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Alternative Splicing of Breast Cancer Associated Gene *BRCA1* from Breast Cancer Cell Line

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Breast cancer is the most common malignancy among women, and mutations in the *BRCA1* gene produce increased susceptibility to these malignancies in certain families. In this study, the forward 1-13 exons of breast cancer associated gene *BRCA1* were cloned from breast cancer cell line ZR-75-30 by RT-PCR method. Sequence analysis showed that nine *BRCA1* splice forms were isolated and characterized, compared with wild-type *BRCA1* gene, five splice forms of which were novel. These splice isoforms were produced from the molecular mechanism of 5' and 3' alternative splicing. All these splice forms deleting exon 11b and the locations of alternative splicing were focused on two parts: one was exons 2 and 3, and the other was exons 9 and 10. These splice forms accorded with GT-AG rule. Most these *BRCA1* splice variants still kept the original reading frame. Western blot analysis indicated that some BRCA1 splice variants were expressed in ZR-75-30 cell line at the protein level. In addition, we confirmed the presence of these new transcripts of *BRCA1* gene in MDA-MB-435S, K562, Hela, HLA, HIC, H9, Jurkat and human fetus samples by RT-PCR analysis. These results suggested that breast cancer associated gene *BRCA1* may have unexpectedly a large number of splice variants. We hypothesized that alternative splicing of *BRCA1* possibly plays a major role in the tumorigenesis of breast and/or ovarian cancer. Thus, the identification of cancer-specific splice forms will provide a novel source for the discovery of diagnostic or prognostic biomarkers and tumor antigens suitable as targets for therapeutic intervention.

Keywords: Alternative splicing, Breast cancer, *BRCA1*, Tumorigenesis

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Introduction

Recent genomewide analyses of alternative splicing (AS) indicate that up to 70% of human genes may have alternative splice forms (Kalnina, *et al*., 2005). Alternative splicing is a widespread process used in higher eukaryotes to regulate gene expression and functional diversification of proteins (Graveley, 2001; Faustino and Cooper, 2003). Alternative splicing of mRNA allows many gene products with different functions to be produced from a single coding sequence, which is one of the most significant components of functional complexity of genome. Alternative splicing is also highly relevant to disease and therapy (Black, 2003; Garcia-Blanco, *et al*., 2004). At least in some cases, changes in splicing have been shown to play a functionally significant role in tumorigenesis, either by inactivating tumor suppressors or by gain of function of proteins promoting tumor development. Thus, the identification of cancer-specific splice forms provides a novel source for the discovery of diagnostic or prognostic biomarkers and tumor antigens suitable as targets for therapeutic intervention (Kalnina, *et al.,* 2005).

Breast cancer is the most common cancer affecting women in the western world. During the past decade, a number of genes associated with breast cancer have been cloned and identified. Among these, *BRCA1* and *BRCA2* are the two major genes (Miki, *et al.,* 1994; Wooster, *et al.,* 1995; Tavtigian, *et al.,* 1996; King, *et al.,* 2003). *BRCA1* gene was cloned in 1994, and from that date, numerous studies have been undertaken with the aim of understanding its function. *BRCA1* gene is composed of 22 coding exons, encoding a protein of 1863 amino acids (Miki, *et al.,* 1994). *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 *BRCA1* gene, numbers of mutations have been found throughout the entire coding sequence (Miki, *et al.,* 1994; King, *et al.,* 2003). Alternative splicing cDNAs of *BRCA1* gene are incidentally detected and characterized in the course of cloning the wild-type *BRCA1*

gene (Lu, *et al.,* 1996), and a few reports pay attention to the alternative splice variants of *BRCA1* gene and their functions. Thus far, only three protein products of *BRCA1* alternative splice variants have been identified (wild-type *BRCA1*, *BRCA1*Δ11b and *BRCA1*-IRIS) at the different levels (Wilson, *et al.,* 1997; ElShamy and Livingston, 2004; Fortin, *et al.,* 2005).

