

# BRCA1 Tumor Suppressor Gene Product Shares Immunoreactive Epitopes with a Protein Present in Seminal Plasma

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**Objectives:** To develop immunofluorometric procedures for measuring BRCA1 protein in various biological fluids and tissue extracts.

**Design and Methods:** Five commercially available monoclonal and polyclonal antibodies against BRCA1 were evaluated for developing competitive and non-competitive immunofluorometric procedures for BRCA1. Biotinylated and nonbiotinylated peptides were used to assess the specificity of the antibodies for blocking experiments and for the competitive immunoassay. Extensive studies to exclude cross-reactivity and non-specific effects in the non-competitive immunoassay were undertaken. Seminal plasmas as well as breast tumor extracts, amniotic fluids and cerebrospinal fluids were analyzed.

**Results:** We designed novel methods for measuring BRCA1 immunoreactivity. One configuration based on the "sandwich-type" immunoassay principle was used for further studies. We discovered that seminal plasma contains an immunoreactive protein which appears to possess the D-20 (aminoterminal) and C-20 (carboxy-terminal) epitopes of BRCA1. Molecular weight identification using gel filtration chromatography has shown that the immunoreactive species has a molecular weight between 660 and 160 KDa.

**Conclusions:** We identified for the first time a protein in seminal plasma that shares immunoreactive epitopes with the BRCA1 tumor suppressor protein. We are currently purifying this protein in order to examine if it is homologous or identical to BRCA1.

**KEY WORDS:** BRCA1; familial breast cancer; ovarian cancer; seminal plasma components; prostate cancer; cancer susceptibility genes.

## Introduction

**B**reast cancer is the most common cancer among North American women. By the year 2000, it is estimated that worldwide, breast cancer will account for 500,000 deaths annually (1). About 10% of breast cancers are due to inherited genes, two of

which, BRCA1 and BRCA2, have now been cloned (2-4). BRCA1, the breast and ovarian cancer susceptibility gene, is localized to human chromosome 17q21. Mutations in this gene account for approximately 95% of families with increased incidence of early onset breast and ovarian cancer and 45% of families with increased incidence of breast cancer alone (5,6). BRCA1 is believed to encode a tumor suppressor protein since transfected BRCA1 gene inhibits the growth of breast and ovarian cancer cells but not of other malignant cells. Most inherited BRCA1 mutations produce truncated proteins that vary in length from 5% to 99% of full-length protein. However, missense mutations that alter cysteines in a motif called the RING finger are also found. The C-terminal end of the BRCA1 protein appears to be essential for normal BRCA1 function in breast epithelial cells because patients inheriting codon 1853 stop mutations, develop very early onset breast cancer. The BRCA1 gene encodes a 1863 aminoacid protein and it is expressed in several tissues including the breast and ovary.

The amino-terminal domain of BRCA1 possesses high sequence homology with a zinc finger topology (3). This topology, C<sub>3</sub>HC<sub>4</sub>, is referred to as the "zinc-ring domain," "RING finger," or A-box and is found in several viral proteins, proto-oncoproteins, and regulatory and transcription factors (7,8).

The BRCA1 zinc-ring domain and its structure and function are of particular interest because it is the location of some of the most frequently occurring mutations linked to breast and ovarian cancer. The 185delAG mutation (deletion of two nucleotide base pairs in exon 2) in the zinc-ring domain has been shown to occur in 1 in 100 Ashkenazi Jewish individuals. This is in comparison with the estimated 1 in 833 frequency of all BRCA1 mutations in the general population (9,10).

The mechanism by which the loss of function of

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BRCA1 causes cancer is unknown. One of the first steps in understanding the biological function of BRCA1 and is to establish its subcellular localization. For BRCA1, in particular, there are conflicting data in the literature while one group suggested that BRCA1 is a secreted protein (11,12), others believe that BRCA1 is a nuclear transcription factor (2,3,13). Yet, a third group postulates that BRCA1 is nuclear in normal cells while it resides in the cytoplasm of cancerous cells (14,15). Unfortunately, these major differences in opinion have not yet been resolved (16,17). These differences in opinion arise because there are no techniques available yet to reliably monitor the BRCA1 protein. Western blotting and immunohistochemistry are not highly specific and cross-reactions of some of the polyclonal antibodies against BRCA1 with the epidermal growth factor receptor and other proteins have been noted (17). Jensen *et al.* (11) and Holt *et al.* (12) have presented evidence that BRCA1 and possibly BRCA2 belong to the granin family of secreted proteins and that they exert their biological function by releasing bioactive peptides with antitumor activities (18). This proposal has been highly contested by groups suggesting that BRCA1 is a transcription factor (17).

