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13. ABSTRACT (Maximum 200 Words) Mutations in BRCA2 are responsible for about 35% of familial breast cancers and also a proportion of familial ovarian cancers. Both BRCA2 and BRCA1 proteins were shown to have transcriptional activation domains and also shown to be associated with RNA polymerase suggesting that these proteins may function as transcriptional factors and have a role in the regulation of transcription. Recent studies on enzymes responsible for histone acetylation and deacetylation revealed that some of the transcriptional factors function as histone acetyl transferases. Since BRCA2 showed transcriptional activation properties, we tested whether BRCA2 is associated with histone acetyltransferase. Our results suggested that BRCA2 is associated with histone acetyltransferase (HAT) activity. We propose to test whether HAT activity plays a role in tumor suppressor activity of BRCA2. We plan to identify the factors that associate with BRCA2 and study the role of these protein-protein interactions in the biochemical and biological properties of BRCA2. We intend test whether transcriptional activation function has any role in the tumor suppressor activity of BRCA2. We intend to identify the BRCA2 target genes and study (in future) their role in the tumor suppressor activity of BRCA2.				
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INTRODUCTION:

Alterations in BRCA1 and BRCA2 tumor suppressor genes have been shown to account up to 90% of familial breast cancers and also a proportion of familial ovarian cancers. Since loss of wild type BRCA2 allele in heterozygous carriers results in breast and ovarian cancers, BRCA2 is regarded as a tumor suppressor gene. Interestingly, both BRCA2 and BRCA1 are expressed coordinately and also in a cell cycle-dependent manner. Two potential functions of BRCA2 were proposed which includes a role in DNA repair and in the regulation of transcription.

Interestingly, BRCA1 proteins were shown to have transcriptional activation function and also shown to be associated with RNA polymerase. These results suggest that both BRCA2 and BRCA1 proteins may function as transcriptional factors and have a role in the regulation of transcription. Recent studies on enzymes responsible for histone acetylation revealed that some of the transcriptional factors function as histone acetyl transferases. Since BRCA2 showed transcriptional activation properties, we tested whether BRCA2 functions as a histone acetyltransferase. Our recent results suggested that BRCA2 is associated with histone acetyl transferase (HAT) (1). As mentioned above, it is possible that BRCA2 also recruit other transcriptional factors which themselves function as HAT proteins. Identification of these recruitment partners will provide in sights into the molecular mechanism of growth and tumor suppressor function of BRCA2. Taking into consideration of the presence of transcriptional activation domain in BRCA2 and also having intrinsic HAT activity, it is not unreasonable to suggest that BRCA2 is a transcriptional factor. This proposal is aimed to strengthen this notion of BRCA2. Our recent finding that BRCA2 interacts with CBP supports this conclusion. It is possible that BRCA2 and CBP/p300 and other factors acetylate histones bound at specific promoters through cooperative acetylation and this could be a mechanism by which BRCA2 activates genes responsible for growth inhibition and differentiation. Therefore, identification of target genes of BRCA2 and its associating factors may provide a clue to the tumor suppressor function of BRCA2. Because of its large size, it is possible that BRCA2 has multicellular functions which include DNA repair, transactivation, HAT activity etc. which may play a role in tumor suppressor function.

BODY:

Several transcriptional factors (CREB, ACTR, SRC-1, P/CAF, TAF 250, nuclear hormone receptors etc.) have been demonstrated to interact with CBP (2). Some of these transcriptional factors (ACTR, SRC-1, P/CAF, TAF 250 etc.) were found to have intrinsic HAT activity and recruit other transcriptional factors (such as CBP/p300 etc) which have intrinsic HAT activity to provide a cooperative regulatory effect on gene expression (3). Since BRCA2 is a transcriptional factor, we hypothesized that BRCA2 may interact with CBP and other factors and acetylate histones bound at specific promoters through cooperative acetylation and this could be a mechanism by which BRCA2 activates genes responsible for growth inhibition and differentiation. To test this hypothesis, we have studied the interaction of BRCA2 and CBP by GST pull-down assay. Briefly, we have cloned BRCA2 cDNA fragment into pcDNA-3 vector and linearized the vector with appropriate restriction enzyme. We have used this linearized expression vector carrying BRCA2 cDNA as a template and carried out in vitro transcription and translation in the presence of ³⁵S-methionine. This radiolabelled BRCA2 protein was incubated with beads containing GST-CBP-1, GST-CBP-2 and GST respectively. CBP-1 (a 451-662) and

