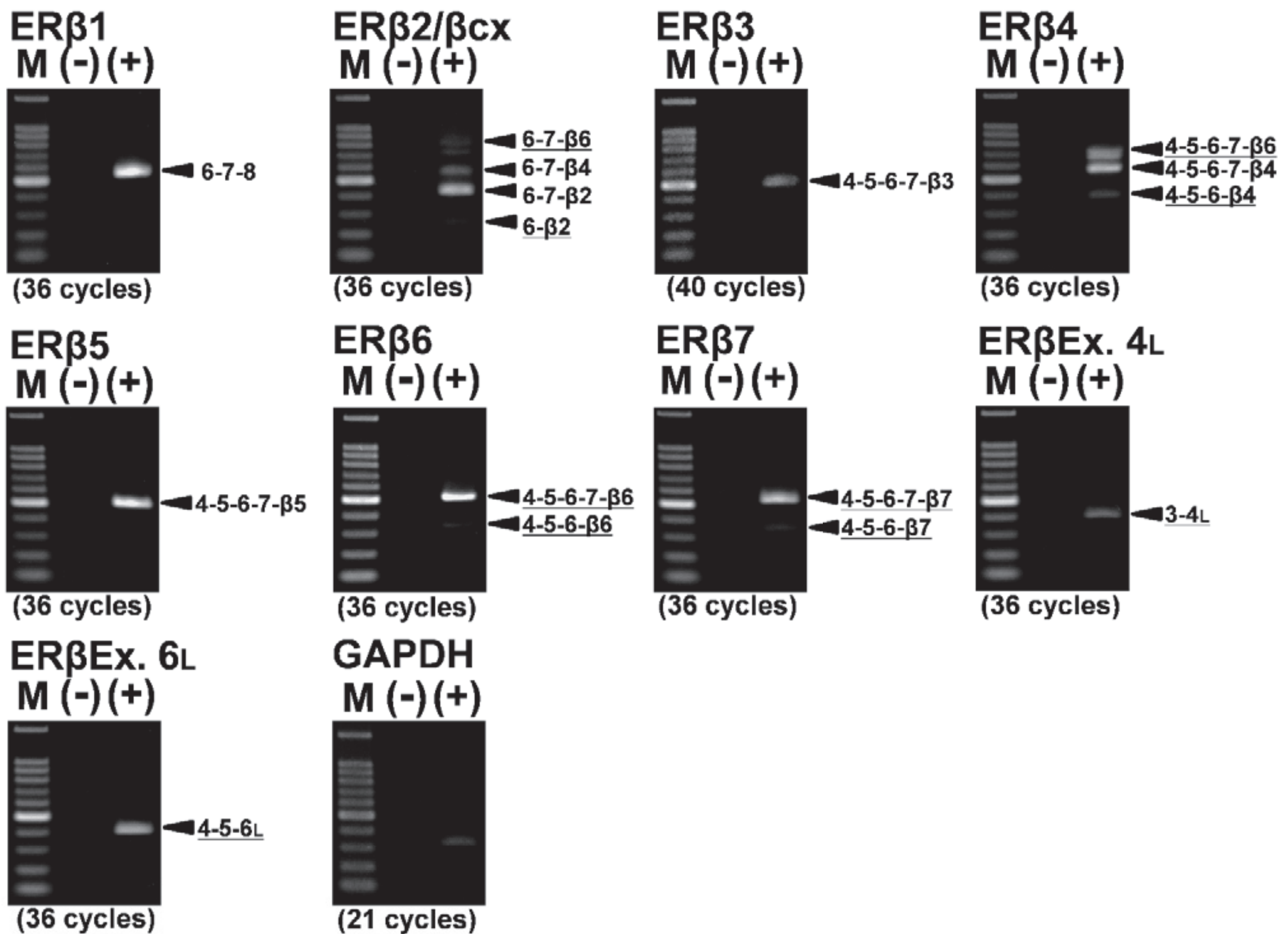

Fig. 3 Expression and splicing profiles of human ERβ transcripts in the testis
Human testis total RNA was subjected to RT-PCR analysis. The forward primers were designed in conventional coding exons, and the reverse primers were located in respective variant-specific sequences. The splicing patterns of the ERβ amplicons are indicated to the right of the respective panels. The novel splicing patterns identified in the present study are underlined. The *GAPDH* gene was amplified as an internal control. The number of PCR amplification cycles used is indicated below each panel. The labels " (+) " and " (–) " on the lanes indicate PCR samples amplified from testis total RNA reacted with and without reverse transcriptase, respectively. M, 100-bp ladder marker.