In this article we present evidence that BRCA1 shares immunoreactive epitopes with a protein present at high levels in seminal plasma. Further work toward the purification and sequencing of this protein will demonstrate if this seminal plasma component represents a molecule that is highly homologous or even identical to BRCA1.

## Materials and methods

### BRCA1 REAGENTS

The following antibodies against BRCA1 were purchased: (1) Polyclonal C-20 antibody generated by immunizing rabbits with the peptide LYQCQ ELDTYLIPQIPHSY (peptide C-20). (2) Polyclonal D-20 antibody generated by immunizing rabbits with the peptide DLSALRVEEVQNVINAMQKI (peptide D-20). Peptide C-20 maps to the carboxy-terminus of human BRCA1 (aminoacids 1843–1862) and peptide D-20 to the aminoterminal (aminoacids 2–21). Both antibodies were affinity purified with peptides immobilized on agarose columns. The antibodies were custom produced by Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA) so that the final product is dissolved in phosphate buffered saline, free of preservatives or other extraneous proteins. The routinely commercially available antibodies are dissolved in a buffer containing gelatin and they are not suitable for labeling. And (3) Monoclonal mouse antibodies against BRCA1 were purchased from Oncogene Research Products, Cambridge, MA, USA. Antibodies MS-13 and MS-110 were raised against recombinant human BRCA1 aminoacids 1–304. Antibody SG-11 was raised against a peptide representing aminoacids 1846–

1863 and recognizes the carboxyterminus of human BRCA1. SG-11 antibody does not cross-react with the epidermal growth factor receptor. These antibodies were purchased as 0.1 mg/mL solutions in gelatin. The peptides C-20 and D-20 were also purchased from Santa Cruz Biotechnology.

Biotinylation of all BRCA1 antibodies and the peptides C-20 and D-20 was performed with NHS-LC-Biotin (Pierce Chemical Co., Rockford, IL, USA) using standard procedures described elsewhere (19, 20). Briefly, we mixed the reagent to be biotinylated with an equal volume of 0.5 M carbonate buffer, pH 9.5, and then added NHS-LC-Biotin dissolved in dimethylsulfoxide (DMSO). In general, the DMSO added was  $\leq 5\%$  of the total biotinylation volume and NHS-LC-Biotin was added at a 100-fold molar excess. Biotinylation was allowed to proceed for 1 h at room temperature. The biotinylated reagents were used without further purification. The biotinylation of antibodies was verified by capturing them on goat anti-rabbit (C-20, D-20 antibodies) or goat anti-mouse (SG-11, MS-13, MS-110 antibodies) coated plates and detecting them with streptavidin-alkaline phosphatase (SA-ALP) conjugates. The biotinylation of the peptides was verified by capturing them on their respective antibodies followed by detection with SA-ALP conjugates as described below.

### SEMINAL PLASMAS

Seminal plasmas were obtained by centrifuging human semen samples submitted for fertility studies. These samples were kept at  $-20^{\circ}\text{C}$  until analysis.

### EXTRACTION OF BREAST TUMORS

Breast tumors were extracted as described elsewhere (21). The extracts were used for analysis of BRCA1 immunoreactivity.

### IMMUNOFUOROMETRIC PROCEDURES

For all assays developed, we used streptavidin-alkaline phosphatase conjugates (SA-ALP) to detect biotinylated moieties. The ALP substrate diflunisal phosphate and  $\text{Tb}^{3+}$ -EDTA chelates were used essentially as described elsewhere (22). Time-resolved fluorometry was used as a detection technique and fluorescence counts are in arbitrary units. Arbitrary fluorescence units may vary between experiments due to use of different instruments and types of microtiter plates during the course of this investigation.

Polystyrene microtiter strips were used throughout. These were coated with goat anti-mouse immunoglobulin (GAMiG plates) or goat anti-rabbit immunoglobulin (GARiG plates) by passive adsorption overnight (100  $\mu\text{L}$ /500 ng per well). For detailed procedures see Diamandis *et al.* (19).

### SANDWICH-TYPE IMMUNOASSAY

A typical "sandwich-type" immunoassay used in this study involves the following steps (Figure 1). (1)

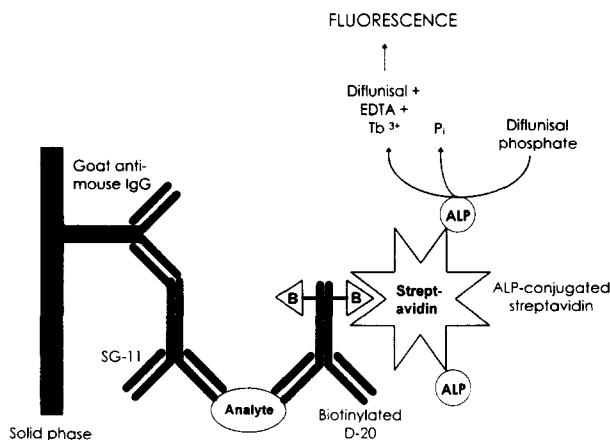


Figure 1 — Sandwich-type immunoassay configuration of BRCA1. SG-11 is a mouse monoclonal antibody against the carboxyterminal end of BRCA1. D-20 is a rabbit polyclonal antibody against the aminoterminal end of BRCA1. B = biotin; ALP = alkaline phosphatase. For more details see text and reference 22.

