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Humoral immunity to human breast cancer: antigen definition and quantitative analysis of mRNA expression

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Abstract

The ability of the immune system to recognize structurally altered, amplified or aberrantly expressed proteins can be used to identify molecules of etiologic relevance to cancer and to define targets for cancer immunotherapy. In the current study, ninety-four distinct antigens reactive with serum IgG from breast cancer patients were identified by immunoscreening breast cancer-derived cDNA expression libraries (SEREX). A serological profile was generated for each antigen on the basis of reactivity with allogeneic sera from normal individuals and cancer patients, and mRNA expression profiles for coding sequences were assembled based upon the tissue distribution of expressed sequence tags, Northern blots and real-time RT-PCR. Forty antigens reacted exclusively with sera from cancer patients. These included well-characterized tumor antigens, e.g. MAGE-3, MAGE-6, NY-ESO-1, Her2neu and p53, as well as newly-defined breast cancer antigens, e.g. kinesin 2, TATA element modulatory factor 1, tumor protein D52 and MAGE D, and novel gene products, e.g. NY-BR-62, NY-BR-75, NY-BR-85, and NY-BR-96. With regard to expression profiles, two of the novel gene products, NY-BR-62 and NY-BR-85, were characterized by a high level of testicular mRNA expression, and were overexpressed in 60% and 90% of breast cancers, respectively. In addition, mRNA encoding tumor protein D52 was overexpressed in 60% of breast cancer specimens, while transcripts encoding SNT-1 signal adaptor protein were downregulated in 70% of these cases. This study adds to the growing list of breast cancer antigens defined by SEREX and to the ultimate objective of identifying the complete repertoire of immunogenic gene products in human cancer (the cancer immunome).

Introduction

The structural characterization of human tumor antigens recognized by the autologous host has provided convincing evidence for immune recognition of cancer, and has led to the identification of attractive targets for vaccine-based approaches to cancer therapy (1). Innovations in immunological methods of gene discovery have led to the molecular definition of a large repertoire of tumor antigens recognized by the cellular and humoral immune systems. The discovery of MAGE antigens by the genetic method of T-lymphocyte epitope cloning (2) inaugurated current efforts to identify antigenic targets recognized by CD8+ T-lymphocytes and to develop antigen-specific cancer vaccines. A number of other human tumor antigens recognized by CD8+ T-lymphocytes have now been identified, including BAGE (3), GAGE (4), tyrosinase (5) and MelanA/Mart1 (6, 7). Progress in the identification of tumor antigens recognized by CD4+ T-lymphocytes has been hindered by the complexity of the MHC class II presentation pathway i.e., a requirement for specialized accessory molecules and processing vesicles, and the variable length of MHC class II binding peptides. In spite of these limitations, peptide epitopes

recognized by CD4+ T-lymphocytes of cancer patients have been identified, including tyrosinase (8) and Melan A/Mart-1 (9), as well as mutated forms of the LDL receptor (10), CDC27, and triosephosphate isomerase (11). With regard to tumor antigens recognized by autologous human antibodies, a method referred to as SEREX (serological expression cloning) has contributed greatly to our understanding of the humoral immune response to cancer (12). To date, more than 200 SEREX-defined antigens derived from a variety of tumor types have been described in the literature (13), and an additional group of approximately 800 antigens are listed in the SEREX database (14). SEREX-defined antigens can be classified into the following categories: (a) *cancer/testis (CT) antigens,* e.g., MAGE-1 (12), NY-ESO-1 (15), HOM-Mel-40/SSX2 (12), CT7 (16) and SCP-1 (17); (b) *mutational antigens,* e.g., p53 (18) and LKB/STK11 (19); (c) *overexpressed/amplified gene products,* e.g., eIF-4gamma (20) and carbonic anhydrase XII (21); (d) *immunogenic splice variants,* e.g., NY-CO-37/PDZ-45 (18) and ING1 (22); (e) *differentiation antigens*, e.g., tyrosinase (12) and NY-CO-27/Galectin-4 (18); and (f) *3p antigens*, encoded by putative tumor suppressor genes on chromosome 3p21.3, e.g., NY-LU-12 (23), NY-REN-9/ LUCA-15 and NY-REN-10/gene 21(19). Serum reactivity toward a number of SEREX-defined antigens, such as NY-CO-9, NY-CO-16, NY-REN-31, NY-REN-32, has been shown to be cancer-related (18, 19, 24), occurring at a variable frequency in cancer patients, but not in normal individuals. This cancer-related humoral immune response against SEREX-defined antigens suggests mutation or aberrant expression as the immunogenic stimulus, but with few exceptions, no mutations have been found and the majority of these SEREX-defined antigens are ubiquitously expressed. A recent study by Brass and colleagues (20) has provided evidence for gene amplification/overexpression as the basis for the immunogenicity of SEREX-defined antigens.