Distribution of Novel ERβ mRNA Variant Transcripts

We analyzed the distribution of ERβ variant mRNAs in human peripheral organs and brain subregions by comprehensive use of RT-PCR (**Fig. 5**). The ERβ1, ERβ2/βcx, and ERβ5 mRNAs were detected after relatively small numbers of PCR amplification cycles (33 and 34 cycles) and were distributed in a broad range of peripheral organs and brain subregions. The ERβ4, ERβ6, ERβ7, ERβEx. 4_L, and ERβEx. 6_L products were amplified by using a large number of PCR cycles (38 cycles) and observed in a few organs. The ERβ3 and exon 7-skipping variant amplicons were detected only in the testis.

Discussion

Nuclear receptor pre-mRNAs are subject to complicated splicing, which contributes to the heterogeneity of mRNAs and encoded proteins[11]. In particular, alternative splicing in the regions encoding C-termini results in variant proteins lacking most or one part of ligand-binding domains and instead possessing variant-specific C-terminal sequences[41]. Nuclear receptor genes including ERβ contain non-conventional terminal exons, and alternative choice of exons yields mRNAs encoding C-terminally truncated variants.

The pioneering studies of Ogawa et al.[20] and Moore et al.[19] described the presence of several non-conventional terminal sequences and C-terminally truncated ERβ variants (ERβ2/βcx, ERβ3, ERβ4, and ERβ5) in humans. In the current study, we reassessed the structure of the human ERβ gene and identified novel non-conventional exons, C-terminally truncated ERβ variants (ERβ6, ERβ7, ERβEx. 4_L, and ERβEx. 6_L), and exon 7-skipping variant forms (ERβ2δ7, ERβ4δ7, ERβ6δ7, and ERβ7δ7). The ERβ 2/βcx, ERβ4, and ERβ6 mRNAs are generated by alterna-

