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Expression Profiles of BRCA1 Splice Variants in Asynchronous and in G1/S Synchronized Tumor Cell Lines

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Disrupting the function of the BRCA1 gene by mechanisms other than germline mutations is suspected to occur in cases of sporadic breast/ovarian cancers. Using ribonuclease protection assay and multiplex RT-PCR, we examined the change of the total BRCA1 mRNA pool and the expression profile of four predominant BRCA1 splice variants in asynchronous and in G1/S synchronized tumor cell populations *compared to normal breast cells.* Experiments were carried out on MCF-7 and MDA-MB-231 breast cancer, OVCAR-5 ovarian cancer, and K562 leukemia cell lines. The ratio of the full length, the Δ(11q), the Δ(9,10), and the Δ(9,10,11q) BRCA1 isoforms showed different expression patterns in the examined breast and ovarian tumor cell lines as compared to the leukemia cell line. This observation raises the possibility that the dysregulation of alternative splicing of the BRCA1 gene could be involved in tumor formation in the breast and the ovary, even in the absence of germline mutations.

Key Words: **BRCA1; alternative splicing; G1/S synchronization; gene expression.**

Germline mutations of the BRCA1 gene are associated with hereditary breast and ovarian cancers (1). Hundreds of distinct mutations of the gene have been identified so far, including numerous founder mutations that are frequently present in certain Eastern and Central European populations (2–8). Somatic mutations of the gene, however, are very rare and have been found mostly in sporadic ovarian rather than in breast cancer cases (9–12), therefore, other mechanisms affecting the normal BRCA1 function have been suggested to play a possible role in sporadic tumor formation. Namely, several groups described a decreased expression level of BRCA1 in sporadic tumors (13, 14) which in some cases was shown to be associated with aberrant promoter methylation pattern (15) or with increased expression of the Brn-3 transcription factor that can strongly repress the BRCA1 promoter in mammary tumor cells (16). These results underline the role of the BRCA1 gene in sporadic tumors even in the absence of germline mutations.

The expression of the BRCA1 gene is tissue specific, being the highest in thymus and testis cells and also relatively high in breast and ovarian epithelial cells (1). The expression is also cell-cycle dependent, starting to increase both at the mRNA and the protein level at the G1/S border, and that level is maintained until the end of the G2 phase (17–19). This phenomenon, however, was examined for the full length BRCA1 mRNA isoform and its corresponding 1863 amino acid long protein species, although other possible isoforms produced by alternative splicing may also play an important role in different cellular mechanisms.

Since its presence was first suggested (20), alternative splicing was found to be a very important level of gene regulation (21, 22) and its existence is now predicted in about one-third of the human genes (23). For the BRCA1 gene, 12 distinct mRNA splice variants have been identified so far (1, 24, 25); 11 of them maintain the original open reading frame (26) having the possiblity to code for a functional protein. Nevertheless, only four of these isoforms are believed to be common in various tissues: the full length BRCA1, the Δ(11q) BRCA1, the Δ(9,10) BRCA1, and the Δ(9,10,11q) BRCA1 mRNAs. However, it has not been fully elucidated whether these splice variants proportionally contribute to the whole BRCA1 mRNA pool expression pattern (24, 27, 28), or they are regulated differently by alternative splicing mechanisms as described for other genes (29, 30). This study is aimed to measure the relative expression levels of these four common BRCA1 mRNA isoforms in different tumor cell lines *compared to normal breast cells and* to unravel

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how this expression profile is altered when the transcription rate of BRCA1 is increased at the G1/S border of the cell cycle.

MATERIALS AND METHODS

Normal breast cell samples, cell cultures, and G1/S synchronizing with mimosine. Normal breast tissues were obtained during operation at the National Institute of Oncology, Hungary. Approximately 0.5 g of pathologist verified normal breast tissues were collected in 1 ml of Solution D (6 M guanidine-thiocyanate, 0.0375 M sodium-citrate (pH 7.0), 0.7% *N*-laurosyl-sarcosyl and 0.005% β-mercapto-ethanol) for subsequent RNA extraction. MCF-7 and MDA-MB-231 breast cancer cells were grown in D-MEM media (GIBCO BRL) supplemented with 10% FBS and 100 U/ml penicillin (Biogal RT, Hungary). OVCAR-5 ovarian cancer cells and K562 leukemia cells were maintained in RPMI-1640 media (GIBCO BRL) containing 10% FBS and 100 U/ml penicillin. Mimosine (Sigma), a plant amino acid derivative, was used to synchronize cells at the G1/S border of the cell cycle as previously described (19). Cells were washed with 1× PBS and collected for RNA isolation in Solution D.

