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Cloning and Functional Identification of Two Novel *BRCA1* Splicing Variants

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Abstract—*BRCA1* is an important tumor suppressor gene associated with inherited breast and ovarian cancers. In this investigation, two novel *BRCA1* splicing variants were cloned from breast cancer cell line ZR-75-30. These transcripts, named *BRCA1*-PI21-Δ2-21 and *BRCA1*-Δ2-14, lacked most exons of full length *BRCA1* gene, but maintained the original reading frame. We also demonstrated the presence of *BRCA1*-PI21-Δ2-21 in several human cell lines. Expression of both variants fused with green fluorescent protein (GFP) showed that they targeted different subcellular compartments in the transfected cells. Viability of the cells expressing both fusion proteins decreased notably compared with the viability of cells expressing only GFP. Fluorescence activated cell sorting assay confirmed that the overexpression of two splicing variants resulted in cell apoptosis. Taken together, the different subcellular localization and cell effects of two *BRCA1* splicing variants imply that they can have different biological functions in breast cancer cells. Elucidating the functions of *BRCA1* splicing variants would help to understand the exact roles of the *BRCA1* gene in tumor suppression.

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Key words: breast cancer, BRCA1, alternative splicing, subcellular localization, apoptosis

Breast cancer is the most common malignant tumor affecting women in the Western world. Most of breast cancers are sporadic, but some are the result of inherited predisposition, principally due to mutations in tumor suppressor genes. During the past decade, a number of genes associated with breast cancer have been cloned and identified. Among them, *BRCA1* and *BRCA2* are the two major genes [1-3]. The *BRCA1* gene is composed of 22 coding exons encoding a protein of 1863 amino acids. The protein product of *BRCA1* contains a zinc-binding RING motif and two tandem BRCT domains [4]. BRCA1 is involved in various fundamental cellular processes, including checkpoint control of cell cycle, DNA repair and recombination, transcriptional regulation, apoptosis, and centrosome duplication [5-8]. However, until now, there has been no agreement on the subcellular localization of BRCA1. Both nuclear and cytoplasmic localization of BRCA1 have been detected in different tissues and cells [9-12].

BRCA1 mutations are found in approximately 50% of patients with inherited breast cancer and up to 90% of families with breast and ovarian cancer susceptibility. Since the cloning of the *BRCA1* gene, many mutations have been found throughout the entire coding sequence [3, 13]. Recently, research on the *BRCA1* gene has mostly focused on the detection of mutations and identification of functions of the full length *BRCA1* gene product (wild type *BRCA1*). Although alternative splicing cDNAs of *BRCA1* gene were incidentally detected and characterized in the course of cloning the wild type *BRCA1* [14, 15], only a few reports paid attention to the alternative splicing variants of the *BRCA1* gene and their functions [16-18].

In the current study, five *BRCA1* splicing variants were cloned and characterized from breast cancer cell line ZR-75-30 by the RT-PCR method. Sequence analysis showed that some transcripts still maintain the original

Abbreviations: FACS) fluorescence activated cell sorting; FCS) fetal calf serum; GFP) green fluorescent protein; PBS) phosphate-buffered saline.

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reading frame, and two of them were previously unidentified. Fluorescence activated cell sorting (FACS) assay showed that they might have important biological functions in breast cancer cells.

MATERIALS AND METHODS

Cells and cell culture. ZR-75-30 cells originated from a 47-year-old premenopausal black women with infiltrating ductal carcinoma (purchased from the American Type Culture Collection: CRL-1504) and breast cancer cells MDA-MB-435S were grown in minimal essential medium (MEM). The cell lines Cos7, K562, HeLa, HLA, Jurkat, HIC, HEL, and H9 were obtained from the China Center for Type Culture Collection (CCTCC, Wuhan). All culture media contained 10% FCS (fetal calf serum) supplemented with ampicillin (100 units/ml) and streptomycin (100 μg/ml). Cells were incubated at 37°C in a humidified incubator with 5% CO_2.

RNA extraction and RT-PCR. ZR-75-30 cells were collected in T-flasks (25 cm^2) at 80% confluency. Total cellular RNA was isolated using TRIZOL reagent (Invitrogen, USA) according to the manufacturer's protocols. About 5 μg of total RNA was used for the synthesis of the first strand cDNA using the SuperScript RNase H^- Reverse Transcriptase (Invitrogen) and oligodT primer under the conditions recommended by the manufacturer. Ten percent of the first strand cDNA was used as template. TaKaRa *LA* Taq^{Tm} (Japan) was used for PCR. The PCR reaction was performed as follows: 94°C for 5 min, then 10 cycles of amplification (94°C for 40 sec, 62-52°C for 45 sec, 72°C for 240 sec) and 24 cycles (94°C for 40 sec, 56°C for 45 sec, 72°C for 240 sec), ending with 72°C for 10 min. The primers and nested primers for RT-PCR were FP1, N-FP1, RP1, and N-RP1 (table). They were designed and synthesized according to the wild type *BRCA1* cDNA sequence. RT-PCR products from breast cell line ZR-75-30 were cloned into the *Eco*RI-*Sal*I-digested pUC18 vector. Clones were sequenced with the universal M13 primers. Sequence analysis was performed with Gene Runner software.

RT-PCR expression analysis of *BRCA1* transcripts from human cell lines. Based on the sequence analysis of *BRCA1* transcripts from breast cancer cell line ZR-75-30, the forward primer FP-I21 was designed and synthesized to detect the expression of *BRCA1*-PI21-Δ2-21 transcripts from human cell lines, pairing with the reverse primer RP1. Total RNA of cell line samples was prepared and RT-PCR was performed according to the above-mentioned method.