Preparation of solid-phase goat-anti-mouse IgG. (2) Immobilization of SG-11 monoclonal antibody by adding into the wells 100  $\mu$ L of SG-11 antibody (stock 0.1 mg/mL) diluted 200-fold (100  $\mu$ L/50 ng per well) in diluent A [diluent A composition: 50 mM Tris, pH 7.40 containing 60 g/L of bovine serum albumin (BSA) and 0.5 g/L of sodium azide]. After incubation for 1 h with shaking the wells are washed 6 times. (3) Adding the analyte (*e.g.*, seminal plasma diluted in diluent A from 10-fold to  $10^6$ -fold) and incubating for 1 h with shaking at room temperature followed by washing 6 times. (4) Adding the biotinylated D-20 antibody (stock 0.50 mg/mL) diluted 1,000-fold (100  $\mu$ L/50 ng per well) in diluent B [diluent B composition: 50 mM Tris, pH 7.40, containing 60 g/L of BSA, 0.5 g/L of sodium azide, 0.5 mol/L of KCl, 5 g/L of Tween 20 surfactant, 0.5 g/L of rabbit IgG and 100 mL/L of goat serum]. After 30 min incubation with shaking, the wells are washed 6 times. (5) Detection of biotinylated moieties with SA-ALP as described elsewhere (22).

#### COMPETITIVE-TYPE IMMUNOASSAY

A competitive-type immunoassay is performed with the following steps: (1) Preparation of solid-phase goat-anti mouse IgG (GAMiG) or goat anti-rabbit IgG (GARiG). (2) Immobilization of SG-11 in GAMiG plates (SG-11 at amounts of 100  $\mu$ L/20 ng per well) or C-20 antibody or D-20 antibodies on GARiG plates (100  $\mu$ L/20 ng per well each). (3) Adding to the wells the tracer peptide (biotinylated C-20 peptide for antibodies SG-11 and C-20 or biotinylated D-20 peptide for the D-20 antibody) at concentrations of 50  $\mu$ L/1.25 ng per well for SG-11, 50  $\mu$ L/0.2 ng per well for D-20 and 50  $\mu$ L/0.6 ng per well of the C-20 antibody. All biotinylated peptides were dissolved in diluent A. We also add to the wells the competing peptide or sample, diluted in diluent

C [diluent C composition: 50 mM Tris, pH 7.40, containing 60 g/L of BSA, 0.5 g/L of sodium azide, 0.5 mol/L of KCl, 5 g/L of Tween 20 surfactant, 50 mL of mouse serum and 100 mL/L of goat serum]. The competing peptide or sample was added simultaneously with the biotinylated peptide at a volume of 50  $\mu$ L. (4) After incubation for 1 h at room temperature with shaking, the wells are washed and the amount of bound biotinylated peptide is detected with SA-ALP.

#### GEL FILTRATION CHROMATOGRAPHY

In order to determine the molecular weight of the immunoreactive species, we used a gel filtration column, Superdex, obtained from Pharmacia Biotech Montreal, PQ. The column was eluted at 0.4 mL/min with a mobile phase consisting of 0.1 mol/L phosphate buffer, pH 7.0, containing 0.15 mol/L of NaCl. Fractions of 0.4 mL were collected for analysis.

#### STANDARDIZATION

Since there is no BRCA1 standard or protein available, we devised an arbitrary system that serves as a comparative method only. We prepared a seminal plasma pool with high immunoreactivity and preliminarily purified it by passing through a Superose gel filtration column (Pharmacia Biotech, Montreal, PQ, Canada) and collecting all fractions with molecular weight >100 KDa. This pool was concentrated with Amicon Centricon 30 units and given an arbitrary concentration of 500,000 U/L. Serial dilution in diluent A produced standards used for comparative purposes.

#### Results

We first evaluated the specificity of the five antibodies against peptides C-20 and D-20 and an irrelevant peptide with sequence YRSPHNGSTYSV. For this experiment, we immobilized antibodies on either goat anti-mouse coated plates (SG-11, MS-13, MS-110) or goat anti-rabbit coated plates (C-20, D-20) and reacted them with each biotinylated peptide. Irrelevant monoclonal and polyclonal antibodies were used as negative controls. The bound biotinylated peptides were detected using streptavidin-alkaline phosphatase (SA-ALP) and time resolved fluorometry. The results are shown in Table 1. Clearly, antibodies SG-11 and C-20 recognize specifically biotinylated peptide C-20 but not biotinylated peptide D-20, and antibody D-20 recognizes specifically biotinylated peptide D-20 but not biotinylated peptide C-20. Anti-BRCA1 antibodies MS-13 and MS-110 do not recognize either C-20 or D-20 biotinylated peptides. These two monoclonals bind to epitopes different from either the aminoterminal or the carboxyterminal end of BRCA1.