In the current study, nine *BRCA1* splice forms were characterized from breast cancer cell line ZR-75-30 by RT-PCR method. Most of *BRCA1* splice variants still kept the original reading frame. Western blot analysis indicated that some *BRCA1* splice variants were expressed in ZR-75-30 cell line at the protein level. These results suggested that breast cancer associated gene *BRCA1* may have unexpectedly a number of splice variants. We hypothesized that alternative splicing of *BRCA1* possibly plays an important role in the tumorigenesis of breast and/or ovarian cancer. Thus, the identification of cancer-specific splice forms will provide a novel source for the discovery of diagnostic or prognostic biomarkers and tumor antigens suitable as targets for therapeutic intervention.

Materials and Methods

Cells and cell culture. ZR-75-30 cells originated from a 47-year-old premenopausal Negro women with infiltrating ductal carcinoma (Purchased from ATCC: CRL-1504) were grown in minimal essential medium (MEM). The culture media contained 10% FCS 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. Breast cancer cell line MDA-MB-435S was grown in MEM culture and was purchased from ATCC, as well as ZR-75-30. The human cell lines K562, Hela, HLA, HIC, H9, HEL and Jurkat were obtained from China Center for Type Culture Collection (CCTCC, Wuhan).

Human tissues. 3-month old healthy human fetus was obtained from accidental abortions in the People's Hospital of Wuhan University. Human breast tumor, liver tumor, colon tumor and stomach tumor samples were obtained from patients at the People's Hospital of Wuhan University. Human blood sample was provided by a healthy person. The study was approved by the hospital ethics committee and the relations. Multiple tissues from the fetus and tumors were separated and cut into 1 mm^3 pieces, quickly frozen in liquid nitrogen, and stored at −80°C until processed.

RNA extraction and RT-PCR. Total RNA of cell lines or tissue samples were prepared using TRIZOL reagent (Invitrogen) according to the manufacture's instruction. In order to detect the quality of total RNA, RNA was electrophoresed on 1.2% agar and was recorded by Gel Imaging System (GENE). About 5 μg of total RNA was used to synthesize the first strand cDNA with the superscript II $RNaseH^-$ reverse transcriptase (Invitrogen) and oligo dT under the conditions recommended by the manufacturer. 10% of the first strand cDNA was used as template. PCR reaction was performed as follows: 94°C for 5 min, then 10 cycles of amplification (94°C for 40 s, 52-62°C for 45 s, 72°C for 120 s) and 24 cycles of amplification (94°C for 40s, 55°C for 45s, 72°C for 120s), ending with 72°C for 10 mins. The primers for RT-PCR were FP and RP (Table 1). They were designed and synthesized according to the wild-type *BRCA1* cDNA sequence.

Cloning and Sequence analysis. The RT-PCR products from breast cell line ZR-75-30 were purified after gel electrophoresis using Gel Extraction Kit (Omega, USA) and the purified products were cloned into pGEM-T easy vector. A lot of positive clones were sequenced with the universal T7 and SP6 promoter primers. Sequence analysis was performed with Generunr software. Multiple sequence alignment (MSA) was carried out with DNAMAN program.

Western blotting analysis. ZR-75-30 cells were collected from the plates and denatured. The samples were subjected to 12% SDS-PAGE and transferred to nitrocellulose membrane for western blotting analysis. The expression of *BRCA1* splice variant proteins was detected by using rabbit C-terminal BRCA1 polyclonal antibody (AB9141, Abcam) as the primary antibody and anti-rabbit IgG (1 : 1000, Sanying Biotechnology) as the secondary antibody. The color reaction was revealed by diaminobenzidine (DAB, Sigma) reagent.

