CANCER GENOMICS & PROTEOMICS *2*: 1-24 (2005)

Genomics, Proteomics and Cancer: Specific Ribosomal, Mitochondrial, and Tumor Reactive Proteins Can Be Used as Biomarkers for Early Detection of Breast Cancer in Serum

J. ALBERTO FERNANDEZ-POL[1,2,3], PAUL D. HAMILTON[1] and DENNIS J. KLOS[1]

[1]*Laboratory of Molecular Oncology, Department of Veterans Affairs Medical Center, St. Louis, MO 63106,*
[2]*Institute of Applied Neurociences, University of Buenos Aires, Facultad de Medicina, BsAs, Argentina, and*
[3]*Metalloproteomics, LLC, Chesterfield, MO 63017, U.S.A.*

Abstract. *We have used genomics and proteomics based technologies to study tissue and serum protein profiles in patients with breast cancer (BC) in comparison to control healthy subjects. One critical objective of this study was to identify and characterize new tissue and serum biomarkers of BC using differential screening of a recombinant cDNA human BC expression library. A second major objective of this study was to evaluate the clinical utility of Metallolpanstimulin (MPS-1/S27 ribosomal) protein as a biomarker for the early detection and monitoring of BC by immunoassay measurements of serum MPS-1 protein levels and to identify MPS-1 protein in sera of BC patients. A third objective was to present data on cloned BC genes denoted protein subgroup-30 (PS-30), consisting of mitochondria, nuclear, and ribosomal proteins which are increased after growth factor stimulation of BC cells in tissue culture. To study in detail MPS-1 protein expression in BC, the MPS-1 concentrations were determined in the blood of 117 females free of any disease, and in 203 female patients diagnosed with primary BC. The results indicate that increased serum MPS-1 levels can be used for the early detection of BC. Normal subjects have low concentrations of MPS-1 protein in sera. Moreover, changes in MPS-1 protein serum levels can be used for the study of BC progression or regression after various types of therapy. In both the low and high value range, MPS-1 is 10-fold more effective than CA-15-3 in modifying the probability of the target condition –breast cancer. The use of HPLC, Western blot, Immuno-Mass Spectrometry, and protein sequencing confirmed the presence of authentic MPS-1 in sera of patients with BC. Negligible levels of MPS-1 protein were detected in sera from normal subjects. We conclude that (1) the increase in serum MPS-1 can be used for the early detection of BC; and (2) MPS-1 proved to be reliable in the follow-up of patients with advanced BC as demonstrated by the close correlation between MPS-1 protein levels and BC progression or regression after various types of therapy. Furthermore, all proteins denoted group -30 (Mr <30,000), consisting of ribosomal, nuclear and mitochondria proteins, were found to be significantly increased in BC tissues in comparison to control tissues, suggesting that these proteins may be useful markers for detection of BC. Finally, several serum reactive proteins such as haptoglobin and C3 complement components provided valuable information on oncogenic activity in BC patients.*

Genomics and Proteomics based technologies have been developed that allow the study of tissue and serum protein profiles in patients with cancer in comparison to control healthy subjects. Here we report the use of these methods to distinguish cancer from control data sets and to identify specific proteomic patterns of potential biomarkers produced or associated with Breast Cancer (BC).

Breast cancer is an increasing medical problem, as demonstrated by its high incidence in numerous countries around the world (1-7). In the USA, breast cancer is the most common female neoplasm and the second most common cause of cancer death (1,2). It is estimated that approximately 186,000 new cases of invasive breast cancer in women will be diagnosed in the USA in 2003 (3). Approximately 46,000 women will die of breast cancer each year. One out of 10 women will suffer from the disease (1,2). For women ages 15 to 54 years, breast cancer is the most prevalent cause of cancer-related death (1-3,7). The incidence rate for female breast cancer has been increasing

Abbreviations: MPS-1,metallopanstimulin/S27 ribosomal protein, H, heat-denatured proteins; HPLC, high pressure liquid chormatogaphy; MS, mass spectroscopy, BC, breast cancer.

Correspondence to: Dr. J. A. Fernandez-Pol, Metalloproteomics, LLC., 437 Hunters Hill Dr, Chesterfield, Missouri 63017-3446, U.S.A. Tel/Fax: (314) 275-7856, e-mail: fernandezpol@earthlink.net

Key Words: Breast cancer, metallopanstimulin, biomarkers.

steadily since the 1960s at approximately 1.5% a year in the USA (1-3). Currently, the majority of cases of breast carcinoma are detected at a time when the tumor has extended beyond the perimeter of the gland, making it incurable (2,3,7).

Breast cancer research has focused on methods to detect breast cancer at its earlier stages, to cure the disease after diagnosis (3,5,6). At present, imaging techniques which exploit the different physical properties of the cancer and normal or non-malignant surrounding tissues are used in the early detection of breast cancer (3,4,7). Imaging techniques are the most efficient way to reduce cancer-related mortality (3,7). For breast cancer screening, mammography is the most effective technology presently available (7). MRI, ultrasound, digital mammography and nuclear medicine techniques are currently used in selected populations. Imaging is also being used as a guide for biopsy. However, the majority of women in the USA (80%) who undergo breast biopsies do not have cancer, indicating the imprecision of imaging techniques (7). In spite of advances in these areas, one third of women who develop breast cancer still die of the disease (7).

Recognition of the limitations of current imaging technologies for the early diagnosis and screening of breast cancer has led to the evaluation of new methods of cancer detection using tumor marker assays (8,9). Thus, a number of laboratories are exploring the possibility of early breast cancer detection by measuring biochemical products (antigens) in the blood, urine, or nipple aspirates (8-10). Genetic testing, a prospective technique, is also being used in women who are at risk for breast cancer (5). More recently, the use of proteomic analysis provides unique information which may be useful for early detection of breast cancer. Because of the high incidence of breast cancer, even small improvements in early detection and treatment may represent a large number of lives saved or prolonged (7).

Tumor markers in bodily fluids are substances secreted or shed by cancer cells or produced by the host cells as a reaction to neoplastic cells (8,9,10). The development of a sensitive and specific test (s) for the early detection of breast cancer has been the focus of a number of laboratories for the past 30 years (8,9). Although tumor markers have enhanced the understanding of the molecular biology and natural history of breast cancer (4,7), the elusive goal of early detection of breast cancer by using tumor-associated antigens in blood has not yet been achieved (8-10). It is thought that early detection of tumor-associated antigens by biochemical means will have a significant impact in the management and outcome of the disease (8-10).

There are a number of biomarker tests for breast cancer which utilize monoclonal antibodies directed against specific breast cancer-associated antigens and which are able to detect these antigens in serum (8-23). The present clinical utility of these markers is, however, limited to the detection of advanced metastatic cancer and in the monitoring of patients with advanced disease (8-23). For example, for more than 20 years the CA 15-3 assay, which measures a mucin (denoted MUC-1) antigen expressed in late breast cancer, has been one of the most valuable tumor marker tests in the staging and monitoring of breast carcinoma (10-23). More recently, the BR27.29 assay which measures the same MUC-1 antigen detected by the CA 15-3 assay, although by means of a different epitope, has also been successfully used for the same purpose (15). The use of the BR27.29 assay is particularly useful to monitor effectiveness of therapy. However as it measures the same antigen as the CA 15-3 assay, it is also ineffective in the early detection of breast cancer (15). These and other data indicate that new biomarkers are needed for the early detection of breast cancer.

The experimental tumor marker test of the present paper evolved from the study of a gene denoted Metallopanstimulin-1 (MPS-1)/S27 ribosomal zinc finger protein, which encodes a multifunctional protein involved in protein synthesis, deletion of mutated mRNAs, DNA repair, ribotoxic responses, and oncogenesis (24-33,35). MPS-1 was identified, cloned and characterized from a cDNA library constructed from a human mammary carcinoma cell line (MDA-MB-468) that was stimulated by epidermal growth factor (EGF) and transforming growth factor (TGF beta) (29). The MPS-1 has been found in high concentrations in cells that are actively dividing (28,29). When MPS-1 is overexpressed, it is secreted into the extracellular space (28). It has been shown that the MPS-1 DNA sequence and the protein can be used in diagnostic methods such as detection of malignant cells associated with numerous tumors (24-35). Subsequently, we found that sera from cancer patients contain the MPS-1 protein, which interact with specific anti-MPS-1 antibodies (25). Atsuta *et al.* have also identified MPS-1 as a tumor associated antigen in breast cancer, confirming our previous results (29) with a different cloning system (36). Recently, Sundblad *et al.* demonstrated a correlation between overexpression of MPS-1 and aggressive breast cancer growth (37).

One major objective of this study was to evaluate the clinical utility of Metallopanstimulin (MPS-1/S27) protein as a biomarker for the early detection and monitoring of breast cancer by immunoassay measurements of serum MPS-1 protein and the identification of MPS-1 protein in sera of breast cancer patients. In this paper, we present (1) the experimental clinical uses of the MPS-1 test for detection, monitoring and management of breast malignancy; (2) the comparison of the test properties of MPS-1, CA-15-3, and CEA for detection of breast cancer; and (3) the identification of MPS-1 in breast cancer sera by

HPLC, Western blot, Mass Spectroscopy, and protein sequencing. The results suggest that this molecular marker may be potentially useful to detect early breast cancer.

A second objective was to screen the same cDNA library from which the MPS-1 gene was isolated, to identify and characterize a group of genes differentially expressed in BC after growth factor stimulation. We found that there are 33 distinct genes encoding for proteins of <30 kDa that are differentially expressed in BC cells after growth factor stimulation. The results suggest that these proteins may be useful to determine the presence or absence of carcinogenic processes in BC. Finally, a third objective was to identify specific tumor-reactive proteins in the sera of BC patients.

Materials and Methods

Clinical specimens. This study included 117 healthy control female subjects who did not have evidence for breast cancer or other malignancy, for the purpose of establishing the reference range for the MPS-1 test in normal women. Patients included 203 women with primary breast cancer (23 % intraductal and 77% intraductal and infiltrative adenocarcinomas) staged according to TNM classification. Furthermore, 40 women with benign breast disease were also studied. Five pregnant women were also included in this study. Male (164 patients) and female (9 patients) subjects with other common neoplastic diseases (ovarian, gastric, lung, prostate and colon cancers) were included for reference purposes. An additional, 40 patients with breast cancer were followed longitudinally for up to 2-3 years to evaluate the efficacy of the marker in monitoring clinical course after chemotherapy. The study was retrospective. The diagnosis for all normal subjects and patients was based on clinical history, containing both laboratory and radiologic information. The groups with disease present had histopathologic information, stage, treatment, and clinical status. Response to therapy was confirmed by nuclear medicine scans and computerized tomography. The age range for all normal subjects and patients studied was 19 to 84 years (Average age 56.33±19 years). All the tumor marker measurements for each individual subject were performed by using aliquots from the same serum sample.

The serum samples corresponding to normal females, and breast cancer patients were obtained from reference laboratories (Bioclinical Partners, MA; Nichols laboratories, CA). In addition, separate serum samples from male and females (normals, patients with non-malignant diseases and other types of cancers), were used to evaluate the performance characteristics of the MPS-1 test. These samples were discarded serum samples obtained at the Immunoassay laboratory, DVA Medical Center. The use of residual blood samples was approved by the Human Studies Subcommittee, DVA Medical center, as "Research Exempt from IRB Review".

Tumor marker tests

MPS-H serum assay. The MPS-H serum radioimmunoassay (RIA) was performed after heat(H)-denaturing serum samples to exposed the N-terminus of MPS-1 (25,27,28). The polyclonal antibody used in this study, denoted anti-MPS-N, is directed towards a unique site on the N-terminus of MPS-1 (amino acids 2-17) (25,28,29). The peptide antisera against the amino terminus of the MPS-1 protein have been prepared as follows. One sequence, PLAKDLLHPSPEEEKR, corresponding to MPS-1 amino acid residues 2-17, was designated as the N-terminus peptide (28,29). It was derived from the N-terminal region of the protein, located between the N-terminus and the zinc finger domain (28,29). The N-terminal peptide was selected so that they would not contain any portion of the zinc finger domain of the MPS-1 protein , because the zinc finger domain is a highly conserved structure and antisera against it might cross-react with other zinc finger proteins. Indeed, we found that antibodies generated against the zinc-finger domain of MPS-1 (amino acids KITTVFSHAQTVVL) cross-reacts with other zinc finger proteins and cannot be use in RIA procedures (data not shown). For quality control, the N-terminus peptide was subjected to mass spectroscopy (Figure 8). The production of anti-peptide polyclonal antibodies is described in detailed elsewhere (28). The IgG antibodies were purified by affinity chromatography on Affi-Gel-Protein A agarose (28). Specificity of the IgG antibodies was determined by peptide-anti-peptide antibody neutralization studies using western blot analysis of human recombinant MPS-1 protein (28).

The target serum MPS-1 protein was released from the carrier proteins, by adding a calcium and magnesium-free buffered diluent (phosphate buffered saline, pH 7.3, 1:3 dilution) to the sample and then heating the sample (100˚C) under controlled conditions (3 minutes) to denature the MPS-1 protein so the N-terminus is exposed. The efficacy of this step to detect MPS-1 was previously determined with recombinant MPS-1 by SDS-PAGE, with and without heating (3 min, 100˚C), under non-denaturing and denaturing conditions and Western blot analysis (28,29).

Quality control. Quality control for MPS-H RIA was done following the NCCLS recommendations (38). Each patient sample was measured in duplicate as were all blanks, controls, and standards. All high serum samples were diluted (25,27).