We have further developed competitive-type assays for epitopes C-20 and D-20 by using solid-phase antibodies, biotinylated peptides C-20 or D-20 as

TABLE 1  
Specificities of BRCA1 Antibodies Against Biotinylated Peptides C-20, D-20, and an Irrelevant Peptide

Capture Antibody	Biotinylated Peptide/Arbitrary Fluorescence Units			
	C-20	D-20	Irrelevant	Biotin only
<b>GAMiG Coated Plates</b>				
SG-11	20,000	400	250	380
MS-13	1,200	800	250	300
MS-110	500	250	200	300
Lutropin (Control)	200	200	250	200
Somatotropin (Control)	250	250	200	250
None (Gelatin)	250	250	250	250
<b>GARIg Coated Plates</b>				
C-20	30,000	400	200	220
D-20	700	38,000	100	100
Rabbit IgG (Control)	200	180	100	100
None (Gelatin)	450	311	180	150

tracers and unlabeled peptides C-20 and D-20 as competing peptides. Calibration curves of the three assays developed are shown in Figure 2. These assays are useful when searching for either the whole molecule or fragmented BRCA1, which may contain the epitopes C-20 (carboxyterminus) or D-20 (aminoterminal). The competitive assay with SG-11 antibody was used for analysis of HPLC fractions for molecular weight identification of immunoreactive species (see below).

We have further attempted to develop a "sandwich-type" immunoassay for BRCA1 by using two different antibodies. Among all the possible combinations of the five antibodies, we excluded the pair of C-20 and SG-11 antibodies since they bind to the same epitope. Additionally, we excluded the combinations of only two monoclonal or only two polyclonal antibodies since these antibodies are supplied commercially in small amounts and as mixtures with gelatin, and their purification before direct coating to microtiter wells would have been costly. Moreover, the direct coating approach requires much higher amounts of antibodies than indirect coating of these very expensive reagents (for further discussion on this issue, see chapter 24 of reference 19). The remaining feasible combinations included the following (SP = solid phase; B = biotin).

1. SP-GARIg-C-20-BRCA1-Mab-B (Mab = MS-13 or MS-110)
2. SP-GARIg-D-20-BRCA1-Mab-B (Mab = MS-13, MS-110 or SG-11)
3. SP-GAMiG-MS-13-BRCA1-PAb-B (PAb = C-20 or D-20)
4. SP-GAMiG-MS-130-BRCA1-PAb-B (PAb = C-20 or D-20)
5. SP-GAMiG-SG-11-BRCA1-D-20-B

Since no BRCA1 standard is known to exist and a donated crude BRCA1 recombinant preparation (a

gift from Dr. R. Jensen, Vanderbilt University, Nashville, TN, USA) appeared to contain no immunoreactive BRCA1, we decided to check the above combinations in fluids that may contain BRCA1. These included 200 breast tumor cytosolic extracts and seminal plasmas. We found that the combinations involving MS-13 and MS-110 antibodies either for detection or coating, did not produce any positive results (see also below). However, a positive signal with diluted seminal plasma was obtained when we used the following two combinations.

1. GARIg-D-20-BRCA1-SG-11-B
2. GAMiG-SG-11-BRCA1-D-20-B

Here, we use BRCA1 as analyte liberally since we do not yet know if the immunoreactive species is indeed BRCA1 or a cross-reactive protein. Among the two configurations, we selected combination number 2 based on preliminary experiments directed toward minimizing nonspecific effects due to the presence of gelatin in these antibody preparations. Our final assay configuration is shown in Figure 1. A few comments on this procedure and its optimization should be useful: (1) GAMiG plates can be prepared by coating overnight with 100  $\mu$ L/500 ng of GAMiG per well in a 50 mM Tris buffer, pH 7.80. (2) SG-11 antibody can then be immobilized by diluting it in diluent A and adding it at a concentration of 100  $\mu$ L/50 ng of SG-11 per well. More SG-11 antibody is not necessary. (3) We have diluted seminal plasma in diluent A, which has very high protein content in order to minimize protein differences in the tested samples. Some seminal plasmas test positive even at dilutions between  $10^4$ - to  $10^6$ -fold. (4) We optimized the diluent of biotinylated antibody D-20 (diluent B) to contain high salt, surfactant and large amounts of rabbit IgG in order to minimize possible nonspecific effects of the detection antibody. With all these precautions in mind, we