In the initial SEREX analysis of human breast cancer (22), 30 different antigens were identified, including CT antigens (HOM-Mel-40/SSX2 and NY-ESO-1), and a new member of the ING (Inhibitor of Growth) family of putative tumor suppressor proteins, ING2. In the present study, a SEREX analysis of 6 additional cases of breast cancer was carried out, and 94 breast cancer antigens were defined and characterized. In addition, the expression levels of mRNA transcripts encoding a subset of these breast cancer antigens were studied by real-time PCR in order to address the relationship between expression level and immunogenicity.

Results

Identification of human breast cancer antigens by SEREX analysis

Serum samples from 6 individuals with breast cancer were used to immunoscreen tumor-derived cDNA expression libraries using the SEREX methodology. In 4 of the 6 cases, autologous patient sera were used to screen cDNA libraries prepared from the corresponding primary breast cancer. In the other 2 cases, allogeneic patient sera were used to screen a cDNA library prepared from the SK-BR-3 cell line. As shown in Table 1, ninety-four serologically-defined breast cancer antigens were identified. They were designated NY-BR-8 through NY-BR-101 (25). These 94 antigens (Tables 2 through 4) represent 74 known gene products and 20 novel proteins, and could be categorized on the basis of cellular localization, subcellular targeting motifs, known function, or sequence similarities. Thirty-nine antigens are known or predicted nuclear proteins, and include DNA binding proteins (e.g., BR-8, -21, -55, and -66), transcription factors (e.g., BR-11, -52, -77, and -79), RNA binding proteins (e.g., BR-17, -18, -74 and -91), and RNA/DNA helicases (BR-65, -78 and -82). Twenty-four antigens are known or predicted cytoplasmic proteins, and include metabolic enzymes (e.g., BR-28, -30, -39 and -58), ribosomal proteins (e.g., BR-31, -32, -64 and -92), cytoskeletal proteins (BR-10, -88 and -101), sorting proteins (e.g., BR-42, -44, -48, and -80), adaptor proteins (BR-34 and BR-41) and proteins involved in the ubiquitin pathway (BR-29 and BR-37). Six antigens localize to the plasma membrane (BR-27, -43, -56, -57, -82 and -90) and 2 are extracellular gene products (BR-35 and BR-36). Twenty-three antigens had no defining structural characteristics. The nucleotide sequences of all novel clones have been deposited in the GenBank database (sequential accession numbers AF308284 - AF308300). The cDNA sequences coding for the 94 antigens identified in this study were also compared to sequences deposited in the SEREX database (14). Examination of this database revealed that 25 of the 94 breast cancer antigens defined in this study (26%) were also identified through SEREX analysis of breast cancer and other tumor types (Tables 2, 3 and 5).

Table 1. Immunoscreening of cDNA expression libraries from breast cancer with autologous and allogeneic patient sera (SEREX).

