EXHIBIT 2

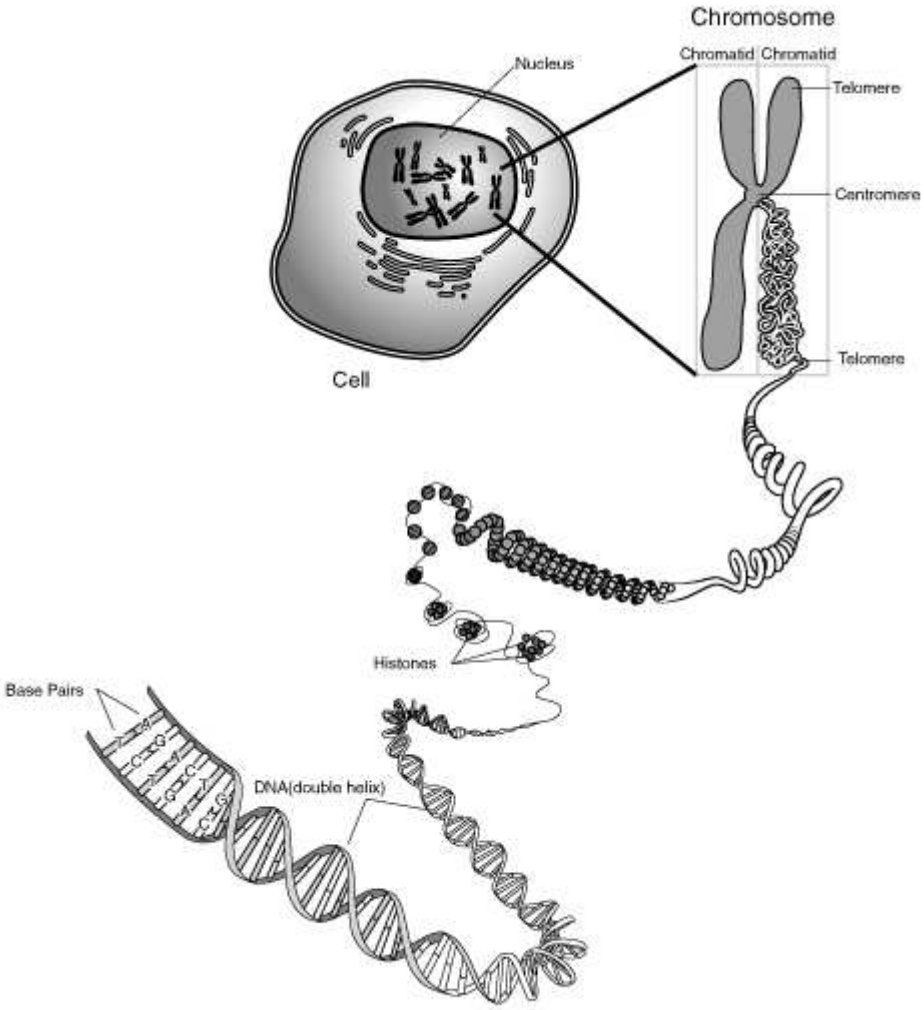
Chromosome
Nucleus
Chromatid
Chromatid
Telomere
Centromere
Telomere
Cell
Histones
Base Pairs
DNA(double helix)

EXHIBIT 3

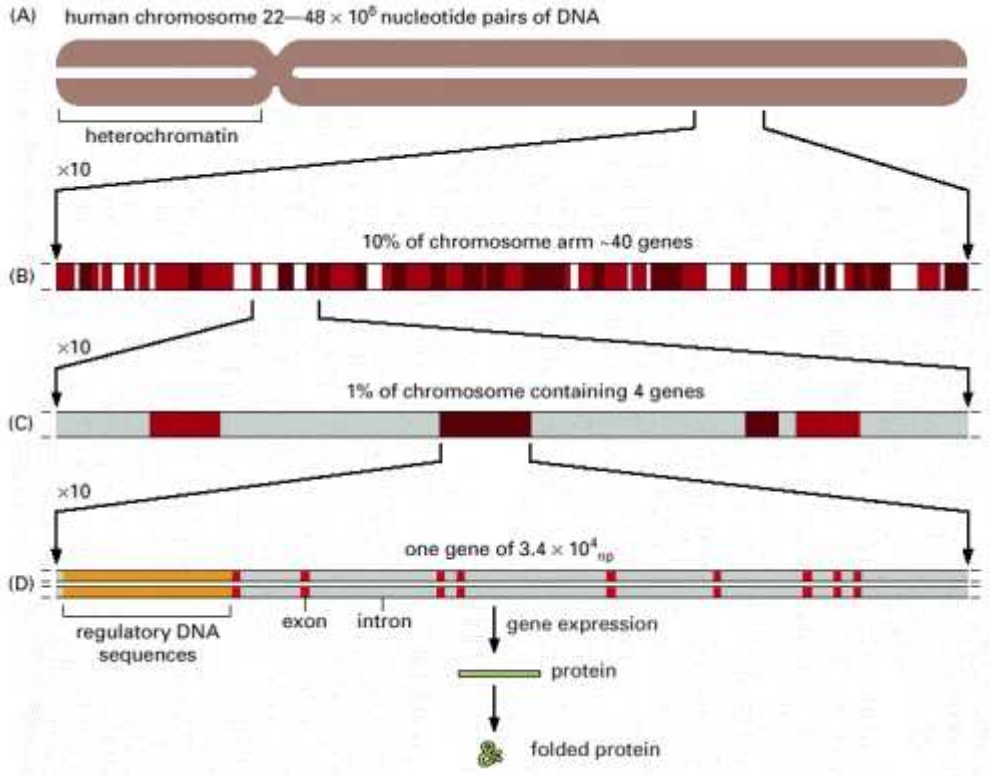

Figure 4-15.