Construction of GFP-*BRCA1* splicing variant fusion vectors. pEGFP-C1 was purchased from Clontech (USA). Two pairs of primers (forward primers, FP2 and FP3; reverse primers, RP2 and RP3) were designed and synthesized to construct two recombinant eukaryotic vectors. Two novel *BRCA1* splicing variants (*BRCA1*-PI21-Δ2-21 and *BRCA1*-Δ2-14) were inserted into pEGFP-C1 plasmid. Both constructs were confirmed by sequencing.

Cell transfection and fluorescence imaging. The constructed plasmids were transfected into Cos7 cells using Lipofectamine^{TM}2000 reagent (Invitrogen) according to manufacture's protocols. pEGFP-C1 was also transfected as a positive control. The amount of DNA used for transfection was 1.6 μg/well. Twenty-four hours after transfection, the expression of GFP and GFP-*BRCA1* splicing variant fusion proteins was recorded using a fluorescence microscopy CCD system and confocal laser scanning microscopy. pDsRed2-Nuc with nuclear marker (Clontech) was used to analyze the colocalization of *BRCA1* splicing variant.

Western blotting analysis. The Cos7 cells transfected with pEGFP-C1 and pEGFP-*BRCA1* splicing variants were collected from the plates and denatured. The samples were subjected to 12% SDS-PAGE and transferred to nitrocellulose membrane for Western blotting analysis as described [19]. The expression of GFP and fusion proteins was detected by using rabbit anti-GFP polyclonal antibody (1 : 1000; Sanying Biotechnology, China) as the primary antibody and donkey anti-rabbit IgG (1 : 1000; Sanying Biotechnology) as the secondary antibody. The color reaction was revealed using alkaline phosphatase reagent.

Trypan blue viability test. The trypan blue viability test was used to determine the viability of cells expressing GFP and GFP-BRCA1 splicing variants fusion proteins. Cos7 cells were seeded onto 12-well culture plates. The next day they were transfected with various constructs. Each sample was replicated in three wells. Twenty-four hours later cells transfected with the constructed vectors were trypsinized and washed twice with PBS (phosphate-buffered saline). Cells were diluted in complete medium without serum to an approximate concentration of $(1\text{-}2)\cdot10^5$ cells per ml. A 0.2-ml portion of 0.4% Trypan Blue stain was added per ml cell suspension, mixed thoroughly, and allowed to stand for 3-5 min at room temperature. Viable and non-viable cells were counted in the random campus visualis.

Flow cytometry analysis. Cells transfected with the constructed vectors were trypsinized and washed twice with PBS. Cells were then fixed with cold ethanol overnight. Fixed cells were pelleted and washed twice with PBS plus 1% FCS. RNA was removed by adding RNase A at 37°C for 30 min. Finally, the cells were stained with propidium iodide. Stained cells were analyzed on a FACSort instrument (Becton Dickinson, USA). The percentage of cells with sub-G1 DNA content was taken as a measure of the apoptotic rate of the cell population.

RESULTS

Sequence analysis of two novel *BRCA1* splicing variants from ZR-75-30. Primers were designed and synthe-

Primers for RT-PCR and construction of GFP-*BRCA1* splicing variant fusion genes

Primer name	Primer sequence (5′-3′)	Location
FP1	TGAGCTCGCTGAGACTTCCTGGACC	exon 1a/21
RP1	GGGCTGAAGTGATTCTCCTGCCTTAG	exon 24/879
N-FP1	GGCGAATTCTTTCTCAGATAACTGGG	exon 1a/64
N-RP1	GGCGTCGACAGTAGCCAGGACAGTAG	exon 24/223
FP2	AGCCTCGAGCTGATGACAGCAAGAAAAC	intron 21/975
FP3	ACCCTCGAGCTCACAGTTGCTCTGGGAG	exon 15/46
RP2	GCGGTCGACTCAGTAGTGGCTGTGGGGGA	exon 24/106
RP3	GCGGAATTCTCAGTAGTGGCTGTGGGGGA	exon 24/106
FPI21	AAGCTACCCACCTTTGCC	intron 21/875

Note: The sequences of restriction endonuclease for cloning are underlined. Primers FP1 and RP1 were used for RT-PCR; primers N-FP1 and N-RP1 – for nested PCR; primers FP2, RP2 and FP3, RP3 were used to construct the pEGFP-C1 fusion vectors for proteins fused with GFP (GFP-PI21-Δ2-21 and GFP-Δ2-14, respectively). The forward primer FP-I21 and the reverse primer RP1 were used in RT-PCR to detect the expression of BRCA1-PI21-Δ2-21.

sized according to the entire *BRCA1* gene sequence (table). Total RNA from breast cancer cell line ZR-75-30 was used as a template to synthesize first-strand cDNA, followed with two serial rounds of nested PCR. A series of products with different size was acquired. These amplified bands were shorter in length than the wild type *BRCA1* cDNA due to the short extension time of the PCR conditions (Fig. 1). Sequence analysis showed that overall five splicing variants of *BRCA1* were obtained. It has been reported that two alternative first exons of *BRCA1* were identified, named exon 1a and exon 1b [20]. In this work, all these splicing variants presented with exon 1a and not exon 1b. Two of these splicing variants, *BRCA1*-PI21-Δ2-21 and *BRCA1*-Δ2-14, were novel (Fig. 2, a and b). In addition, both splicing forms accorded with the GT–AG rule (Schemes 1 and 2). Most exons of the full length *BRCA1* gene were skipped out in the alternative splicing events. The nucleotide sequences have been deposited in GenBank with accession numbers DQ333387 and DQ363751, respectively.