CA 15-3 serum assay. CA 15-3 was measured by a competitive inhibition RIA (Centocor, Malvern, PA). The CA 15-3 RIA is a test for the quantitative determination of MUC-1 mucin in serum of patients previously treated for stage II or III breast cancer. The MUC-1 molecule is a glycoprotein (10,11). All high CA 15-3 serum samples were diluted. The College of American Pathologists Ligand Assay Survey recommendations for quality control were followed (38). The upper limit of normal (40 U/mL) for CA 15-3 was determined in this study from the mean value plus 3 standard deviations for 117 apparently healthy female subjects.

Carcinoembryonic antigen assay(CEA). CEA was measured by the Abbot CEA assay (Abbot, Abbott Park, IL). The upper limit of normal (10 U/mL) for CEA (22) was also determined in this study from the mean value plus 3 standard deviations for 117 apparently healthy female subjects.

Method of statistical analysis for comparison of test properties of serum MPS-H, CA 15-3, and CEA. The statistical procedures utilized for this analysis were performed as described by Sackett *et al.*, (39) and Jaeschke *et al.* (40). A total of 320 subjects, 203 with known breast cancer, and 117 apparently normal, age-matched subjects were evaluated. The number and proportion of patients in each group, disease (breast cancer) *Present*, and disease (breast cancer) *Absent* were determined for various value ranges of MPS-H, CA-15-3 and CEA. The true positive (TP) ratio is defined as

the proportion of patients with disease in each test value range that is the number in each range divided by the total in the disease *Present* group. The false positive (FP) ratio is defined as the proportion of patients without disease in each test value range that is the number in each range divided by the total in the disease *Absent* group. The likelihood ratio is defined as the TP ratio divided by the FP ratio, or sensitivity divided by 1 minus specificity. The TP ratio (sensitivity), FP ratio (1-specificity) and likelihood ratio (LR) were calculated for each value range of MPS-H, CA-15-3 and CEA shown. Operating cut-off values for positive MPS-H, CA-15-3 and CEA test results, which represent a positive test result for disease presence were 20, 40 and 10 Units/mL, respectively.

Identification of serum MPS-1 protein by HPLC, Western blot analysis, and sequencing. Identification of serum MPS-1 was achieved by heat-denaturing pooled metastatic breast cancer sera (3 min; 1:3 dilution), desalting the sera in a G-150 gel filtration column, with the subsequent application of HPLC and Western blot analysis as described in (28). Chromatography was performed using a Waters HPLC Protein PAK DEAE 5P 088044 column according to manufacture's specifications. Western blot analysis was done using the same anti-peptide antibodies (N-terminus of MPS-1; amino acids 2-17) that were used in all RIA procedures reported in this paper. The standard PVDF protein transfer membrane procedure was used to prepare the serum MPS-1 protein band for sequencing. Sequencing was performed at the Protein Chemistry Laboratory, Washington University School of Medicine, St. Louis, MO.

Identification of MPS-1 protein by Mass Spectroscopy (MS). Mass spectroscopic analyses were used to identify in serum samples chemically or heat-denatured MPS-1 proteins. Ionized proteins were detected and their mass accurately determined by Time-of-Flight Mass Spectrometry (TOFMS). Samples were run multiple times.

Solid-phase immunoassay and Mass Spectroscopy. For the solid-phase immunossay, the MPS-1 or structurally related molecules from heat-denatured human sera were dissociated from any material to which they may be bound in the serum as described elsewhere (Fernandez-Pol, US patents Re.35,585 (1997), 5,668,016 (1997), 5,668,01, 5,955,287 (1999), and 6,589,753 (2003). The serum MPS-1 was bound to an absorbent paper support containing covalently bound rabbit polyclonal anti-MPS-1 IgG antibodies. After washing the paper strip to remove the non-specifically bound materials, the specifically bound MPS-1 protein was eluted from the absorbent support utilizing a chaotropic agent. The eluted MPS-1 was detected and measured by mass spectrometry, micro-array proteomics, and immunological methods.

Preparation of cDNA clones and Sequencing. DNA clones were retrieved from storage at -70˚C. These clones were from a differential hybridization experiment done with the MDA-468 library. They were inserted in the pcDNAII plasmid which confers ampicillin resistance. These clones were grown overnight by streaking on LB agar plates plus 50 μg/mL Ampicillin. Individual colonies were then chosen and grown overnight in 5 mL of LB broth plus 50 μg/mL Ampicillin. Chart 1 and the adjacent Table VII, show the clones analyzed and the results obtained, respectively.

Plasmid DNA was isolated using a Qiagen mini plasmid kit (Qiagen, Valencia, CA). Plasmid DNA was resuspended in 25 μL of TE buffer, pH 8.0, and 1.0 or 0.5 μL of plasmid per lane was then run on a 0.8 agarose mini-gel at 60V in TBE buffer for 2 hours, stained with ethidium bromide, and then analyzed using an ITTI video analysis system. A 1 Kb DNA ladder was included at 2 μg/lane (Chart 1). The 1.6 Kb band is present at 10% of the total DNA. It was set at 200 ng and the plasmid DNA was quantitated against it. The concentration of the plasmid DNA was adjusted to 100 ng/mL, and 32 μL of each plasmid was sent to Research Genetics (Hunsville, AL) for sequencing.

Results

Performance characteristics of the MPS-H assay. The normal reference ranges for the MPS-H test were established from data presented in Table I. The mean value for MPS-H in females was 8.63±4.23 SD with the upper limit of normal established as 21.33 (mean plus 3 SD). Table I shows that there is no significant difference in MPS-H levels between healthy male and female subjects. The results of studies with healthy female subjects indicate an MPS-H reference range of non-detectable to 15 ng/mL (90% of the healthy population). Six percent of the subjects in this group had MPS-H levels in the range of 16-20 ng/mL. In our series of 117 healthy female individuals, only two or 1.7% of the subjects showed MPS-H levels >20 ng/mL (Table V).

The reliability of the MPS-H RIA procedure was evaluated by examining the reproducibility of measurements on selected samples that represent the range of values found in heat-denatured human sera, with particular reference to the low end of the RIA curve (10-20 ng/mL). Data for intra- and inter-assay precision are presented in Table II. Intraassay precision (within run) was determined by assaying samples containing various concentrations of MPS-H immunoreactive control sera. Each sample was assayed 20 times in a single assay. The intraassay coefficient of variation was 6.8 to 12.6%. Interassay precision (between run) was determined by duplicate measurements of low, medium, high and very high level pooled patient samples in a series of 10 individual assays. The intraassay coefficient of variation was 9.6 to 12.4%. The coefficients of variations for the low level control showed a higher variability in the inter-assay variation (12.4%) compared with the intra-assay variation (11.3%). Overall, the precision data reflects good duplications for a non-automated assay.

Sensitivity and specificity of the THE MPS-H assay. Table III shows the results for the number and percent of breast cancer patients with elevated MPS-H levels. These studies demonstrated that the majority (91% or 185/203) of the active primary breast cancers were detected, when a cutoff value of >15 ng/mL MPS-H was used. Thus, in patients

Table I. *Normal reference range for the MPS-H assay.*

Population	No.	MPS-H (ng/mL) Mean±SD	Range (Mean±3SD)
Females	117	8.63±4.23	21.33
Males	196	6.59±3.9	18.2
Total	313	7.6±4.0	19.8

Table II. *Precision data for the MPS-H assay.*

Intraassay precision (within run)				
	Low	Medium	High	Very high
Control serum samples	1	2	3	4
No. of replicates	20	20	20	20
Mean (ng/mL)	12.4	27.8	45.6	375
SD	1.4	3.5	3.9	25.6
CV (%)	11.3	12.6	8.5	6.8

Interassay precision (between run)				
	Low	Medium	High	Very high
Control serum samples	1	2	3	4
No. of assays	10	10	10	10
Mean (ng/mL)	11.3	25.1	47.1	459
SD	1.4	2.7	4.5	45
CV (%)	12.4	10.8	10	9.6

The samples were grouped into low (12.5 ng/mL), medium (25 ng/mL), high (50 ng/mL) and very high (400 ng/mL), and the coefficient of variation (CV) was determined within each of these groups after repeated assays.

Table III. *Abnormal MPS-H levels in breast cancer patients*

	MPS-H (ng/mL) Mean±SD	Number and percent of patients with abnormal MPS-H levels (>15 ng/mL)	
Benign breast disease	8.02±3.1	0 /20	0%
Primary breast cancer	95.1±96.1	185/203	91%
Stage 0 breast cancer	93.8±74.7	16/17	94%
Stage I breast cancer	62.8±76.5	42/53	79%
Stage II breast cancer	104.6±110	68/72	94%
Stage III breast cancer	94.2±82	23/24	95%
Stage IV breast cancer	124.3±100	37/37	100%

Table IV. *Abnormal MPS-H levels in common malignant diseases.*

Type of cancer		MPS-H (ng/mL) Mean±SD	Number and percent of patients with abnormal levels (>15 ng/mL)	
Ovarian		231±274	3./5	60%
Gastric		132±113	6./6	100%
Lung	T3,T4	117±255	12./12	100%
	M1	323±255	4./4	100%
Colon	T3,T4	39±18	17./17	100%
	M1	174±181	10./10	100%
Prostate	T3,T4	53±55	84/84	100%
	M1b	174±226	42/42	100%

Primary tumor (T): Stages T3 and T4; Distant metastasis: M1; Metastasis to bone: M1b.

suspected of having breast cancer, MPS-H elevations greater than 15 ng/mL may indicate the existence of an active oncogenic process (24,25). Importantly, none of the 20 patients with benign breast disease exhibited elevated MPS-H levels. Data for early stage primary breast cancer (stage 0- stage II) showed that 126 of 142 (88%) of these patients were detected. This data suggests that the MPS-H marker has the required sensitivity for the routine detection of early stage breast cancer. Data for late stage primary breast cancer (stage III-IV) show that 60 of 61 (98%) patients were detected. Results for abnormal MPS-H concentrations in women and men with other common malignant conditions are summarized in Table IV. The results show that the MPS-H test also detects other malignancies, demonstrating that the test lacks specificity for detection of breast cancer.

Comparison of test properties of MPS-H, CA 15-3, and CEA for breast cancer. Figure 1 shows the correlation of serum MPS-H and CA 15-3 levels in untreated patients with different stages of breast cancer. Figure 2 shows the MPS-H, CA 15-3, and CEA distribution in our study population of patients with breast cancer and normal subjects. The ability of MPS-H to identify patients with breast cancer compared to CA 15-3 and CEA is graphically characterized by the minimal overlap of MPS-H values in the two study populations (normal *versus* breast cancer) (Figure 2).

Table V compares the test properties of MPS-H, CA-15-3 and CEA for detection of breast cancer. When breast cancer is considered the target condition and only those patients with proven breast cancer are included in the disease *Present* group, patients with MPS-H values less than 10 are only 0.05 times as likely to have breast cancer as an individual with a value

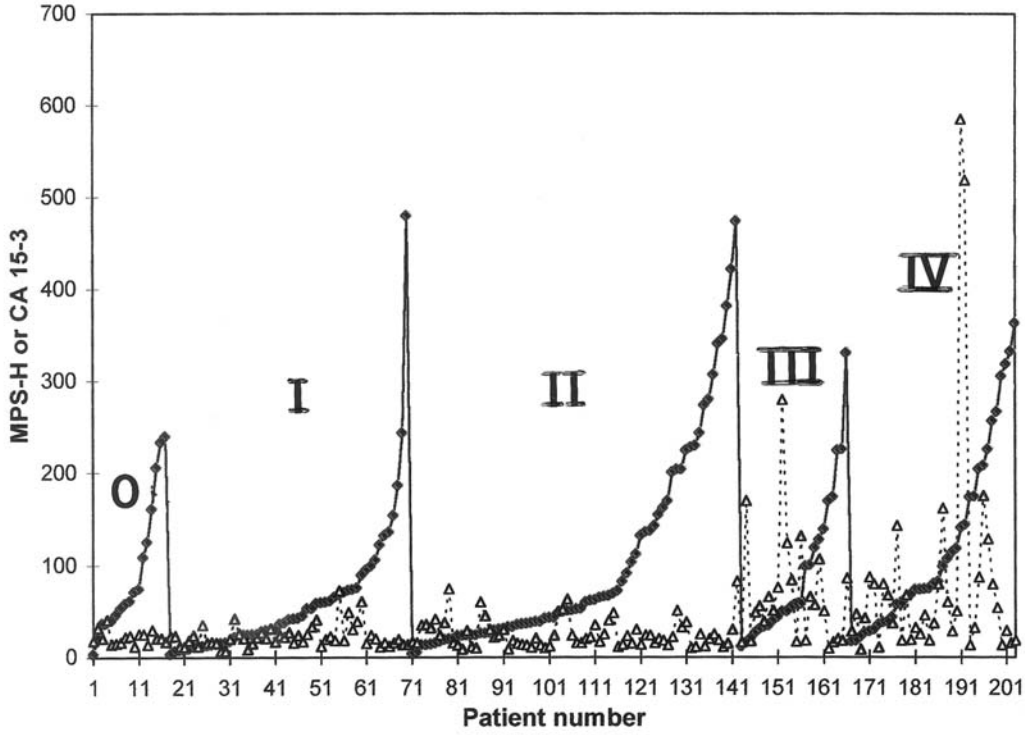


Figure 1. *Serum MPS-H and CA 15-3 levels in untreated patients with different stages of breast cancer. The MPS-H levels are organized in ascending order. The individual values plotted in the chart for each marker correspond to measurements done in the same patient sample. The vertical lines separate from left to right breast cancer stages 0, I, II, III and IV. Stage 0 (T_{is}, noninvasive carcinoma in situ); Stage I (T_1), tumor is < 2 cm; Stage II (T_2), tumor is >2 cm and <5 cm; Stage III (T_3, T_4), tumor is >5 cm; Stage IV (M_1), distant metastasis present. Ordinate, MPS-H (ng/mL) or CA 15-3 (Units/mL); Abscissa, patient number.*

