PJN
ISSN 1680-5194

PAKISTAN JOURNAL OF NUTRITION

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Pakistan Journal of Nutrition 1(1) : 56-63, 2002

Cloning and Expression of Bovine BRCA1

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Abstract: Breast ovarian cancer susceptibility (BRCA) proteins appear to be involved in cell cycle regulation, DNA repair or genome integrity and induction of apoptosis in a variety of cells from humans and laboratory animals. The BRCA gene and protein have not been identified in cattle. The pattern of BRCA1 gene expression during normal mammary gland development and involution has not been examined in detail in any mammalian species. Therefore, the purpose of the present study was to clone the BRCA1 gene in Holstein dairy cattle and determine if the BRCA1 gene is differentially expressed through various stages of mammary gland development. We also localized immunoreactive BRCA1 protein in bovine mammary cells and milk fat globule membrane. Bovine BRCA1 cDNA was highly conserved to the human. Five hundred base pairs of exon 11 (+3384/ + 3866, human BRCA1 cDNA position) and the C-terminus 1 kb were identical to the human. Seven hundred base pairs of the N-terminus, which contain two ring domains, showed 90 % homology to human BRCA1. In bovine tissues, the degree of BRCA1 gene expression, from highest to lowest , was as follows: liver, spleen, mammary tissues and kidney. The mammary tissues of early pregnancy heifers (3 months) showed much higher mammary gland BRCA1 mRNA expression than other mammary developmental stages. BRCA1 expression declined during the remainder of pregnancy and remained low for lactating cows. However, its expression increased when involution proceeded. Immunohistochemical studies showed that immunoreactive BRCA1 was localized in the nucleus and cytoplasm of mammary epithelial cells from lactating cows. It was not present in myoepithelial cells. The protein was also localized in the milk fat globule membranes. Our data suggest that BRCA1 is involved in bovine mammary gland development and/or differentiation, is specifically localized in secretary epithelial cells and is likely a secreted protein during normal lactation.

Key words: BRCA1, mammary, development, gene expression, cow, cells, involution

Introduction

Breast ovarian cancer susceptibility (BRCA) genes and their expressed proteins are currently being studied in human cells and tissues, as well as laboratory animals (Chen *et al.*, 1996; Futreal *et al.*, 1994; Hall *et al.*, 1990; Jensen *et al.*, 1996; Marquis *et al.*, 1995; Miki *et al.*, 1994; Paterson, 1998). These proteins appear to be involved in cell cycle regulation; thereby influencing tissue growth and /or differentiation (Chen *et al.*, 1996; Marquis *et al.*, 1995; Rajan *et al.*, 1996; Ruffner and Verma, 1997). They have also been implicated in DNA repair or genome integrity and induction of apoptosis in a variety of cells (Paterson, 1998; Shao *et al.*, 1996). Moreover, the BRCA1 gene has been identified as a tumor suppressor gene (Paterson, 1998). The gene is mutated in sporadic breast cancer and there are also inherited mutant forms of the gene that show a familial predisposition to cancer (Futreal *et al.*, 1994; Hall *et al.*, 1990; Miki *et al.*, 1994).

The BRCA1 gene encodes a 190-220 kDa protein consisting of 1,863 amino acids (Chen *et al.*, 1996; Paterson, 1998). The protein has sequence homology and biochemical analogy to the granin protein family (Jensen *et al.*, 1996). Both BRCA1 and the granins, are localized in secretory vesicles and are secreted by a regulated pathway. They are posttranslationally glycosylated, and are responsive to hormones (Romagnolo *et al.*, 1998).

Two putative nuclear localization signals and a potential ring-finger motif suggest that the BRCA1 protein is a nuclear protein. Chen *et al.*,1996 showed that BRCA1 was a nuclear phospo-protein in normal and non-breast /ovarian cancer cells, while in breast/ ovarian cancer cells BRCA1 was exclusively localized in the cytoplasm. They suggested that abnormalities of nuclear transport in sporadic tumors might influence BRCA1 cellular localization. Scully *et al.*, 1996; Ruffner and Verma 1997, however, reported that BRCA1 was predominantly localized in the nuclei of normal as well as breast /ovarian cancer cells.

It is very difficult to obtain non malignant or "normal" human breast tissue from females over various stages of mammary development, lactation and involution for studies focusing on differential gene expression. Therefore, the pattern of BRCA1 gene expression in normal mammary tissue, over various stages of development, has not been examined in detail. Cattle, however are useful for studies that are designed to describe changes in gene expression throughout various physiological states. They are convenient for multiple tissue biopsy and blood sampling. Furthermore, much of what we know about mammary gland biology has been derived from cattle and other ruminants. The purpose of the present study was to clone the BRCA1 gene in Holstein dairy cattle and determine if the BRCA1 gene is differentially expressed through various developmental stages of the mammary gland. We also localized immunoreactive BRCA1 protein in mammary cells and milk fat globule membrane.

Materials and Methods

Cloning and Sequencing of Bovine BRCA1: Total RNA was isolated from 0.5 g of mammary tissue of a non-lactating cow obtained from the slaughter house. Tissue was homogenized by a Polytron homogenizer in Tri reagent (Molecular Research Center) according to the manufacturer's recommendations. Total RNA (1 μg) was used for reverse transcription (RT) with a Cycle Kit (Invitrogen). RT procedures were according to manufacturer's recommendations, and 1/5 of the RT product (4 μg) was used for PCR amplification. The PCR steps were performed as follows: 95°C for 3 min, then 30 cycles of amplification (95°C X 30 sec, 52°C X 60 sec, 68°C X 4 min.), ending with 68 °C X 8 min. Advanced Taq polymerase (Clontech) was used for amplification. The amplified products, 5.5 kb and 2.1 kb, were cloned into Topo-XL Cloning Kit (Invitrogen). The procedures used were according to the manufacturer's instruction. The cloned elements were sequenced at the Cornell University sequencing facility. All primers used are shown in Table 1.

Northern blot analysis: For Northern blot analysis, 15 μg of total RNA was mixed with formaldehyde loading buffer (40mM Mops pH 7, 10 mM sodium acetate, 1mM EDTA, 50 % formamide, 5% formaldehyde), and loaded on a denatured agarose gel (40 mM Mops pH 7, 10 mM sodium acetate, 1 mM EDTA, 5% formaldehyde, 1% agarose). After electrophoresis, samples were transferred onto nylon membranes (Gene Screen; Dupont New England Nuclear) and UV crossed-linked. Prehybridization, hybridization, and washing steps were performed, as described by Sambrook *et al.*, 1989.