Results

RT-PCR was carried out to isolate the 5' part (exons: 1-13) of *BRCA1* cDNA sequence from breast cancer cell line ZR-75-30. The forward primer FP (5'-GGGTTTCTCAGATAACTGGG-3') was located at the 38th nt of the wild-type *BRCA1* cDNA sequence (Genbank Number: U14680) in exon 1a, while the reverse primer RP (5'-GCTGTTAGAAGGCTGGCTCCCATG-3') was sited at the 4403th nt of *BRCA1* full length encoding region in exon 13. In the course of RT-PCR, the amplified DNA fragment was only about 1.0 kb long, which was very shorter than that of corresponding wild-type *BRCA1* cDNA sequence (Fig. 1). The RT-PCR products were cloned into pGEM-T vector. When PCR was used to screen the positive clones for sequencing analysis, some positive clones with different foreign fragments were detected (Fig. 2). 12 positive clones were sequenced by T7 promoter and SP6 promoter primers. Sequence analysis showed that these 12 positive clones contained the different size cDNA sequences of *BRCA1* gene

Table 1. Primers for RT-PCR to isolate exons 1-13 of breast cancer associated gene *BRCA1*

Primers name	Primer sequence (5'-3')	Length	Location
FP	GGGTTTCTCAGATAACTGGG	20 bp	exon 1a, 38
RP	GCTGTTAGAAGGCTGGCTCCCATG	24 bp	exon 13, 4403

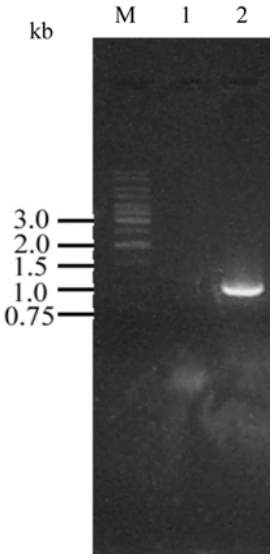

Fig. 1. RT-PCR result for amplifying exons 1-13 of breast cancer associated gene *BRCA1*. Lane M: 1 kb DNA ladder; Lane 1: the negative control of RT-PCR; Lane 2: the amplified products of RT-PCR.

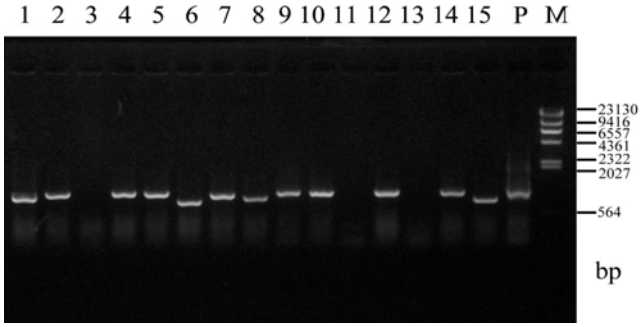

Fig. 2. Screening the positive clones of *BRCA1* exons 1-13 by PCR method. Lanes 1-15: the different positive and negative clones F1-15, respectively; Lane P: the positive control; Lane M: 1 kb DNA ladder.

and several novel splice sites of *BRCA1* gene were detected.

F1 positive clone deleted not only exons 9-10 by the molecular mechanism of exon skipping, but also the largest 3' part of exon 11 (Named exon 11b) by the molecular mechanism of 5' alternative splicing. This kind of alternative splicing site was also reported in NM-007305. Three positive clones (F2, F5 and F14) deleted the last 6 nucleotides GTAAAG of exon 1a and entire exon 11b by the molecular mechanism of 5' alternative splicing. This kind of splice site was consistent with that of the reported *BRCA1* variant BC072418.1. F4 positive clone was the longest variant out of all isolated *BRCA1* forms from our investigation and only deleted exon 11b, the splice site of which was also presented in the *BRCA1* variant NM-007304.2. Sequence analysis showed that F6 positive clone lacked exon 2, 9, 10 and 11b, and also deleted the +3 nucleotides CAG of exon 8 by the molecular mechanism of 3' alternative splicing. F7 positive clone lacked both exon 3 and exon 11b. F8 positive clone still deleted the last 6 nucleotides GTAAAG of exon 1a by the 5' alternative splicing,

Fig. 3. The structural maps of the wild-type *BRCA1* exons 1-13 and nine *BRCA1* splice forms cloned from breast cancer cell line ZR-75-30. Five splice forms (F6: Del-E2 + CAG + E9 + E10 + E11b; F7: Del-E3 + E11b; F8: Del-GTAAAG + E9 + E10 + E11b; F9,12: Del-CAG + E11b; F15: Del-E2 + E10 + E11b) were novel. Numbers corresponded to different exons and the missing exons are shown by red connecting lines. The capital letters are exon nucleotides, while the small letters are corresponding to intron nucleotides.