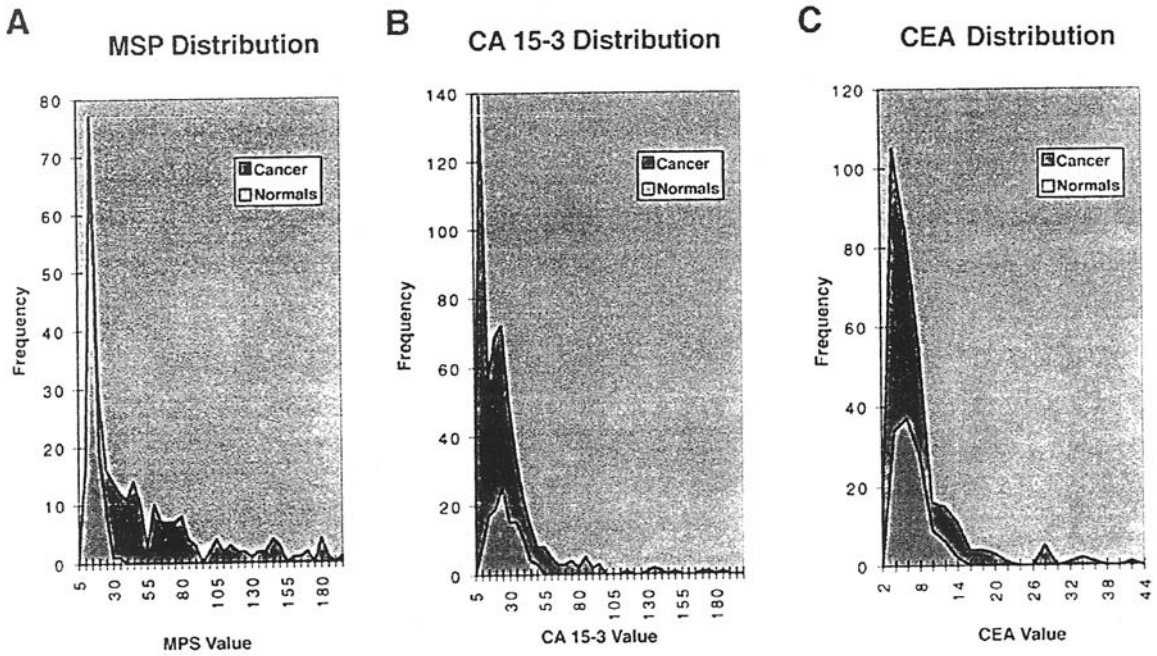


Figure 2. *MPS-H, CA 15-3, and CEA distribution in our study population of patients with breast cancer and normal subjects. The ability of MPS-H to distinguish breast cancer from normal subjects compared to CA 15-3 and CEA is characterized by the minimal overlap of MPS-H values in the two populations compared to the other two markers.*

Table V. *Comparison of test properties of MPS-H, CA-15-3, and CEA in breast cancer.*

MPS	Value range CA-15-3	CEA	Breast cancer Present No.	Proportion	Absent No.	Proportion	Likelihood ratio
0-5	-	-	2	2/203 = 0.009852217	22	22/117 = 0.188034188	0.05
6-10	-	-	6	6/203 = 0.02955665	72	72/117 = 0.615384615	0.05
11-15	-	-	10	10/203 = 0.049261084	14	14/117 = 0.11965812	0.41
16-20	-	-	11	11/203 = 0.054187192	7	7/117 = 0.05982906	0.91
20+	-	-	174	174/203 = 0.857142857	2	2/117 = 0.017094017	50.14
Total			203		117		

MPS	Value range CA-15-3	CEA	Breast cancer Present No.	Proportion	Absent No.	Proportion	Likelihood ratio
-	0-5	-	0	0/203 = 0	8	8/117 = 0.068376068	0.00
-	6-10	-	12	12/203 = 0.0591133	20	20/117 = 0.170940171	0.35
-	11-15	-	36	36/203 = 0.177339901	20	20/117 = 0.170940171	1.04
-	16-20	-	41	41/203 = 0.201970443	23	23/117 = 0.196581197	1.03
-	21-40	-	77	77/203 = 0.379310345	42	42/117 = 0.358974359	1.06
-	40+	-	37	37/203 = 0.18226601	4	4/117 = 0.034188034	5.33
Total			203		117		

MPS	Value range CA-15-3	CEA	Breast cancer Present No.	Proportion	Absent No.	Proportion	Likelihood ratio
-	-	0-2.5	103	103/203 = 0.507389163	47	47/117 = 0.401709402	1.26
-	-	2.6-5	48	48/203 = 0.236453202	52	52/117 = 0.444444444	0.53
-	-	6-10	24	24/203 = 0.118226601	16	16/117 = 0.136752137	0.86
-	-	10+	28	28/203 = 0.137931034	2	2/117 = 0.017094017	8.07
Total			203		117		

higher than 10. In contrast, patients with a MPS-H value greater than 20 are 50 times as likely to have breast cancer as a patient with a value lower than 20. A similar analysis for CA-15-3, and CEA reveals that the test properties for these two markers are not as valuable for detection of breast cancer as MPS-H. While CA-15-3 values below 5 are not associated with any false negatives, patients with CA-15-3 values above 40 are only 5.33 time as likely to have breast cancer as patients with values lower than 40. Performance characteristics for CEA are similarly poor in our study population (Table V).

The likelihood ratios indicate by how much a given diagnostic test result will raise or lower the pretest probability of the target disorder. There are several advantages for determination of likelihood ratios rather than sensitivity and specificity: 1) Likelihood ratios need not change with changes in prevalence or pretest probability; 2) Likelihood ratios can be calculated for multiple levels of the sign, symptom, or lab test result; and 3) They can be used to shorten the list of diagnostic hypotheses since the pretest odds for target disorder multiplied by the likelihood ratio = posttest odds for the target disorder. For example, if the pretest probability of breast cancer is low, moderate or high (0-20%, 21-80%, or 81-100%), the post-test odds can be determined by multiplying the pretest odds by the likelihood ratio. In our data, a patient with a low pretest probability (*e.g.* 20% and pretest odds of 0.25), and a MPS-H value of less than 10 would have a post-test odds of 0.25 x 0.05 or 0.0125. This converts to a posttest probability of 1.0%. The posttest minus pretest probability difference is 19%. Similarly, a patient with a moderate pretest probability (*e.g.* 40% and pretest odds of 0.66), and a MPS-H value greater than 20 would have a post-test odds of 0.66 x 50.1 or 33. This converts to a posttest probability of 97%. The posttest minus pretest probability difference is 57%.

Other posttest minus pretest probability differences can be computed for varying levels of pretest probability and likelihood ratios that depend on the value range of the patient's test result. In general, likelihood ratios greater than 10 or less than 0.1 generate large and often conclusive changes from pretest to posttest probability (40). Likelihood ratios of 5-10 and 0.1 to 0.2 generate moderate shifts in pretest to posttest probability. Likelihood ratios of 2 to 5 and 0.5 to 0.2 generate small changes in probability. Based on these considerations, only MPS-H would appear to be effective in modifying the probability of the target condition - breast cancer in both the low and high value range.

Correlation of clinical course with tumor markers. Twenty patients were followed sequentially to determine the correlation between tumor marker data and clinical course of the disease (Figure 3). Clinical evaluation was established by objective clinical data and tumor marker levels corresponding to initial and final measurement. An increase or decrease over 20% in tumor marker levels was considered to be significantly correlated with the therapeutic response ($p<0.01$). The data demonstrates that MPS-H levels accurately indicated disease regression in 4 of 4 cases, disease stability in 6 of 6 cases, and disease progression in 10 of 10 cases (Figure 3). In contrast, CA 15-3 levels increased in 2 of 4 cases with disease regression; increased significantly in 3 of 6 cases with stable disease; and remained unchanged or decreased in 6 of 10 cases with disease progression (Figure 3). This demonstrates a significant advantage of MPS-H over CA 15-3 (100% *versus* 55% concordance, respectively) in detecting activity of the oncogenic process. In all cases studied, changes in MPS-H levels, as determined by the slope of the curves, were much faster than changes in CA 15-3 levels. Thus, the detection of regression or progression is more efficient with MPS-H than with the CA 15-3 tumor marker.

Longitudinal studies. MPS-H and CA 15-3 were used to serially monitor patients in a longitudinal study which included 20 patients. Two representative examples from this group are presented to illustrate the potential of the MPS-H test in following the course of breast cancer patients.

- Increased levels of MPS-H antigen indicate cancer progression. Figure 4A shows an example of changes in MPS-H serum antigen(s) in a postmenopausal patient at high risk for recurrence. This 55-year old patient presented with poorly differentiated adenocarcinoma stage III, grade 3, estrogen receptor negative, with axillary node metastasis. Baseline levels for tumor markers were not available at the time of biopsy. Figure 4A shows that one year later (after biopsy and initial therapy), MPS-H was highly abnormal (74.5 ng/mL, day 1; or >50-fold likelihood ratio for "*cancer present*", Table V) indicating cancer progression. Chemotherapy (Adriamycin) was initiated at day 1 (Figure 4A). Subsequently, the levels of MPS-H continue to increase (Figure 4A), indicating progression of the disease after ineffective therapy. Bone metastasis (vertebral bodies, coxa) were documented 9 months after initiation of chemotherapy at day 1 (Figure 4A). In this case, MPS-H provided an advance warning of impending metastatic disease 9 months before bone metastasis were confirmed by standard procedures. Although CA 15-3 increased progressively during the time period studied (Figure 4A), it did not provide early indication of recurrent disease as demonstrated by the values at days 1 and 66 which were within normal limits; the value at day 189 for CA 15-3 (54 U/mL) was scarcely higher than normal (40 U/mL). Thus, the rate of increase in MPS-H levels was much faster than the increase in CA-15-3, demonstrating the usefulness of MPS-H in predicting early cancer recurrence.
- Decreased levels of MPS-H antigen indicate cancer remission. Figure 4B illustrates the decrease in MPS-H and CA 15-3 levels in a 49-year old postmenopausal patient with primary breast cancer successfully treated by chemotherapy and adjuvant therapy after recurrence. The patient was diagnosed with stage III, grade 2, primary breast cancer designated as poorly differentiated, estrogen receptor positive, infiltrating ductal adenocarcinoma with lymphatic metastasis. At the time of diagnosis (Day 1), the patient was treated with mastectomy, chemotherapy and adjuvant therapy (Tamoxifen). Baseline levels for tumor markers were not available at the time of biopsy (Day 1). Figure 4B shows that 714 days after the initial treatment, MPS-H was highly abnormal (100 ng/mL at day 714; or >50-fold likelihood ratio for "*cancer present*", Table V) indicating cancer recurrence. Clinical evaluation demonstrated metastatic disease to the bones. After chemotherapy (cytoxan and 5-flurouracil), initiated at day 714, the MPS-H levels decreased progressively from 100 ng/mL (day 714) to 31.2 ng/mL (day 939) after treatment (Figure 4B), which correlated with clinical evidence of successful therapy. The CA 15-3 also progressively decreased but at a much slower rate than MPS-H (Figure 4B). The patient showed improvement in metastatic disease and the lower tumor marker values were entirely consistent with the clinical findings. However, the abnormal levels of both MPS-H (31.2 ng/mL) and CA 15-3 (56 ng/mL) at the time of the last measurements (Figure 4B, day 939), suggest residual active cancer disease after chemotherapy.

In summary, the results presented in Figure 4A and B, indicate that a persistent elevation in circulating MPS-H levels following treatment or increase in an otherwise lower level is strongly suggestive of recurrent or residual cancer and poor therapeutic response. A declining MPS-H value is generally indicative of a good response to treatment and a more favorable clinical outcome.