CBP-2 (1680-1891) represent amino and carboxy-terminal domains that were shown to interact with a variety of transcriptional factors. CBP-1 was shown to interact with CREB, Sap-1a, c-Myb and c-Jun etc where as CBP-2 is shown to interact with E1A, P/CAF, c-Fos, MyoD etc. In vitro translated [S]³⁵-Methionine labeled BRCA2 (aa 1-500) was bound to GST-CBP-2 but not to GST-CBP-1 and GST itself. These preliminary results suggested that BRCA2 interacts with CBP *in vitro*. We proposed to confirm these results. As per the proposal we repeated these experiments twice and confirmed that BRCA2 interacts with CBP. Our recent results suggest that BRCA2 appears to be an unstable enzyme that lose irreversibly HAT activity with storage. Such rapid and irreversible loss of HAT activity was also observed in the case of other HAT enzymes namely GCN5, PCAF etc. We have also observed such loss of HAT activity with CBP on storage.

Our preliminary results suggested that BRCA2 and BRCA2a interacts with p53. We repeated these experiments using GST pull down assay and confirmed that indeed p53 directly interacts with the BRCA2 and BRCA2a. We repeated GST pull down assays and confirmed that p53 interacts with aminoterminal region of BRCA2 (aa 189-500).

In order to determine the BRCA2 domain responsible for interaction with p53, we have made carboxy-terminal deletion of BRCA2 and BRCA2a and expressed in bacteria and purified by affinity column. We repeated our preliminary results and confirmed that p53 showed very weak binding to carboxyl-truncated BRCA2 and BRCA2a suggesting that amino acid 189-500 is essential for interaction with p53.

Since BRCA2 physically interacts with p53, we tested what effect BRCA2 will have on the transcriptional activation properties of p53. pcDNA-BRCA2 or pcDNA BRCA2a either alone or together with p53 was cotransfected into Saos-2 cells (p53 null Osteosarcoma cells) together with p53 reporter plasmid (pG13-CAT). Our results suggest that the expression of BRCA2 and BRCA2a had no effect on the expression of p53-specific reporter in the absence of exogenous wild type p53. As expected, expression of wild type p53 resulted in transcriptional activation of p53 reporter plasmid (pG13-CAT) (a gift from Dr. Vogelstein). However, co-transfection of BRCA2 (or BRCA2a) and p53 resulted in a dramatic increase in CAT activity of pG13-CAT suggesting that BRCA2 and BRCA2a cooperates with p53 in the transcriptional activation of p53 reporter plasmid. Therefore, our preliminary results suggest that BRCA2 and BRCA2a function as transcriptional co-factor of p53. We intend to confirm these results by repeating these experiments (at least three to four times) with increasing concentrations of BRCA2 and BRCA2a. Of course, we plan to use appropriate empty vector controls. These results may shed new clues in understanding the function of BRCA2.

KEY RESEARCH ACCOMPLISHMENTS:

BRCA2 and BRCA2a interact with CBP carboxyterminal region. BRCA2 and BRCA2a also interact with p53 in vitro. We confirmed that p53 interacts with the aminoterminal region of BRCA2 (aa 189-500).

REPORTABLE OUTCOMES:

None

CONCLUSIONS:

Our research on this project suggests that BRCA2 and BRCA2a interact with CBP carboxyterminal region suggesting that BRCA2 and BRCA2a may undergo posttranslational modification. These posttranslational modifications may have profound effect on the function of BRCA2 and BRCA2a. Therefore, identifying what type of translation modification takes place as a result of protein-protein interaction of BRCA2/BRCA2a and CBP is important for future work. Understanding the effect protein-protein interaction of p53 and BRCA2/BRCA2a on p53-mediated transcriptional activity may provide new clues to the role of BRCA2 in tumor growth suppressor function.