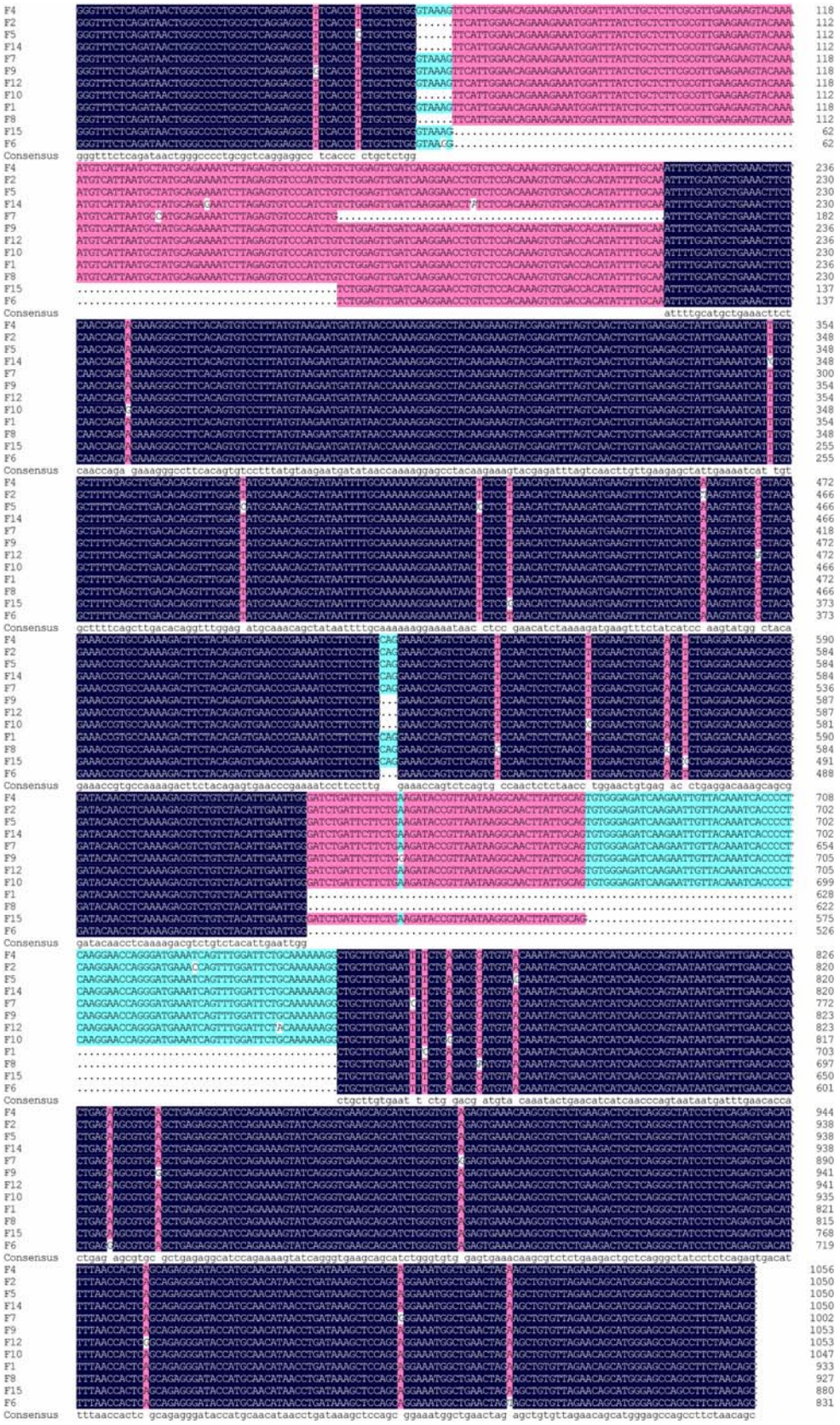

Fig. 4. Multiple sequence alignment of 12 positive clones (F4, F2, F5, F14, F7, F9, F12, F10, F1, F8, F15 and F6) encoding exons 1-13 of breast cancer associated gene *BRCA1*. Dots show the missing parts of exon sequences.

compared with F1 clone. F9 and F12 positive clones deleted the +3 nucleotides CAG of exon 8 at the basis of F4 variant. Besides exon 11b, F10 positive clone missed both the last 6 nucleotides GTAAAG of exon 1a and the +3 nucleotides CAG of exon 8. The splice site of F10 positive clone was similar to the *BRCA1* sequence BC085615.1. F15 positive clone deleted three exons 2, 10 and 11b. Additionally, all these splice variants presented a lot of mutations, compared with the cDNA sequence of the wild-type *BRCA1* gene. All these results of sequence analyses were shown in Fig. 3 and Fig. 4.

As shown in Fig. 4, nine kinds of splice forms of *BRCA1* gene were detected and identified from the breast cancer cell line ZR-75-30, five splice forms (F6: Del-E2 + CAG + E9 + E10 + E11b; F7: Del-E3 + E11b; F8: Del-GTAAAG + E9 + E10 + E11b; F9,12: Del-CAG + E11b; F15: Del-E2 + E10 + E11b) of which were novel except for four splice forms (F1: Del-E9 + E10 + E11b; F2,5,14: Del-GTAAAG + E11b; F4: Del-E11b; F10: Del-GTAAAG + CAG + E11b). All these nine splice forms deleted exon 11b by the molecular mechanism of 5' alternative splicing, which was the longest exon (3456 nucleotides) of the 24 exons of *BRCA1*. The parts of exon skipping were collected in exons 2-3 and exons 9-10. The 5' alternative splicing site was mainly focused on the