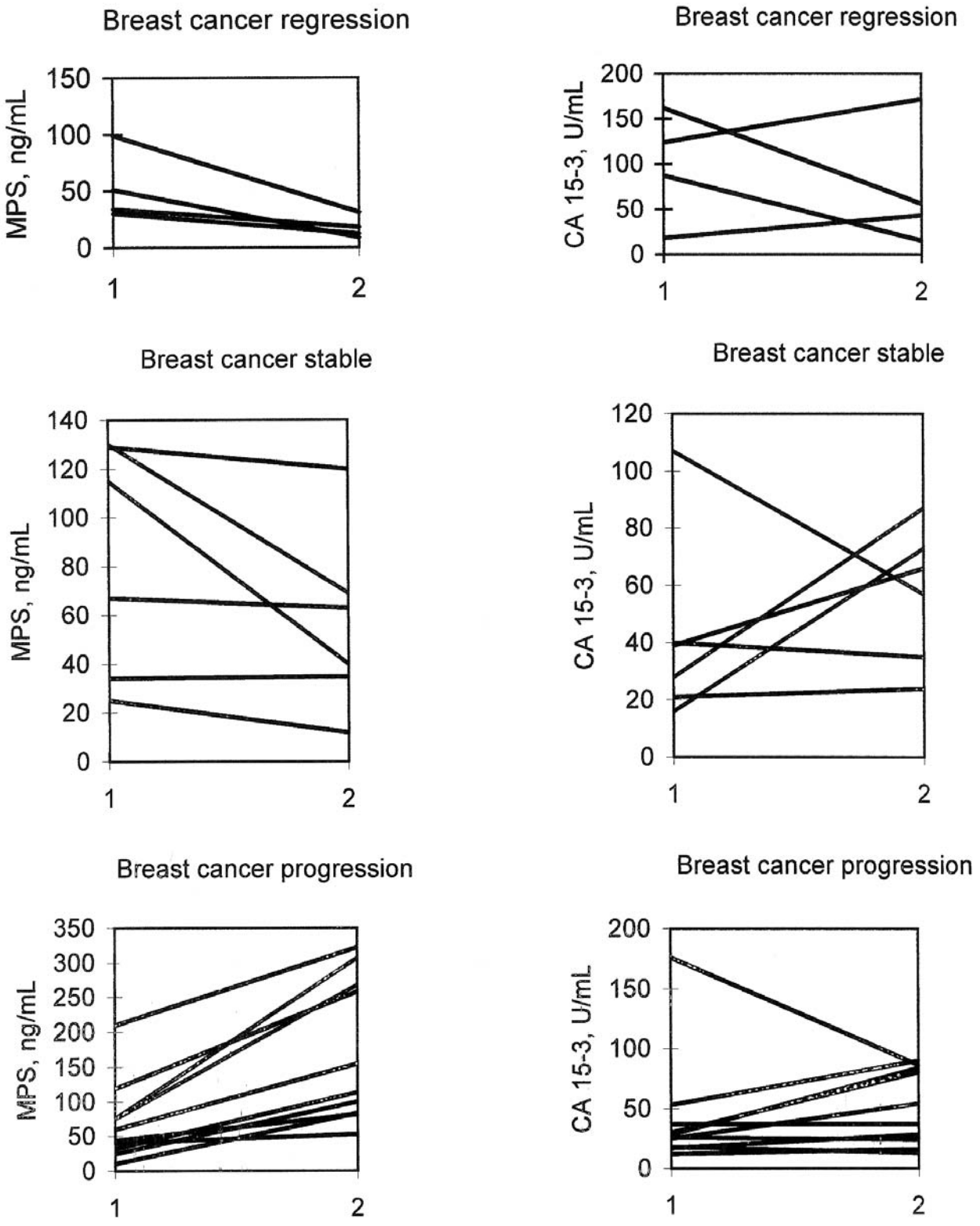


Figure 3. *Initial and final (4 months) measurements of MPS-H and CA 15-3 in patients with breast cancer. The ability of MPS-H to detect regression, progression, or stable disease is characterized by congruent changes in MPS-H levels. (1) initial measurement; (2) final measurement.*

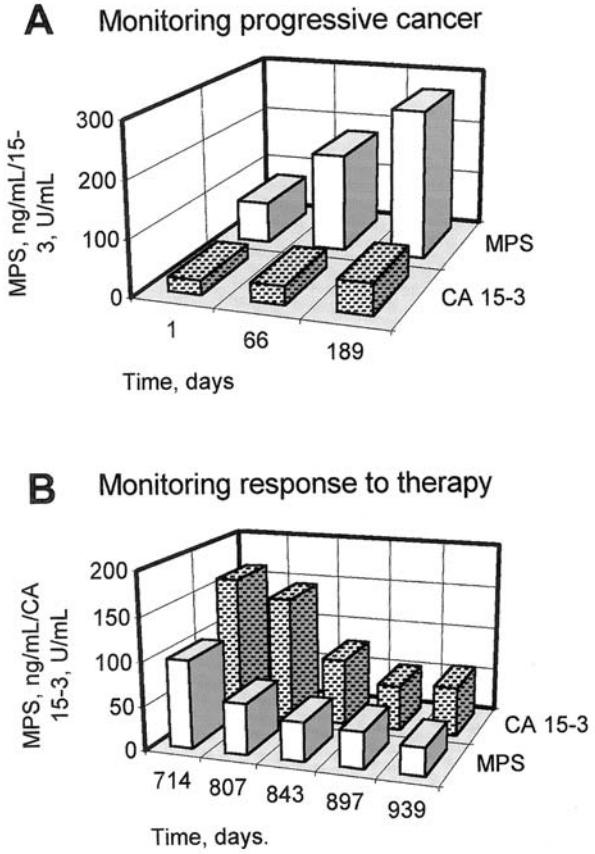


Figure 4. *Monitoring breast cancer progression and response to therapy using MPS-H and CA 15-3 tumor markers. (A) Recurrence after therapy correlates with a progressive increase in MPS-H levels in a patient with metastatic breast carcinoma. Note the faster increase in MPS-H levels in comparison to CA 15-3 levels. (B) Successful therapy correlates with a progressive decline in MPS-H levels in a patient with metastatic breast carcinoma. Note the faster decline in MPS-H levels in comparison to CA 15-3 levels.*

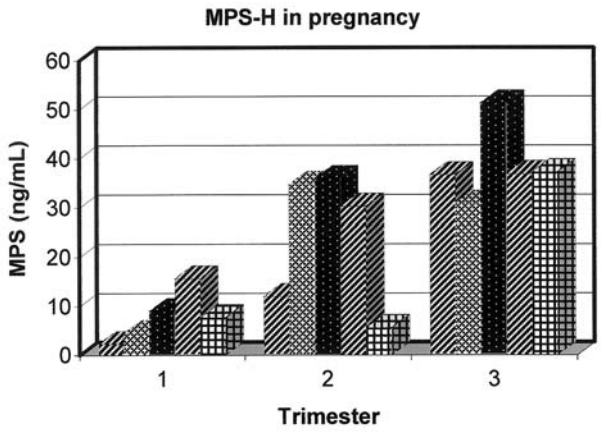


Figure 5. *Increased MPS-H levels in pregnancy. All five subjects studied had increased MPS-H levels by the 3rd trimester.*

False positive results during pregnancy. Because the MPS-1 protein is produced by epithelial cell of the rat embryo and it is also synthesized by the invading trophoblastic cells of the developing human placenta (24), the MPS-H protein is present in the sera of pregnant women. Figure 5 shows that the MPS-H levels were normal (<15 ng/mL) in 5 of 5 (100%) women during the first trimester of pregnancy. Data for the second trimester show that 3 of 5 pregnant women exhibited elevated MPS-H levels (> 30 ng/L). Whereas data for the third trimester show that all women exhibited elevated MPS-H levels (Figure 5). Thus, the MPS-H test cannot be used to detect breast cancer in pregnant women.

False positive and false negative results in various pathological conditions. A total of 120 separate patients with various extreme non-malignant conditions were studied to determine the reliability of the test under those conditions. As with any other medical test that determines the concentration of proteins in the human serum, severe/extreme cases of organ failure such as liver, kidneys, massive brain damage, massive muscle damage, septicemia and in terminal patients, the MPS-H test can show falsely positive or negative results (data not shown). For example, acute or chronic renal insufficiency may impair the normal excretion mode of MPS-H and lead to false-positive results (data not shown).

Identification of MPS-1 in sera of patients with metastatic breast cancer. Analysis were performed to determine if the MPS-H immunoreactive material present in cancer patient sera contained authentic MPS-1 protein. The pooled sera from metastatic breast cancer patients showing high levels of MPS-H (>100 ng/mL) by RIA, were analyzed by HPLC and Western blot analysis (Figure 6). The heat-denatured serum fractions (void volume) from the gel filtration step were reconstituted in 0.1% TFA in water and chromatographed on a DEAE HPLC column using a linear gradient of NaCl (0-0,8M) (Figure 6). RIA analysis of all column fractions showed that only fraction 40 contained a peak of MPS-H immunoreactive material (180 ng/mL) (Figure 6). Western blot analysis of aliquots of fraction 40 with anti-peptide N-terminus antibodies identified one band of Mr =10,000 under reducing conditions (Figure 6, lane A, arrow, peak 4). Aliquots of fraction 40 were electrophoresed, transferred to PVDF protein sequencing membrane, and the protein corresponding to the Mr =10,000 was extracted and sequenced in the first 12 amino acids. The results showed that the protein of Mr =10,000 (Figure 6, Lane A, peak 4, arrow) was identical to human recombinant MPS-1. Thus, MPS-1 protein of Mr =10,000

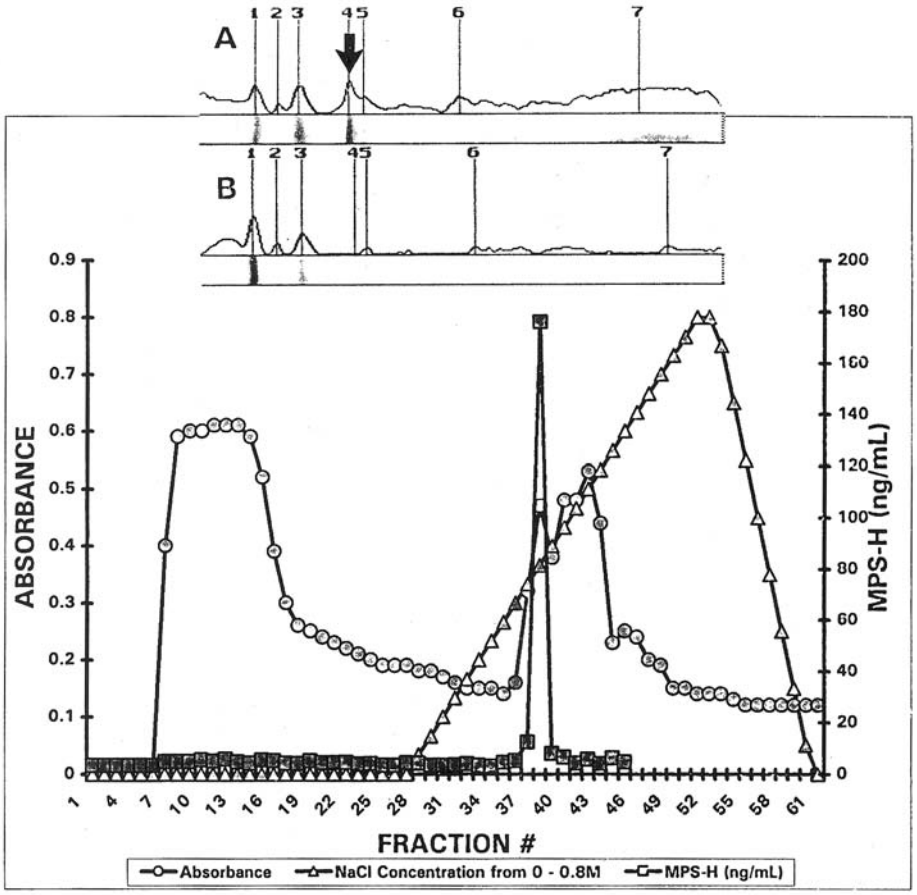


Figure 6. *Identification of MPS-1 in Sera of Metastatic Breast Cancer Patients by HPLC and Western blot analysis. The tracings show the results for HPLC. After SDS-PAGE and transfer, Western blot analysis was performed with anti-MPS-N antibodies. The pooled MPS-H lyophilized column fractions from the previous purification step (gel filtration, void volume) were reconstituted in 2 mL of 0.1 TFA. Then, the sample was chromatographed on a DEAE HPLC column. The sample was eluted with a linear gradient of 0 to 0.8 M NaCl at a flow rate of 0.8 mL/min. Then, fractions were collected, lyophilized and reconstituted; aliquots from each fraction were subjected to RIA to detect MPS-H. Inset, top: Western blot analysis of fraction 40, which contained the peak of MPS-H (180 ng/mL) immunoreactivity by RIA, showed a protein of Mr = 10,000 in sera from metastatic BC. Inset: Lane A, arrow, peak 4, indicates the position of MPS-1 protein of Mr = 10,000. Lane B shows the pooled sera from normal women (control, no evidence of cancer) chromatographed under identical conditions as sample in Lane A. The MPS-1 protein is undetectable in control sera at position 4 (Figure 6, lane B). The high molecular weight immunoreactive protein bands labeled as 1,2 and 3 are common to both A and B blots. The molecular weight markers used for calibration of Mr were: ovalbumin (Mr 43,000), carbonic anhydrase (Mr 29,000), beta-lactoglobulin (Mr 18,400), lysozyme (Mr 14,300), bovine trypsin inhibitor (Mr 6,200), and insulin chains A and B (Mr 2,300-3400). SDS-PAGE was performed on a 15% acrylamide gel under reducing conditions.*

detected in metastatic breast cancer patient sera unequivocally correspond to the N-terminus portion of recombinant human MPS-1 protein (28,29). Pooled sera from normal women (control, no evidence of cancer) chromatographed under identical conditions as cancer sera samples showed that the MPS-1 protein was undetectable by Western blot analysis at position 40 (Figure 6, lane B). The protein bands labeled as 1,2, and 3 were common to both A and B blots (Figure 6) and correspond to small amounts of heat-denatured immunoreactive MPS-1 covalently bound (-S-S- groups) to fragments of serum carrier proteins (our unpublished data).