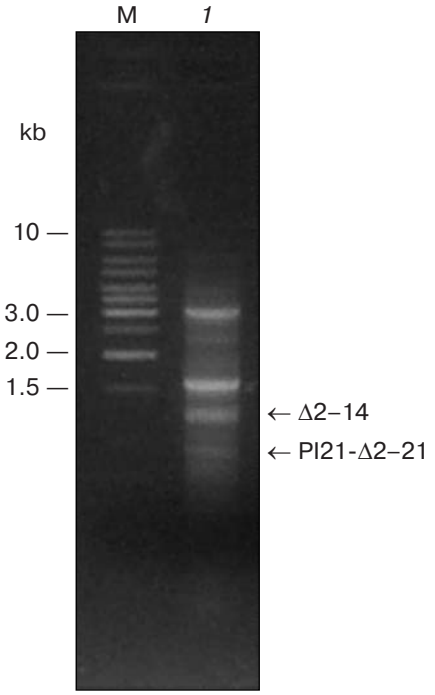

Fig. 1. RT-PCR results for amplifying breast cancer-associated gene *BRCA1*. RT-PCR was carried out for amplification of *BRCA1* splicing variants from ZR-75-30 cells. Lanes: M) 1-kb marker; *1*) products of nested RT-PCR.

BRCA1-PI21-Δ2-21 lacks exons 2 through 21, but inserts 129 nucleotides (nt) in frame between exon 1a and exon 22. Exon 1a directly links to these 129 nt, and then the 129 nt are fused to exon 22. These 129 nucleotides are generated from intron 21 (nucleotides 873 to 1001). This transcript is called *BRCA1*-PI21-Δ2-21 (partial intron 21 and deleted exons 2-21) due to its structure. This alternative splicing form keeps the original open reading frame (ORF), which constitutes 291 bp and putatively codes 96 amino acids. The start codon of *BRCA1*-PI21-Δ2-21 lies in the 129 nt of intron 21, and the stop codon is not changed compared with the known wild type *BRCA1* gene (Fig. 2a and Schemes 1 and 2). The full length BRCA1 comprises a zinc-binding RING motif at the N-terminus and two tandem BRCT motifs (carboxyl-terminal domain of BRCA1) at the C-terminus [4, 12]. *BRCA1*-PI21-Δ2-21 is the shortest alternative splicing variant of *BRCA1* gene found so far, which only maintains the last BRCT motif of BRCA1 protein. *BRCA1*-Δ2-14 lacks exons 2 through 14, and exon 1a splices directly to exon 15, which is designated as *BRCA1*-Δ2-14 (deleted exons

2-14). The coding region of *BRCA1*-Δ2-14 also misses a large portion of the 5′ region, but this transcript still maintains the original reading frame (354 aa), which comprises residues 1510-1863 of the full length BRCA1 protein (Figs. 2b and Schemes 1 and 2).

RT-PCR expression analysis of *BRCA1*-PI21-Δ2-21 transcript containing intron sequence in human cell lines. *BRCA1*-PI21-Δ2-21 inserts 129 nucleotides in frame between exon 1a and exon 22. Exon 1a directly links to the 129 nt, and then the 129 nt are fused to exon 22. As far as some intron sequences became exon sequences in the splicing variant *BRCA1*-PI21-Δ2-21, forward primers locating in these intron sequences were designed and synthesized to detect its expression in several human cell lines by the RT-PCR method (table).

RT-PCR analysis was carried out on cDNA samples from eight human cell lines (K562, HeLa, HLA, Jurkat, HIC, MDA-MB-435S, HEL, and H9). As shown in Fig.

a

```
TTTCTCAGATAACTGGGCCCCTGCGCTCAGGAGGCCTTCACCCTCTGCTC   50
TGGGTAAAGCAAAAGCTACCCACCTTTGCCTCCTGTGCCTGCTTCTGCCC  100
AGGGACTTAGGTCCTCTTACACCTTAGAGAAAGGCCTTAGCATCTGGTCA  150
CAGGCAGATGGATGACAGCAAGAAAACCTGGCTGCAATATCAACTGGAAT  200
        M  D  D  S  K  K  T  W  L  Q  Y  Q  L  E    14
GGATAGTACAGCTGTGTGGTGCTTCTGTGGTGAAGGAGCTTTCATCATTC  250
W  I  V  Q  L  C  G  A  S  V  V  K  E  L  S  S  F   31
ACCCTTGGCACAGGTGTCCACCCAATTGTGGTTGTGCAGCCAGGTGCCTG  300
 T  L  G  T  G  V  H  P  I  V  V  V  Q  P  G  A  W  48
GACAGAGGACAATGGCTTCCATGCAATTGGGCAGATGTGTGAGGCACCTG  350
  T  E  D  N  G  F  H  A  I  G  Q  M  C  E  A  P    64
TGGTGACCCGAGAGTGGGTGTTGGACAGTGTAGCACTCTACCAGTGCCAG  400
V  V  T  R  E  W  V  L  D  S  V  A  L  Y  Q  C  Q   81
GAGCTGGACACCTACCTGATACCCCAGATCCCCCACAGCCACTACTGACT  450
 E  L  D  T  Y  L  I  P  Q  I  P  H  S  H  Y  *     96
GCAGCCAGCCACAGGTACAGAGCCACAGGACCCCAAGAATGAGCTTACAA  500
AGTGGCCTTTCCAGGCCCTGGGAGCTCCTCTCACTCTTCAGTCCTTCTAC  550
TGTCCTGGCTACT                                        563
```