The organization of genes on a human chromosome

(A) Chromosome 22, one of the smallest human chromosomes, contains 48 × 10^6 nucleotide pairs and makes up approximately 1.5% of the entire human genome. Most of the left arm of chromosome 22 consists of short repeated sequences of DNA that are packaged in a particularly compact form of chromatin (heterochromatin), which is discussed later in this chapter. (B) A tenfold expansion of a portion of chromosome 22, with about 40 genes indicated. Those in *dark brown* are known genes and those in *light brown* are predicted genes. (C) An expanded portion of (B) shows the entire length of several genes. (D) The intron-exon arrangement of a typical gene is shown after a further tenfold expansion. Each exon *(red)* codes for a portion of the protein, while the DNA sequence of the introns *(gray)* is relatively unimportant. The entire human genome (3.2 × 10^9 nucleotide pairs) is distributed over 22 autosomes and 2 sex chromosomes (see Figures 4-10 and 4-11). The term *human genome sequence* refers to the complete nucleotide sequence of DNA in these 24 chromosomes. Being diploid, a human somatic cell therefore contains roughly twice this amount of DNA. Humans differ from one another by an average of one nucleotide in every thousand, and a wide variety of humans contributed DNA for the genome sequencing project. The published human genome sequence is therefore a composite of many individual sequences. (Adapted from International Human Genome Sequencing Consortium, *Nature* 409:860–921, 2001.)

EXHIBIT 4

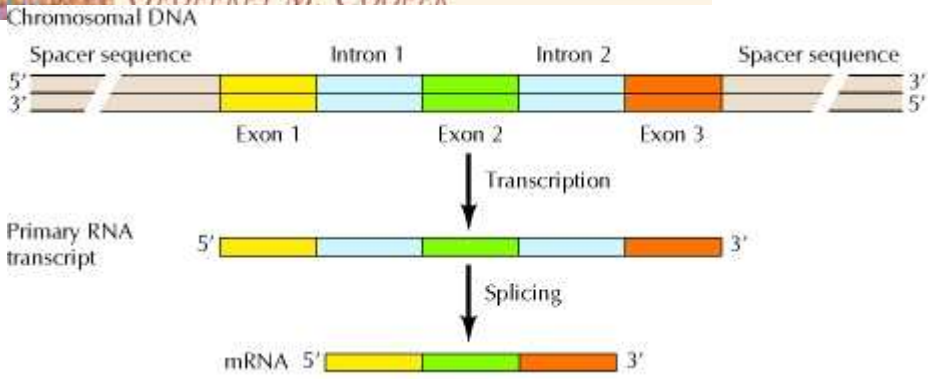

Figure 4.2.

The structure of eukaryotic genes

Most eukaryotic genes contain segments of coding sequences (exons) interrupted by noncoding sequences (introns). Both exons and introns are transcribed to yield a long primary RNA transcript. The introns are then removed by splicing to form the mature mRNA.

EXHIBIT 5

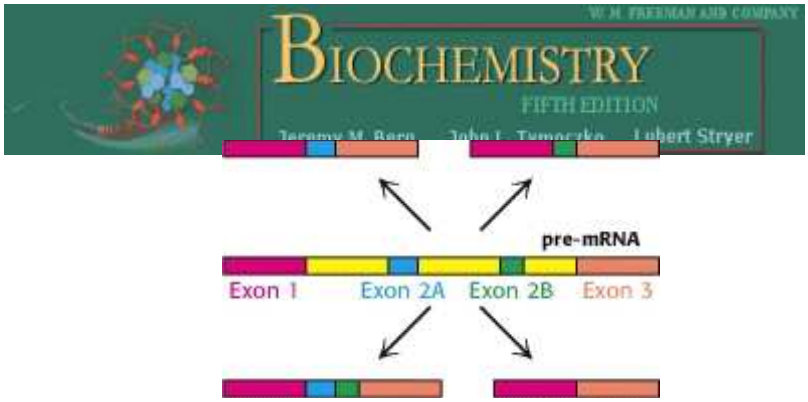

Figure 28.33.

Alternative Splicing Patterns

A pre-mRNA with multiple exons is sometimes spliced in different ways. Here, with two alternative exons (exons 2A and 2B) present, the mRNA can be produced with neither, either, or both exons included. More complex alternative splicing patterns also are possible.

EXHIBIT 6

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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'-GGGTTTCTCAGATAACTG GG-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'-GCTGTTAGAAGGCTGGC TCCCATG-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

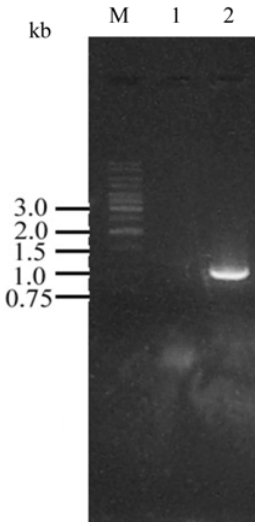

Fig. 1. RT-PCR result for amplifying exons 1-13 of breast cancer associated gene *BRCA1*. Lane M: 1 kb DNA ladder; Lane 1: the negative control of RT-PCR; Lane 2: the amplified products of RT-PCR.

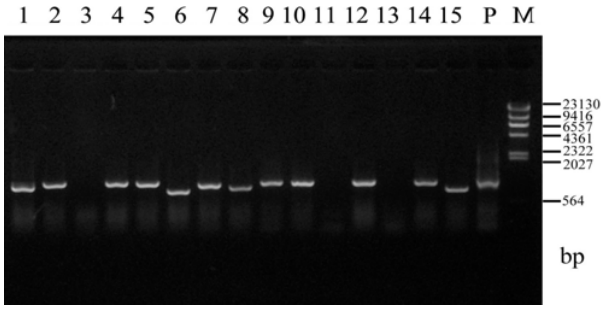

Fig. 2. Screening the positive clones of *BRCA1* exons 1-13 by PCR method. Lanes 1-15: the different positive and negative clones F1-15, respectively; Lane P: the positive control; Lane M: 1 kb DNA ladder.