Illustrative examples of the identification of MPS-1 serum protein in four separate cases of patients with metastatic BC are shown in Figure 7. The BC samples which contained high levels of MPS-H (>100 ng/ml) were chromatographed as described above. Figure 7 shows only the region of the Western blot corresponding to the peak of Mr 10,000. As illustrated in Figure 7, Lanes 1,2,3 and 4, show that the anti-MPS-N antibodies recognized a protein of Mr 10,000 only in sera from BC patients but not in sera from normal subjects. The recognition of the Mr 10,000 protein by the anti-MPS-N antibodies was neutralized by incubation of the antibody with the peptide antigen (amino acid sequence: 2-

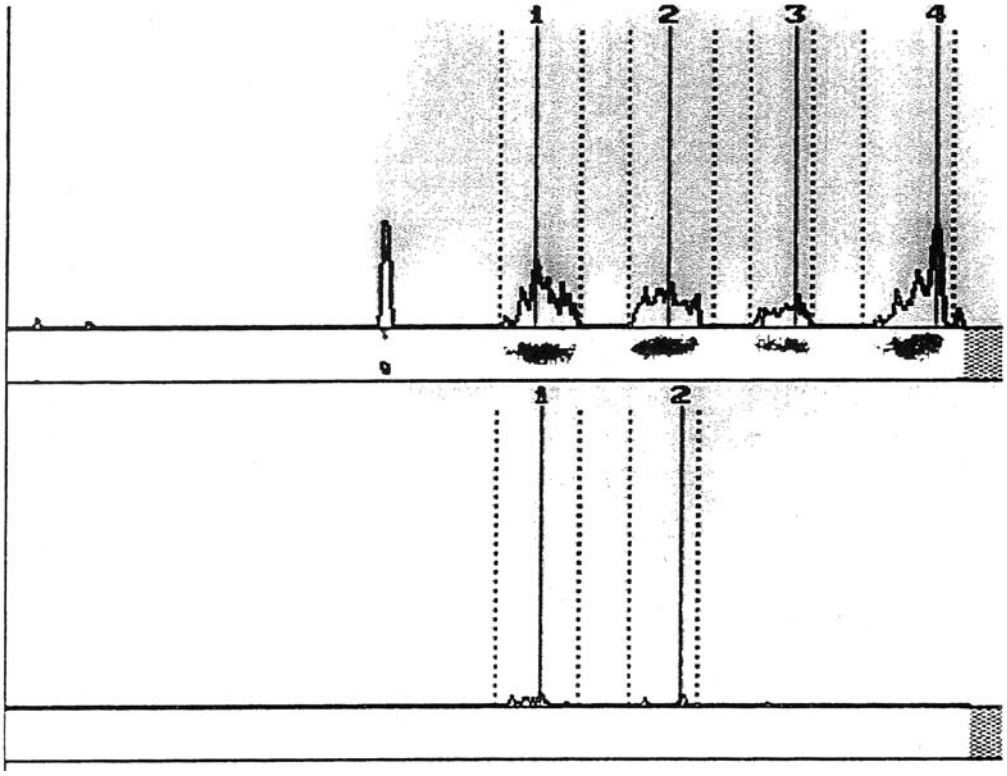


Figure 7. *Western blot analysis of patients with metastatic breast cancer. The extracts from the chromatography steps, were electrophoresed (70 μg protein/lane), and transferred for Western blot analysis. Only the Western bands corresponding to the 10,000 Dal region are shown. Bands 1 to 4 (top right quadrant) corresponding to breast cancer sera from separate patients were exposed to anti-MPS-N antibodies (1:5000 dilution). All samples show a protein of Mr =10,000. Bands 1 to 4 (bottom right quadrant) corresponding to normal sera from separate women (control, no evidence of BC cancer) did not show the band of Mr 10,000 after exposure to anti-MPS-N antibodies. Western immunoblot analysis of specific neutralization of anti-MPS-N antibodies by peptide-N (amino acids 2-17) is shown in the left upper and lower quadrants. MPS-N antibody was incubated with peptide-N at a molar ratio of 1:100 (antibody:antigen) for 30 min at room temperature. The mixture was added to the transfer lanes containing equal amounts of protein as those of lanes 1 to 4 (70 μg protein/lane). The Western blots were subsequently processed as indicated in Materials and Methods. Note that no bands are observed (upper and lower quadrant), showing the specificity of anti-MPS-N antibodies for the detection of the N-terminus of MPS-1. Molecular weight standards were the same as those of Figure 6.*

17) which was used to raised the antibody (Figure 7 and 8). These results clearly show that the anti-MPS-N antibody is highly specific for detection of MPS-1 protein species. Thus, the results presented here indicate that authentic MPS-1 protein present in the sera of metastatic BC patients is most likely responsible for the positivity of the RIA test. The MPS-1 serum protein was undetectable in four separate control sera from normal women(Figure 7).

Identification of MPS-1 in sera of patients with cancer by Mass Spectroscopy (MS). Pooled MPS-1 serum samples were tested by MS to confirm the presence and relative quantity of the MPS-1 protein in patients with cancer containing different levels of MPS-1 protein (Figure 9). Pooled sera from patients with cancer with extra high (XHCPS), high (HCPS), medium (MCPS), and low (LCPS) concentrations of MPS-1 were previously determined by RIA. Figure 9 shows the verification of reproducibility of results with RIA by MS. The detection of a known protein in a known relative concentration is shown. The pooled serum samples analyzed by MS displayed a protein peak which corresponds to the estimated molecular weight of translationally modified MPS-1 of 10068 Da whose concentration correlated in a semi-quantitative fashion with the measurements by RIA in the same samples. In pooled control sera from normal subjects MPS-1 was undetectable (Figure 9).

The molecular weight of recombinant human MPS-1 by SDS-PAGE and Western blot analysis with antibodies against the N-terminus of MPS-1 (amino acids: 2-17) have been previously determined as approximately 10,000±50 Dal (27-29). The molecular weight of MPS-1 present in

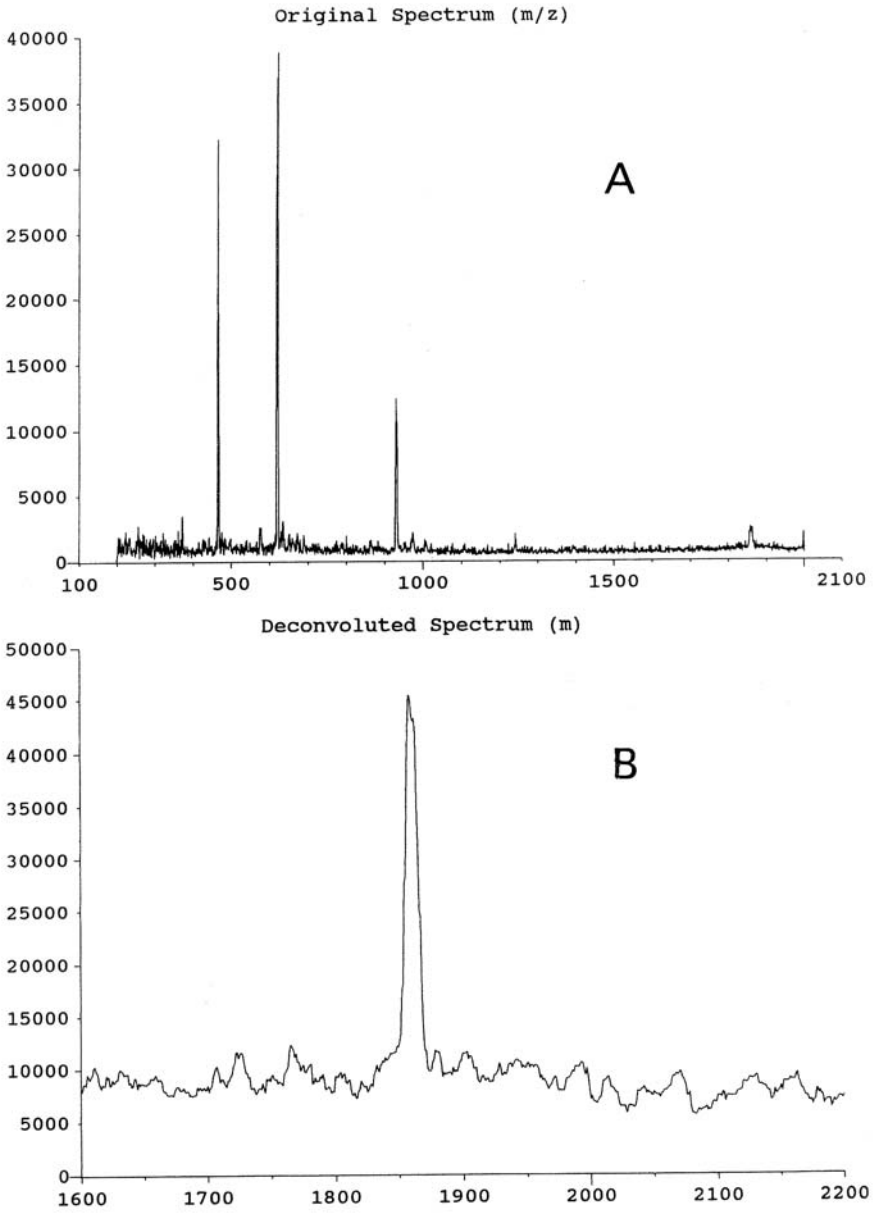


Figure 8. *Mass spectrometry of synthetic MPS-1 peptide. Peptide A without cysteine, average Mr= 1859.31 was utilized for immunoassays. m/z, mass to charge ratio. (A) Original spectrum; (B) Deconvoluted spectrum (m).*

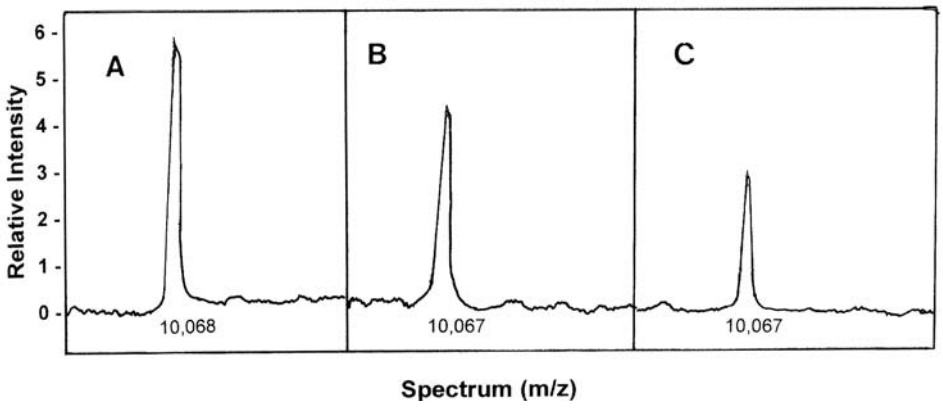


Figure 9. *Mass spectrometry of pooled serum samples from patients with cancer containing different concentrations of authentic MPS-1 protein as determined by RIA. The relative intensity of the MS peaks of Mr=10,070 Da correlate with the concentrations of MPS-1 protein measured by RIA in the same pooled samples. Concentrations of MPS-1: (A) HCPS, high control pooled sera; (B) MCPS, medium; and (C) LCPS, low. m/z, mass to charge ratio.*

cancer patient sera and isolated by HPLC, was determined by SDS-PAGE and Western blot analysis as approximately 10,000 Dal (Figure 6 and 7). The molecular weight of the MPS-1 protein detected by MS in metastatic cancer patients sera was approximately 10,067,9+H to10,068.5+H Dal (Figure 9).

Solid-phase immunoassay and Mass Spectroscopy (MS). Figure 10 shows that the MPS-1 protein can be detected in heat denatured sera using an absorbent paper support containing covalently bound rabbit polyclonal anti-MPS-1 IgG antibodies directed against the N-terminus of MPS-1. After solid-phase immunoassay, the eluted MPS-1 protein was detected by MS (Figure 10 B). When the anti-MPS-1 antibody is neutralized with peptide A, no protein peak corresponding to MPS-1 was detectable (data not shown). These data provides conclusive proof that the protein peak at 10,068 Da is authentic MPS-1 protein.

Identification by differential hybridization of growth factor stimulated genes in breast cancer cells. After DNA sequencing we found several clones encoding ribosomal proteins, such as L4,L5,L30,L37 and S18 (Table VII). Expression levels of these ribosomal genes was increased with respect to control, unstimulated cells. Table VII also shows that the expression of numerous other genes was increased after growth factor stimulation of MDA-468 cells, in comparison to control unstimulated cells.

Identification of host reactive proteins in sera of patients with breast cancer (BC). A systematic time-dependent analysis of heated serum samples by Western blot analysis and sequencing showed the presence of a differential pattern of proteins in normal sera in comparison to sera from BC patients (Figure 11 and 12). Sequence analysis of these bands showed the following proteins in BC sera after heat activation. One of the bands that reacted with anti-MPS-1 antibodies was C3 of complement (analphylotoxin) of Mr 9,100 (Figure 11). This band has sequence similarity withMPS-1 at the N-terminal portion that clearly shows crossreactivity with the anti-MPS-N antibodies. Figure 11 shows a Western blot analysis of heated human serum analyzed by anti-MPS-N antibodies. The results of sequencing showed that band A, is identical to C3a of complement (analphylotoxin), which has sequence homology to the N-terminus of human MPS-1 protein (Figure 12).

Since complement components are expressed by neoplasias and are present in the serum (51,52), measurements of inhibition of precipitation by RIA of MPS-1 in heated serum from control and BC patient samples were performed to determine if any of the complement component may interfere with the MPS-1 test. The following complement components at saturating concentrations were tested by addition to the incubation mixtures of the RIA for MPS-1: CH, CI, C1s, and C1q (64,65). None of these complement components had any effect on the MPS-1 RIA test (data not shown). The displacement of C3 (anaphylotoxin) on recombinant MPS-1 measured by the RIA test was less than 1%, indicating that the MPS-1 test accurately measures authentic MPS-1 protein in serum.