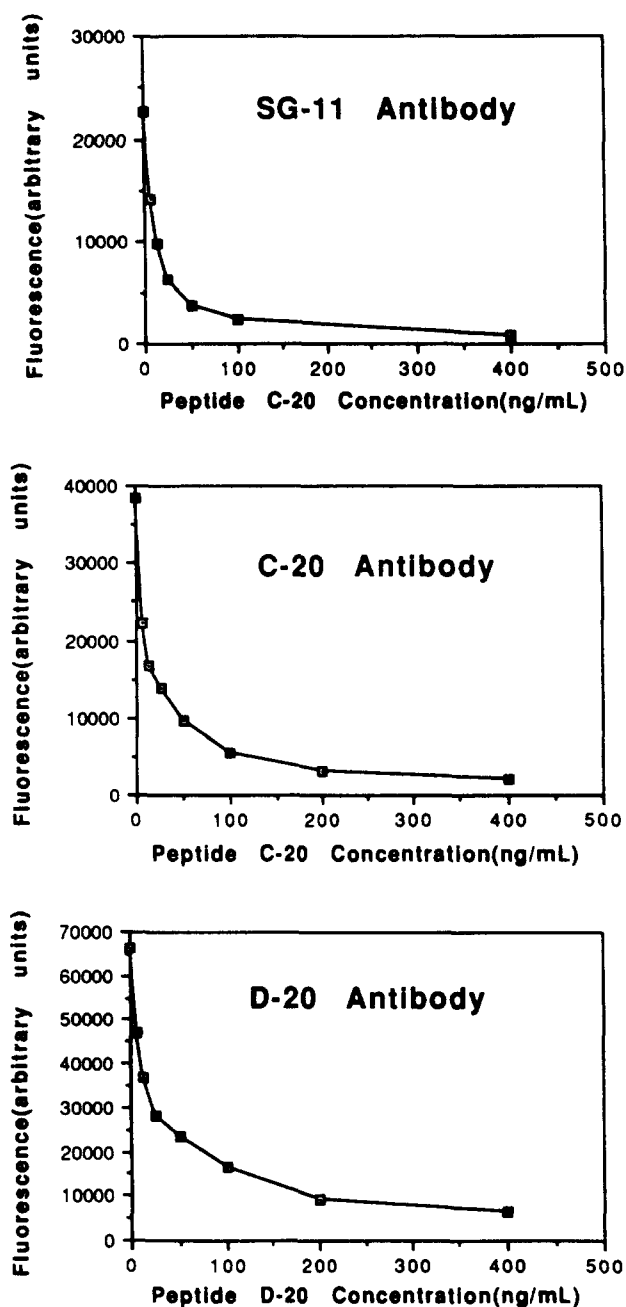


Figure 2 — Competitive-type immunoassay for BRCA1 with three different antibodies. The calibration curves were obtained with biotinylated peptides as labeled molecules. The competing molecules are unlabeled peptides at the concentrations shown. For more details see text.

proceeded to establish the specificity of this configuration for the epitopes recognized by SG-11 and D-20 antibodies.

In Table 2, we present data showing that SG-11 coating antibody but not irrelevant monoclonal antibodies, of the same class or subclass, is necessary to generate a signal in the assay design shown in Figure 1. Clearly, the presence of SG-11 antibody is necessary for signal generation, suggesting that the epitope recognized by SG-11 is present in the immu-

noreactive species of seminal plasma. Further support of the above data came from an experiment aiming to show that blocking of the binding site of SG-11 by peptide C-20, abolishes the signal. For this experiment, we prepared GAMIG plates coated with SG-11. We then reacted the coated SG-11 antibody with either BSA alone (no blocking) or with BSA containing peptides C-20 or D-20 or the irrelevant peptide at concentrations of 1  $\mu\text{g/mL}$ . After 30 min, we washed out excess peptides and completed the immunoassay as usual. The data are shown in Table 3. Clearly, SG-11 antibody blocked by peptide C-20 did not bind the immunoreactive substance in seminal plasma. None of the other two peptides had any effect. These data further support the proposal that SG-11 antibody is necessary for signal generation in the assay design of Figure 1, and that the immunoreactive species of seminal plasma binds to the SG-11 epitope and not to other parts of this antibody.