Briefly, the 5' region of BRCA1 cDNA, 1.2kb, was used as a

Table 1: Primers used to amplify various regions of bovine BRCA1 cDNA by PCR

Name of Primer pairs[a]	Position[b]
85104S/0414A	+85/+5669
YJ1S/YJ2A	+225/+809
85104S/yj2A	+85/+809
792S/4214A	+792/+4214
LAIIS/925IIA	+3384/+3847
LAIS/925IIA	+2757/+3847
RG2S/0614A	+4661/+5584

[a]All primers were 20 nucleotides in length. S for sense strand and A for antisense strand. [b]Primer position is based on the human BRCA1 cDNA sequence. NID is g555931.

template to make a cDNA probe. The cDNA probe was labeled with P^{32}-dATP by a random primer DNA labeling kit (Life Technologies, Inc.). Prehybridization was carried out in hybridization buffer (6X SSPE, 5X Danhart's solution, 0.1 % SDS, 50% formamide, and 100 μg/ml Salmon sperm DNA) for 1 hour, and $1x10^6$ cpm/ml of the labeled probe were added and incubated over night at 42 °C. The membrane was washed twice with 2x SSPE/0.1 % SDS for 30 min at 65 °C, and 1x SSPE /0.1 % SDS for 1 hour at 65 °C prior to autoradiography exposure.

Western Blot Analysis: Bovine mammary tissue from a lactating cow (.5 g) was homoginized in a Polytron with 2 ml of 0.25 M sucrose containing 0.1 M Tris-HCl (pH 7.4) and the following protease inhibitors: 1mM PMSF, 25 μg/ml leupeptin, 25 μg/ml apotinin, 1 mM benzamidine and 10 μg/ml soybean trypsin inhibitor (Sigma Chemical Co.). The homogenates were then centrifuged at 36,500 rpm for 20 min at 4 °C. After centrifugation, the supernatant was collected and quantified for protein concentration using the Bradford method (Biorad). The supernatant (120 μg) was run on a 5% SDS-PAGE, transferred to a PVDF membrane, the blot was blocked with phosphate-buffered saline (PBS, pH 7.2) containing 3% BSA and 0.05% Tween-20. BRCA1 immunoblots were performed using the BRCA1-8F7 monoclonal antibody (GeneTex), or BRCA1-Ab3 (Oncogene Research), diluted 1: 50 in 1% BSA in PBS. Binding was visualized by incubating the membrane with a horseradish peroxidase conjugated rabbit anti-mouse antibody (Sigma). The immunocomplexes were detected by color development with 4-chloro-1-naphtol (Sigma) and hydrogen peroxide, or LumiGLO chemiluminescent substrate (KPL).

Distribution of BRCA1 in bovine tissues

Ribonuclease Protection Assay (RPA): The expression patterns of BRCA1 mRNA in various bovine tissues and in mammary tissue across various physiological stages and involution (see below) were measured by RPA and a Multi-NPA hybridization procedure (Ambion). Liver, spleen, kidney, and mammary gland (.5g of each tissue) were isolated from three non pregnant cows in the fifth and seventh month of lactation. Briefly, total RNA was extracted from liver spleen kidney and mammary tissue using Tri reagent (Molecular Research Center), as described by the supplier. The template to generate a ribo-probe was prepared by PCR from bovine BRCA1 cDNA. BRCA1 primer pair, forward (+85/+104): 5'tctgctctgggtaagttca 3' and backward (+640/+620): 5'atttaggtgacactatagaatgatgttgtatccgctgctt 3' was used for preparation of template. The reverse primer contains the SP6 RNA polymerase binding site (underlined in the above sequence) to make antisense ribo-probe. A 500 nt biotin labeled antisense ribo-probe was synthesized by MAXI Script (Ambion) according to the manufacturer's recommendations. The synthesized probe was gel purified and hybridized with 15 μg of total RNA to protect a fragment of the expected length. After nuclease digestion, the protected fragment was electrophoresed and transferred to a nylon membrane by electro-transfer. Protected fragments were detected by Ambion's Bright star bio-detection kit. BRCA1 mRNA was quantified by Photo Image IS1000 with arbitrary units corrected for the expression of the control, actin mRNA (BRCA1/Actin).

Differential expression of BRCA1 in mammary tissue

Mammary biopsy: Mammary tissue was obtained by biopsy from conscious female Holstein cattle in the following physiological stages (primiparous and three and eight months pregnant; multiparous and lactating for 3-5 months (early lactation); multiparous and lactating for 6-8 months (mid lactation); multiparous and lactating for 9-11 months (late lactation); and multiparous and undergoing mammary involution (three, six, and eight weeks). Three animals were biopsied per each developmental stage.

Animals were brought into a surgery suite and the point of tissue excision was anesthetized with lidocane. Five ml lidocane was also injected around the excision site. Approximately 10 grams of tissue was removed containing parenchymal elements. Blood vessels were cauterized or sutured to prevent further bleeding after biopsy removal. The excision site was closed with self-dissolving suture. Animals were given an injection of penicillin and returned to the Cornell University dairy farm.

Total RNA was extracted from the mammary biopsy samples according the procedures described above. The expression patterns of BRCA1 mRNA in mammary tissue across various physiological stages and involution were measured by RPA and a Multi-NPA hybridization procedure (Ambion) as described above.

Identification of BRCA1 in mammary tissue, milk fat globule membrane and MCF-7 breast cancer cells

Mammary tissue: Mammary tissue (25 grams) was obtained from a lactating Holstein cow at slaughter. The tissue was cut into 10 mm3 sections, fixed with Bouin's fixative and embedded in paraffin and blocked. After cutting serial sections (6 μm), they were mounted on plain glass slides. Slides were deparaffinized through a series of xylene baths and then rehydrated. Finally, slides were placed in a Microprobe holder. The slides were dipped and blotted one time in 0.5% hydrogen peroxide in methanol, and then dipped and left for 10 minutes to block endogenous peroxidase activity. Slides were incubated with normal blocking serum (goat) for 10 min at room temperature in a humid chamber and then blotted. The slides were incubated with 1: 50, 1:200 affinity purified rabbit polyclonal antibody to BRCA1 (I-20, Santa Cruz Biotechnology) or 1: 50, 1:200 mouse monoclonal antibody to BRCA1 (Oncogene Science) in a humid chamber for 2 hours at 37 °C. Non-immune serum was used for control slides. After incubation, biotinylated secondary anti-rabbit or anti-mouse IgG antibody (Zymed) were applied to slides for 20 min at room temperature in a humid chamber. After incubation and washing slides were incubated again with streptavidin/peroxidase conjugate for 10 min at room temperature in a humid chamber. Slides were removed from the Microprobe holder and incubated (30 S) with chromogen/substrate solution (Zymed) at room temperature . Slides were then washed in tap water and counterstained with Gill's #1 hematoxylin for 10-30 seconds. The slides were washed in tap water and blotted. Permount was added to each slide and the cover slips were attached. Staining was visualized using a binocular microscope.