Haptoglobin was found in increased quantities in heat-activated BC sera (53,54). Haptoglobin did not react with anti-MPS-N antibodies (data not shown). Figure 12 shows haptoglobin degradation bands corresponding to the alpha and beta subunits.

Discussion

Genomics and Proteomics based technologies have been developed that allow the study of tissue and serum protein profiles in patients with cancer in comparison to control healthy subjects (46-50,63). Here we report the use of these methods to distinguish cancer from control data sets and to identify specific proteomic patterns of potential biomarkers produced or associated with Breast Cancer (BC).

Initially, genes differentially expressed in BC were identified, cloned and characterized from a cDNA library constructed from a human mammary carcinoma cell line (MDA-MB-468) that was stimulated by epidermal growth factor (EGF) and transforming growth factor (TGF beta) (28,29,60-62). We found that when MPS-1/S27 ribosomal protein is overexpressed in BC cancer cells, it is released into the extra-cellular space. Using the same cDNA library we have identified a group of genes differentially expressed in BC cells after stimulation with EGF plus TGF beta (Table VII).

The basic science studies were translated into the clinic to investigate the potential use of the growth factor stimulated genes for early diagnosis of cancer. Initially, methods for detecting MPS-1, derivatives thereof or structurally related compounds in serum samples were developed (59). The essential steps of this technology consisted of dissociating MPS-1 from serum binding proteins, binding the MPS-1 to an absorbent support, eluting the MPS-1 from the absorbing support utilizing chemical or physical agents and detecting the eluted MPS-1 by immunological methods, mass spectrometry, microarray proteomics, or electrophoresis. Table VI delineates the important developmental milestones in the study of MPS-1/S27 ribosomal protein.

Our studies concerning the use of MPS-1 in the detection of breast cancer demonstrate that this empirical marker has

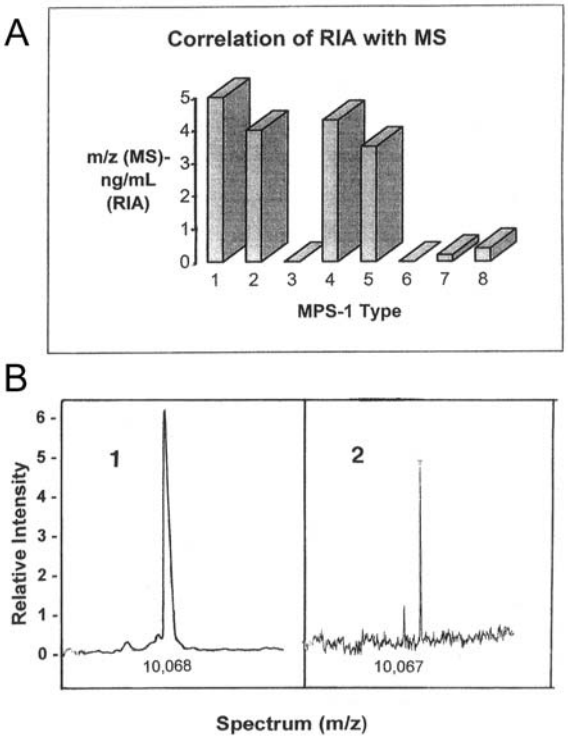


Figure 10. *Solid phase immunoassay and mass spectrometry performed on both recombinant MPS-1 protein and MPS-1 protein present in cancer patient sera. Heat-denatured serum proteins were absorbed to a paper support containing covalently bound anti-MPS-N antibodies. After binding, the proteins were eluted and MPS-1 proteins were detected by radioimmunoassay (RIA) and mass spectroscopy (MS). (A 1, 2) Recombinant MPS-1 protein analyzed by RIA (1) and MS (2); (A 4, 5) HCPS, high MPS-1 cancer patient pooled sera analyzed by RIA (4) and MS (5); (A 7, 8) Normal pooled sera analyzed by RIA (7) and MS (8); (3, 6) buffer, control. (Figure B 1 and 2) Proteins of Mr = 10,068 (Recombinant MPS-1; panel 1) and Mr = 10,067 (serum MPS-1; panel 2) detected by MS.*

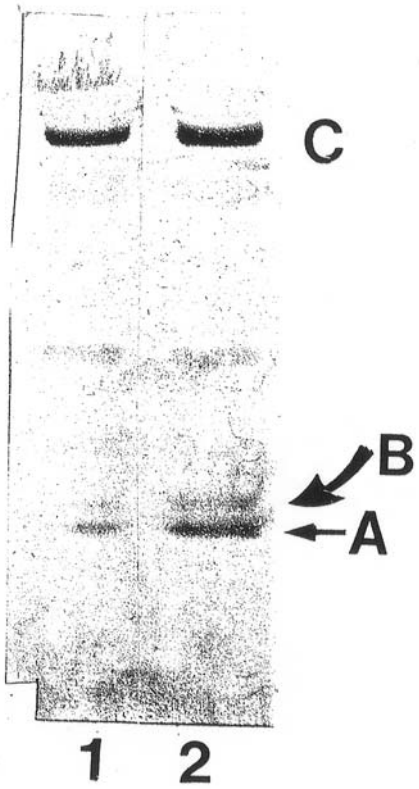


Figure 11. *Western blot analysis of heated human serum from a patient with metastatic cancer. After electrophoresis and transfer of two aliquots of heat activated cancer patient serum, Western blot analysis was performed with anti-MPS-N antibodies. Lane 1, anti-MPS-N antibodies neutralized with the N-peptide. Note that no MPS-1 band is observed, showing specificity of anti-MPS-N antibodies for the N-terminus of MPS-1. Lane 2, anti-MPS-N antibodies alone react with two bands, A and B. Band A of 9,100 Da, is the C3a component of the complement (anaphylotoxin), which has sequence homology to the N-terminus of human MPS-1 as follows: VQLTEKRMDKV; Band B, of 10,000 Da, was identified by sequencing as MPS-1; Band C, corresponds to MPS-1 covalently bound to carrier proteins of high molecular weight. For details see the text.*

the required sensitivity for the detection of early breast cancer as demonstrated by the detection of stage 0, I and II adenocarcinomas. We have identified the MPS-1 protein by HPLC and sequencing in the sera of patients with metastatic breast cancer (Figure 6 and 7). Mass spectroscopy also demonstrates the presence of MPS-1 protein in cancer patient sera but not in control sera (Figure 9).

Atsuta et al have recently verified that the MPS-1 protein is a tumor associated antigen in patients with breast cancer (34). Moreover, Sundblad et al demonstrated that MPS-1 protein is a histological marker for breast cancer (37). However, since the antigen is produced by many tumors, it is not specific for breast cancer (Table IV; 24,25,27). Thus, the clinical utility of MPS-1 in detecting breast cancer is limited to the detection of a carcinogenic and/or oncogenic process in a patient suspected of having breast cancer.

For follow up and monitoring, the MPS-1 antigen has been shown to correlate well with tumor burden, development of metastasis and response to treatment (chemotherapy, hormonotherapy or irradiation). The role of MPS-1 in the management of breast cancer patients may be related to the ability of this marker to detect early disease recurrence in high risk individuals. This early detection of recurrence may provide sufficient lead time for treatment decisions. In this consideration, numerous longitudinal studies indicate that tumor markers usually provide the first sign of disease recurrence (9-27). In our studies, MPS-1 provided a lead time of 9-12 months before

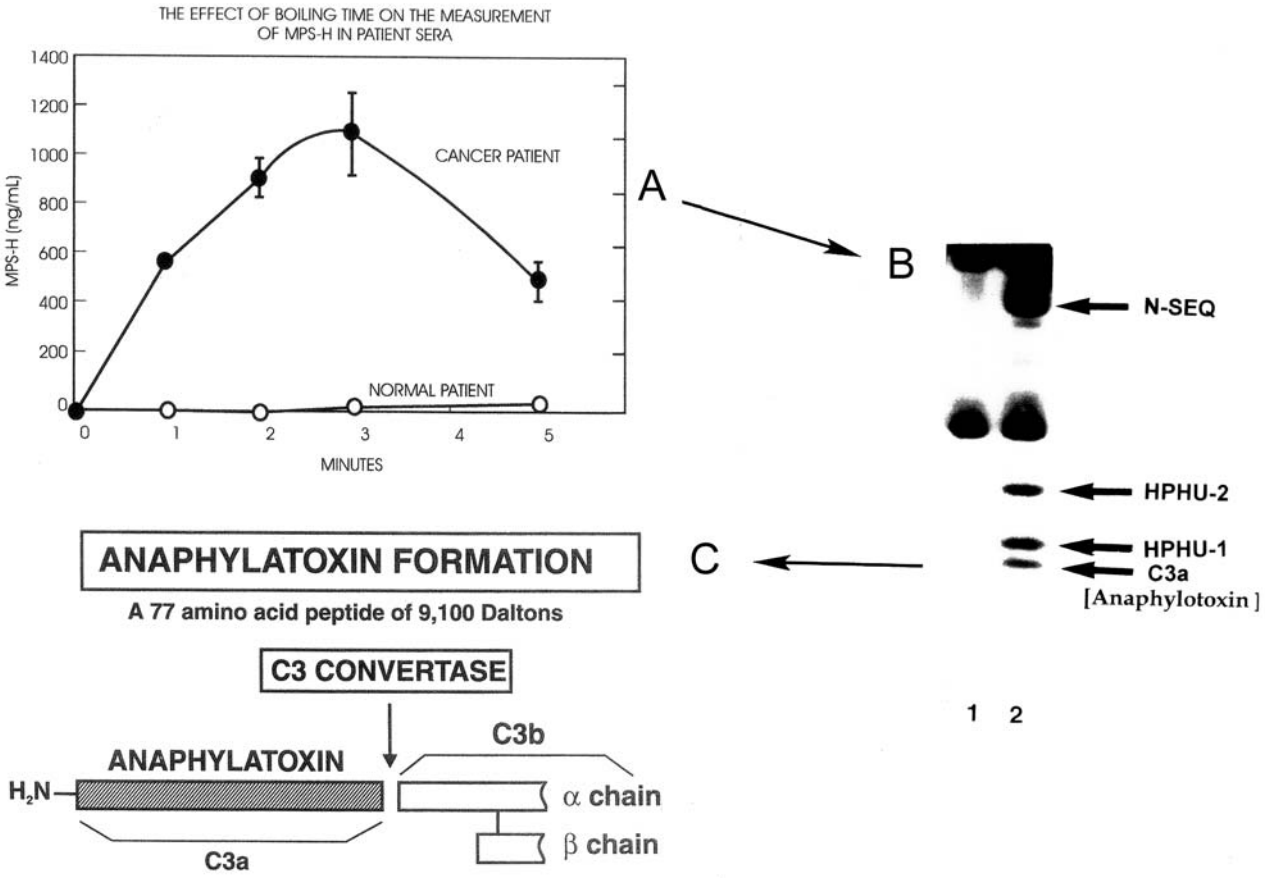


Figure 12. *Identification by sequencing of heat resistant serum proteins from a patient with metastatic cancer. After heating of sera, purification, electrophoresis and transfer, the protein bands were stained and sequenced. Lane 1: Control, normal human serum. No bands are seen in the low Mr region. Lane 2: Cancer patient serum contains complement C3a (anaphylotoxin), and Haptoglobin subunits. Band A: complement C3a (anaphylotoxin). Bands B and C: Haptoglobin subunits (sequence identified: Alpha-1 chain, VDSGNDVTDI). For details see the text.*

metastases were confirmed by conventional procedures. Thus, serial measurements of MPS-1 concentrations may be an important indicator for monitoring patients with breast cancer and in determining the actual effectiveness of surgery and/or other treatments. Patients initially presenting with high MPS-H levels that decrease after various therapeutic modalities predictably experience a favorable response to therapy. In contrast, a positive, persisting or increasing tumor marker level is associated with a high probability of recurrent disease (Table V), usually in the form of metastatic disease. However, correlations are not always exact. For example, as with any other tumor marker (9,21), in late cancer (within the last 4 weeks of life), when organ failure and massive metastatic disease occurs, the marker is less reliable, producing false positive or false negative results.

The results presented here with breast cancer further verify prior results obtained with the use of the MPS-1 test in other malignancies (25,27,55-57). An extensive clinical study with 632 individuals including healthy subjects, active cancerous diseases, non-malignant diseases, and premalignant diseases has previously provided important information about the use of MPS-1 in the detection of various types of cancer (26). In general, the MPS-1 test was found to be useful in: 1) Detection of primary disease in previously undiagnosed individuals; and 2) Detection of cancer recurrence in previously diagnosed and treated patients (24,25,27,55-57).