Fig. 2. cDNA and the deduced amino acid sequences of *BRCA1*-PI21-Δ2-21 (a) and *BRCA1*-Δ2-14 (b) splicing variants. The predicted protein sequence is shown below the nucleotide sequence. The primers for RT-PCR are underlined. The initial codon ATG and terminal codon TGA are highlighted in gray color.

b

```
TTTCTCAGATAACTGGGCCCCTGCGCTCAGGAGGCCTTCACCCTCTGCTCTGGGTAAAGGTCATCC   66
CCTTCTAAATGCCCATCATTAGATGATAGGTGGTACATGCACAGTTGCTCTGGGAGTCTTCAGAAT  132
                                    M  H  S  C  S  G  S  L  Q  N   10
AGAAACTACCCATCTCAAGGGGAGCTCATTAAGGTTGTTGATGTGGAGGAGCAACAGCTGGAAGAG  198
 R  N  Y  P  S  Q  G  E  L  I  K  V  V  D  V  E  E  Q  Q  L  E  E   32
TCTGGGCCACACGATTTGACGGAAACATCTTACTTGCCAAGGCAAGATCTAGAGGGAACCCCTTAC  264
 S  G  P  H  D  L  T  E  T  S  Y  L  P  R  Q  D  L  E  G  T  P  Y   54
CTGGAATCTGGAATCAGCCTCTTCTCTGATGACCCTGAATCTGATCCTTCTGAAGACAGAGCCCCA  330
 L  E  S  G  I  S  L  F  S  D  D  P  E  S  D  P  S  E  D  R  A  P   76
GAGTCAGCTCGTGTTGGCAACATACCATCTTCAACCTCTGCATTGAAAGTTCCCCAATTGAAAGTT  396
 E  S  A  R  V  G  N  I  P  S  S  T  S  A  L  K  V  P  Q  L  K  V   98
GCAGAATCTGCCCAGGGTCCAGCTGCTGCTCATACTACTGATACTGCTGGGTATAATGCAATGGAA  462
 A  E  S  A  Q  G  P  A  A  A  H  T  T  D  T  A  G  Y  N  A  M  E  120
GAAAGTGTGAGCAGGGAGAAGCCAGAATTGACAGCTTCAACAGAAAGGGTCAACAAAAGAATGTCC  528
 E  S  V  S  R  E  K  P  E  L  T  A  S  T  E  R  V  N  K  R  M  S  142
ATGGTGGTGTCTGGCCTGACCCCAGAAGAATTTATGCTCGTGTACAAGTTTGCCAGAAAACACCAC  594
 M  V  V  S  G  L  T  P  E  E  F  M  L  V  Y  K  F  A  R  K  H  H  164
ATCACTTTAACTAATCTAATTACTGAAGAGACTACTCATGTTGTTATGAAAACAGATGCTGAGTTT  660
 I  T  L  T  N  L  I  T  E  E  T  T  H  V  V  M  K  T  D  A  E  F  186
GTGTGTGAACGGACACTGAAATATTTTCTAGGAATTGCGGGAGGAAAATGGGTAGTTAGCTATTTC  726
 V  C  E  R  T  L  K  Y  F  L  G  I  A  G  G  K  W  V  V  S  Y  F  208
TGGGTGACCCAGTCTATTAAAGAAAGAAAAATGCTGAATGAGCATGATTTTGAAGTCAGAGGAGAT  792
 W  V  T  Q  S  I  K  E  R  K  M  L  N  E  H  D  F  E  V  R  G  D  230
GTGGTCAATGGAAGAAACCACCAAGGTCCAAAGCGAGCAAGAGAATCCCAGGACAGAAAGATCTTC  858
 V  V  N  G  R  N  H  Q  G  P  K  R  A  R  E  S  Q  D  R  K  I  F  252
AGGGGGCTAGAAATCTGTTGCTATGGGCCCTTCACCAACATGCCCACAGATCAACTGGAATGGATG  924
 R  G  L  E  I  C  C  Y  G  P  F  T  N  M  P  T  D  Q  L  E  W  M  274
GTACAGCTGTGTGGTGCTTCTGTGGTGAAGGAGCTTTCATCATTCACCCTTGGCACAGGTGTCCAC  990
 V  Q  L  C  G  A  S  V  V  K  E  L  S  S  F  T  L  G  T  G  V  H  296
CCAATTGTGGTTGTGCAGCCAGATGCCTGGACAGAGGACAATGGCTTCCATGCAATTGGGCAGATG1056
 P  I  V  V  V  Q  P  D  A  W  T  E  D  N  G  F  H  A  I  G  Q  M  318
TGTGAGGCACCTGTGGTGACCCGAGAGTGGGTGTTGGACAGTGTAGCACTCTACCAGTGCCAGGAG1122
 C  E  A  P  V  V  T  R  E  W  V  L  D  S  V  A  L  Y  Q  C  Q  E  340
CTGGACACCTACCTGATACCCCAGATCCCCCACAGCCACTACTGACTGCAGCCAGCCACAGGTACA1188
 L  D  T  Y  L  I  P  Q  I  P  H  S  H  Y  *                      354