Isolated MFGM: Milk fat globule membranes (MFGM) were prepared as described by Spitsberg *et al.*,1997 with minor modifications. Milk was obtained from a healthy mastitis free cow in early lactation. Cream was isolated by centrifugation of milk at 3,000 x g for 50 min. It was then suspended in two volumes of 50 nmol/L Tris-HCL, pH 7.5, containing 0.15 mmol/L NaCl (TBS), and homogenized in a Waring blender for 1 min. The homogenate was centrifuged at 100,000 x g for 90 min at 4 °C. The MFGM pellet was washed once and resuspended in a small volume of TBS, followed by recentrifugation at 100,000 x g for 90 min and stored in a -70 °C freezer. No somatic cells were found in the MFGM, as determined by light microscopy.

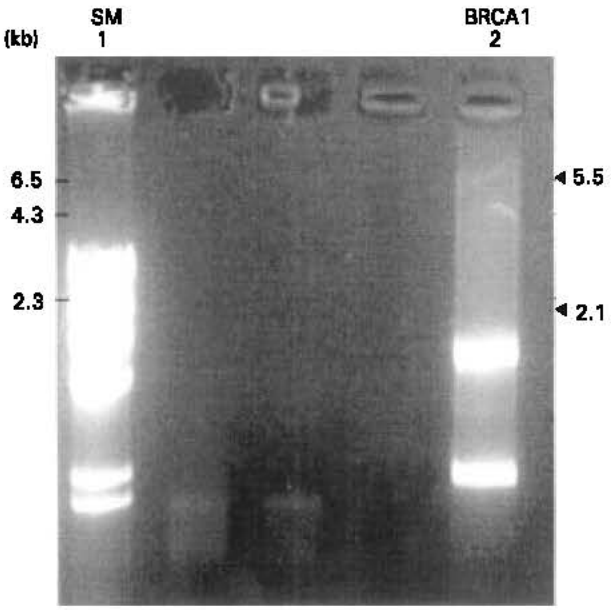


Fig. 1: PCR Amplification of Bovine BRCA1 cDNA. Left lane 1: A HindIII DNA size marker. Right lane 2: Amplified bovine BRCA1 cDNAs. The top band represents 5.5 kb. The bottom band (2.1 kb) is an alternating splicing variant of BRCA1.

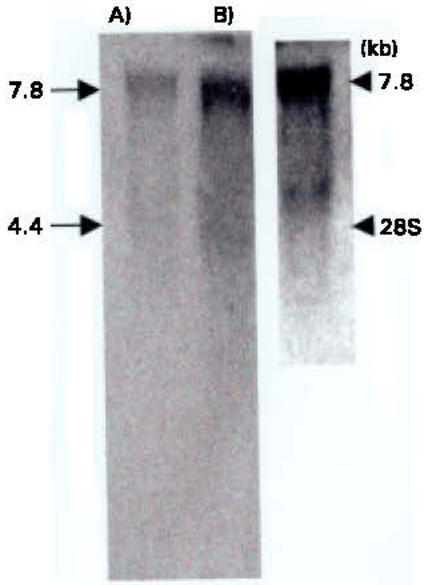


Fig.2: Northern blot analysis of bovine BRCA1. (A) Each lane contains 10 and 20 μg, respectively of total RNA extracted from a lactating bovine mammary gland. Arrows indicate an approximate 7.8 kb transcript of BRCA1 and the 28S position. (B) 20 μg of total RNA was blotted and hybridized with the N-terminus probe of bovine BRCA1. The top arrow indicates the 7.8 kb transcript and the bottom arrow shows the 4.4 kb splicing variant transcript.

Microscope slides were coated with L-lysine and left for 10 minutes to dry. MFGM pellets were then smeared on the slides as thin as possible. After fixation of the MFGM coated slides, in 3.7% formalin in PBS (pH 7.4) for 30 min, they were washed in PBS, 3 x for 5 min. The slides were treated with PBS + 0.1% Triton-X-100 for 5 min to permeabilize the membranes and the slides were dried . Immunostaining was carried out as described above for mammary tissue.

MCF-7 breast cancer cells: BRCA1 is expressed in MCF-7 human breast cancer cells (Paterson, 1998). Therefore, we isolated an MCF-7 total cell lysate, as a positive control for experiments identifying BRCA1 in bovine tissues and MFGM. MCF-7 cells were cultured and protein extracts were prepared using cell lysis buffer (Promega). MFGM was prepared as described above. MCF-7 protein extracts (100 μg) and MFGM (200 μg) were electrophoresed on a 5% SDS-PAGE, transferred to a PVDF membrane. The blot was blocked with phosphate-buffered saline (PBS, pH 7.2) containing 3% BSA and 0.05% Tween-20. The BRCA1 immunoblot was performed using BRCA1-17F8 monoclonal antibody (GeneTex), BRCA1-Ab3 (Oncogene), I-20 (Santa Cruz biotech) diluted 1: 50 in 1% BSA in PBS. Binding was visualized by reacting with a horseradish peroxidase conjugated secondary antibody (Sigma). The immunocomplexes were detected by peroxide, or LumiGLO chemiluminescent substrate (KPL).