The CA 15.3 is a test for the determination of the DF3 antigen in serum and plasma of patients previously treated for stages II or III breast cancer when they recur (stages III and IV). Stages 0, I and II have very low levels of CA 15.3 and stages III and IV show high levels of this antigen. The serum marker CA 15-3 is an established marker in the monitoring of breast cancer patients with stages III-IV (10-19;20-23;41,42). Serum CA 15-3 levels depend on stage of

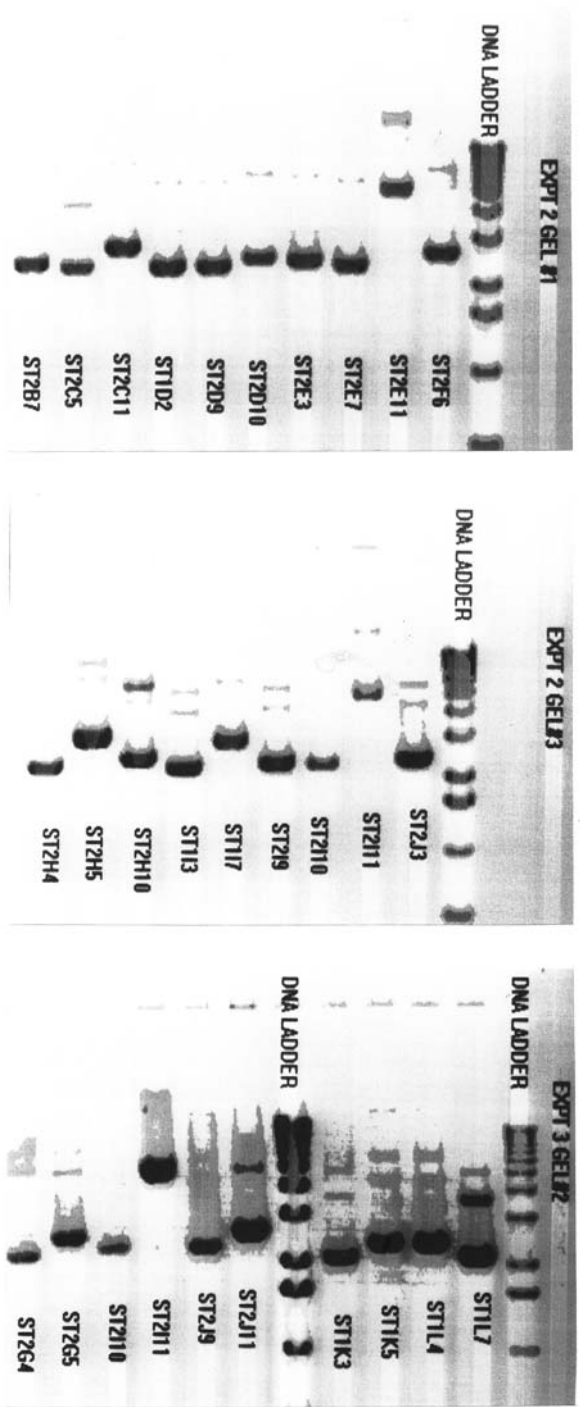
Table VI. *Translating basic research to the clinic: important developmental milestones.*

1990. Isolation and sequencing of a GF-inducible gene denoted MPS-1 from breast cancer cells.
1991. MPS-1 gene encodes a multifunctional ribosomal protein S27 with zinc finger structure.
1992. MPS-1 is overexpressed in carcinogenic processes.
1992. Characterization of recombinant MPS-1 protein.
1994. Mass spectrometry (MALDI-TOF) of MPS-1 proteins.
1996. MPS-1 as a novel tumor marker in sera of patients with various types of cancer.
1999. Isolation and sequencing of 33 growth factor-induced genes from breast cancer cells

Table VII. *Genes differentially expressed in human breast cancer cells after growth factor stimulation.*

Homo sapiens ribosomal proteins: S16, S6, S25, S7, S14, S18, L4, L5, L7a, L23a and L37
Human elongation factor 1-alpha
Nuclear encoded 26S rDNA genomic partial sequence
mRNA related to *Gallopavo* microsatellite repeats
Human mRNA for KIAA0136 gene
Human Carbonyl reductase 1
H. sapiens mRNA for myosin regulatory light chain
H. sapiens mitochondrial DNA, complete genome
Mitochondria 16S ribosomal RNA gene
Dynein light intermediate chain
Human RA023A gene
H. sapiens signal recognition particle 9kD (SRP9) mRNA
Human hsc 70 gene for 71kDa heat shock protein
Telomerase protein
Human ferritin heavy chain mRNA complete
H. sapiens mitochondrial CoxII mRNA
Erythroblastosis virus oncogene homolog 2 protein (ets) gene complete sequence
H. sapiens mitochondrial phosphate carrier
mRNA for dexametasone inducible protein
Human G-protein couple receptor (CPR2)
Human calpain
Human cytochrome c oxygenase
Human casein kinase

cDNA isolation, sequencing and BLAST sequence results on the query sequence are described in the text



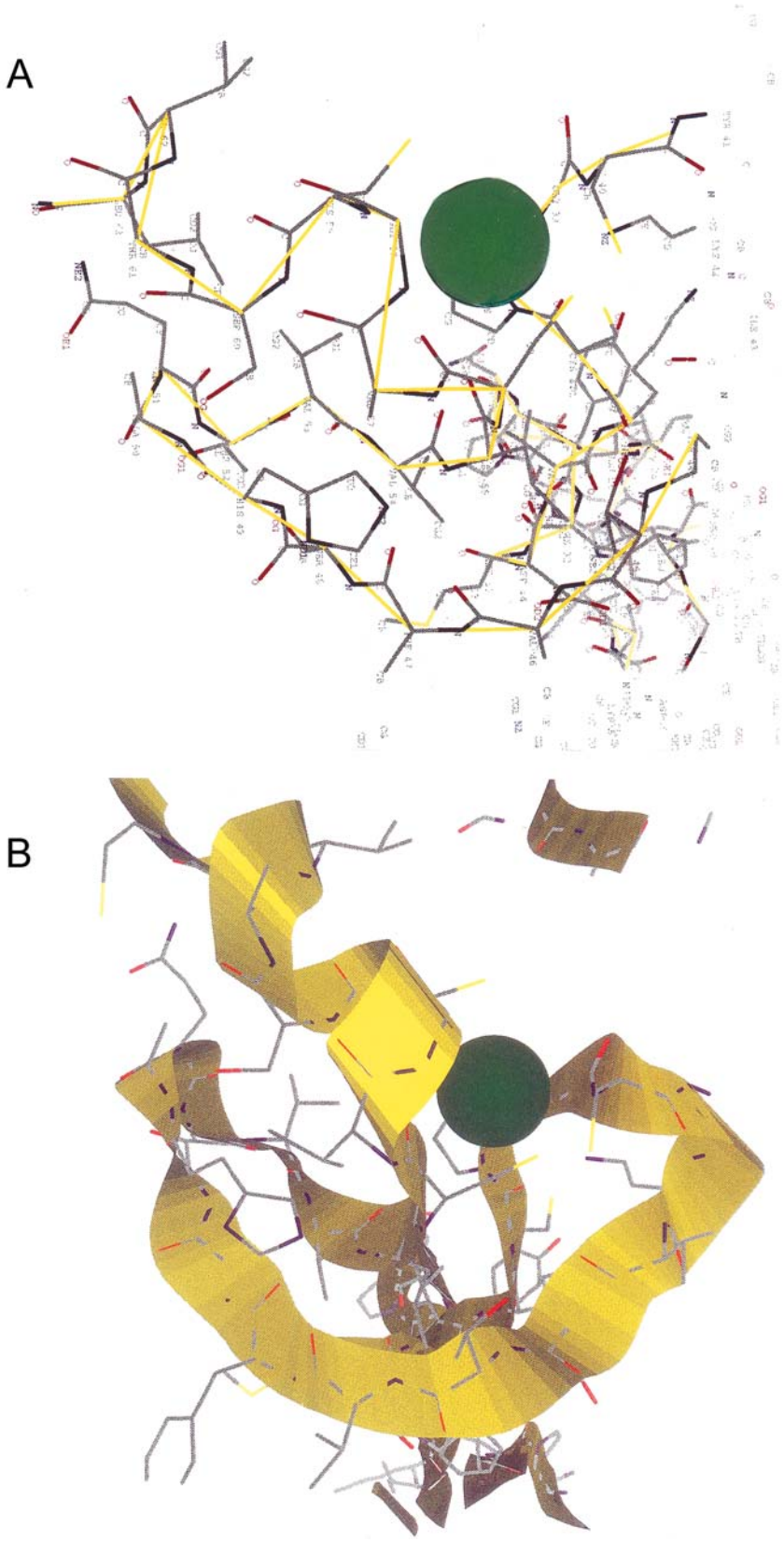


Figure 13. *Molecular modeling of MPS-1 protein. A, Backbone model; B, space distribution model. Green circle, Zn^{2+} atom.*

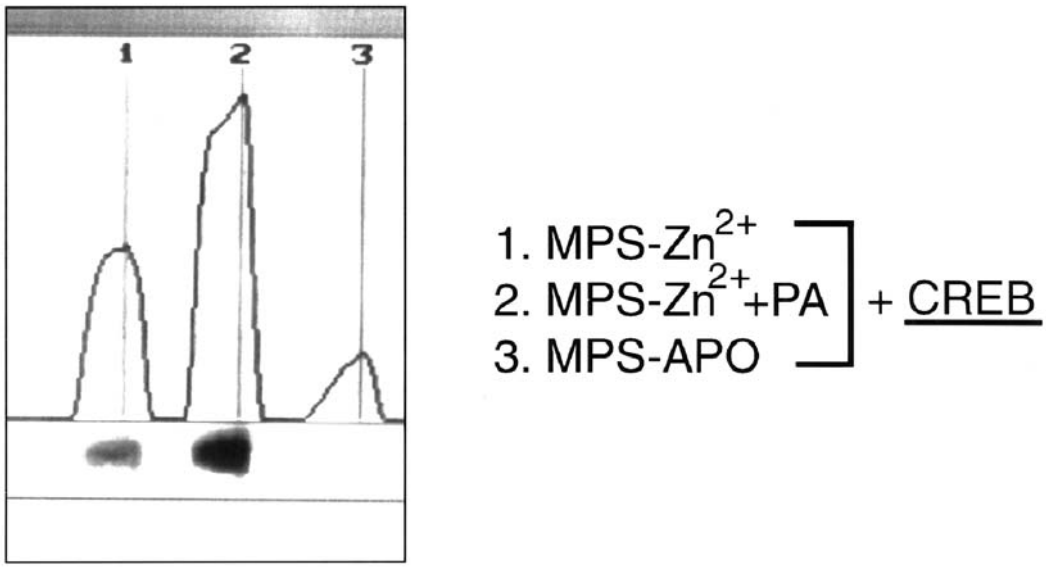


Figure 14. *Zinc-dependent binding of MPS-1 to DNA. Gel shift assays were performed with CREB DNA sequence. 1, MPS-1 plus Zn^{2+}; 2, MPS-1 plus Zn^{2+} with 1 mM picolinic acid (PA); 3, MPS-APO, MPS-1 without Zn^{2+}.*

breast tumor and tumor activity. For follow up and monitoring, the CA 15-3 antigen has been shown to correlate well with tumor burden, development of metastasis and response to treatment (chemotherapy, hormonotherapy or irradiation). Improved clinical status and response to therapy are associated with decreasing CA 15-3 levels. In contrast, lack of response to therapy or disease progression, correlates with increases in levels of CA 15-3 with a lead time of 4 to 12 months (21,23). However, the CA 15-3 and CEA perform poorly under all circumstances examined in our study population (Figures 1-4; Table V), and they cannot be used for early detection of breast cancer because of their inability to detect stages 0, I or II (Figure 1). The CA 15.3 is a test for breast cancer that is not specific for breast cancer. It is worth noting that the CA 15.3 is positive in 34.4% of patients having other malignancies (14). Furthermore, the BR27.29 as a marker for breast cancer is positive in numerous tumors (up to 30%) in lung and other cancers. In addition, CA 14.3 or BR27.29 cannot differentiate between normal subjects and subjects with benign neoplasms.

Recently, serum HER-2/neu antigen was studied as potentially useful to detected breast cancer antigens (41). Serum HER-2/neu antigen concentrations have been reported to correlate with increased tumor volume in patients with breast cancer (41). Serum HER-2/neu protein was found to be increased in 30% of the patients and CA-15-3 was increased in 60% of the patients (41). Ali et al concluded that the combination of both of these markers predict a worse prognosis than CA-15-3 alone (41).

The MPS-1 antigen was clearly superior to CA 15-3 or HER-2/neu antigens in detecting stages 0, I and II. In stages III and IV, MPS-1 was also a better marker because it detected a larger percentage of tumors and the likelihood ratio of MPS-1 was 10-fold greater than CA 15-3 (Table V). Moreover, the MPS-1 levels changed much faster than CA 15-3 in response to therapy in all cases studied (*e.g.* Figure 4A,B). These kinetic results with MPS-1 in breast cancer are similar to the changes observed in prostate cancer after successful therapy, in which the MPS-1 antigen changed much faster than prostatic specific antigen (PSA) (24,25,27). It should be noted that in numerous instances the results of MPS-1 and CA 15-3 are incongruent (Figure 3). This should not be surprising for at least two reasons: 1) the biological properties of the two markers are entirely different; (2) paradoxical patterns have been observed with certain tumor markers after therapy such as early rises, followed by decreases (21); and (3) MPS-1 is able to detect cancer much earlier than CA 15-3, indicating entirely different tumor marker kinetics. Thus, in all the cases studied, the MPS-1 test is more sensitive in detecting recurring disease than the CA 15-3 assay.

The data presented here with MPS-1 indicates that there is a clear cut difference between benign (20 patients) and malignant processes (203 patients). The benign processes never exceeded a cut off of 11 ng/mL which overlaps with the normal range. This is in agreement with the data that MPS-1 is a marker for malignancy and does not detect benign tumors. However, a larger number of cases will have to be studied in order to substantiate this preliminary data.