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,

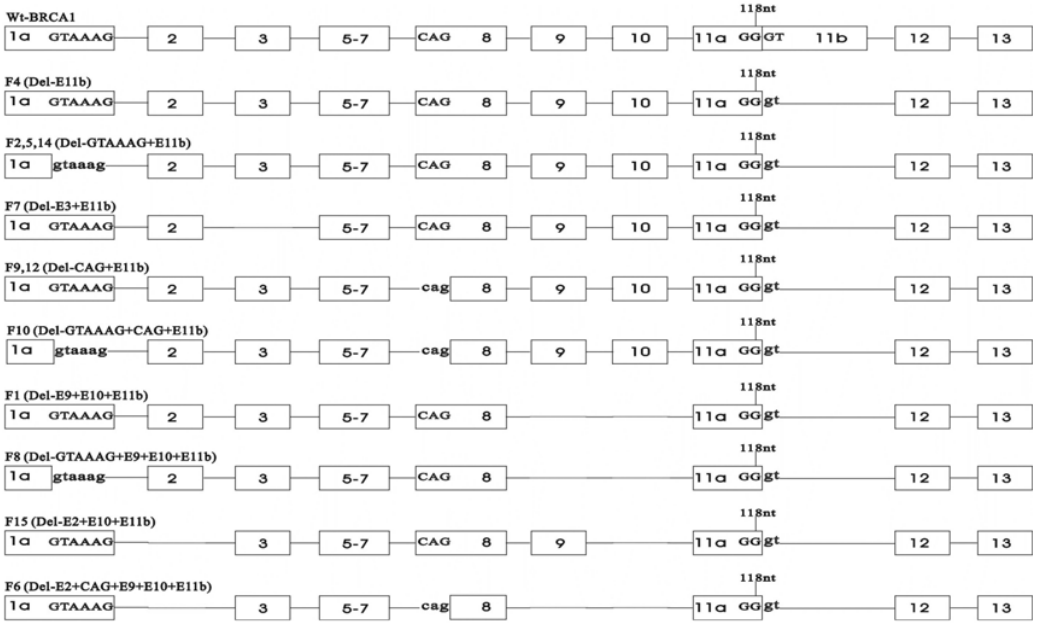

Fig. 3. The structural maps of the wild-type *BRCA1* exons 1-13 and nine *BRCA1* splice forms cloned from breast cancer cell line ZR-75-30. Five splice forms (F6: Del-E2 + CAG + E9 + E10 + E11b; F7: Del-E3 + E11b; F8: Del-GTAAAG + E9 + E10 + E11b; F9,12: Del-CAG + E11b; F15: Del-E2 + E10 + E11b) were 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.

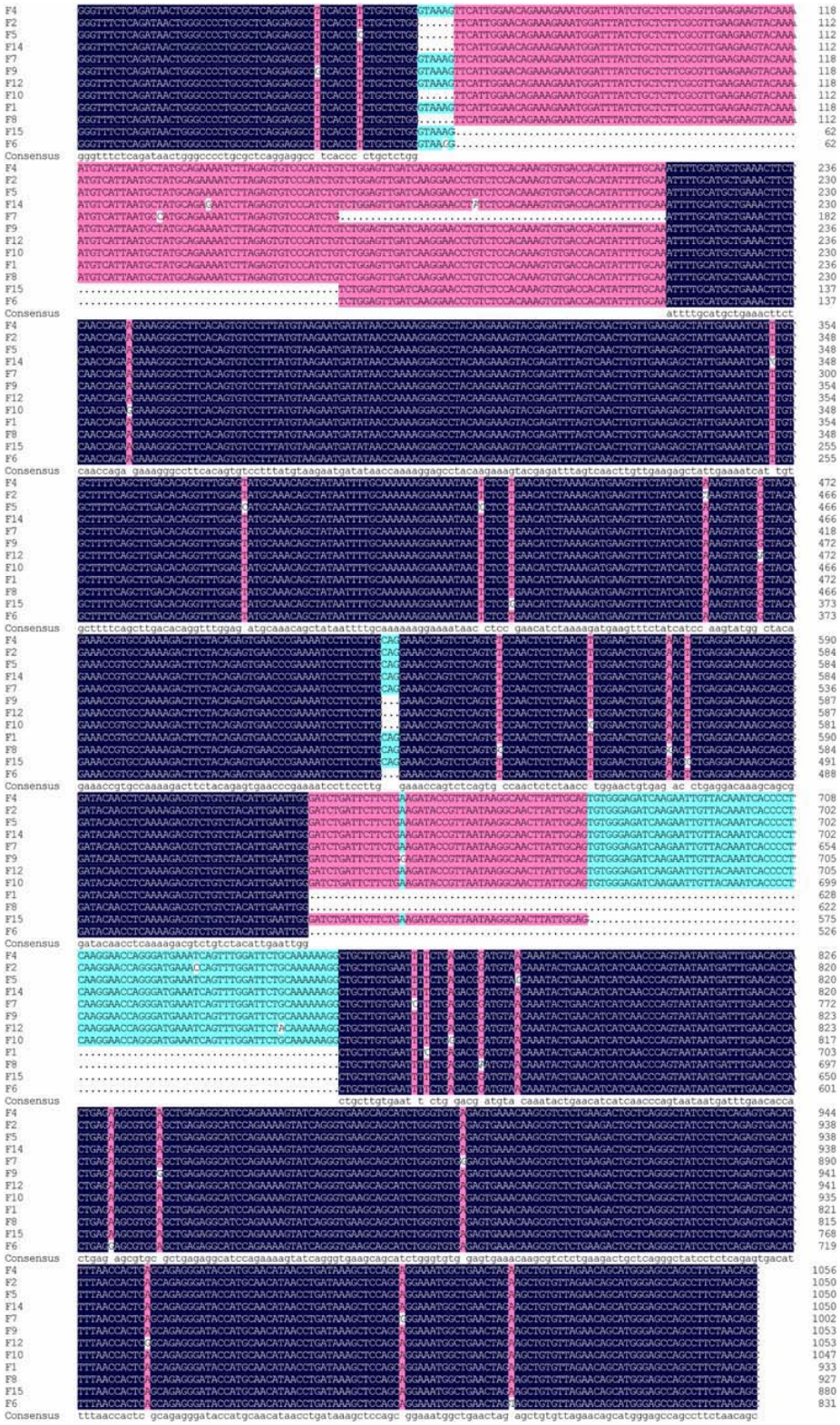

Fig. 4. Multiple sequence alignment of 12 positive clones (F4, F2, F5, F14, F7, F9, F12, F10, F1, F8, F15 and F6) encoding exons 1-13 of breast cancer associated gene *BRCA1*. Dots show the missing parts of exon sequences.