Results and Discussion

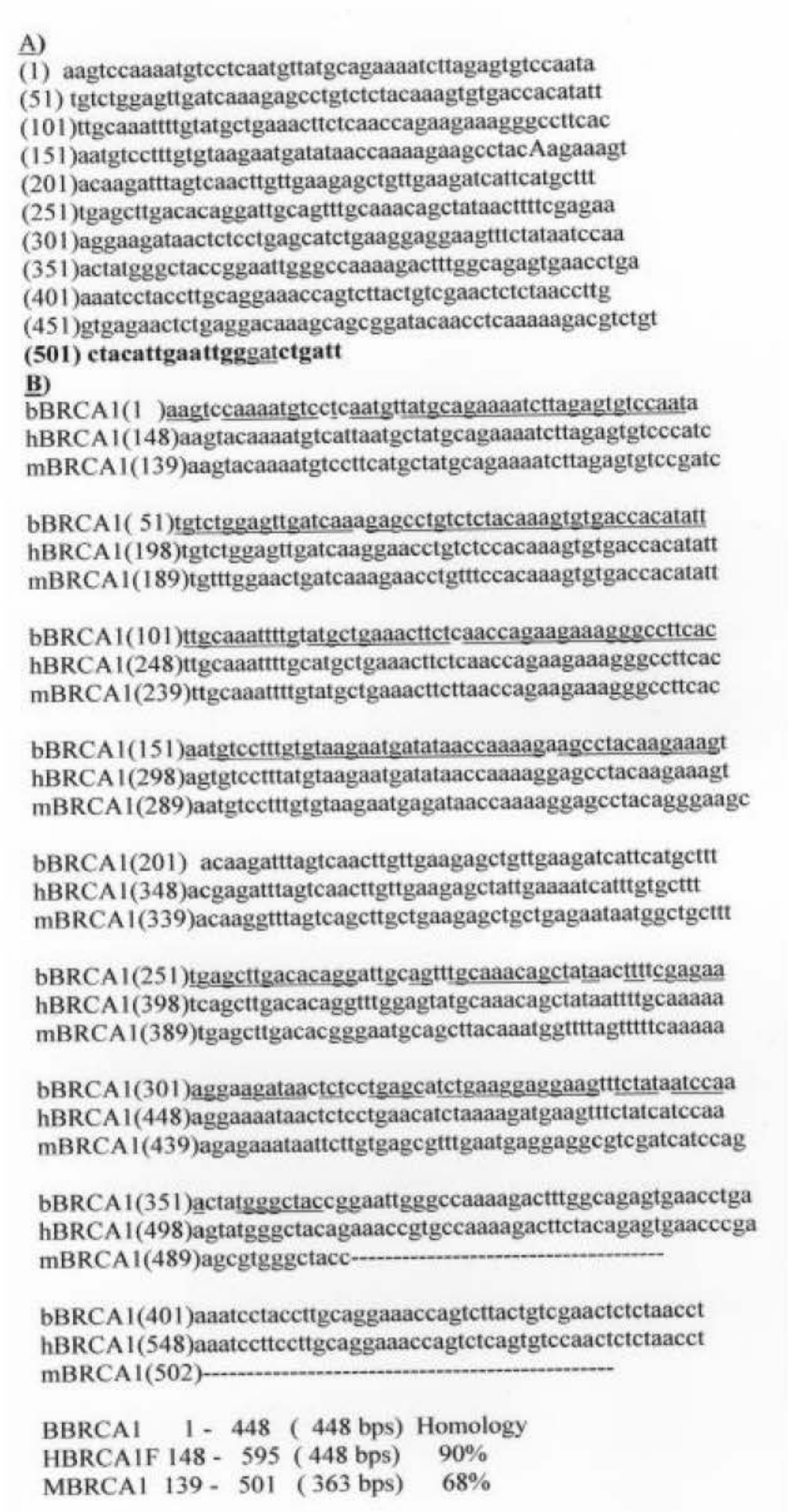
Cloning and sequencing of bovine BRCA1 : RT-PCR was performed with RNA from bovine mammary tissue, using primers derived from exons 3 and 24 of human BRCA1. Two PCR products (Fig. 1) were observed that would have derived from full length BRCA1 (i.e., about 5.5 kb, and 2.1 kb) as in human. Miki *et al.*, 1994 reported that a breast tissue-derived BRCA1 cDNA clone in which exons 9, 10, 11 were spliced out. These exons are 47, 76, and 3427 nucleotides in length, respectively. The small fragment was considered as exon 11 deletion splicing variant. This expectation was confirmed by northern analysis in which two transcripts were observed: one band around at 7.8-kb and the other one is at 4.4-kb (Fig. 2). Studies to determine whether splicing variants are functionally active are ongoing.

Bovine BRCA1 cDNA was highly conserved to the human. Five hundred base pairs of exon 11 (+3384/ + 3866, human BRCA1 cDNA position) and the C-terminus 1 kb were identical to the human. Seven hundred base pairs of the N-terminus, which contains two ring domains, showed 90 % homology to human BRCA1(Fig. 3). The sequence of 120 nt splicing junction, beginning at nucleotide 515, was as follows: GGgatc. (rest of 120 nt)YCTGC (lower case letters represent the 120 nt that are spliced spliced out). The Western blot analysis (Fig. 4) showed that size of bovine BRCA1 was quite similar to human's (i.e., 190-220 kDa).

Distribution of BRCA1 in bovine tissues : The distribution of bovine BRCA1 mRNA in spleen, kidney, liver and mammary tissues were analyzed by the RNase protection assay (Fig. 5). A probe spanning exons 2 to 8, as described in the methods section, was used for the RPA assay. Tissues were obtained from a cow in early lactation. The level of BRCA1 mRNA expression in all tissues was similar to that found in the mouse. The degree of bovine BRCA1 expression from highest to lowest was as follows: liver, spleen, mammary tissues and kidney.

Differential expression of BRCA1 in mammary tissue: BRCA1 mRNA expression was examined over developmental stages from biopsied mammary tissue by RNase protection assay (Fig. 6). The mammary tissues of early pregnancy heifers (3 months) showed much higher mammary BRCA1 mRNA expression than other mammary developmental stages. BRCA1 expression declined during the remainder of pregnancy and remained low for lactating cows. However, its expression increased when involution proceeded.