Total RNA isolation. Total RNA was isolated from approximately 10^7 cells using the acidic phenol/chloroform extraction method (31). RNA pellets were suspended in deionized formamide and run on a 1% formaldehyde agarose gel to verify their integrity. Concentration of the RNA samples were measured by spectrophotometry.

Ribonuclease protection assay (RPA). Exon 20 of the BRCA1 gene was used to construct an expression plasmid vector from which antisense BRCA1 RNA probe could be generated using T7 phage RNA polymerase. Since this exon is intact in all described splice variants, the total BRCA1 mRNA pool could be examined using this probe. For a control reaction, we used a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA plasmid construct (nt 689–823, GenBank Accession No. NM_002046), provided by Dr. R. A. Jensen (Vanderbilt University School of Medicine, Nashville, TN). BRCA1 and GAPDH plasmids were linearized with *Hin*dIII and *Eco*RI restriction endonucleases respectively, then Riboprobe *In Vitro* Transcription System Kit (Promega) was used to generate radioactively labelled antisense RNA probes according to the manufacturer's instructions. For RPA, RNase Protection Assay System Kit (Promega) was used. Briefly, purified RNA probes were coprecipitated with 20 μg of total RNAs, and hybridized overnight at 55°C in the total volume of 20 μl. The solution was treated with RNase ONE ribonuclease for 1 h at 25°C. Protected fragments were then ethanol precipitated and run on a denaturing 6% polyacrylamide gel. The gel was dried and radioactive signals were detected using PhosphorImager 445 SI (Molecular Dynamics). Quantitative analysis was carried out using the ImageQuant program (Molecular Dynamics).

Reverse transcription polymerase chain reaction (RT-PCR). For the detection of the four predominant BRCA1 mRNA isoforms, we carried out a triple-primer RT-PCR method (TP-PCR) described earlier (32). The following primers were used for the reactions: 5′-CCAACTCTCTAACCTTGGAACTGTG-3′ (Primer 1, sense primer hybridizing to exon 8 of BRCA1 cDNA), 5′-CTTCCAGCCCATCTGTTATGTTG-3′ (Primer 2, antisense primer hybridizing to exon 11 of BRCA1 cDNA) and 5′-GATGACCTTTCCACTCCTGGTTC-3′ (Primer 3, antisense primer hybridizing to the junction of exons 14 and 15 of BRCA1 cDNA). Using the two antisense primers and double amount of the sense primer in the same reaction tube, we coamplified four cDNA products using GeneAmp RNA PCR Kit (PE Biosystems, USA) according to the manufacturer's instructions. A Perkin Elmer DNA Thermal Cycler 480 was used for the experiments. Primer 1 and Primer 2 coamplify the full length and the Δ(9,10) BRCA1 mRNAs, whereas Primer 1 and Primer 3 coamplify the Δ(11q) and the Δ(9,10,11q) BRCA1 mRNAs. The identity of each amplicon was verified by DNA sequencing using an ABI PRISM 310 Genetic Analyzer (PE Biosystems, USA). The structure of the four splice variants and the localization of the used primers are shown on Fig. 1.