last 6 nucleotides GTAAAG of exon 1a, while the 3' alternative splicing site was collected in the +3 nucleotides CAG of exon 8, which possibly indicated that special mechanism controlled the sites of alternative splicing. These splice forms accorded with GT-AG rule. All the other 11 positive clones still kept the original open reading frame of the wild-type *BRCA1* gene, except for F15 splice variant with early terminus (the +9 nucleotides TGA of exon 11). Sequence analysis showed that the translational initial codon ATG of F6 variant and F15 variant was located at the +8 nucleotide of exon 5, resulting from the missing of exon 2. F6 splice variant still maintained the wild-type ORF because of the deleted exons 9-10 for encoding even 41 aa residues, and so this variant deleted the upstream 47 aa residues of exons 2-3 coding region and 41 aa residues of exons 9-10 coding region. F15 splice variant was early terminated, because of deleting 76 nucleotides of exon 10, and its terminal codon TGA was sited at the +9 nucleotides of exon 11a.

In order to further investigate the expression of *BRCA1* splice variants, western blotting was performed utilizing the specific rabbit BRCA1 polyclonal antibody. Western blotting analysis under reducing conditions revealed that ZR-75-30 cell line expressed some *BRCA1* splice variants, the size of which was lower than that of the wild-type BRCA1 protein (Fig. 5). The result confirmed that the natural splice variants of *BRCA1* occurred at the protein level, which was consistent with the alternative splicing of *BRCA1* gene at the RNA level.