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APPENDICES:

1. Siddique, H., Zou, J.P., Rao, V.N., and Reddy, E.S.P. The BRCA2 is a histone acetyltransferase. *Oncogene*. *16*: 2283-5, 1998. (manuscript attached)
2. Zou, J.P., Hirose, Y., Siddique, H., Rao, V.N. and Reddy, E.S.P. Structure and expression of variant BRCA2a lacking the transactivation domain. *Oncology Reports*. *6*: 437-440, 1999. (manuscript attached)

SHORT REPORT

E Shyam P Reddy

The BRCA2 is a histone acetyltransferase

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Patients carrying mutations in *BRCA1* or *BRCA2* tumor suppressor genes have shown to have high risk in developing breast and ovarian cancers. Two potential functions of *BRCA2* were proposed which includes role in the regulation of transcription and also in DNA repair. Forty-five-amino acid region encoded by exon 3 of *BRCA2* was shown to have transcriptional activation function. Recent studies of the several enzymes involved in acetylation and deacetylation of histone residues have revealed a possible relationship between gene transcriptional activation and histone acetylation. Since *BRCA2* appear to function as a transcriptional factor, we have tested for Histone acetyl transferase (HAT) activity of *BRCA2*. Here, we present evidence that *BRCA2* has intrinsic HAT activity, which maps to the amino-terminal region of *BRCA2*. Our results demonstrate that *BRCA2* proteins acetylate primarily H3 and H4 of free histones. These observations suggest that HAT activity of *BRCA2* may play an important role in the regulation of transcription and tumor suppressor function.

Keywords: *BRCA2*; histone acetyl transferase; protein-protein interaction; tumor suppressor

Alterations in *BRCA1* and *BRCA2* tumor suppressor genes have been shown to be involved in 90% of familial breast cancers (Newman *et al.*, 1988; Miki *et al.*, 1994; Easton *et al.*, 1993; Wooster *et al.*, 1994; Wooster and Stratton, 1995). Recent studies revealed that both *BRCA1* and *BRCA2* are involved in ovarian and prostate cancers. Interestingly, *BRCA2* was found to be more associated with male breast cancer compared to *BRCA1* (Wooster *et al.*, 1994). Patients with *BRCA2* mutations were also found to be at a higher risk with a variety of other cancers including carcinomas of pancreas, prostate and colon (Thorlacius *et al.*, 1996; Phelan *et al.*, 1996; Gudmundsson *et al.*, 1995; Tonin *et al.*, 1995). The *BRCA2* gene is composed of 27 exons and encodes a protein of 3418 amino-acids with no significant homology to any known protein (Wooster *et al.*, 1995; Bork *et al.*, 1996). *BRCA2* and *BRCA1* proteins have been shown to interact with Rad 51 which suggests that they play a role in DNA repair (Scully *et al.*, 1997; Sharan *et al.*, 1997; Zhang *et al.*, 1998). *BRCA1* was also shown to induce apoptosis suggesting that *BRCA* proteins may

play a role in the regulation of apoptosis of cells (Shao *et al.*, 1996; Rao *et al.*, 1996). It remains to be seen whether *BRCA2* plays a similar role in apoptosis.

Interestingly, both *BRCA1* and *BRCA2* gene products are regulated in a cell cycle-dependent manner and have a potential transactivation function (Rajan *et al.*, 1996; Vaughn *et al.*, 1996; Chapman and Verma, 1996; Monteriro *et al.*, 1996; Milner *et al.*, 1997; Wang *et al.*, 1997; Cui *et al.*, 1998a). Recently, we have shown that *BRCA1* proteins interact with transcriptional co-activator CBP suggesting that *BRCA1* has a role in the regulation of transcription (Cui *et al.*, 1998b). Exon 3 of *BRCA2* was found to have weak homology with transcriptional factor *c-jun* and also shown to activate transcription in mammalian cells (Milner *et al.*, 1997). These results suggest that *BRCA2r* has a role in the regulation of gene expression.