We then proceeded to examine the specificity of reaction of biotinylated D-20 detection antibody. This antibody generates a signal by binding to seminal plasma components already bound by antibody SG-11. In Table 4, we present data generated with biotinylated D-20 detection antibody or with irrelevant biotinylated antibodies. Apart from a minor nonspecific binding of biotinylated gelatin, no significant signal was generated with any biotinylated detection rabbit antibody, except D-20. Further verification that the signal generated by biotinylated D-20 antibody is specific, comes from experiments with blocking peptide D-20. When we preincubated biotinylated detection antibody D-20 with peptide D-20 before adding it to the assay, the signal was abolished (Table 5). The data presented with the biotinylated D-20 detection antibody strongly suggest that this antibody reacts with the D-20 epitope in an immunoreactive protein present in seminal plasma. The combined data suggest that our assay configuration shown in Figure 1 detects a protein present in seminal plasma that contains in its structure the epitopes C-20 and D-20. These two epitopes represent the aminoterminal and carboxy-terminal end of BRCA1.

#### MOLECULAR WEIGHT CHARACTERIZATION

We have injected a seminal plasma sample into a gel filtration chromatography column and collected the fractions. These fractions were then analyzed for BRCA1 immunoreactivity using the "sandwich-type" and "competitive-type" assay with SG-11 antibody. The column was calibrated with molecular weight standards. The results are shown in Figure 3. Both assays detect an immunoreactive substance with a molecular weight between 660–160 KDa.

#### ANALYSIS OF CLINICAL SAMPLES

We have analyzed BRCA1 immunoreactivity with the "sandwich-type" immunoassay in 16 amniotic

TABLE 2  
Fluorescence Signal with the Proposed "Sandwich-Type" Assay in the Presence of Various Capture Antibodies<sup>a</sup>

Capture Antibody <sup>b</sup>	Antibody Subclass	Fluorescence, Arbitrary Counts <sup>c</sup>		
		0 $\mu$ L	500 $\mu$ L	2,000 $\mu$ L
SG-11	IgG2	1,069	15,700	17,200
MS-13	IgG1	1,400	1,400	1,700
MS-110	IgG1	680	840	1,004
Lutropin	unknown	550	670	810
Prolactin	IgG2a	430	530	714
Thyrotropin	IgG1	420	411	607
Progesterone	IgG2a	912	862	1,650
Thyroxine	IgG2b	560	476	814
Gelatin <sup>d</sup>		820	760	920
None <sup>e</sup>		850	680	850

<sup>a</sup>Assay configuration shown in Figure 1.

<sup>b</sup>All antibodies were mouse IgGs.

<sup>c</sup>Seminal plasma diluted in diluent A was used to produce arbitrary standard solutions.

<sup>d</sup>Diluent of SG-11, MS-13, and MS-110 contains 0.2% gelatin.

<sup>e</sup>No capture antibody to assess GAMIg nonspecific effects.

fluids, 16 breast tumor cytosolic extracts and 16 cerebrospinal fluids (all diluted 1:1 with diluent A). All were negative except two breast tumor extracts that showed traces of immunoreactivity. We then analyzed seminal plasmas diluted to varying degrees in diluent A the "sandwich-type" assay. All 16 seminal plasmas were positive at dilutions as high as 50,000-fold.

## Discussion

Although there is no controversy that the BRCA1 gene is involved in the pathogenesis of familial breast cancer, the biological function of the encoded protein is unknown. Three different opinions exist regarding the subcellular localization of this protein. Currently, there is no firm agreement whether BRCA1 is nuclear, cytoplasmic, or secreted protein. Part of the controversy was created because the methods for studying BRCA1 protein are not reliable. Immunohistochemistry and Western blotting rely on the reaction of one primary antibody with

fixed tissues or membranes and usually, under conditions where the proteins are denatured. Moreover, it is now known that a number of antibodies directed against the carboxyterminal end of BRCA1 interact on Western blots with the epidermal growth factor receptor (17).

The most specific method for quantifying any high molecular weight protein is the "sandwich-type" assay, which relies on signal generation after binding of the analyte to two separate antigenic determinants. Appropriately designed, such assays will bind the native, nondenatured analyte in an "ELISA-type" format (19). Such assays for BRCA1 do not currently exist.

We here describe the design of the first "sandwich-type" and "competitive-type" assays for BRCA1 by using commercially available monoclonal and polyclonal antibodies raised against peptides representing BRCA1 sequences. By using such assays, we discovered that the epitopes SG-11 (carboxytermi-

TABLE 3  
Fluorescence Signal with the Proposed "Sandwich-Type" Assay in the Presence or Absence of Blocking Peptides

BRCA1 Immunoreactive Concentration (Arbitrary U/L) <sup>a</sup>	Peptide Added/Fluorescence (Arbitrary Units)			
	None	C-20	D-20	Irrelevant Peptide
0	1,300	1,100	1,600	1,300
20	3,200	1,400	3,200	3,350
500	21,000	3,500	21,500	22,000
2,000	30,500	4,800	29,000	27,000

<sup>a</sup>Prepared by diluting seminal plasma as described in the **Methods** section.