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

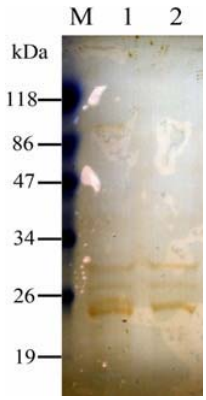

Fig. 5. Western blotting for the expression of BRCA1 splice variants from ZR-75-30. M: Middle molecular weight protein marker with color; 1 and 2: the lysates of the breast cancer cell line ZR-75-30.

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

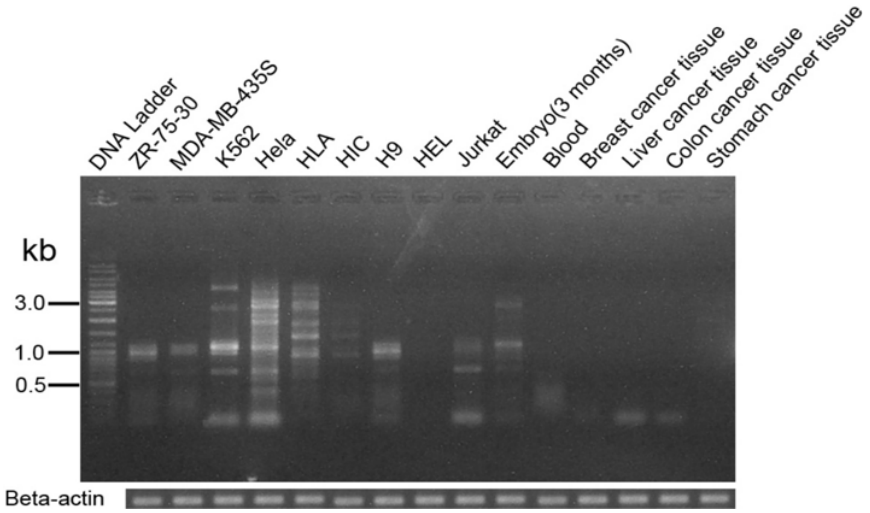

Fig. 6. Half quantitative RT-PCR analysis for amplifying exons 1-13 of breast cancer associated gene *BRCA1* from human cell lines and tissues. DNA ladder was 2-Log DNA Ladder from NEB.

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.

Acknowledgments This work was supported by the grants from R&D Infrastructure and Facility Development Program of China (Number: 2004DKA305400-4).

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EXHIBIT 7

[CANCER RESEARCH 59, 2546–2550, June 1, 1999]

Advances in Brief

Increased Level of Exon 12 Alternatively Spliced *BRCA2* Transcripts in Tumor Breast Tissue Compared with Normal Tissue[1]

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Abstract

The breast cancer susceptibility gene *BRCA2* is expressed in a wide range of tissues as an 11-kb mRNA transcript encoding a 3418-amino acid protein, which is involved in the response to DNA damage. To obtain a better molecular characterization of *BRCA2* expression in breast tissue, we analyzed full-length *BRCA2* mRNA by means of reverse transcriptase-PCR with a panel of primer pairs encompassing the entire cDNA sequence. We report the identification of an exon 12 alternatively spliced *BRCA2* transcript (Δ12-*BRCA2*) in normal human breast tissue, in a wide variety of other normal human tissues, and in several mouse tissues. The deletion observed in this transcript (96 bp) preserves the open reading frame, and translation of the transcript would result in a *BRCA2* isoform lacking 32 amino acids between codons 2280 and 2311. The analysis of matched normal and primary tumor breast tissues from 12 patients showed that the expression level of the Δ12-*BRCA2* transcript was higher in 4 of 12 (33%) tumor tissues compared with their normal breast tissues. Overproduction of the Δ12-*BRCA2* variant was associated with steroid receptor-negative tumors ($P = 0.0005$). These data suggest that the mechanisms generating the *BRCA2* mRNA variant exist in normal breast tissue and may be dysregulated in steroid receptor-negative breast tumor tissues.

Introduction

Breast cancer, one of the most common life-threatening diseases in women, occurs in hereditary and sporadic forms. Two major breast cancer susceptibility genes, *BRCA1* and *BRCA2*, have recently been isolated (1, 2). Both behave as classic tumor suppressor genes in familial breast cancers, in which loss of both alleles is required for the initiation of malignancy. Recent studies suggest that BRCA1 and BRCA2 proteins, by interaction with RAD51, a protein involved in the repair of double-strand DNA breaks, play a role in response to DNA damage as well as in mitotic and meiotic recombination (3).

A number of germ-line mutations in the *BRCA1* and *BRCA2* genes have been identified in families that are prone to breast cancer (4–6) but not in sporadic breast cancers (7–9). Several laboratories are now working on the molecular characterization of *BRCA1* and *BRCA2* expression and function in breast cancer. The identification of a major mRNA splice variant of *BRCA1* lacking the majority of exon 11 is, therefore, of considerable importance (10–12). The full-length BRCA1 product and the *BRCA1* variant lacking exon 11 may have distinct roles in cell growth regulation and tumorigenesis (11).

To obtain a better molecular characterization of *BRCA2* expression, we analyzed full-length *BRCA2* mRNA by using a panel of six primer pairs that together encompass the entire cDNA sequence (exons 1–27). We describe here a splice variant lacking exon 12 in both normal and tumor breast tissue, the expression level of which was higher in tumor tissue than in normal breast tissue, especially in steroid receptor-negative tumors.

Materials and Methods

Patients and Samples. Thirty-eight primary breast carcinomas were obtained at Center René Huguenin (St-Cloud, France). The samples were examined histologically for the presence of tumor cells. A tumor sample was considered suitable for this study if the proportion of tumor cells was >60%. Adjacent normal breast tissue was also taken from 12 of the 38 patients. The patients included in this study met the following criteria: primary unilateral breast carcinoma for which complete clinical, histological, and biological information was available and no other primary cancers. None of the 38 patients had received radiation therapy or chemotherapy before surgery.