Recent studies of the several enzymes involved in acetylation and deacetylation of histone residues have revealed a possible relationship between gene transcriptional activation and histone acetylation (Brownell *et al.*, 1996; Parthun *et al.*, 1996; Yang *et al.*, 1996; Orgyzko *et al.*, 1996; Mizzen *et al.*, 1996; Roth and Allis, 1996; Wade and Wolffe, 1997; Pazin and Kadonaga, 1997; Wolffe, 1997). This view is supported by the identification of Histone acetyl transferase (HAT) activity associated with several transcription factors including p300/CBP, GCN5-related factors, p/CAF, SRC-1 and TAF_{II} 250. These results suggest that some transcriptional activators operate by disrupting the nucleosomal structure through acetylation of histones leading to the activation of gene expression.

Here, we report for the first time that the amino-terminal region of *BRCA2* has intrinsic HAT activity from which it may be inferred that *BRCA2* joins the above list of transcriptional activators/factors that possess HAT activity. This intrinsic *BRCA2*-HAT activity may play a key role in the tumor suppressor function of *BRCA2*.

Recently, we have cloned an alternatively spliced isoform, *BRCA2a*. This variant *BRCA2a* lacks a transcriptional activation domain (exon 3) as a result of alternative splicing (our unpublished results). In order to test the HAT activity of *BRCA2*, we have expressed the amino-terminal region of *BRCA2* (aa 1–500) and its isoform *BRCA2a* (aa (1–18)-(105–500)) as GST-fusion proteins in bacteria by cloning appropriate *BRCA2* cDNA fragments into a GST expression vector (Our unpublished results). Purified recombinant proteins of *BRCA2* and *BRCA2a* were assayed for histone acetyl transferase activity. Amino-terminal domains of both *BRCA2* and *BRCA2a* clearly demonstrated histone acetyl transferase activity (Figure 1). Control samples where *BRCA2* or *BRCA2a* was replaced with bovine serum albumin (BSA) showed no

damaged DNA for DNA repair. Patients with mutations in HAT and/or transactivation domains of BRCA2 may show a loss of gene expression which are critical for growth inhibition and differentiation and result in a subset of familial breast and prostate cancers. One can use BRCA2-HAT assay for screening patients with BRCA2 mutations.

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Structure and expression of variant BRCA2a lacking the transactivation domain

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Abstract. BRCA1 and BRCA2 are tumor suppressor genes shown to be involved in 90% of familial breast cancers and also known to be involved in ovarian and prostate cancers. Both BRCA1 and BRCA2 gene products are regulated in a cell cycle-dependent manner and have potential transactivation function. Here, we show that BRCA2 undergoes differential splicing giving rise to a novel variant protein BRCA2a, lacking putative transcriptional activation domain. Both BRCA2a and BRCA2 are expressed at high levels in thymus and testis but moderate levels in mammary gland and prostate suggesting that BRCA2a and BRCA2 may have a role in the development and differentiation of these tissues.

Introduction

Germ-line mutations in autosomal dominant susceptibility genes are responsible for up to 10% of all breast cancers (1,2). Mutations in breast cancer susceptibility genes, BRCA1 and BRCA2, could account for up to 90% of familial breast cancers (3-5). Recently BRCA1 and BRCA2 have also been shown to be associated with ovarian and prostate cancers. Interestingly, unlike BRCA1, BRCA2 is associated with male breast cancer (4). The BRCA2 gene is composed of 27 exons and encodes a protein of 3418 amino-acids with no significant homology to any known protein (6,7). Expression of both BRCA1 and BRCA2 was shown to be cell cycle regulated and expressed at high levels in late G1 and S-phase (8,9). Recently exon 3 of BRCA2 was shown to function as a transcriptional activation domain suggesting that BRCA2 may have a functional role in the regulation of transcription (10). Similar transcriptional activation function was also shown to be present in BRCA1 (11-13). Previously we have shown that BRCA1 is trans-ported into the nucleus in the

absence of serum and interacts with transcriptional factor E2F, cyclins and cdks suggesting a role for BRCA1 in cell cycle regulation (14). BRCA2 and BRCA1 proteins were shown to interact with Rad 51 suggesting that they may play a role in DNA repair (15-18).