Marquis *et al.*, 1995; Rajan *et al*, 1996 showed that BRCA1 is broadly expressed in tissues of the early embryo, and is expressed



A)
(1) aagtccaaaatgtcctcaatgttatgcagaaaatcttagagtgtccaata
(51) tgtctggagttgatcaaagagcctgtctctacaaagtgtgaccacatatt
(101)ttgcaaattttgtatgctgaaacttctcaaccagaagaaagggccttcac
(151)aatgtcctttgtgtaagaatgatataaccaaaagaagcctacAagaaagt
(201)acaagatttagtcaacttgttgaagagctgttgaagatcattcatgcttt
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(351)actatgggctaccggaattgggccaaaagactttggcagagtgaacctga
(401)aaatcctaccttgcaggaaaccagtcttactgtcgaactctctaaccttg
(451)gtgagaactctgaggacaaagcagcggatacaacctcaaaaagacgtctgt
(501) ctacattgaattgggatctgatt

B)
bBRCA1(1)aagtccaaaatgtcctcaatgttatgcagaaaatcttagagtgtccaata
hBRCA1(148)aagtacaaaatgtcattaatgctatgcagaaaatcttagagtgtcccatc
mBRCA1(139)aagtacaaaatgtccttcatgctatgcagaaaatcttagagtgtccgatc

bBRCA1(51)tgtctggagttgatcaaagagcctgtctctacaaagtgtgaccacatatt
hBRCA1(198)tgtctggagttgatcaaggaacctgtctccacaaagtgtgaccacatatt
mBRCA1(189)tgtttggaactgatcaaagaacctgtttccacaaagtgtgaccacatatt

bBRCA1(101)ttgcaaattttgtatgctgaaacttctcaaccagaagaaagggccttcac
hBRCA1(248)ttgcaaattttgcatgctgaaacttctcaaccagaagaaagggccttcac
mBRCA1(239)ttgcaaattttgtatgctgaaacttcttaaccagaagaaagggccttcac

bBRCA1(151)aatgtcctttgtgtaagaatgatataaccaaaagaagcctacaagaaagt
hBRCA1(298)agtgtcctttatgtaagaatgatataaccaaaaggagcctacaagaaagt
mBRCA1(289)aatgtcctttgtgtaagaatgagataaccaaaaggagcctacagggaagc

bBRCA1(201) acaagatttagtcaacttgttgaagagctgttgaagatcattcatgcttt
hBRCA1(348)acgagatttagtcaacttgttgaagagctattgaaaatcatttgtgcttt
mBRCA1(339)acaaggtttagtcagcttgctgaagagctgctgagaataatggctgcttt

bBRCA1(251)tgagcttgacacaggattgcagtttgcaaacagctataacttttcgagaa
hBRCA1(398)tcagcttgacacaggtttggagtatgcaaacagctataattttgcaaaaa
mBRCA1(389)tgagcttgacacgggaatgcagcttacaaatggttttagttttcaaaaa

bBRCA1(301)aggaagataactctcctgagcatctgaaggaggaagtttctataatccaa
hBRCA1(448)aggaaaataactctcctgaacatctaaaagatgaagtttctatcatccaa
mBRCA1(439)agagaaataattcttgtgagcgtttgaatgaggaggcgtcgatcatccag

bBRCA1(351)actatgggctaccggaattgggccaaaagactttggcagagtgaacctga
hBRCA1(498)agtatgggctacagaaaccgtgccaaaagacttctacagagtgaacccga
mBRCA1(489)agcgtgggctacc----------

bBRCA1(401)aaatcctaccttgcaggaaaccagtcttactgtcgaactctctaacct
hBRCA1(548)aaatccttccttgcaggaaaccagtctcagtgtccaactctctaacct
mBRCA1(502)----------

BBRCA1	1 -	448	(448 bps)	Homology
HBRCA1F	148 -	595	(448 bps)	90%
MBRCA1	139 -	501	(363 bps)	68%

Fig.3: Ring finger domain (N-terminus) of Bovine BRCA1 cDNA Sequence. (A) The nucleotide sequence of bovine BRCA1 corresponds to human BRCA1 exon 2 to 8. The 120 bp splicing point is underlined. (B) Bovine BRCA1 sequence comparison with human and mice

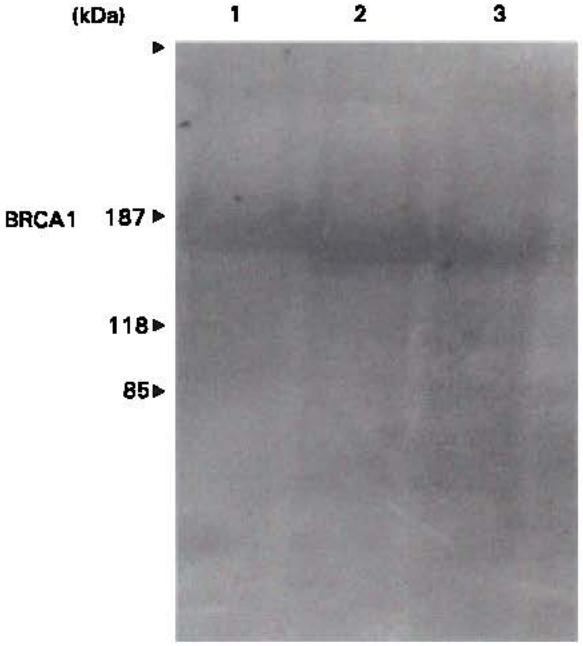

Fig.4: Western blot analysis of bovine BRCA.Each lane contains 120 μg of crude extracts prepared from lactating bovine mammary tissue. The extracts were electrophoresed on 5% SDS-PAGE and then transferred to PVDF membranes. Protein was detected using N-terminal BRCA1 monoclonal antibody (BRCA1-8F7).

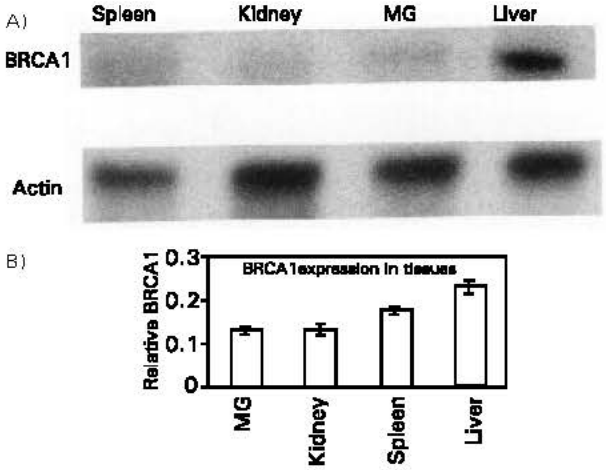

Fig.5: Tissue distribution of BRCA1 in a lactating cow. (A) Total RNA was isolated from a cow in early lactation and BRCA1 mRNA expression was analyzed by RNase protection assay, using a biotin labeled antisense BRCA1 probe. (B) Quantification of BRCA1 mRNA expression in (A). Relative BRCA1 mRNA expression to ß-actin mRNA expression is shown. Representative radiographs are shown in A. Means and standard errors are shown for three separate electrophoretic runs in B.