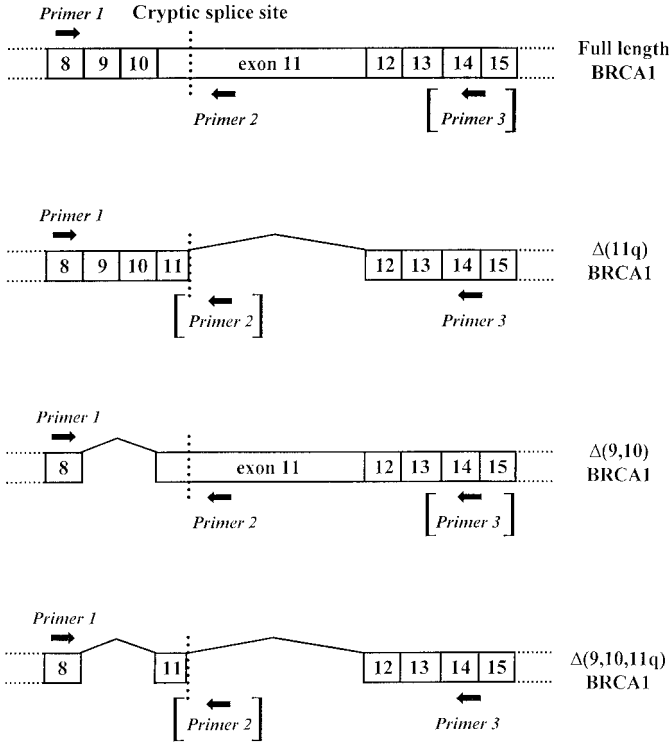

FIG. 1. The structure of the detected BRCA1 splice variants and the location of the cDNA primers used to amplify them. In the conditions applied, these primers do not amplify genomic DNA due to the long interspersed intronic sequences. Primers in brackets do not result in the amplification of a given cDNA species either because it would produce a very long amplicon or because the sequences they would anneal to are missing from that particular variant. Primers 1 and 2 coamplify the full length and the Δ(9,10) BRCA1 mRNAs, whereas primer 1 and 3 coamplify the Δ(11q) and the Δ(9,10,11q) BRCA1 isoforms, respectively. The Δ(11q) isoform is called in different names in the literature, some of which could be confusing such as the name Δ(11) because not the entire exon 11 is missing from this splice variant. Here we used a terminology similar to that applied to chromosomes such that the long portion of exon 11 missing from this variant was called 11q, hence the name: Δ(11q) BRCA1. Exon sizes are not drawn to scale.

Quantification of RT-PCR results. The RT-PCR products were run on a 2% nondenaturing agarose gel and quantification was done by analyzing the scanned picture of the gel using a Personal Densitometer SI (Molecular Dynamics). Although RT-PCR is not suitable to compare RNA species from different samples quantitatively, it can be used to determine the relative proportions of RNAs in the same reaction if the same primer pairs are used to coamplify them (32). This was the case for Primer 1 and 2, and Primer 1 and 3, separately, since they amplify two mRNA species at the same time. To test whether this comparison could also be applied for amplicons where only the sense primer (Primer 1, Fig. 1) is common in the reaction, thus for the full length and the Δ(11q) BRCA1, and for the Δ(9,10) and Δ(9,10,11q) BRCA1 species, respectively, we used individual amplicons (excised from the agarose gel and purified), mixed in different concentrations, and coamplified them using the same primer sets. It turned out that the final ratio of the amplicons depended solely on the initial concentrations of the mixed cDNAs

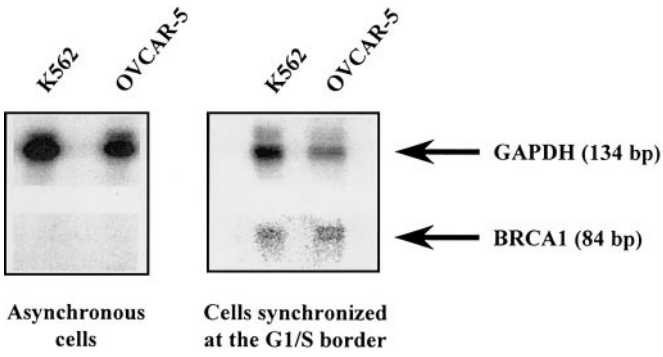

FIG. 2. Ribonuclease protection assay on asynchronous and on G1/S synchronized cell populations. Weak BRCA1 signals were detected in both synchronized K562 and OVCAR-5 cells (right panel), whereas no specific signals were detected for BRCA1 in either of the asynchronous cell populations even after much longer exposure time (left panel).

and there was no bias against any of the amplicons during this reaction (data not shown).