Seven normal breast tissue specimens obtained from women undergoing cosmetic breast surgery were used as sources of normal RNA. Total RNA from a pool of six normal human breast tissues was also purchased from Clontech (Palo Alto, CA).

RNA Extraction. Total RNA was extracted from samples by the acid-phenol guanidium method (13). The quality of the RNA samples was determined by electrophoresis through denaturing agarose gels and staining with ethidium bromide, and the 18S and 28S RNA bands were visualized under UV light. The yield was quantified spectrophotometrically.

cDNA Synthesis. Reverse transcription was performed in a final volume of 20 μl containing 1× RT[3]-PCR buffer [1 mM each dNTP, 5 mM $MgCl_2$, 50 mM KCl, and 10 mM Tris-HCl (pH 8.3)], 20 units of RNase inhibitor, 50 units of Moloney murine leukemia virus RT (PE. Applied Biosystems, Foster City, CA), 2.5 μM random hexamers, and 1 μg of total RNA. The samples were incubated at 20°C for 10 min and 42°C for 30 min, and RT was inactivated by heating at 99°C for 5 min and cooling at 5°C for 5 min.

Primers and PCR Conditions. Single-stage PCR was carried out in a final volume of 50 μl containing 2 μl of the RT reaction mix, 400 nM each primer, 200 μM each dNTP, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, and 1 unit of AmpliTaq DNA polymerase (PE. Applied Biosystems).

The positions of the primers on the *BRCA2* gene are shown in Fig. 1, and their nucleotide sequences were as follows: (*a*) BRC1U (113U, 5′-GTGAGGGGACAGATTTGTGA-3′) and BRC1L (1099L, 5′-GGACATTTGGCATTGACTTT-3′) for exons 1–10 (PCR product of 987 bp); (*b*) BRC2U (6974U, 5′-CCACACATTCTCTTTTTACA-3′) and BRC2L (7939L, 5′-CCTTACCAAAATAATCTTCA-3′) for exons 11–16 (PCR product of 966 bp); (*c*) BRC3U (7767U, 5′-AAAAACATCCACTCTGCCTC-3′) and BRC3L (8776L, 5′-CCTTTTCTTCCTCTCTTTCA-3′) for exons 15–20 (PCR product of 1010 bp); (*d*) BRC4U (8663U, 5′-GAGGAAATGTTGGTTGTGTTGA-3′) and BRC4L (9508L, 5′-ACAAATAGACGAAAGGGGCA-3′) for exons 19–25 (PCR product of 846 bp); (*e*) BRC5U (9453U, 5′-AGGATTTGTCGTTTCTGTTGT-3′) and BRC5L (10009L, 5′-CATCAATCTCTTTCTCCCCTT-3′) for exons 24–27 (PCR product of 557 bp); (*f*) BRC6U (983U, 5′-ACAGTGAAAACACAAATCAAAGAG-3′) and BRC6L (7252L, 5′-GACGTTCCTTAGTTGTGCGA-3′) for exons 9–13 (PCR product of 6270 bp); (*g*) BRC7U (7048U, 5′-GGAGAGCCCCTTATCTTAGTGG-3′) and

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[3] The abbreviations used are: RT, reverse transcriptase; WT, wild-type.

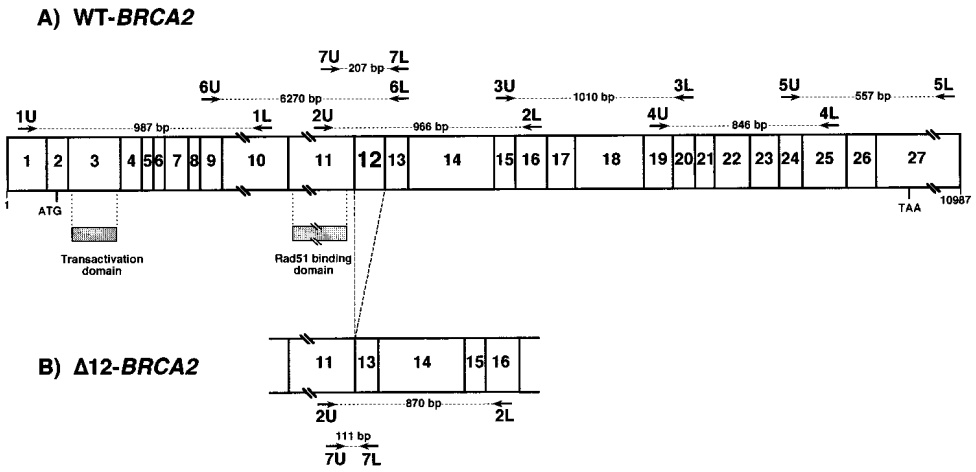

Fig. 1. Location of primer pairs used for *BRCA2* gene analysis. ▨, known functional domains of BRCA2 protein.

BRC7L (7254L, 5′-TTGACGTTCCTTAGTTGTGCGA-3′) for exons 11–13 (PCR product of 207 bp).

Primers were chosen with the aid of the Oligo 4.0 (National Biosciences, Plymouth Meeting, MN) computer program. Primers are designated by the nucleotide position (relative to *BRCA2* GenBank accession no. U43746) corresponding to the 5′ position, followed by the letter U for upper (*i.e.*, sense) strand or L for lower (*i.e.*, antisense) strand. The PCR procedure comprised: initial denaturation at 94°C for 5 min; 32 cycles of 1 min at 94°C, 1 min at 55–65°C, and 1.5 min at 72°C; and a final extension step of 10 min at 72°C, using a Perkin-Elmer 9600 DNA thermocycler. Aliquots (2 μl) of the appropriately diluted PCR products were added to 2.5 μl of deionized formamide containing 0.3 μl of a molecular size marker (Genescan 2500 ROX; PE. Applied Biosystems). The mixtures were denatured by heating and 2.5-μl aliquots were loaded onto 6% polyacrylamide gels containing 8 M urea and run for 6 h at 1200 V on the Applied Biosystems model 373A DNA sequencing system (PE. Applied Biosystems). The resulting gel data were analyzed for fragment size and peak area by using the Genescan 672 Fragment Analysis software (PE. Applied Biosystems).