in an epithelial-specific fashion in the mammary gland of mice. The highest BRCA1 expression was found in the terminal end bud structures which contain rapidly dividing, relatively undifferentiated cell types that give rise to the differentiated mammary epithelial tree during ductal morphogenesis, during

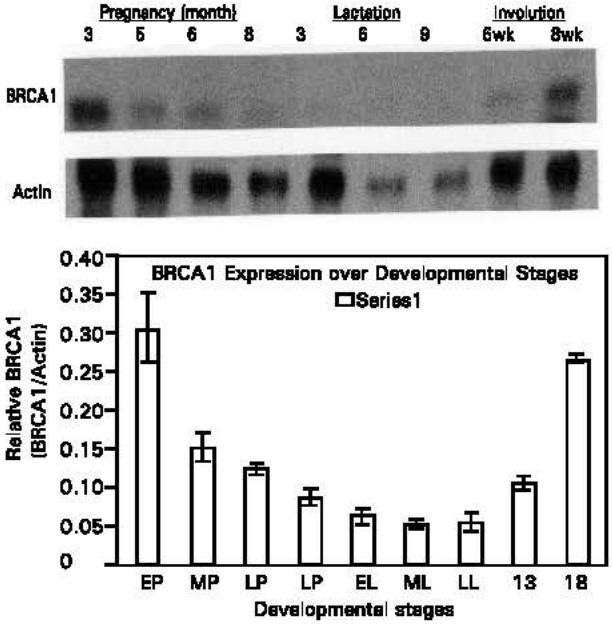

Fig.6: Bovine mammary gland BRCA1 expression over various developmental stages.(A) RNase protection assay (RPA) was used to quantify bovine BRCA1 mRNA expression. Top and mid-panel are RPA hybridized to the 550 nt, 500 nt bovine BRCA1 antisense riboprobe and 250 nt actin riboprobe, respectively. Each lane contains 15μg of total RNA extracted from indicated developmental stage of bovine mammary tissue. (B) Quantification of BRCA1 mRNA expression showed in (A). Relative BRCA1 mRNA expression to ß-actin mRNA expression is shown. EP (Early Pregnancy): 3 months; MP (Mid Pregnancy): 5 months; LP (Late Pregnancy): 6 months; LP (Late Pregnancy): 8 months; EL (Early Lactation): 3 months; ML (Mid Lactation): 6 months; LL (Late Lactation): 9 months; 6wk Involution, and 8wk Involution. Representative radiographs are shown in A. Means and standard errors are shown for three separate electrophoretic runs of three animals at each developmental stage in B.

puberty and in developing alveoli during pregnancy. Taken together, BRCA1 is considered to regulate cellular proliferation and differentiation.

Differentiation of the mammary epithelial cell is completed in mid-pregnancy in the bovine. Early pregnancy tissue (3 month) showed the highest BRCA1 expression, as expected. The expression of BRCA1 was maintained high until mid-pregnancy and then decreased until late lactation. So, if we consider these expression patterns, BRCA1 may serve as a regulator of cell proliferation in bovine mammary tissue. In other words, through all of pregnancy, BRCA1 may regulate the cell cycle and/or repair DNA. In addition, the interesting point is that BRCA1 expression increased over involution as in mice. The involution period includes a tissue remodeling process with wide ranging apoptosis taking place. Shao *et al.*,1996 showed that serum deprivation or calcium ionophore treatment of BRCA1 transfectants resulted in programmed cell death in NIH3T3 and MCF-7 cell lines. So far, there is no direct evidence that BRCA1 is directly involved in apoptosis during involution. Needless to say, further research is needed to answer it clearly. Marquis *et al.*, 1995 reported the mammary glands of parous mice that had undergone four weeks of postlactational regression express higher levels of BRCA1 mRNA than the mammary glands of age-matched virgin control mice.