RESULTS

In the present study, we applied RPA to investigate the expression pattern of the total BRCA1 mRNA pool in G1/S synchronized cells compared to asynchronous cell populations on OVCAR-5 ovarian cancer and K562 leukemia cells. We detected an increase in the expression level due to G1/S synchronization by mimosine in both cell lines (Fig. 2). To our knowledge, this is the first time when this phenomenon is proved for this particular ovarian cancer cell line and for any leukemia cells. We then carried on examining the relative proportions of the four predominant BRCA1 mRNA splice variants to the whole BRCA1 mRNA pool and to investigate whether these splice variants are equally responsible for the increased expression level at the G1/S border of the cell cycle.

Additionally to OVCAR-5 and K562 cell lines, MCF-7 and MDA-MB-231 cell lines, *as well as normal breast cells* were also studied for the BRCA1 splice variants. For the two latter breast tumor cell lines, previous experiments demonstrated the similar overall BRCA1 expression pattern in G1/S synchronized cells (18, 19). Although RPA is a sensitive method to measure gene expression, it still failed to detect BRCA1 mRNAs in non-synchronized tumor cells. Therefore, we used a PCR-based method, TP-PCR (32), to detect BRCA1 mRNA isoforms present in very small quantities in these samples. We demonstrated that the four predominant BRCA1 splice variants are present in normal breast cells and in all cell lines examined, although the percentage of each splice variant was different in asynchronous cell populations of all cell types (Fig. 3). We did not detect other previously described splice variants in the samples, however, we showed that the ratios of the four predominant mRNA species changed due to G1/S synchronization in a cell line dependent manner (Fig. 4).

The full length BRCA1 isoform was shown to make up the largest proportion of the total BRCA1 mRNA pool in normal breast cells and in the examined cell lines, and its ratio increased due to mimosine treatment in breast and ovarian cancer cells but not in leukemia cells (Fig. 4A). The Δ(11q) is the only isoform the expression pattern of which showed a similar tendency in all cell types: its ratio to the total BRCA1 mRNA pool decreased due to G1/S synchronization (Fig. 4B). The ratios of the Δ(9,10) and the Δ(9,10,11q) isoforms showed more cell type specific patterns, however, their profile in the leukemia cells were notably different from that measured in the breast and the ovarian cells (Figs. 4C and 4D). It is important to note that the Δ(9,10,11q) BRCA1 isoform was found to be the least abundant mRNA species in all cell types examined, including normal breast cells. Moreover, its percentage relative to the other BRCA1 splice variants changed quite in the opposite way in the two examined breast tumor cell lines: In MCF-7 it fell below the detection level, whereas in MDA-MB-231 it appeared due to G1/S synchronization (Fig. 4D).

DISCUSSION

In the present work, we demonstrated that the expression of the overall BRCA1 mRNA pool increases due to G1/S synchronization on an ovarian tumor cell line (OVCAR-5) and, shown for the first time, a leukemia cell line (K562). This latter finding is of special interest since it underlines a general, cell type-independent role of the BRCA1 gene in proliferating cells. This is in good agreement with the results of others who described a similar phenomenon in MCF-7, in MDA-MB-231 and in other breast tumor cell cultures (18, 19). The question, however, still remained that to what extent the common BRCA1 splice variants contribute to this expression change, therefore we investigated leukemia, breast, and ovarian tumor cell lines to examine this problem.

Our expression studies by RPA strengthen the fact that the transcription rate of BRCA1 increases at the G1/S border of the cell cycle. However, as pointed out earlier (30), the ratio of the differently spliced isoforms of a certain gene is not affected by merely changing the rate of transcription, and its tissue specific alternative splicing pattern is strongly influenced by the possible *trans*-acting factors that are present in different cell types. Here, using the TP-PCR method, we provided evidence that four of the twelve previously described BRCA1 splice variants are present in different proportions in the examined tumor cell lines *as well as in normal breast cells*. Moreover, when the transcription rate of BRCA1 increased, their ratio to the total