Quantitative Δ12-*BRCA2* Transcript Analysis. Coamplification of all *BRCA2* transcripts (Δ12-*BRCA2* and WT *BRCA2* transcripts) with the same primer pair (BRCA2U/BRCA2L) provided a quantitative competitive RT-PCR method, in which the internal control was the coamplified WT *BRCA2* mRNA, and comparative expression of Δ12-*BRCA2* and WT *BRCA2* mRNA could be determined for each sample. According to Pannetier *et al.* (14), the ratios between these two *BRCA2* transcripts were similar regardless of the number of cycles.

The sensitive Applied Biosystems model 373A DNA sequencing system (PE. Applied Biosystems) was used, and the different fragments were quantified with Genescan 672 Fragment Analysis software (PE. Applied Biosystems), which calculates peak size and area.

For a given sample, the final result was expressed as a proportion ($P\Delta12$, in percentage) of Δ12-*BRCA2* mRNA over all *BRCA2* mRNAs, was determined as follows:

$$P\Delta12 = 100 \times \frac{\Delta12\text{-}BRCA2 \text{ (peak area)}}{\Delta12\text{-}BRCA2 + \text{WT } BRCA2 \text{ (peak areas)}}$$

The reproducibility of the quantitative measurements was evaluated by three independent replicate cDNA synthesis and PCR runs. The means of the replicated measurements and their 95% confidence intervals were calculated.

Sequencing of PCR Products. PCR products were resolved in a 1.5% low-melting point agarose gel (Life Technologies, Inc., Gaithersburg, MD), stained with ethidium bromide. DNA fragments were eluted from gels and purified using the QIAquick PCR purification kit (Qiagen, Santa Clareta, CA). The purified fragments were sequenced using the ABI PRISM Dye Terminator Cycle Sequencing kit (PE. Applied Biosystems) on the Applied Biosystems model 373A DNA sequencing system (PE. Applied Biosystems).

Statistical Analysis. Biological parameters were compared using the χ^2 test. Differences between the two populations were judged significant at confidence levels of >95% ($P < 0.05$).

Results

Identification of a Major *BRCA2* Variant in Normal Human Breast Tissue. We analyzed *BRCA2* mRNA expression in normal breast tissue samples by RT-PCR), using five primer pairs (BRC1U/BRC1L–BRC5U/BRC5L) that encompass the entire cDNA sequence of full-length *BRCA2*, except the large exons 10 and 11 (Fig. 1). The samples comprised seven normal breast tissue specimens obtained from women undergoing cosmetic breast surgery and a pool of six normal human breast tissues. All primer pairs yielded a major RT-PCR fragment that was exactly the size predicted from the published *BRCA2* cDNA sequence (GenBank accession no. U43746). However, besides the expected band, the BRC2U/BRC2L primer pair yielded an additional band of smaller size (~10% of the WT *BRCA2* transcript amount; Table 1). Other primer pairs (BRC1U/BRC1L and BRC3U/3L) yielded abnormal bands, but at very low levels (<3% of the WT *BRCA2* transcript). Finally, primer pairs BRC4U/BRC4L and BRC5U/BRC5L did not yield additional abnormal bands.

The additional band of smaller size (870 bp) than the expected band at 966 bp obtained with the BRC2U/BRC2L primer pair (exons 11–16) was observed in all of the normal breast samples examined. This smaller transcript had a 96-bp deletion, presumably resulting from exon 12 alternative splicing of the *BRCA2* mRNA. PCR ampli-

Table 1 *Proportion of Δ12-BRCA2 transcripts in human breast tissues*

Patient no.	Normal tissue	Tumor tissue
Individual normal breast tissues		
P1	13.2 ± 1.2[a]	
P2	10.5 ± 0.8	
P3	14.1 ± 0.9	
P4	8.9 ± 1.1	
P5	8.6 ± 0.6	
P6	10.1 ± 1.5	
P7	8.6 ± 0.9	
Pool of six normal breast tissues	9.1 ± 0.6	
Matched normal and tumor breast tissues		
P8	7.2 ± 1.1	35.3 ± 0.9
P9	6.6 ± 0.9	7.1 ± 1.2
P10	11.8 ± 0.6	27.2 ± 1.3
P11	11.1 ± 0.8	23.9 ± 0.6
P12	9.8 ± 1.8	11.1 ± 1.9
P13	7.3 ± 1.4	21.4 ± 0.8
P14	11.4 ± 0.7	11.6 ± 0.9
P15	14.7 ± 1.1	12.9 ± 0.9
P16	13.2 ± 0.4	15.0 ± 0.6
P17	8.1 ± 0.7	7.9 ± 1.5
P18	7.7 ± 1.1	8.2 ± 0.8
P19	6.0 ± 0.9	6.8 ± 1.1

[a] For each sample, the mean proportion (%) of *BRCA2* transcripts was calculated as the mean of the proportions found for each measurement. Triplicate cDNA and PCRs were performed for each sample. Data represent 95% confidence intervals of the means.

fication with primer pair BRC7U/BRC7L, which encompasses only exon 12 (Fig. 1), and sequence analysis (Fig. 2) confirmed the existence of a splice variant lacking exon 12.

Putative alternative splicing involving large exons such as exons 10 (1116-bp) and 11 (4932-bp) was studied by Northern blot analysis. This analysis showed a single 11-kb *BRCA2* transcript in all samples tested (data not shown), confirming a previous study (15). However, because *BRCA2* is remarkably similar to *BRCA1*, both having a large exon 11 (3426 bp for *BRCA1* and 4932 bp for *BRCA2*), and because several authors have shown the existence of an mRNA variant of *BRCA1* with deletion of exon 11 (10–12), we confirmed our Northern blot results by RT-PCR analysis using a primer pair (BRC6U/BRC6L) encompassing exons 10 and 11 of *BRCA2* (Fig. 1). This primer pair not only did not yield the expected 6270-bp fragment because of the inefficiency of RT-PCR over such a length of sequence, but it also did not yield the putative 222- and 1338-bp fragments when exon 11 (with and without exon 10, respectively) had been spliced out.