GAGCCACAGGACCCCAAGAATGAGCTTACAAAGTGGCCTTTCCAGGCCCTGGGAGCTCCTCTCACT1254
CTTCAGTCCTTCTACTGTCCTGGCTACT                                        1282
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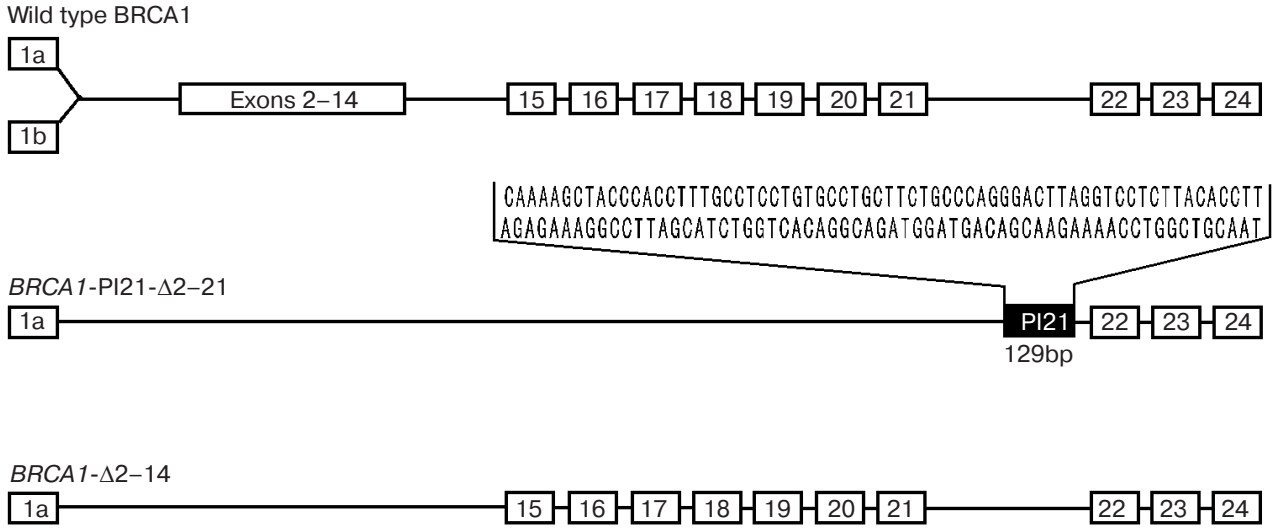
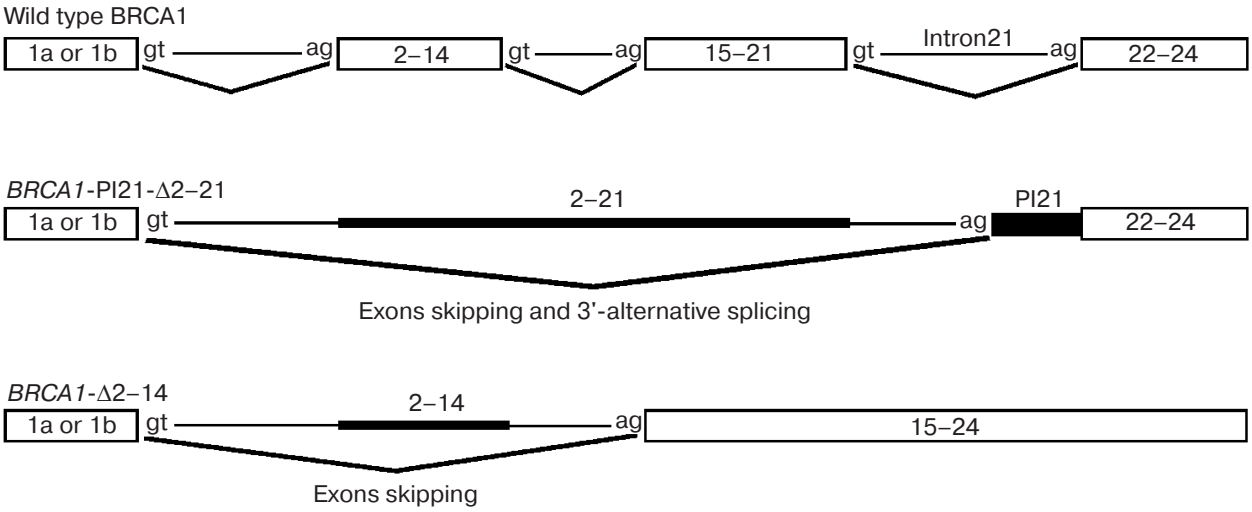
Fig. 2. (Contd.)



BRCA1 splicing variant map. The scheme represents two novel *BRCA1* splicing variants and full length *BRCA1*. The marked nucleotides are generated from introns (correspondence to full length *BRCA1* is shown). The initial codon ATG of *BRCA1*-PI21-Δ2-21 is bolded

Scheme 1



The alternative splicing forms of *BRCA1*-PI21-Δ2-21 and *BRCA1*-Δ2-14 variants. Lines represent introns and rectangles represent exons. The skipping exons are showed with bold lines (2-21 and 2-14). The partial intron 21 becomes the exon (black rectangle)

Scheme 2

3, the new transcript *BRCA1*-PI21-Δ2-21 was widely expressed in K562, HeLa, HLA, and HIC cell lines, but Jurkat, MDA-MB-435S, HEL, and H9 cell lines and human normal blood hardly expressed this transcript. This result showed that *BRCA1*-PI21-Δ2-21 splicing variant isolated from ZR-75-30 existed in some human cell lines. The orderliness of the transcript from cell lines needed more samples to be analyzed. It is probably difficult to investigate expression of *BRCA1*-PI21-Δ2-21 in clinical samples because the cells expressing *BRCA1*-PI21-Δ2-21 are usually rapidly eliminated by apoptosis (further shown in Fig. 7). We collected samples of several human tumors (from patients with breast, liver, colon, and stomach cancer) and showed with RT-PCR that there was no expression of *BRCA1*-PI21-Δ2-21 (data not shown). In addition to the expected band of *BRCA1*-PI21-Δ2-21, there are other transcript forms expressed in these cell lines, which showed the complexity of *BRCA1* alternative splicing.

Subcellular localization of *BRCA1* transcript variants. The subcellular localization of BRCA1 and its splicing variants is still unclear. To investigate this issue, two GFP

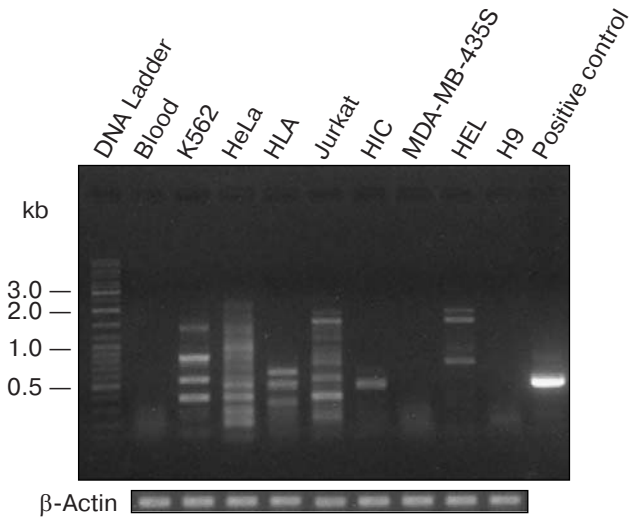


Fig. 3. Semi-quantitative RT-PCR analysis of *BRCA1*-PI21-Δ2-21 splicing variant from human cell lines. The DNA ladder is a 2-Log DNA Ladder from New England Biolabs (USA).

fusion expression constructs (pEGFP-PI21-Δ2-21 and pEGFP-Δ2-14) were introduced into the cultured Cos7 cells. Twenty-four hours after transfection, fluorescence of the randomly selected transfected cells was captured using a fluorescence microscopy CCD system (Fig. 4a; see color insert) and confocal microscopy (Fig. 4b). Fluorescence imaging showed that GFP and GFP-BRCA1 splicing variant fusion proteins were highly expressed. As shown in Fig. 4b, green fluorescence of cells expressing GFP-Δ2-14 as well as the positive control GFP was distributed both