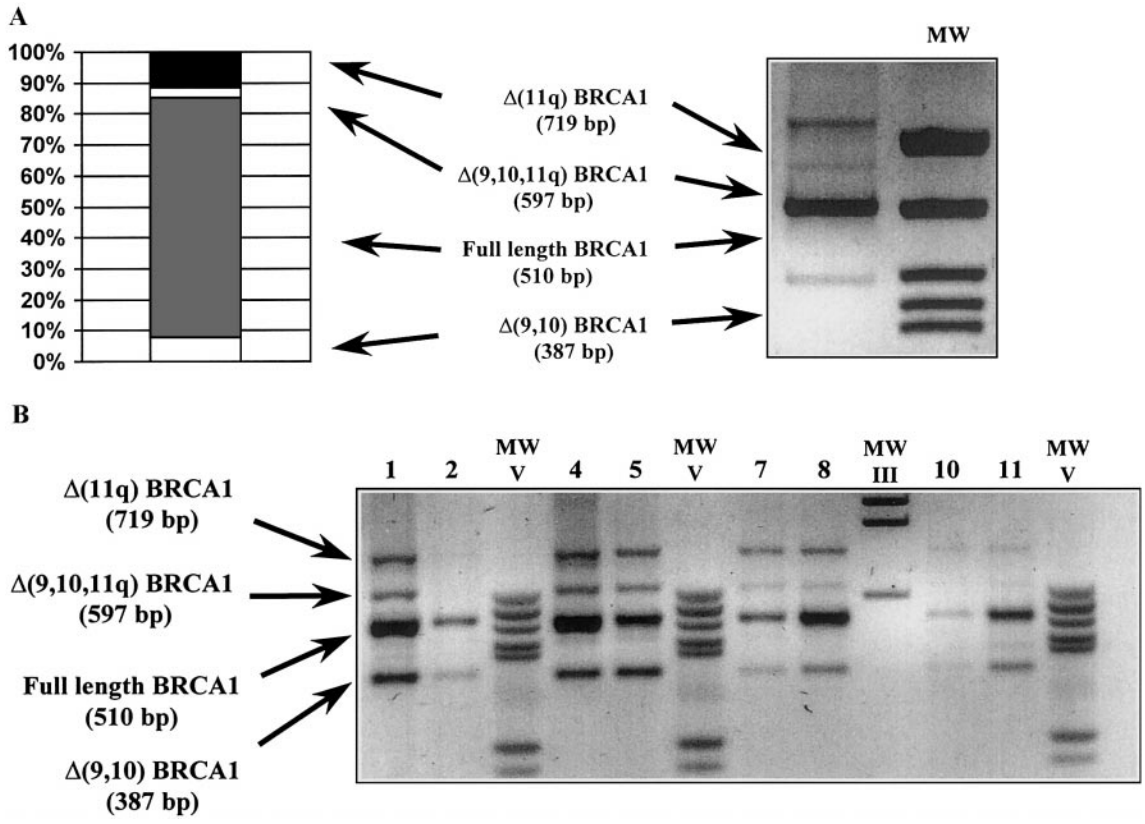

FIG. 3. Examples of RT-PCR products run on a 2% agarose gel. Arrows point to different amplicons, representing different mRNA isoforms. (A) One representative experiment carried out on normal breast cells. The left panel indicates the ratios of the splice variants in the total BRCA1 mRNA pool. In normal breast cells, the full-length variant is significantly more abundant (approx. 77%) compared to the other isoforms than in any of the asynchronous tumor cell lines (between 35–50%, see also Fig. 4). MW: molecular weight marker. (B) Experiments carried out on the four different tumor cell lines. Lanes 1 and 2: samples of asynchronous and G1/S synchronized MCF-7 cells; lanes 4 and 5: samples of asynchronous and G1/S synchronized K562 cells; lanes 7 and 8: samples of asynchronous and G1/S synchronized OVCAR-5 cells; lanes 10 and 11: samples of asynchronous and G1/S synchronized MDA-MB-231 cells. MW III and MW V: molecular weight markers (Boehringer Manheim).