Detection of the Δ12-*BRCA2* Variant in Other Normal Human Tissues. Expression of the Δ12-*BRCA2* mRNA variant in a variety of normal human tissues, including peripheral blood leukocytes, kidney, smooth muscle, stomach, colon, skin, liver, bone marrow, ovary, placenta, and prostate, was examined.

The Δ12-*BRCA2* variant was observed in all tested tissues, with a proportion of ~10%, compared with the WT *BRCA2* transcript.

Detection of the Δ12-*BRCA2* Variant in Mouse Tissues. To verify the presence of the Δ12-*BRCA2* alternative splicing variant in mouse tissues, we carried out RNA RT-PCR with primers encompassing exon 12. One transcript isoform of the expected size, comparable to the human profile (96 bp smaller than the WT *BRCA2* PCR product), was amplified from all mouse tissues tested (smooth muscle, placenta, and liver). The mouse isoform showed a deletion of exactly the same 96 nucleotides of exon 12 as in the human counterpart.

Increased Level of the Δ12-*BRCA2* Variant in Tumor Breast Tissue Compared with Normal Tissue. Because *BRCA2* is responsible for the development of a subset of familial breast tumors, the possible role of Δ12-*BRCA2* alternative splicing in sporadic breast tumorigenesis warrants investigation. We examined the expression level of the Δ12-*BRCA2* variant in tumor breast tissue compared with normal tissue. We analyzed matched normal and primary tumor breast tissues from 12 patients (patients P8–P19; Table 1). For each sample, three different cDNA and PCRs were performed, and the mean value was determined.

To check the reliability of our molecular analysis, we used several controls: (*a*) triplicate PCRs were carried out, one for 30 cycles, another for 32 cycles, and the last for 35 cycles, to check whether 32 PCR cycles corresponded to the exponential phase of the reaction; (*b*) PCR was carried out with the two primer pairs BRC2U/BRC2L and BRC7U/BRC7L, which both reveal the Δ12-*BRCA2* variant (Fig. 1); (*c*) PCR was carried out using three DNA polymerase conditions: AmpliTaq DNA polymerase (PE. Applied Biosystems), AmpliTaq DNA polymerase (PE. Applied Biosystems) plus TaqStart Antibody (Clontech), and AmpliTaq Gold DNA polymerase (PE. Applied Biosystems). These three controls were used to check whether the two PCR fragments were equally amplified. Indeed, in certain PCR conditions, smaller fragments could be more efficiently amplified than larger fragments. For each sample, the qualitative and quantitative measurements in different PCR conditions were not significantly different from each other, showing that the smaller fragments were not more efficiently amplified, therefore confirming the accuracy of the molecular analysis.

Because the levels of Δ12-*BRCA2* variant in the 12 matched normal samples, together with the 8 previously tested normal samples (7 normal breast tissue specimens obtained from women undergoing cosmetic breast surgery and a pool of 6 normal human breast tissues), consistently fell between 6 and 15 PΔ12 value, values of 20 or more were considered to represent overproduction of the Δ12-*BRCA2* variant in individual breast cancer tissue. Among the 12 patients in whom both primary breast tumors and matched normal breast tissue were investigated, 4 (P8, P10, P11, and P13; 33.3%) clearly showed a higher proportion of the Δ12-*BRCA2* variant in tumor than in normal tissue (Table 1).

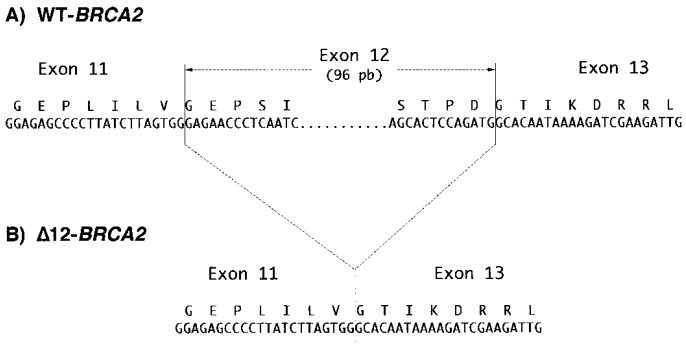

Fig. 2. A comparison of the nucleotide sequences of WT *BRCA2* and its variant lacking the entire exon 12 in human breast tissue. Corresponding amino acid sequences (in single-letter code) are shown. Loss of exon 12 generates a BRCA2 protein lacking 32 amino acids between codons 2280 and 2311.

Overproduction of the Δ12-BRCA2 Variant Is Associated with Steroid Receptor Status. *BRCA2* expression is regulated by estrogen in human breast cancer cell lines (16), so it was important to determine whether a high level of Δ12-*BRCA2* variant is associated with the steroid receptor status of the tumors. We analyzed Δ12-*BRCA2* mRNA expression in 26 additional breast tumors selected on the basis of steroid receptor status: half ($n = 13$) were both estrogen receptor and progesterone receptor negative and other half were both estrogen receptor and progesterone receptor positive. The level of the Δ12-*BRCA2* variant ranged from 5.9 to 41.9% in this additional series. Fig. 3 represents tumors in which the proportion of Δ12-*BRCA2* variant was 5.9% (T444), 22.6% (T182), and 41.9% (T327). Overproduction of the Δ12-*BRCA2* variant was found in 9 (34.6%) of these 26 tumors and was significantly associated with steroid receptor negativity ($P = 0.0005$). None of the 13 steroid receptor-positive tumors showed overproduction of the Δ12-*BRCA2* variant, compared to 9 of the 13 (69%) that were steroid receptor negative.