in cytoplasm and nuclei. In contrast, fluorescence of product of GFP-PI21-Δ2-21 was clearly colocalized with nuclear marker (Fig. 4b), which showed that *BRCA1*-PI21-Δ2-21 splicing variant located in the nucleus. To further confirm the distribution of green fluorescence, these constructs were introduced into cultured HeLa and MCF7 cells. The same distribution of green fluorescence was also detected (data not shown). The result of fluorescence imaging showed that GFP-PI21-Δ2-21 and GFP-Δ2-14 located in different subcellular compartments, irrespective of examined cell types.

To further investigate the overexpression of GFP-BRCA1 variant fusion proteins, Western blotting analysis of cell lysates was performed utilizing the specific rabbit anti-GFP polyclonal antibody. It revealed a band of about 30 kD (Fig. 5, lane *1*) for GFP positive control and about 40- and 69-kD bands for GFP-BRCA1 variant fusion proteins (Fig. 5, lanes *2* and *3*, respectively). The molecular weights of GFP-BRCA1 variant fusion proteins were the same as deduced from the sequences and were larger than that of GFP positive control. The result of Western blotting analysis clearly showed that the GFP-BRCA1 splicing variant fusion proteins were correctly expressed.

It was reported that various constructs of BRCA1 were established to gain insight into the subcellular localization and the key sequence connecting the localization. Cytoplasm and nuclei localization of BRCA1 protein were both found in different tissues and cells. The BRCA1 localization is still a debating focus [9-11]. Recently it was

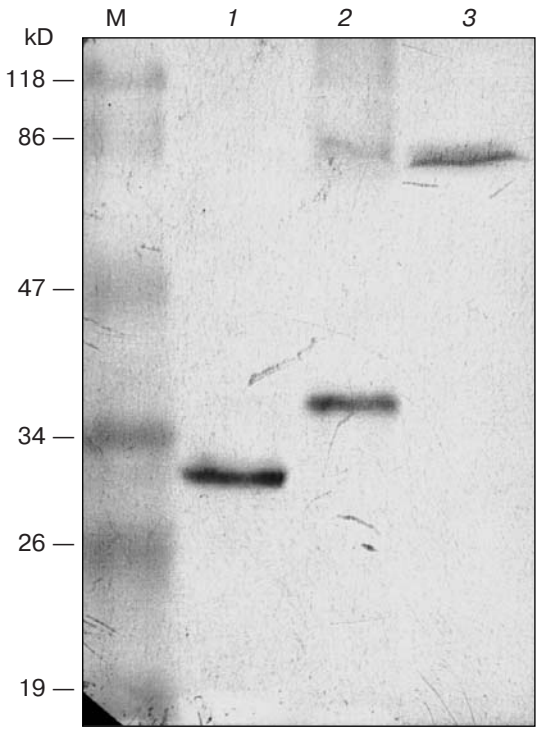


Fig. 5. Overexpression of GFP-BRCA1 splicing variant fusion proteins by Western blotting analysis. The Cos7 cells were transfected with either GFP-*BRCA1* splicing variants or EGFP control. Twenty-four hours later total lysates were prepared and separated by 12% SDS-PAGE. Samples were immunoblotted with GFP antibodies. Lanes *1-3* represent GFP, GFP-PI21-Δ2-21, and GFP-Δ2-14, respectively.

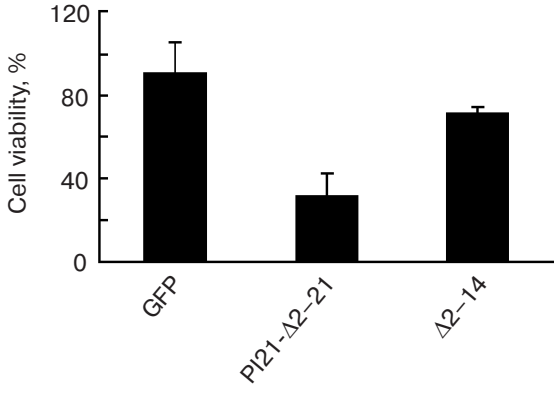


Fig. 6. Viability of cells expressing GFP and GFP-*BRCA1* splicing variant fusion proteins by the trypan blue viability test.

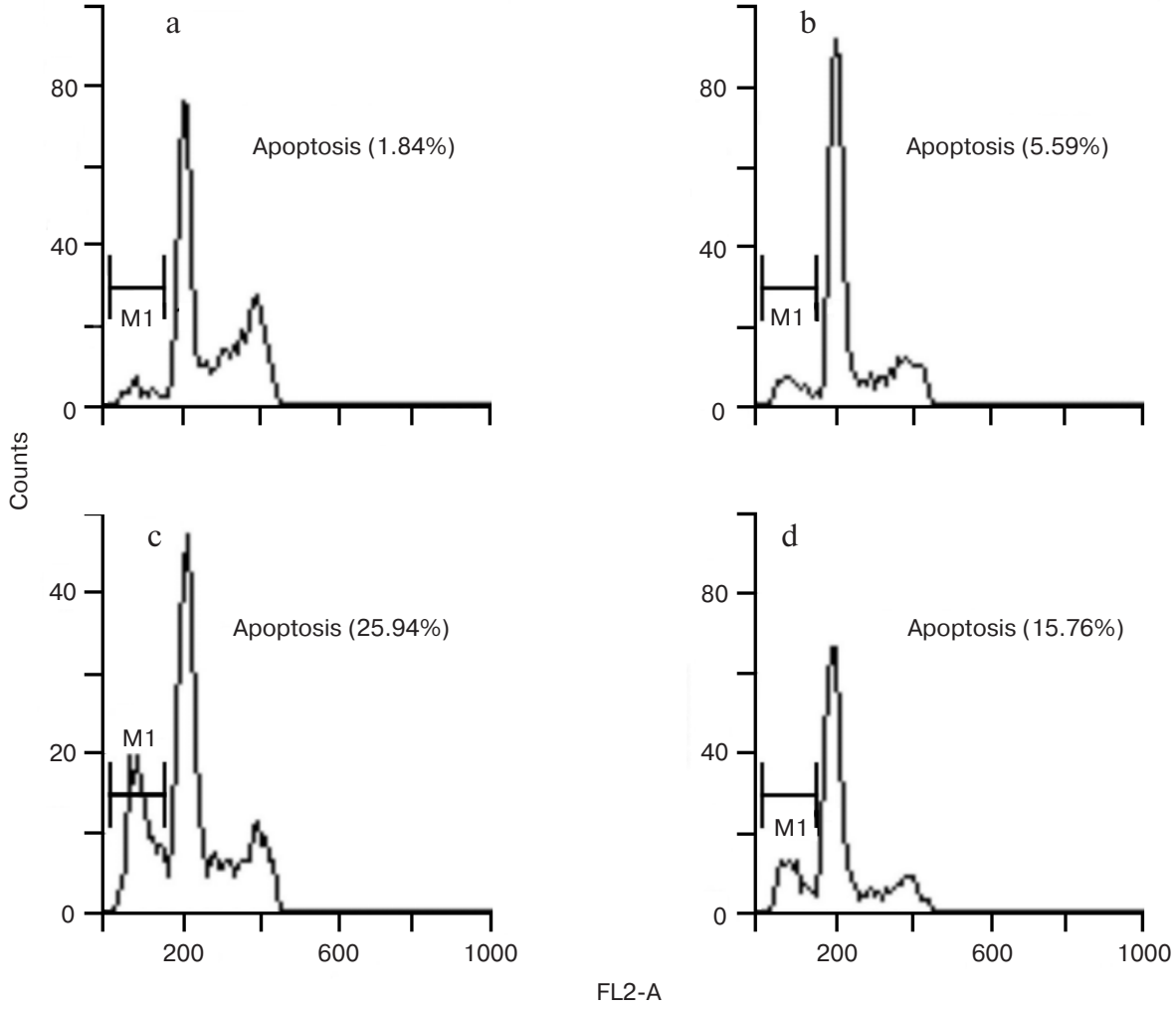


Fig. 7. Overexpression of *BRCA1* splicing variants induces apoptosis: a-d) control, pEGFP-C1, pEGFP-PI21-Δ2-21, and pEGFP-PI21-Δ2-14, respectively.

demonstrated that mutations within the BRCT domains altered BRCA1 localization, causing it to be excluded from the nucleus [12].