BRCA1 mRNA pool varied differently, depending upon the cell type. As the transcription of the BRCA1 gene depends on the cell cycle rather than the cell type (e.g., increase at the G1/S border), the alternative splicing of the gene seems to have cell cycle and cell type specific regulatory mechanisms. Potential tissue specific *trans*-acting factors and their presumably cell-cycle dependent distribution could well explain the different expression profiles of these BRCA1 splice variants and also their characteristically different expression pattern observed in breast and ovarian tumor cells as compared to leukemia cells. Based on our results it could be hypothesized that the disturbance of these currently unknown regulatory pathways could lie behind the tumor formation in breast and ovarian epithelial cells.

It is difficult to assess the role and the significance of the detected BRCA1 mRNA isoforms without the knowledge of their proper function. They all keep the original reading frame of the protein, having the potential to code for functional, yet smaller proteins. In fact, their presence at the protein level was suspected by several groups but proved only for the Δ(11q) isoform (28, 33, 34). In mice, a Brca1 transcript lacking the region homologous to fragment 11q was proved to exist, showing evolutionary conservation of this splice variant and possibly indicating evolutionary conserved function(s) (35). The exact role of these mRNA isoforms, however, still remains to be elucidated.

All functions that have so far been described for the full length brca1 protein include roles in transcriptional activation, in DNA repair and in recombination processes (36, 37) and it is still not clear why the malfunctions of such a gene lead to tumor formations almost exclusively in the breast and the ovary. The observation that the proportion of the full length BRCA1 variant compared to the other isoforms in normal breast cells is significantly higher than in any of the examined cell lines may anticipate that the proportional decrease of this variant may be associated with tumorigenesis. Also, the potential functions of the