TABLE 4  
Fluorescence Signal with the Proposed "Sandwich-Type" Assay in the Presence of Various Biotinylated Detection Antibodies

Biotinylated Detection Antibody	BRCA1 Immunoreactive Concentration (Arbitrary Units/L)/ Fluorescence (Arbitrary Units)			
	0	20	500	2,000
D-20	980	3,500	17,500	19,000
C-20	510	500	520	550
Rabbit anti-PSA	1,100	1,400	1,300	1,400
Rabbit IgG in gelatin	1,500	1,500	3,500	3,400
Gelatin	1,000	1,100	2,700	3,000
Biotin (no antibody)	310	320	310	300

TABLE 5  
Fluorescence Signal with the Proposed "Sandwich-Type" Assay in the Presence or Absence of Blocking Peptide D-20

BRCA1 Immunoreactive Concentration (Arbitrary U/L)	Peptide Added/Fluorescence (Arbitrary Units)		
	None (+SG-11) <sup>a</sup>	None (-SG-11)	+D-20 (+SG-11)
0	1,200	1,100	800
5	1,750	1,000	700
20	11,000	1,200	820
500	17,000	1,200	1,300

<sup>a</sup>Assay was done in the presence (+SG-11) or absence (-SG-11) of capture antibody.

nus) and D-20 (aminotermisus) are present on a protein that it is abundant in seminal plasma. The finding was verified by conducting experiments to confirm the specificity of the fluorescence signal. Non-BRCA1 antibodies fail to generate a signal and the signal could be effectively blocked after the BRCA1 antibodies are reacted with their corresponding peptides. We have thus concluded that the protein under discussion in seminal plasma may represent either BRCA1 or a highly homologous protein. We are now in the process of purifying this protein for sequence analysis.

Important biological questions may be asked if indeed the immunoreactive seminal plasma component proves to be BRCA1. How does BRCA1 enter the seminal plasma at such high concentrations? Which is the tissue that is secreting it? What is the function of BRCA1 in seminal plasma? The vast amounts of immunoreactive BRCA1 in seminal

plasma will, no doubt, help identify a biological role for this protein, which may serve as a paradigm to examine its role in the breast and ovarian cancer. Since seminal plasma contains several potent proteases, it will be interesting to examine if BRCA1 is further fragmented by these enzymes along the lines proposed by Jensen *et al.* (11) and Holt *et al.* (12).

Our group has previously identified another major seminal plasma component, prostate specific antigen (PSA), which is produced in large amounts by the breast (23). We demonstrated a connection between PSA presence in the breast and breast cancer risk (24), as well as prognosis (25). Based on data presented in the literature, we speculated recently that the roles of PSA and BRCA1 may be linked to each other and that may constitute an enzyme/substrate pair (26). We now present strong evidence that these two molecules coexist in seminal plasma.

We believe that our finding represents a major step toward the understanding of the biological function of BRCA1. The newly developed assays will become instrumental in facilitating further crucial studies directed toward the quantification of BRCA1, its pattern of secretion in tissues and cell lines, and its metabolism and hormonal regulation. At this point, the major priority would be the purification of the immunoreactive protein of seminal plasma to homogeneity and its sequencing, to prove that it represents BRCA1 and not a homologous cross-reacting protein.

## References

1. Pisani P, Parkin DM, Ferlay J. Estimates of the worldwide mortality from eighteen major cancers in 1985: implications for prevention and projections of future burden. *Int J Cancer* 1993; **55**: 891-903.
2. Miki Y, Swensen J, Shattuck-Eidens, *et al.* A strong candidate for the breast and ovarian cancer susceptibility gene BRCA-1. *Science* 1994; **266**: 66-71.
3. Futreal PA, Liu Q, Shattuck-Eidens, *et al.* BRCA-1 mutations in primary breast and ovarian carcinomas. *Science* 1994; **266**: 120-122.
4. Wooster R, Bignell G, Lancaster J, *et al.* Identification of the breast cancer susceptibility gene BRCA-2. *Nature* 1995; **378**: 789-792.
5. Narod SA, Ford D, Devukee P, *et al.* An evaluation of genetic heterogeneity in 145 breast-ovarian cancer families. *Am J Hum Genet* 1995; **56**: 254-264.
6. Easton DF, Bishop DT, Ford D, Crockford GP. Genetic