Effects of overexpression of two novel splicing variants on transfected Cos7 cells. Twenty-four hours after transfection, the cellular nuclei and cells expressing GFP-PI21-Δ2-21 and GFP-Δ2-14 fusion proteins become disrupted and disorganized, which showed that the overexpression of these constructs might be toxic to cells. To clarify this question, trypan blue staining was performed for assessment of cellular viability. This test showed that viability of the cells expressing GFP fusion proteins decreased notably compared with the viability of cells expressing only GFP (Fig. 6). FACS assay was performed to ensure that overexpression of both splicing variants resulted in cell death by cell apoptosis or necrosis. The results showed that the part of DNA content of cells transfected with GFP-PI21-Δ2-21 distinctly appeared as a sub-G1 peak. The DNA content of cells transfected with GFP-Δ2-14 also presented a sub-G1 peak, although less prominent than that of samples transfected with GFP-PI21-Δ2-21 (Fig. 7). Collectively, these results supported the indication that the overexpression of these *BRCA1* splicing variants resulted in apoptosis of the cells.

DISCUSSION

By examining the expression pattern of the *BRCA1* gene, more and more evidence is gathered indicating that there is a large number of splicing variants presented in different tissues with remarkably different expression patterns. Several studies have claimed that four splicing variants—the full length, Δ(9,10), Δ(11q), and Δ(9,10,11q)—are expressed in a variety of tissues under different conditions [21]. Recently, two other novel splicing variants have been detected (BRCA1-IRIS and BRCA1 exon 13A-containing transcript). BRCA1-IRIS is a new *BRCA1* gene

transcript product encoded by an open reading frame that extends from codon 1 of the known *BRCA1* open reading frame to a termination point 34 triplets into intron 11 [17]. Exon 13A-containing transcript is generated by the insertion of 66 nucleotides, which come from intron 13, between exon 13 and 14 [18]. In the two transcripts of *BRCA1*, a part of the intron sequence appears in the cDNA due to alternative splicing. In this study, we characterized two novel *BRCA1* splicing variants cloned from breast cancer cell line ZR-75-30. An additional exon was detected in *BRCA1*-PI21-Δ2-21 because intron 21 was alternatively spliced and comprised a part of the cDNA. Since the wild type *BRCA1* gene is composed of 22 coding exons and distributed over roughly 100 kb of genomic DNA [1], there could exist a number of *BRCA1* cDNA variants resulting from the molecular mechanism of alternative splicing to be identified in different tissues and cells. Alternative splicing is a widespread process used in higher eukaryotes to regulate gene expression and functional diversification of proteins. It also has been found that aberration of alternative splicing without genomic mutation is one of the important causes for the development of cancer [22, 23]. Alternative splicing is the major source of proteome diversity in humans and thus is highly relevant to disease and therapy [24, 25]. Studies on the mRNA variants of *BRCA1* and their functions might help to understand the development of breast cancer.