Discussion

Alternative mRNA splicing is a common mechanism for regulating gene expression in higher eukaryotes, and there are many examples of development-, tissue-, and tumor-specific differences in splicing events. To search systematically for the existence of alternative *BRCA2* variants, we carried out RT-PCR with primer pairs covering the whole sequence. We used only a single-stage PCR strategy to avoid the overexpression of shorter PCR products frequently observed by nested PCR. It is interesting to note that Xu *et al.* (12), using a nested RT-PCR strategy, identified multiple variants of the *BRCA1* gene. Here, we report the identification of a major alternative *BRCA2* transcript with an expression level of ~10% in normal breast tissue relative to the WT *BRCA2* transcript. Sequencing of this alternative transcript shows that it arises from alternative splicing of exon 12. We cannot rule out the existence of other important *BRCA2* variants. Indeed, we observed several *BRCA2* variant transcripts in the NH_2-terminal and central domains (primer pairs BRC1U/BRC1L and BRC3U/BRC3L), but they had very low expression levels (<3%) relative to the WT *BRCA2* transcript (data not shown). Thus, we

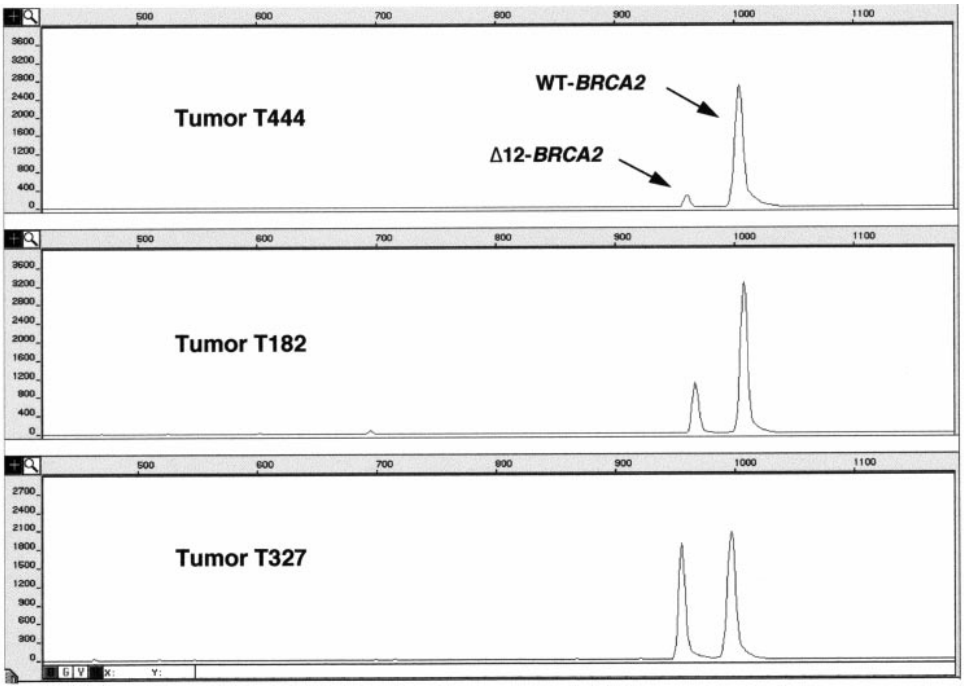

Fig. 3. Δ12-*BRCA2* transcript level in three breast tumor samples. PCR products of Δ12-*BRCA2* and WT *BRCA2* cDNA give two different peaks of fluorescence. The area of each peak correlates with the amount of PCR product. Results were expressed in percentage as the ratio between the Δ12-*BRCA2* peak area value over the sum of the two peak area values (Δ12-*BRCA2* and WT *BRCA2*). Each tumor sample was analyzed in triplicate, but the data for only one analysis are shown here. The proportions of Δ12-*BRCA2* variant were 5.9% in tumor T444, 22.6% in T182, and 41.9% in T327.

observed the Δ3-*BRCA2* variant reported by Siddique *et al.* (17). We did not find any variants in the COOH-terminal domain (exons 19–27; primer pairs BRC4U/BRC4L and BRC5U/BRC5L).

The number of nucleotides (96 bp) missing from the Δ12-*BRCA2* transcript isoform is a multiple of three, suggesting the maintenance of the open reading frame; translation of this transcript would result in a BRCA2 protein lacking 32 amino acids between codons 2280 and 2311. The function of this region of BRCA2 protein is unknown. Indeed, several different domains have been recognized in BRCA2. A coding sequence for a transcriptional activation domain was identified in the 5′ end of the transcript (exon 3; Ref. 18) and a domain that interacts with RAD51 through its BRC repeats located at the 5′ portion of exon 11 (19). None of these functional domains is encoded by exon 12. However, several of our results suggest that the Δ12-*BRCA2* transcript may give rise to a protein with an important biological function. (*a*) The Δ12-*BRCA2* transcript was observed in a large variety of normal adult human tissues, including peripheral blood leukocytes, and it is conserved in the mouse. (*b*) The expression of the Δ12-*BRCA2* transcript was higher in tumor tissue than in normal breast tissue, suggesting the existence of mechanisms generating the *BRCA2* mRNA variant in normal breast tissue and the possibility that these may be dysregulated in breast tumor tissues. It is not clear why or by what mechanism the *BRCA2* mRNA variant level is increased, but it could be caused by factors at the transcriptional or posttranscriptional level or both. (*c*) Overproduction of the Δ12-*BRCA2* variant is associated with steroid receptor-negative tumors. Spillman and Bowcock (16) have previously shown that estrogen regulates *BRCA2* mRNA expression mediated by the estrogen receptor. Taken together, these results suggest that the Δ12-*BRCA2* transcript isoform must make a significant contribution to overall *BRCA2* function.

In conclusion, we have identified an alternatively spliced *BRCA2* transcript that is widely expressed in all normal tissues examined. This Δ12-*BRCA2* transcript is overexpressed in steroid receptor-negative breast tumor tissue, suggesting that dysregulation of the Δ12-*BRCA2* isoform may contribute to progression in human breast cancer. Characterization of the functional properties of the protein derivative and direct assessment of protein produced in various cell types will be necessary to determine the significance of this variant. Alternative splicing of the *BRCA2* gene in lymphocytes may also have an important practical implication: in some cases, it may complicate the detection of germ-line *BRCA2* mutations based on the screening of lymphocyte RNA.

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EXHIBIT 8

Tobin/Dusheck, Asking About Life, 2/e
Figure 13.7