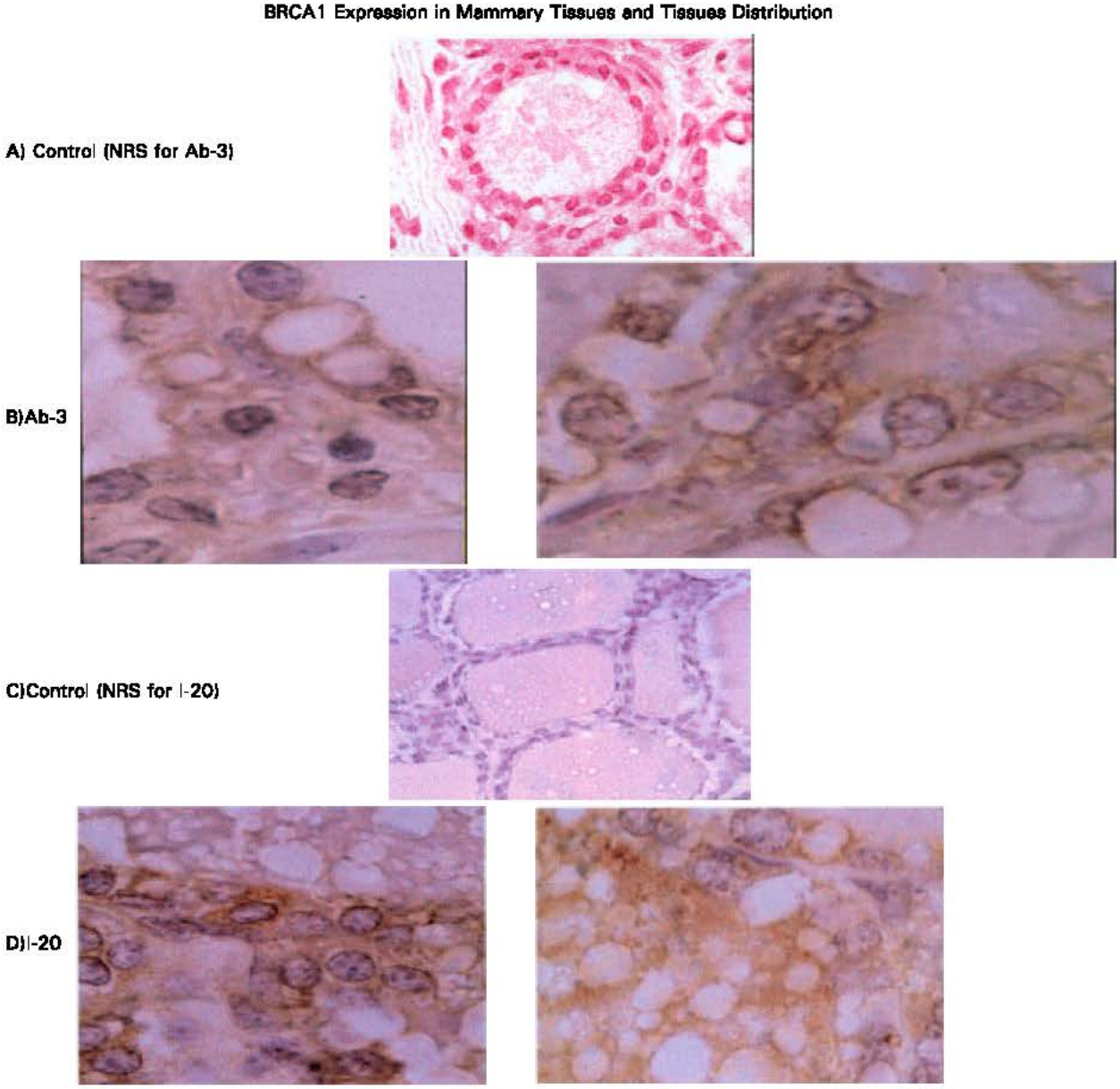

Fig. 7: Localization of BRCA1 in lactating bovine mammary tissue. All slides were processed using the Ab-3 (A, B) and I-20 (C, D) BRCA1 antibodies and immunohistochemistry procedures described in materials and methods. BRCA1 signals were photographed in tissue at 200X (A, C) and at 1,000X (B,D). Note that myoepitheial cells seen with elongated nuclei surrounding alveolar cells are not immunostained.

Murine mammary gland BRCA1 expression varies through all stages of mammary differentiation. It was reported that BRCA1 mRNA expression is very low during lactation in mice (Marquis *et al.*, 1995; Rajan *et al.*, 1996). Bovine BRCA1 mRNA expression is very similar to that of the mouse. BRCA1 protein expression, however, was quite high during lactation compared to other developmental stages. We feel that the elevated levels of BRCA1 protein seen during lacation are a result of regulatory mechanism(s) involved in translation, or RNA stability.

Identification of BRCA1 in mammary tissue, milk fat globule membrane and MCF-7 breast cancer cells

Mammary tissue: We used two antibodies in immunohistochemistry (IHC) to detect immunoreactive bovine BRCA1 in lactating mammary tissues. Both I-20, and Ab-3 antibodies localized BRCA1 in the nucleus, cytosol, and milk fat globule membrane (MFGM) (Fig. 7). The significance of BRCA1 localization in the MFGM shows that it is most likely a secreted protein. This finding supports the hypothesis for BRCA1 localization in the endoplasmic reticulum (ER)/golgi (Jensen *et al.*, 1996).

Our immuno-localization results showed the staining as very intense in epithelial cells, but not in myoepithelial cells, or fibroblasts (Fig. 7). This implies that the signal is cell specific.

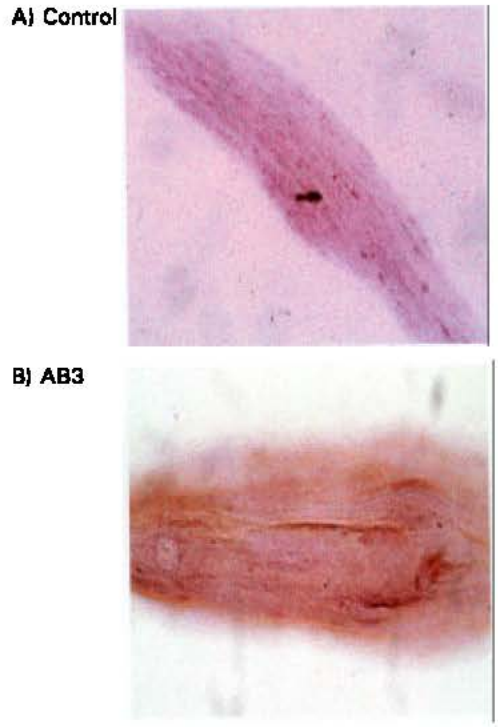

Fig. 8: Identification of BRCA1 in MFGM. All slides were processed using the Ab-3. (A, B) BRCA1 antibody an immunohistochemistry procedures were as described in materials and methods.

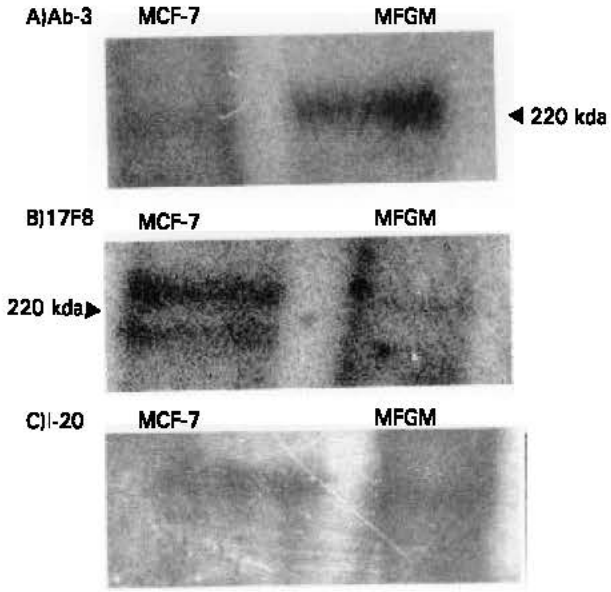

Fig. 9 Western analysis of BRCA1 from MCF-7 cell extracts and MFGM. 100 μg of isolated MCF-7 lysate and 200 μg of MFGM were loaded on 5 % SDS PAGE and then transferred on to a PVDF membrane. Protein was detected using Ab-3 (A), 17F8 (B), and I-20 (C) BRCA1 antibodies

Isolated MFGM: In addition, we observed intense BRCA1 staining in isolated MFGM (Fig. 8). The Ab-3 monoclonal antibody used in immuno-localization studies gives strong support for the secretion of BRCA1 in lactating tissues. It can be arguable that our isolated MFGM might be contaminated from secreted epithelial cells and proteins from dead cells. First, we can not rule out that there was contamination. However, we are sure through our experience that most of cells centrifuged at 3,000 x g for 50 min. This was confirmed under the microscope at the end of the procedure. We do not believe that cell contamination (membrane debris) contributed to our results, because a physiological buffer was used to isolate the MFGM which was isotonic [TBS (pH 7.2)]. We do not believe that isotonic TBS can precipitate free proteins at 100,000 x g for 90 min at 4 C. So, we ruled out the possibility of contamination during MFGM suspension and washing steps. From our data, we believe that bovine BRCA1 is a secreted tumor suppressor protein during lactation. Our next approach is to investigate the molecular mechanism(s) regulating BRCA1 synthesis and secretion.