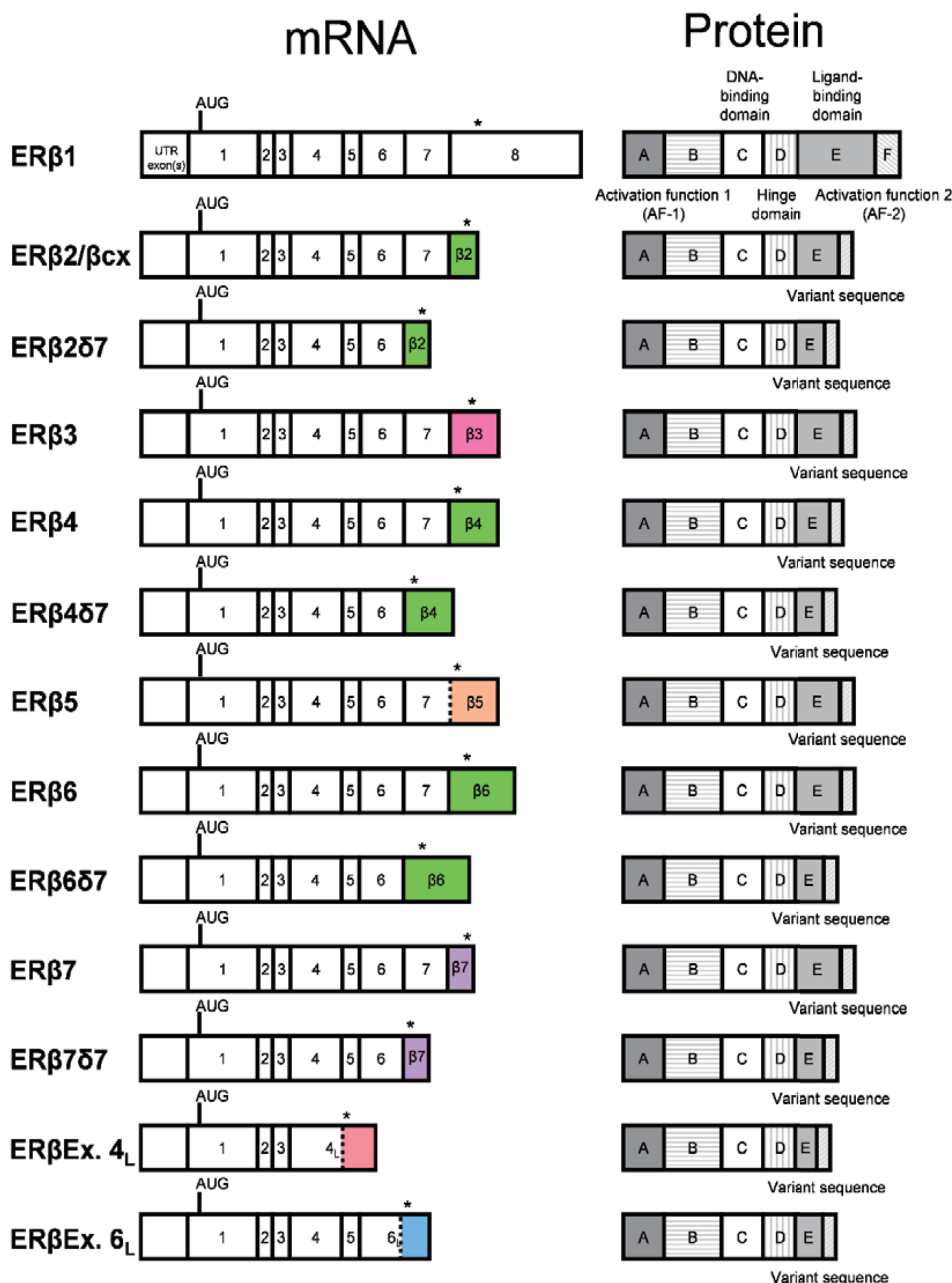

Fig. 4 mRNA and protein structures of human C-terminally truncated ERβ variants
The structures of human C-terminally truncated ERβ variant mRNAs (left) and their potentially encoded proteins (right) are represented schematically. The "AUG"s and asterisks in mRNA panels indicate translational initiation and termination sites, respectively.

tive choice of splice acceptor sites in exon β6. The novel sequences of the ERβEx. 4_L and ERβEx. 6_L variants correspond to the 3′-elongated intronic regions of exons 4 and 6, respectively. In particular, the generation pattern of the ERβEx. 4_L variant is similar to those of human, mouse, and rat CTERP-1 variants[32–35,42]. Although the human, mouse, and rat ERα genes contain non-conventional internal and terminal exons in intronic regions between coding exons[32–35], the non-conventional sequences in the human ERβ gene involve 3′-elongation of conventional exons or are located downstream of a conventional terminal exon (exon 8). Moore et al.[19] reported that the ERβ5