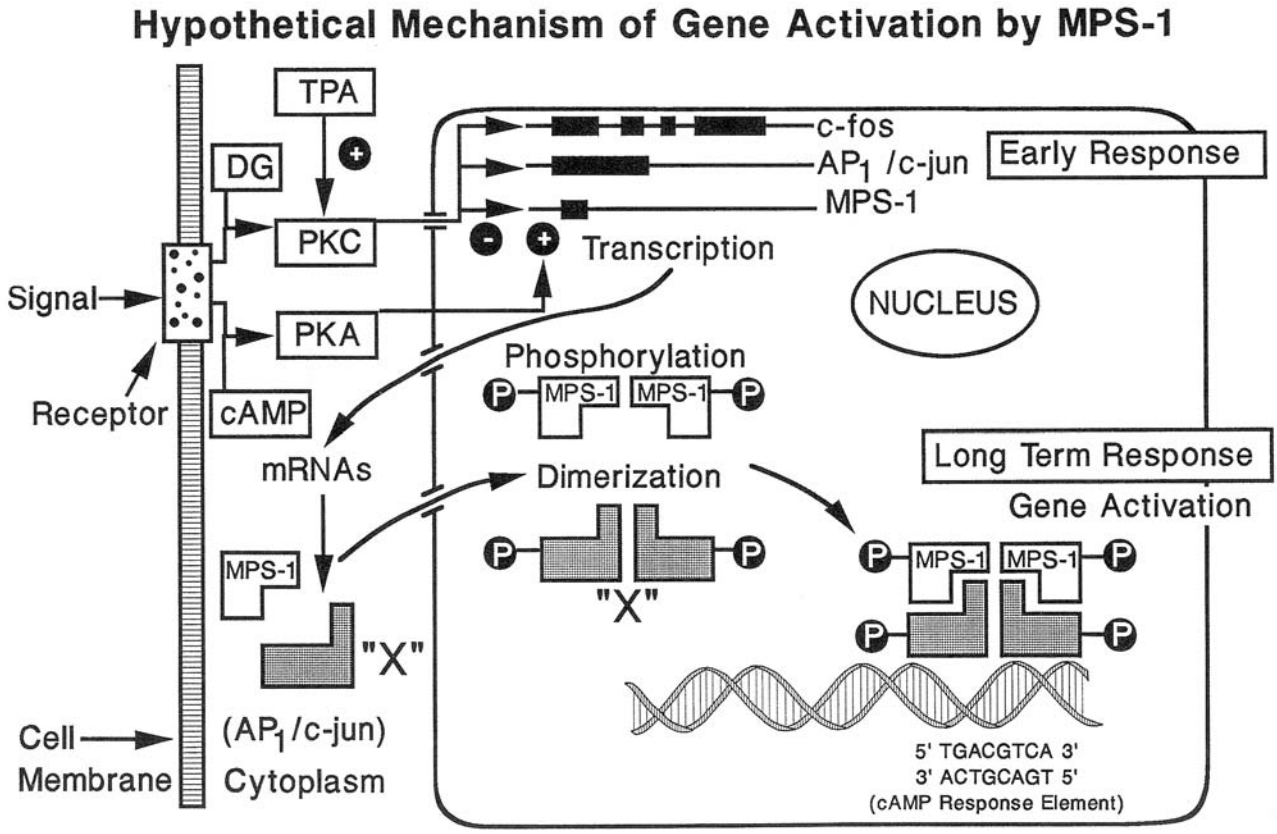


Figure 15. *Hypothetical Mechanism of gene activation (or suppression) by MPS-1 protein. DG, diacylglycerol; TPA, 12-O-tetradecanoylphorbol-13-acetate; PKC, protein kinase C; PKA, protein kinase A; cAMP, cyclic adenosine monophosphate; mRNAs, messenger RNA; c-fos, AP1/c-jun, transcription factors; P, phosphate*

The MPS-1 protein present in sera of breast cancer patients has an estimated molecular weight of 10,050 kDal (Figure 6). These results were verified by mass spectroscopy (Figure 8;56). This protein interacts with anti-MPS-N antibodies and is responsible for the positivity of the MPS-1 test. The MPS-1 protein was cloned from the human breast cancer cell line MDA-MB-468 (29). Furthermore, the MPS-1 protein is present in human breast cancer tissue (34,37). Thus, it is reasonable to proposed that the MPS-1 protein detected by the anti-MPS-N antibodies in both cancer tissues and cancer sera is produced by breast cancer cells and released into the circulation when breast cancer cells overexpress the MPS-1 protein.

There are many reports indicating a connection between overexpression of genes encoding some ribosomal proteins and carcinogenesis (24,31,66). Several ribosomal proteins have functions that are separate from their roles in ribosome and protein synthesis (24,31). The MPS-1/S27 ribosomal protein is involved in DNA repair following DNA damage induced by UV light exposure or chemical carcinogenesis, and in the deletion of mutated mRNAs induced by carcinogens (28,32). The zinc finger motif of MPS-1/S27 and other zinc finger ribosomal proteins allows the binding to nucleic acids which may results in interference with transcription and translation (24,31). Ribosomal protein S27a, which is unrelated to MPS-1/S27, is ubiquitinilated and overexpressed in human colon cancer, and is involved in cell cycle control and DNA replication (24,31). Ribosomal protein L10 is homologous to the Jun-binding protein and to the Wilm's tumor suppressor gene and has oncogenic functions (24). Vaarala et al showed that ribosomal proteins L7a and L37 are overexpressed in prostate cancer (43). Furthermore, genotoxic stress induced by carcinogens induces ribotoxic responses which involved numerous ribosomal proteins (24,31). Taken together, these findings indicate that ribosomal proteins have oncogenic, tumor-suppressor, or cell cycle functions and indicate that certain ribosomal proteins are overexpressed in the process of carcinogenesis or oncogenesis (24,31).

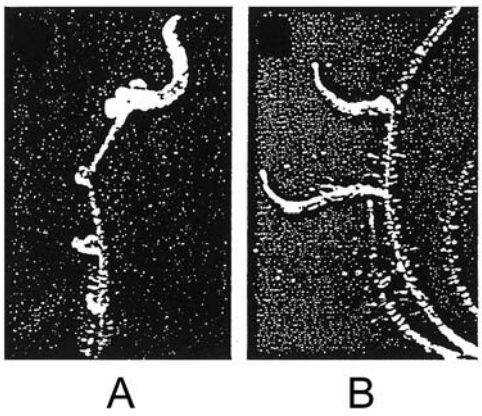


Figure 16. *Phenotype of the ars27A mutant (A) in comparison to wild-type (B) Arabidopsis thaliana roots. Adapted from Revenkova et al (Ref. 32; with permission from Revenkova, 1996). Note the formation of tumors (A) in place of roots (B) when the MPS-1 gene is deleted.*

We found that, in addition to MPS-1/S27, other ribosomal zinc finger proteins are overexpressed in growth factor stimulated breast cancer cells (Table VII). Zinc-finger motifs are characteristic of a large number of ribosomal proteins allowing them to bind nucleic acids. By computer molecular modeling, we have shown that the zinc finger domain of the type CCCC, present in the MPS-1 amino acid sequence, binds Zn^{2+} (Figure 13). The zinc atom is essential to maintain MPS-1 in a configuration that allows the binding of the MPS-1/zinc complex to RNA or DNA. Furthermore, a significant portion of the electronic outer shell of the Zn^{2+} atom is exposed to the solvent environment (*e.g.* water) (Figure 13), allowing the Zn^{2+} atom to interact with certain cellular ligands such as picolinic acid, a product of trytophane metabolism (62). The requirement for Zn^{2+} and the reaction with picolinic acid can be detected by electrophoresis of MPS-1 under non-denaturing conditions (Figure 14). Figure 15 shows a hypothetical model of gene activation (or suppression) by MPS-1 proteins in mammalian cells. Anti-carcinogenic functions of MPS-1 genes were clearly defined by Revenkova *et al.* (32) in the plant Arabidopsis thaliana (Figure 16). Overexpression of some ribosomal proteins is common to numerous malignancies including breast cancer (Table VII). Taken together, the data suggest that overexpression of ribosomal proteins may interfere with both transcriptional and translational processes, possibly contributing to carcinogenesis and/or oncogenesis.

Other genes differentially expressed in human breast cancer cells are listed in Table VII. These group of proteins are involved in transcription (*e.g.*, microsatellite repeats, telomerasa, etc), translation (ribosomal proteins 16S-L37), signal transduction (G-proteins), energy production (mitochondrial proteins), and apoptosis (calpain, heat shock 71kDa protein). They may be valuable tools to study oncogenic processes and characterize new serum biomarkers in BC patients (58).

In a separate series of studies to detect potential biomarkers in serum samples of BC patients and in patients having other types of cancers, the serum samples were analyzed after heating under controlled conditions (25). A systematic analysis of heated serum samples by SDS-PAGE and Western blot analysis showed the presence of a differential pattern of proteins in normal subjects in comparison to cancer patients (Figure 11 and 12). Sequence analysis of the protein bands increased in the sera of cancer patients showed that: (i) One of the bands, which reacts with anti-MPS-1 antibodies is C3 of the complement (anaphylotoxin) of 9,100 Da (Figure 11; 51,52,64,65). This band has sequence similarity with MPS-1 at the N-terminal portion (Figure 12; 34). Since other complement-related proteins are present in the sera of cancer patients (52), measurement of anaphylotoxin in heated sera from patients with breast cancer may be useful to detect BC cancer activity; (ii) Two other bands, which did not react with anti-MPS-1 antibodies were identified by sequencing as haptoglobin, alpha and beta subunits (Figure 12; 53,54). The function of Haptoglobin is to combine with free plasma hemoglobin, preventing loss of iron trough the kidneys and protecting the kidneys from damage by hemoglobin (53). The haptoglobin-hemoglobin complex is degraded by the liver, where haptoglobin is also synthesized. Since haptoglobin is a protein reactive to hemoglobin release, the increased level of Haptoglobin in BC is most likely due to cancer bleeding. Thus, it is unlikely that haptoglobin will be a useful marker for early detection of BC cancer.

It is reasonable to think that any technology that detects cancer disease at an earlier stage, will reduce the morbility and mortality rates (1-6,50,59). The results obtained with breast cancer serum samples presented here are congruent with previous results obtained with various sera from other types of tumors (24,25,27,51,55-57). Taken together, these results indicate that: 1) the MPS-1 test reduces the uncertainty about the presence or absence of an active carcinogenic or oncogenic process; 2) the serial measurement of serum MPS-1 may be a useful indicator in the prognosis and management of patients with tumors that produce MPS-1 proteins. Thus, the MPS-1 antigen may be potentially useful in the evaluation of clinical problems in the area of oncogenesis.

Conclusion

Several proteins of Mr<30,000 were found to be significantly increased in BC sera in comparison to control sera. The results presented here demonstrate that MPS-1 serum protein is a useful marker for early detection of BC cancer activity.

The combination of MPS-1, host tumor reactive proteins such as anaphylotoxin and haptoglobin, with the protein products of the genes listed in Table VII, suggest that identification of BC tumor type and/or oncogenic activity of BC by protein profiling may be feasible. Mass spectroscopy-based protein profiling is a useful tool for screening protein markers in the serum (49,50). However, confirmation of protein identity with specific antibodies or by sequencing of the specific proteins is critical for clinical applications in the diagnostic area (47,48,56). In this paper, the identity of numerous BC-associated tissue and serum proteins was determined by bioinformatics, sequencing, mass spectroscopy and immunoassays. Taken together, the data indicate that overexpression of growth factor-induced BC genes and BC-reactive proteins may be useful as biomarkers for both early detection of breast carcinogenesis and progression of BC.

The findings presented here illustrate the potentially powerful approach for early diagnosis of BC using a combination of data derived from the analysis of differential screening of breast cancer cDNA libraries and the identification of the corresponding proteins in the sera. An illustrative example of this approach is the successful use of recombinant MPS-1 protein and specific anti-MPS-1 antibodies to measure MPS-1 in sera of BC patients by immunoassay and the detection of MPS-1 protein in sera by mass spectroscopy. The possibility of identification of proteomic signatures for the detection of specific cancers and the identification of tumor subtypes is suggested by the studies presented here.

Cancer screening is a means of detecting early cancer in generally asymptomatic people (44,45). Screening tests are for the most part not diagnostic but are useful for investigating patients suspected of having an active oncogenic process. Definite diagnosis is most often made following a work-up with a biopsy or other specific procedure. As suggested by the results obtained with stages 0, I and II, breast cancer, the clinical value of the MPS-1 serum test may be in the early detection of breast cancer. It is conceivable that elevated MPS-1 levels along with abnormal mammography's (44,45) or clinical evaluation, may diagnose breast cancer with a sufficient lead time, which would allow more effective therapy. The use of powerful technologies such as mass spectrometry, in combination with anti-MPS-N antibodies, or other proteomic technologies (7,47,49,50), suggest that these techniques may be utilized to detect MPS-1 in the sera of patients suspected of having breast cancer or for screening purposes.

When MPS-1 serum antigen detection was used in other types of cancer it correctly identified cancer patients but was not found in any of the healthy volunteers (25,27,51). We believe that the data presented here could form the basis for a breast cancer screening program, along with breast examination procedures, by detecting the MPS-1 molecule in serum samples.

Acknowledgements

A portion of this work was supported by DVA Medical Center Research Funds to Dr. Fernandez-Pol from 1990-1999, and from Research Funds from Metalloproteomics LLC. We thank Mark W. Crankshaw, Director of Protein and Nucleic Acid Chemistry Laboratories, Washington University School of Medicine, St. Louis, MO, for synthesizing the MPS-1 peptides, for mass spectroscopic analysis and for sequencing of proteins.

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Received November 24, 2004
Accepted December 9, 2004