Recently we have shown that both BRCA1 and BRCA2 interact with CBP *in vivo* and *in vitro* suggesting that both these proteins may regulate transcription through CBP (19,20). Because of coordinated expression of BRCA1 and BRCA2 genes and association of these genes in the common breast cancer phenotype, it appears that BRCA1 and BRCA2 may function in a similar pathway. Recently, we have shown that BRCA1 induces apoptosis suggesting a novel function in the regulation of apoptosis of cells (21,22). It remains to be seen whether BRCA2 plays a similar role in apoptosis. Both BRCA1 and BRCA2 proteins may have multicellular functions such as transcriptional activation, DNA repair and regulation of apoptosis.

In this study, we have cloned alternatively spliced BRCA2 cDNA and characterized them by nucleotide sequence analysis. We demonstrate that this differentially spliced BRCA2a transcript has lost transcriptional activation domain as a result of alternative splicing giving rise to BRCA2a with potential dominant negative pathophysiology. Interestingly, BRCA1 was also shown to encode multiple products as a result of alternative splicing (13,14,21,23-25).

Materials and methods

Molecular cloning of BRCA2 and BRCA2a cDNAs. cDNAs were obtained by the reverse transcription of total RNA from BT-474 cells using cDNA kit (Takara). BRCA2 cDNAs were amplified by PCR using appropriate 5' and 3' primers and cloned into a pcDNA3 vector. These cDNAs were characterized by restriction mapping and nucleotide sequence analysis.

RNase protection assay. RNase protection assay was carried using Ribonuclease Protection assay kit (Ambion Inc., Austin TX) as described by the manufacturer. Briefly, the templates were subcloned, linearized and transcribed in 20 μ l of *in vitro* transcription mixture containing 5 μ l of α -³²P-rUTP to obtain radiolabelled probes. These radiolabelled RNA probes were purified by gel electrophoresis. Approximately 5x10⁵ cpm of the probe was mixed with 20 μ g of human breast, prostate, testis and thymus RNA (Clontect, Palo Alto, CA) and the

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Key words: BRCA2a, BRCA2, alternative splicing, transcriptional activation, tumor suppressor, HAT activity

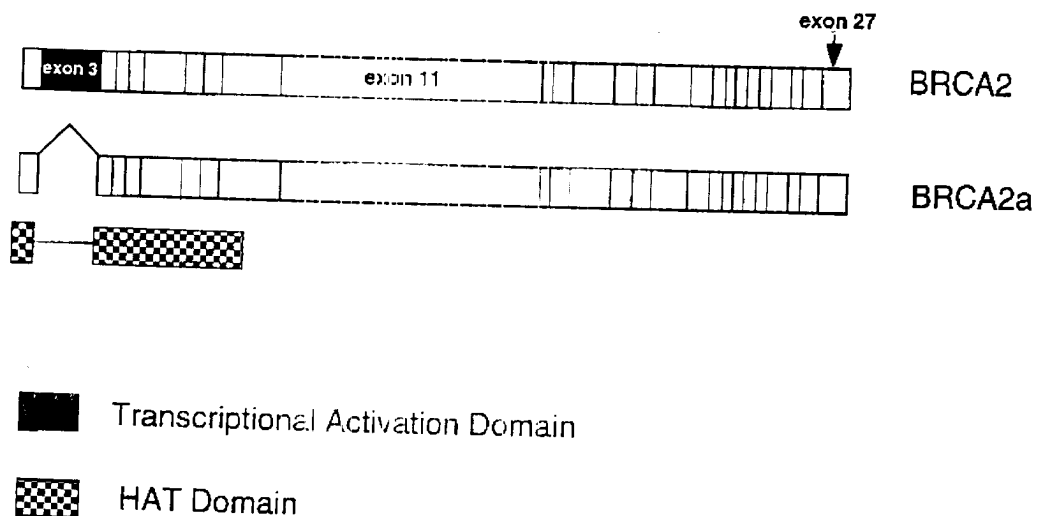


Figure 1. Deletion of BRCA2 transcriptional activation domain in BRCA2a as a result of alternative splicing. Figure is not drawn to scale.