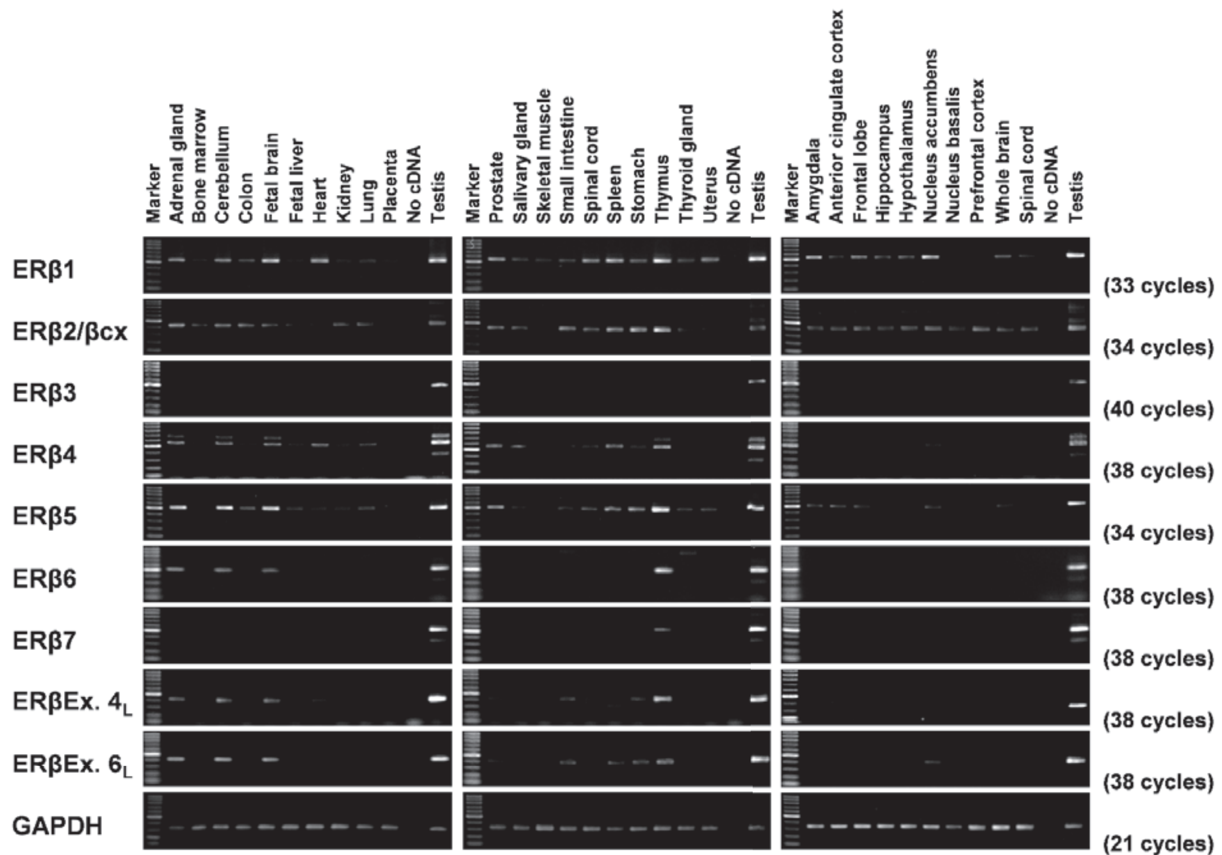

Fig. 5 Distribution of human C-terminally truncated ERβ variant mRNAs in human organs
Expressions of human ERβ variant transcripts were analyzed in a wide variety of human organs and brain subregions by using RT-PCR. The number of PCR amplification cycles used is indicated on the right bottom of each panel. Testicular cDNA and water ("No cDNA") were used as positive and negative controls, respectively.

transcript was produced by 3′-elongation of exon 7 and splicing to exon β2. However, later research did not confirm the splicing profile[22,23], and we could not detect the pattern in the 3′-RACE and ERβ2/βcx amplicons. Recently, a clone encoding the human ERβ5 protein was registered in the public database (Accession #: AB209620), and the clone corresponds to 1-2-3-4-5-6-7-intron-8. Thus, the ERβ5 variant mRNA results from retention of the intron between exons 7 and 8 rather than from 3′-elongation of exon 7 and splicing to exon β2.

The human ERβ1, ERβ2/βcx, and ERβ5 mRNAs were widely distributed and observed after a relatively small number of PCR amplification cycles (33-34 cycles), whereas the other variants exhibited limited expression and required a large number of PCR cycles for detection. The Δexon 7 variants were barely detectable, except in the testis. Thus, our RT-PCR results suggest that the ERβ 1, ERβ2/βcx, and ERβ5 variants are the predominant isoforms in normal human organs. The human testis exhibited abundant and complicated expression profiles of the ERβ variants. Recent discovery of the well validated antibody against human and rodent ERβ proteins indicates that the abundant expression of the ERβ gene in the adult testis is specific to humans[10,43]. Thus, the expression profiles of human ERβ variants imply human-specific modulatory roles in testicular estrogen-signaling pathways.

We deduced that the expression levels of the newly identified variants in normal organs except the testis were lower than those of the ERβ1, ERβ2/βcx, and ERβ5 variants. Thus, the physiological significance of these variants remains unclear. However, an association of the human ERβ variants with clinical and pathological conditions has been suggested[44]. Therefore, future studies should examine the precise roles of the human C-terminally truncated ERβ variants.

In conclusion, this is the first study to show the genomic organization of the human ERβ gene and characterize novel structurally diverse ERβ variants that naturally occur in normal human tissues. Although the physiological and pathophysiological relevance of the C-terminally truncated ERβ variants is unknown, our find-

ings provide useful and fundamental information for further research on ER variants.

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Conflict of Interest: None declared

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