RT-PCR analysis was carried out on cDNA samples from nine human cell lines (ZR-75-30, MDA-MB-435S, K562, Hela, HLA, HIC, H9, HEL and Jurkat), a 3-months human fetus, a health blood and four tumor tissues (Breast, liver, colon and stomach). As shown in Fig.6, most cell lines (MDA-MB-435S, K562, Hela, HLA and H9) and human fetus presented a stong 1 kb DNA band as well as breast cancer cell line ZR-75-30, but this DNA band was not be detected in HEL cell lines, human blood sample and four tumor tissues (breast, liver, colon and stomach). In addition, Jurkat and HIC cell lines showed the weak DNA band with about 1 kb length. We confirmed the presence of these new transcripts of *BRCA1* gene in ZR-75-30, MDA-MB-435S, K562, Hela, HLA, HIC, H9, Jurkat and human fetus samples. It seemed that human tumor and normal tissues were hardly expressed these new transcripts deleted exon 11b, which needed more kinds of tissues and sample quantities to be confirmed. In this experiment, semiquantitative PCR was performed to simply quantify the expression of these splicing variants. The difference of expression from human cell lines and tissues was evidently presented in Fig. 6.

Discussion

Since its presence was first suggested, alternative splicing was found to be a very important level of gene regulation (Lopez, 1998) and was widely accepted as an important source of genetic diversity (Graveley, 2001). The *BRCA1* gene, a tumour suppersor gene, was cloned in 1994, and from that date, numerous studies have been undertaken to understand its function (Miki, *et al.,* 1994; Wooster, *et al.,* 1995; Quinn, *et al.,* 2003; Thangaraju, *et al.,* 2000; Venkitaraman, 2002; King, *et al.,* 2003; Lane, 2004; Au and Henderson, 2005). By examining the expression pattern of theb *BRCA1* gene, more and more evidences were gathered indicating that there are a large number of splice variants present in different tissues and cell types, with remarkably different expression patterns. More than thirty distinct mRNA splice variants have been identified so far (Orban and Olah, 2003), most of which maintained the original open reading frame having the possibility to code for a functional protein. Several studies claimed that four mRNA variants called predominant splice variants - the full length, the Δ(9,10), Δ(11b) and the Δ(9,10,11b) variants - were expressed in a variety of tissues, under different conditions (Orban and Olah, 2003). Recently, two other novel splice variants have been detected (*BRCA1*-IRIS and *BRCA1* exon 13A-containing transcript) (ElShamy and Livingston, 2004). Here, we isolated and identified nine *BRCA1* splice forms from breast cancer cell line ZR-75-30, five of which were novel. Moreover, all the remained 11 clones still kept the original open reading frame of the wild-type *BRCA1* gene, except for F15 splice form with early termination. Although it was difficult to assess the role and significance of the detected *BRCA1* mRNA isoforms without the knowledge of their proper function, most these splice variants kept the original reading frame of the protein and some variants were detected and presented at the protein level by several groups, which suggests that these splice variants have some important and crucial cellular function to be

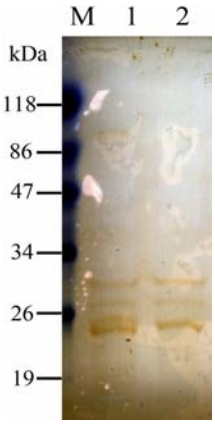

Fig. 5. Western blotting for the expression of BRCA1 splice variants from ZR-75-30. M: Middle molecular weight protein marker with color; 1 and 2: the lysates of the breast cancer cell line ZR-75-30.

elucidated. Since the wild-type *BRCA1* gene was composed of 22 coding exons and distributed over roughly 100 kb of genomic DNA (Miki, *et al.,* 1994), there could exist a large number of *BRCA1* cDNA isoforms resulted from the molecular mechanism of alternative splicing to be identified in different tissues and cells. Thus, *BRCA1* gene should be a good candidate gene for researching the molecular mechanism of alternative splicing.