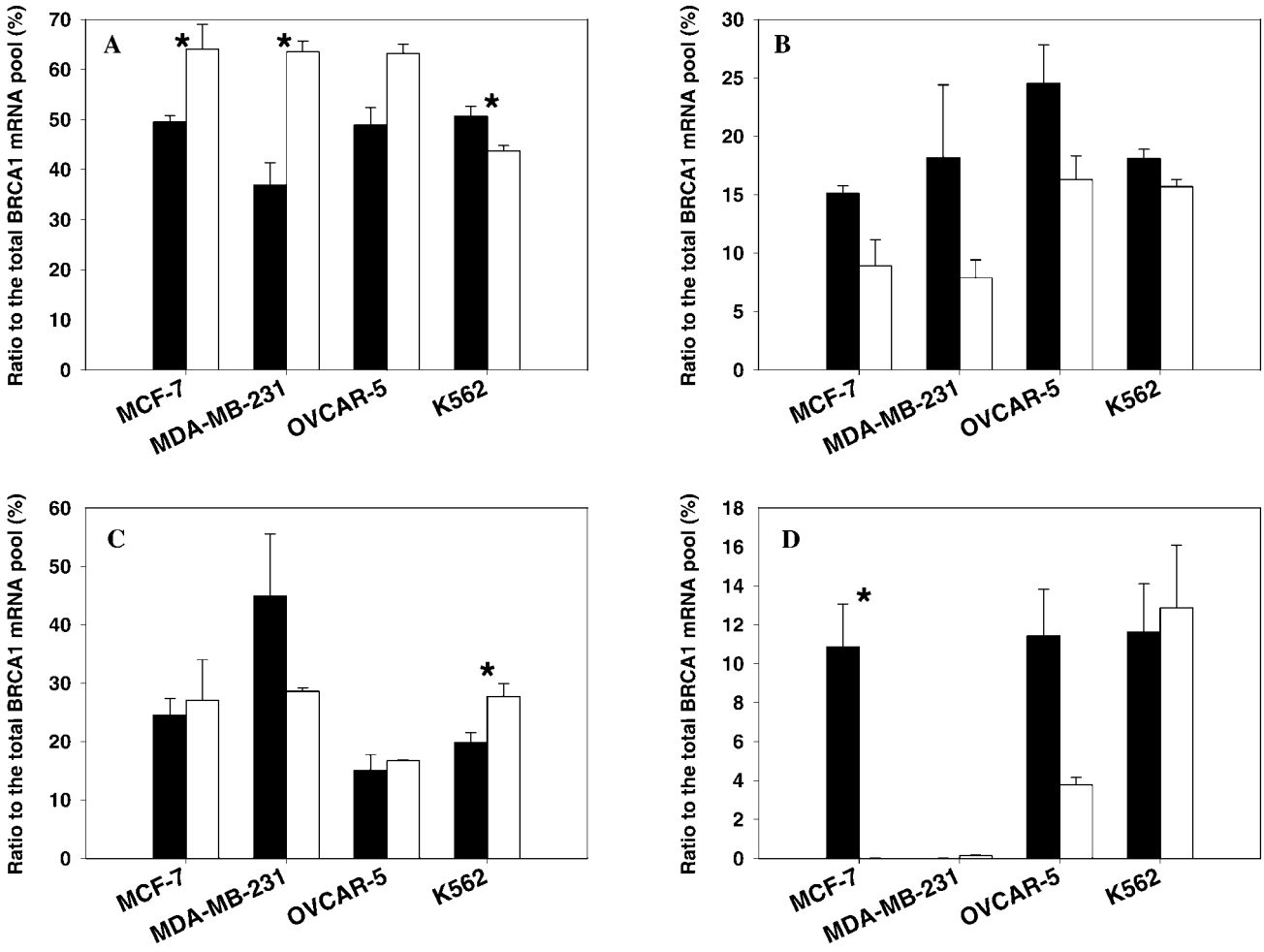

FIG. 4. The ratios of the four predominant splice variants compared to the total BRCA1 mRNA pool in asynchronous and in G1/S synchronized cell populations. The average of three independent measurements were used to calculate the given values. SE values of the mean are indicated with error bars, asterisks indicate changes significant at the $P < 0.05$ level as calculated by one-way analysis of variance. Filled boxes: measurements on samples from asynchronous cells; open boxes: measurements on samples from mimosine treated, G1/S synchronized cells. (A) full length BRCA1 variant; (B) Δ(11q) BRCA1 variant; (C) Δ(9,10) BRCA1 variant; (D) Δ(9,10,11q) BRCA1 variant.

BRCA1 gene are all associated with its presence in the nucleus. In isoforms Δ(11q) and Δ(9,10,11q), the region containing the nuclear localization signal sequences (25) are missing from the mature protein (coded by the missing 11q part), therefore all the above mentioned functions are inevitably abolished by their cytoplasmic localization. The missing 11q part means that other important domains are also missing from the potential protein species such as the RAD51 interacting region (38), further implicating their possible cytoplasmic role.

It is currently unknown what possible functions are associated with the region encoded by exons 9 and 10, which are missing from variants Δ(9,10) and Δ(9,10,11q). The observation that the latter BRCA1 isoform showed different behavior in the two breast tumor cell lines is of unknown significance. Metastatic potentials and estrogen receptor statuses are different in MCF-7 and in MDA-MB-231 cells which could reflect different regulatory mechanisms that might act on the Δ(9,10,11q) isoform. Our results somewhat differ from what Favy *et al.* described for MCF-7 and MDA-MB-231 cell lines (27) because they measured that splice variants containing exon 11 make up the vast majority of the total BRCA1 mRNA pool. Here we describe that in these cell lines, the Δ(11q) and Δ(9,10,11q) isoforms are also present in a significant level, although the Δ(11q) species is not as abundant as Wilson *et al.* suggested (28). These differences may be due to the use of different methods. It is also of special interest that some potential phosphorylation sites, Ser1423, Ser1497, and Ser1524 (39, 40) are retained in all detected isoforms which means that they all could be regulated posttranslationally. Nevertheless, here we proved that the regulation of the alternative splicing of these mRNA species are different from one another, strongly suggesting the presence of active regulatory mechanisms for their maturation.

Malfunctions occuring at any level of regulation of gene expression could contribute to the unproper function of BRCA1, leading to uncontrolled cell proliferation. Here we provide evidence that one possible regulatory level could be at the alternative splicing of the gene, the disruption of which could result in altered function of this tumor suppressor. By further studying its currently undeciphered mechanism, it would definitely help us to understand the proper function(s) of the gene and thereby understanding the impact of the disturbance of these functions in cancer formation.

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