volume of the reaction mixture was adjusted to 30 μ l by 1X hybridization buffer. The hybridization mixture was heated to 95°C for 5 min and then incubated at 45°C overnight. RNase digestion was performed for 1 h at 37°C and the reaction was stopped by the addition of RNase inactivation buffer (kit). The protected fragments were extracted by centrifugation. The pellets were suspended in gel loading buffer, heated to 90°C for 3 min. The reaction products were analyzed by polyacrylamide gel electrophoresis using 5% polyacrylamide/8M urea gels. The gel was dried and subjected to autoradiography.

Results and Discussion

In order to understand the function of BRCA2, we have cloned several cDNAs by RT-PCR and characterized these cDNAs by nucleotide sequence and restriction map analysis. Our results demonstrate that one of the cDNAs (BRCA2a) showed alternative splicing resulting in the deletion of exon 3 (Fig. 1). Previously this exon was shown to contain a potential transcriptional activation domain, which suggested that BRCA2 may function as a transcriptional factor (10). Similar potential for transcriptional activation was attributed to BRCA1 proteins (11-13). Since BRCA2a has lost transcriptional activation domain, it might compete with native BRCA2 in terms of DNA binding or interaction with other transcriptional factors resulting in dominant negative effect on transcription activation function of BRCA2. Such dominant negative variants are also seen in other transcriptional activators (26). Therefore, BRCA2a may represent a potential dominant negative variant which may regulate the putative transcriptional activation properties of BRCA2 proteins. Alternatively, BRCA2a may have other functions which do not need transactivation function.

We performed RNase protection analysis to study the expression of BRCA2 and BRCA2a in different types of tissues. For this, we have used the 459 nucleotide probe (Fig. 2a). The predicted 313 nucleotide fragment (corresponding to BRCA2a) and the 255 and 58 nucleotide

fragments corresponding to BRCA2 were observed in thymus and testis (Fig. 2, lanes 3 and 4). However, moderate to low level of expression was observed in the case of mammary gland and prostate (Fig. 2, lanes 5 and 6). It appears both BRCA2 and BRCA2a are expressed at similar levels in the tissues tested suggesting both forms of BRCA2 may have a functional role in cell growth and differentiation of testis, mammary gland, prostate and thymus.

In summary, we have presented the results supporting that BRCA2 is alternatively spliced, giving rise to a variant BRCA2a protein which lacks transactivation domain. To our knowledge this is the first report demonstrating the presence of variant BRCA2 protein. Since BRCA2a variant lacks transcriptional activation domain, it can potentially interfere with transcriptional activation properties of BRCA2 by competing with BRCA2 for protein-protein interactions and/or DNA binding. Such variant proteins were also seen in the case of other transcriptional factors. It is possible that BRCA2a may regulate the functional properties of BRCA2. Therefore, it becomes important to study the patient DNA samples for mutations outside the coding region (introns, promoters etc.) as they may alter differential splicing pattern of BRCA2 leading to overexpression of BRCA2a. This overexpression of BRCA2a may interfere with normal BRCA2 function and result in cellular transformation. In support of this hypothesis, large deletions that disrupt exon 3 of BRCA2 were observed in patients of breast and ovarian cancers (27).