All functions that have so far been described for the full length BRCA1 protein included roles in transcriptional activation, in DNA repair, in recombination processes (Lane, 2004) and in cell apoptosis (Thangaraju, *et al.,* 2000; Quinn, *et al.,* 2003), and it was still not clear why the malfunctions of such a gene lead to tumor formation almost exclusively in the breast and the ovary. It had also been found that aberration of alternative splicing without genomic mutation was one of the important cause for cancer development (Graveley, 2001; Faustino and Cooper, 2003). Studies on the mRNA variants of *BRCA1* and their functions might be vital to understand the development of breast cancer. In fact, the observation that proportion of the full length *BRCA1* variant compared to the other isoforms in normal breast cells was significantly higher than in any of the examined cell lines anticipated that the proportional decrease of this variant may be associated with tumorigenesis (Orban and Olah, 2001). The cell line dependent expression pattern of *BRCA1* variants indicated that the breast and the ovarian cells might share some common regulatory pathways in alternative splicing as compared with the leukaemia cell line, and the disturbance of such pathways might be associated with breast and ovarian tumorigenesis (Orban and Olah, 2001).

Chen *et al.* reported that BRCA1 was a nuclear protein in normal cells, whereas the protein was aberrantly located in the cytoplasm in breast and ovarian cancer cells (Chen, *et al.,* 1995). In contrast, Scully found that BRCA1 was exclusively a nuclear protein regardless of cell type (Scully, *et al.,* 1996). Using several monoclonal or polyclonal antibodies under different technical conditions on human breast cell lines, both nuclear and cytoplasmic BRCA1 protein staining was detected. Chambon concluded that BRCA1 protein localization was highly dependent on the immunohistochemical conditions, irrespective of the type of antibody and concentration used, and the type of cells examined (Chambon, *et al.,* 2003). It was also reported that BRCA1 was present not only in a dot like pattern in the nucleus but also associated with a channel-like system of cytoplasm and endoplasmic reticulum invaginating into the nucleus (Wilson, *et al.,* 1999). Clearly, there was still an ongoing debate concerning the cellular localization of BRCA1 protein in breast cancer. We thought that the reason

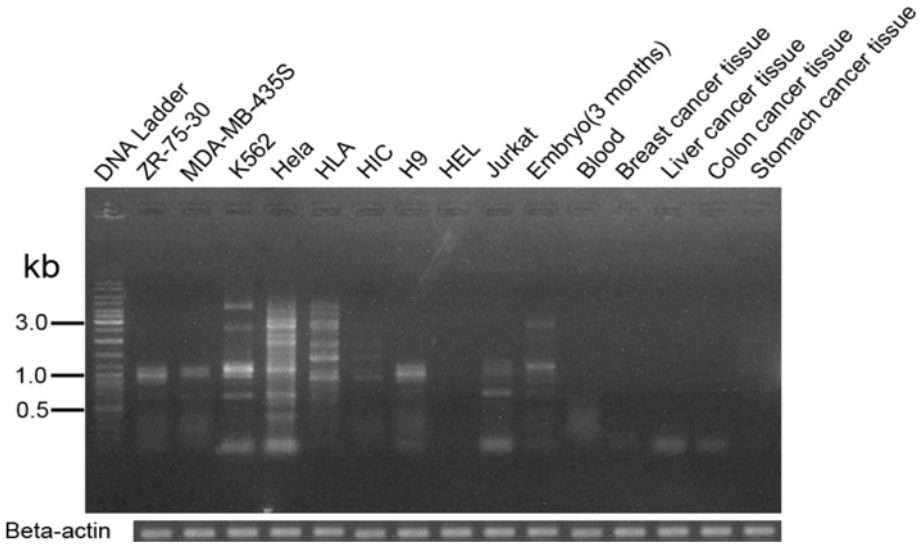

Fig. 6. Half quantitative RT-PCR analysis for amplifying exons 1-13 of breast cancer associated gene *BRCA1* from human cell lines and tissues. DNA ladder was 2-Log DNA Ladder from NEB.

for ongoing debate concerning the cellular localization of BRCA1 protein was possibly due to the existence of large numbers of *BRCA1* splice variants in breast cancer cells.

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