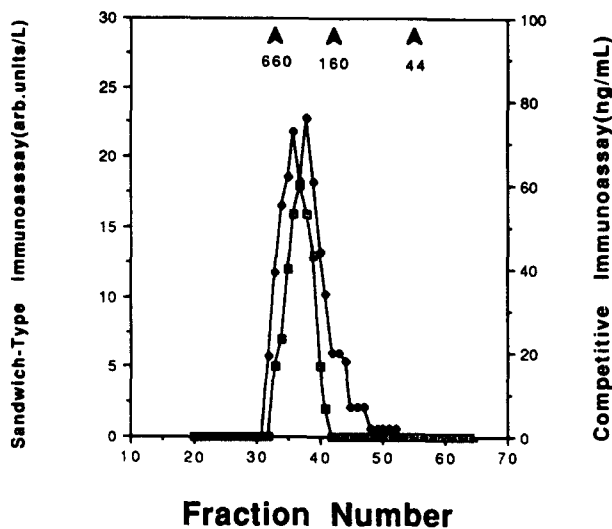


Figure 3 — Separation of a seminal plasma sample on a gel filtration column and analysis of fractions by the competitive immunoassay (◆) and the noncompetitive immunoassay (□) for BRCA1. The concentrations of immunoreactive BRCA1 are in arbitrary units per liter (fractions were diluted  $\times 500$ ) for the non-competitive assay and in ng/mL equivalents of unlabeled C-20 peptide (used for calibration) in the competitive assay. Numbers and arrows on top indicate the molecular weight of eluted standard proteins. BRCA1 immunoreactivity elutes at a molecular weight between 660 and 160 KDa.

- linkage analysis in familial breast and ovarian cancer. Results from 214 families. *Am J Hum Genet* 1993; **52**: 678-701.
7. Von Arnim AG, Deng XW. Ring finger motif of *Arabidopsis thaliana* COPI defines a new class of zinc-binding domain. *J Biol Chem* 1993; **268**: 19626-19631.
  8. Lovering R, Hanson IM, Borden KLB, *et al.* Identification and preliminary characterization of a protein motif related to the zinc finger. *Proc Natl Acad Sci USA* 1993; **90**: 2112-2116.
  9. Goldgar DE, Reilly PR. A common BRCA-1 mutation in the Ashkenazim. *Nat Gen* 1995; **11**: 113-4.
  10. Struwing JP, Abeliovich D, Peretz T, *et al.* The carrier frequency of the BRCA-1 185delAG mutation is approximately 1% in Ashkenazi Jewish individuals. *Nat Gen* 1995; **11**: 198-200.
  11. Jensen RA, Thompson ME, Jetton TL, *et al.* BRCA1 is secreted and exhibits properties of a granin. *Nat Gen* 1996; **12**: 303-308.
  12. Holt JT, Thompson ME, Szabo C, *et al.* Growth retardation and tumour inhibition by BRCA1. *Nat Gen* 1996; **12**: 298-302.
  13. Bradley A, Sharan SK. BRCA-1 products: antibody specificity, functional motifs and secreted tumor suppressors. *Nat Gen* 1996; **13**: 268-269.
  14. Chen Y, Chen CF, Riley DJ, *et al.* Aberrant subcellular localization of BRCA-1 in breast cancer. *Science* 1995; **270**: 789-791.
  15. Scully R, Ganesan S, Brown M, *et al.* Location of BRCA1 in human breast and ovarian cancer cells. *Science* 1996; **272**: 123-125.
  16. Anonymous. Meeting to sort out BRCA-1 confusion. *Science* 1996; **272**: 799.
  17. Various authors. *Nat Gen* 1996; **13**: 264-272.
  18. Steeg PS. Granin expectations in breast cancer? *Nat Gen* 1996; **12**: 223-225.
  19. Diamandis EP, Christopoulos TK (Eds.) *Immunoassay*. San Diego, CA: Academic Press, 1996.
  20. Diamandis EP, Christopoulos TK. The biotin-(strept)avidin system: principles and applications in biotechnology. *Clin Chem* 1991; **37**: 625-636.
  21. Hassapoglidou S, Diamandis EP, Sutherland DJA. Quantification of p53 protein in tumor cell lines, breast tissue extracts and serum with time-resolved immunofluorometry. *Oncogene* 1993; **8**: 1501-1509.
  22. Christopoulos TK, Diamandis EP. Enzymatically amplified time-resolved fluorescence immunoassay with terbium chelates. *Anal Chem* 1992; **64**: 342-346.
  23. Diamandis EP, Yu H. New biological functions of prostate specific antigen? *J Clin Endocrinol Metab* 1995; **80**: 1515-1517.
  24. Sauter ER, Daly M, Lenahan K, *et al.* Prostate specific antigen in nipple aspirate fluid correlate with breast cancer risk. *Cancer Epidemiol Biomarkers Prev* 1996; **5**: 967-970.
  25. Yu H, Giai M, Diamandis EP, *et al.* Prostate specific antigen is a new favourable prognostic indicator for women with breast cancer. *Cancer Res* 1995; **55**: 2104-2110.
  26. Diamandis EP. BRCA1 protein products: antibody specificity, functional motifs and secreted tumour suppressors. *Nat Gen* 1996; **13**: 268.