Our recent results have shown that both BRCA2 and BRCA2a have histone acetyltransferase activity (HAT) (20). These results suggest that domains responsible for HAT activity and the transcriptional activation function of BRCA2 do not overlap (Fig. 1). Since BRCA2 and BRCA2a are HAT proteins, it is possible both BRCA2 and BRCA2a function as transcriptional co-factors. BRCA1 was shown to function as a transcriptional co-factor of p53 (28,29). Recently, we have observed that BRCA2 binds to CBP and function as transcriptional co-factors of p53 (Siddique and Reddy, unpublished results). It is possible that some transcriptional

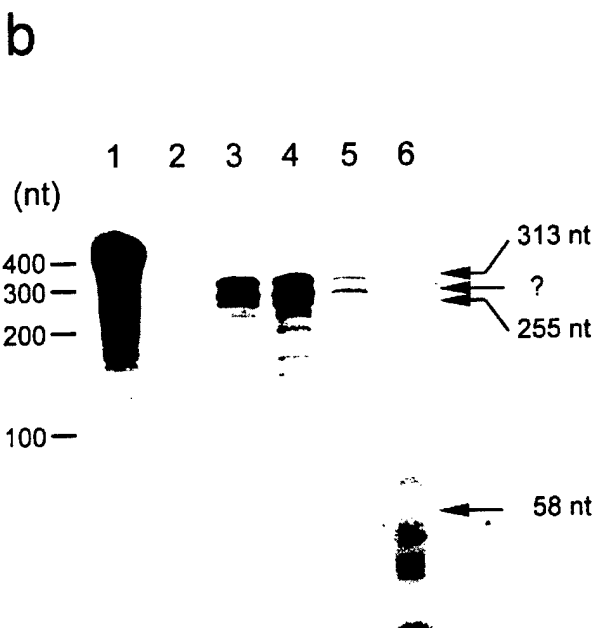
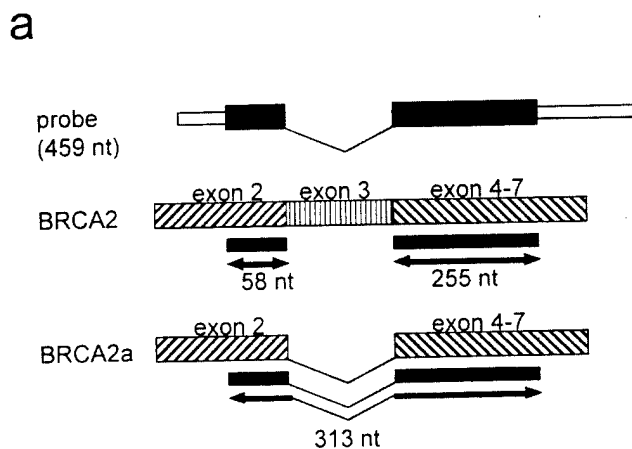


Figure 2. a) Schematic representation of antisense RNA probe used for RNase protection assay. The expected protected fragments from BRCA2 and BRCA2a transcripts are shown. b) Analysis of the expression of BRCA2a in different human tissues by RNase protection assay. This assay was carried out on 20 μ g of total RNA using Ambion RPA kit. Lane 1, probe; Lane 2, tRNA; Lane 3, thymus; Lane 4, testis; Lane 5 mammary gland; Lane 6, prostate. Lanes 1-4 are the result of short exposure (1 day) and lanes 5 and 6 are of longer exposure (3 days). Protected 313 nucleotides (corresponding to the alternatively spliced BRCA2a) and 255 and 58 nucleotide fragments (corresponding to the BRCA2) are shown by arrows.

activators may use BRCA2 and BRCA2a as a transcriptional co-factors and utilize their HAT activity for the activation of gene expression. It is also possible that other transcriptional activators may use BRCA2 but not BRCA2a as a transcriptional co-factor because of the absence of the transcriptional activation domain in BRCA2a. Recent studies revealed that BRCA2 interacts with DNA repair protein Rad 51 (15-17). Earlier, we suggested that BRCA2-Rad 51 complex may disrupt nucleosomal structure using HAT activity of BRCA2 and thereby recognize damaged DNA prior to DNA repair process (20). For such a mechanism, there may not be a need for transactivation function. Therefore, BRCA2 and BRCA2a may have distinct

functions. Rad 51-BRCA2a (which lacks transactivation domain) complex may play a role in DNA repair whereas BRCA2 may play a role in the regulation of transcription. It remains to be seen whether both isoforms of BRCA2 play a role in this DNA repair phenomenon. Alternatively, BRCA2 proteins may participate in DNA repair and tumor suppression through BRCA2 target genes.

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