The cDNA of the wild type *BRCA1* gene encodes a protein of 1863 amino acids, which is identified as a 220 kD phosphoprotein [1]. Based on its predicted structure, including a zinc finger domains near the NH_2-terminus, an acidic COOH-terminal domain, and two putative nuclear location signals in exon 11, BRCA1 might be mainly a nuclear protein [1, 26, 27]. In addition, the sequence homology as well as biochemical analogy to the granin protein family have suggested that BRCA1 might also be a cytoplasmic protein [28]. Chen et al. reported that BRCA1 is a nuclear protein in normal cells, whereas the protein is aberrantly located in the cytoplasm in breast and ovarian cancer cells [9]. In contrast, Scully found that BRCA1 is exclusively nuclear regardless of cell type [10]. It was also reported that phosphorylation of specific residues of BRCA1 after DNA damage also affects its localization [11]. Recently two nuclear export signals in BRCA1 were detected, which shows that BRCA1 is a nuclear–cytoplasmic shuttling protein, and that its nuclear localization is regulated by the combined action of nuclear localization signal (NLS) and nuclear export signals (NES). In addition, mutations in BRCT domains altered its localization [12, 27, 29]. Taken together, these data reveal a discrepancy that still exists concerning the subcellular localization of BRCA1. The current study indicated that two novel *BRCA1* splicing variants missing most of the full length *BRCA1* mRNA might have different subcellular localization *in vivo*. Undoubtedly, further studies will be required to fully understand the localization of the wild type BRCA1 and its splicing variants. We thought that the reason for the ongoing debate concerning the cellular localization of BRCA1 protein was possibly due to the existence of large numbers of *BRCA1* splicing variants in different cell lines and tissues [15].

Although a number of *BRCA1* splicing variants have been cloned, the functions of their products are unknown. Two novel splicing variants of *BRCA1* lose the major part of the full length *BRCA1*. When they were introduced into the cultured Cos7 cells, the cells expressing GFP-PI21-Δ2-21 did not survive. FACS assay indicated that the overexpression of *BRCA1* splicing variants leads to apoptosis of the cells. It was reported that BRCA1 acts as a differential modulator of chemotherapy-induced apoptosis and BRCA1 phosphorylation regulates caspase-3 activation in UV-induced apoptosis [5, 7]. Taken together, these data indicate that BRCA1 might play an important role in apoptosis.

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