Identification of BRCA1 in MCF-7 breast cancer cells: We isolated an MCF-7 total cell lysate isolates, as a positive control for our BRCA1 immuno-histochemistry studies since the protein is expressed in human MCF-7 breast cancer cells. We used three different antibodies for Western blot analyses. The three antibodies, I-20, Ab-3, and BRCA1 17F8, developed from exon 11, were used. Our Western blot results showed that all three antibodies, against BRCA1, were detected in the MCF-7 lysate (Fig. 9). This further suggested that BRCA1 was present in bovine tissues and the MFGM and helps confirm our cloning procedures. Even though our results are limited, with regard to unequivocaly proving that BRCA1 is a secreted protein, we speculate that a functional shift of epithelial cells to differentiated secretory alveolar cells allow BRCA1 secretion to occur in response to hormonal influences during lactation.

Bovine BRCA1 cDNA was highly conserved to the human. Five Bhundred base pairs of exon 11 (+3384/ + 3866, human BRCA1 cDNA position) and the C-terminus 1 kb were identical to the human. Seven hundred base pairs of the N-terminus, which contains two ring domains, showed 90 % homology to human BRCA1. The degree of bovine BRCA1 expression from highest to lowest was as follows: liver, spleen, mammary tissues and kidney.

The mammary tissues of early pregnancy heifers (3 months) showed much higher mammary BRCA1 mRNA expression than other mammary developmental stages. BRCA1 expression declined during the remainder of pregnancy and remained low for lactating cows. However, its expression increased when involution proceeded. Immuno-histochemical studies showed that BRCA1 was localized in the nucleus and cytoplasm of mammary epithelial cells from lactating cows. BRCA1 was not found in myoepithelial cells. The protein was also localized in the milk fat globule membranes. Our data suggests that normal bovine mammary tissue contains immuno-reactive BRCA1 protein and that the BRCA1 gene is differentially expressed through various stages of mammary development and involution. The protein appears to be a secreted protein in normal mammary tissue, since immuno-reactive BRCA1 is present in the milk fat globule membrane. Even though our results are limited, with regard to unequivocaly proving that BRCA1 is a secreted protein, we speculate that a functional shift of epithelial cells to differentiated secretory alveolar cells allow BRCA1 secretion to occur in response to hormonal influences during lactation.

Further studies are necessary to determine the role of this protein in bovine mammary gland development and involution.

References

Chen, Y., A.A. Farmer, C.F Chen, D.C. Jones, P.L. Chen and W.H. Lee, 1996. BRCA1 is a 220-kDa nuclear phosphoprotein that is expressed and phosphorylated in a cell cycle-dependent manner [published erratum appears in Cancer Res. 1996 Sep. 1;56(17):4074] Cancer Res., 56:3168-72.

Futreal, P.A., Q. Liu, D. Shattuck-Eidens, C.J. Cochran, K. Harshman, S. Tavtigian, L.M. Bennett, A. Haugen-Strano, J. Swensen, Y. Miki, K. Eddington, M. McClure, C. Frye, J. W Weaver-Feldhaus, Z. Ding, P. Gholami, L. Söderkvist, A. Kamb, and R.W. Wiseman, 1994. BRCA1 mutations in primary breast and ovarian carcinomas. Science, 266:120-122.

Hall J.M., M.K. Lee, B. Newman, J. E. Morrow, L.A. Anderson, B. Huey and M. C. King, 1990. Linkage of early-onset familial breast cancer to chromosome 17q21. Science, 250:1684-1689.

Jensen R.A., M.E. Thompson, T.L. Jetton, C.I. Szabo, R. van der Meer, B.Helou, S.Tronick, M.C. King, D.L. Page and J.T. Holt, 1996. BRCA1 is secreted and exhibits properties of a granin. Nat. Genet., 12: 303-308.

Marquis S.T., J.V. Rajan, B. A. Wynshaw, J. Xu, G.Y. Yin, K.J Abel, B.L.Weber, and L.A. Chodosh, 1995.The developmental pattern of Brca1 expression implies a role in differentiation of the breast and other tissues Nat. Genet., 11: 17-26.

Miki, J. Swensen, D. Shattuck-Eidens, P.A. Futreal, K. Harshman, S. Tavtigian, Q. Liu, C. Cochran, L.M. Bennett, and W. Ding, 1994. A strong candidate for the breast and ovarian cancer susceptibility gene BRCA-1. Science, 266: 66-71.

Paterson J.W., 1998. BRCA1: a review of structure and putative functions. Dis Markers., Feb., 13:261-274.

Rajan J.V., M. Wang, S.T. Marquis and L.A. Chodosh, 1996. BRCA2 is coordinately regulated with BRCA1 during proliferation and differentiation in mammary epithelial cells. Proc. Natl. Acad. Sci. U. S. A., 93:13078-83.

Romagnolo, D. Annab and T.E. Thompson, 1998. Estrogen upregulation of BRCA1 expression with no effect on localization. Mol. Carcinogenesis, 22: 102-109.

Ruffner, H.and I.M.Verma, 1997. BRCA1 is a cell cycle-regulated nuclear phosphoprotein. Proc. Natl. Acad. Sci. USA., 94: 7138-7143.

Sambrook, J., E.F. Fritsch, S. Maniatis and T. Hrsg, 1989. Molecular Cloning - A Laboratory Manual, 2 nd Edition. Cold Spring Habour Laboratory Press, New York.

Scully, R., S. Ganesan, M. Brown, J.A. DeCaprio, S.A. Cannistra, J. Feunteun, S. Schnitt and D.M. Livingston, 1996. Technical Comment: Location of BRCA1 in human breast and ovarian cancer cells. Science , 272:123-125.

Shao N.S., Y.L. Chai, E. Shyam, P. Reddy and V.N. Rao,1996. Induction of apoptosis by the tumor suppressor protein BRCA1. Oncogene, 13:1-7.

Spitsberg, V.L. and R.C. Gorewit, 1997. In Vitro phosphorylated bovine milk fat globule membrane proteins. Nutritional Biochemistry, 8: 181-189.