Tumor Type	Serum Source	Breast Cancer cDNA Library Designation	Number of Distinct Antigens Identified by SEREX (Known[1]/Novel[2])	Antigens with Non-Cancer Related Serological Profiles	Antigens with Cancer-related Serological Profiles
Invasive ductal carcinoma	Autologous	184	17 (12/5)	13	4
Invasive ductal carcinoma	Autologous	297	6 (6/0)	5	1
Invasive ductal carcinoma	Autologous	257	9 (8/1)	4	5
Invasive pleomorphic lobular carcinoma	Autologous	263	13 (9/4)	5	8
SK-BR-3 (established breast cancer cell line)	Allogeneic-1	9993	34 (27/7)	14	20
SK-BR-3	Allogeneic-2	9993	15 (12/3)	9	6
Totals			**94 (74/20)**	**50**	**44**

[1]Identical to GenBank Entries.
[2]Identities limited to expressed sequence tags (ESTs).

Table 2. SEREX-defined breast cancer antigens: Reactivity with sera from normal individuals.

NY-BR-Antigen	Identity/Similarities (Unigene cluster)	SEREX Database ID Number[1] of Equivalent Isolate (Tumor Source[2])	Reactivity with Normal Sera	Reactivity with Breast Cancer Sera
Known Gene Products				
8	H2K binding factor 2 (Hs.278573)	439 (RC), 1201 (MEL), 8 (GC)	1/16	2/10
13	Zuotin related factor 1 (Hs.82254)	6 (GC)	3/12	3/10
17	NP220 (Hs.169984)	1531 (OC)	1/12	2/10
22	Protein phosphatase 1B (Hs.5687)		1/12	1/10
23	DKFZp434L0117 (Hs.63795)		1/12	1/10
27	MHC Class I HLA Cw-0304 (Hs.277477)		4/25	2/25
29	Proteosome subunit p112 (Hs.3887)		6/12	4/10
30	GPDH (Hs.169476)		4/12	3/10
32	Ribosomal protein L34 (Hs.250895)		2/25	2/25
35	VEGFB (Hs.78781)		1/12	2/10
36	COL6A2 (Hs.4217)		4/19	6/20

42	Vacuolar sorting protein 45B (Hs. 6650)		1/17	1/25
43	Defender against cell death-1 (Hs.82890)		8/25	5/25
50	Sphingosine-1-phosphate lyase 1 (Hs.186613)		7/12	5/10
60	HIP-55 (Hs. 180766)		2/25	1/25
61	TGFB1 anti-apoptotic factor-1 (Hs.75822)	1057 (BC)	1/25	2/25
66	FBI-1 (Hs.104640)		1/20	2/20
68	Heme oxygenase 2 (Hs.63908)		2/20	2/20
70	KIAA0713 protein (Hs.88756)	159 (LC)	1/20	4/20
71	KIAA1002 protein (Hs.102483)		2/20	4/20
74	U2 snRNP auxiliary factor-2 (Hs.103962)	786 (HD), 430 (RC)	5/16	1/20
78	RNA helicase-related protein (Hs.8765)		3/25	1/25
80	TCP1 ring complex pp 5 (Hs.1708)		1/20	1/20
82	KIAA0801 protein (Hs.17585)		1/20	3/20
88	Talin (Hs.278559)	608 (PC)	1/20	1/20
89	Candidate of metastasis 1 (Hs.8603)		1/20	1/20
90	NAP-22 (Hs.79516)		1/20	2/20
95	KIAA0376 protein (Hs.4791)	1777 (CC), 357 (RC), 551 (GC), 955 (BC)	11/20	11/20
100	BRACA-1 AP 2 (Hs.122764)	1785 (CC), 1158 (RC), 190 (PC), 1616 (OC), 807 (BC)	2/19	2/20
Novel Gene Products				
15	Putative nuclear protein (Hs.178175)		1/12	1/10
16	Similar to Ankyrin (Hs.190251)	1792 (CC)	4/12	6/10
18	RNA splicing factor similarity (Hs.11065)		2/12	1/10
20	Similar to calmodulin (Hs.239812)		1/12	1/10
24	Similar to CDC 10 proteins (Hs.99741)		1/12	1/10
40	None (Hs.149190)		1/12	2/10
81	Phospholipase C beta similarity (Hs.75280)		1/20	1/20
86	Oxysterol binding protein similarity (Hs.233495)	319 (GC)	2/17	2/20
87	2 possible reading frames (Hs.18946)		1/25	1/25

[1] SEREX database ID number from http://www.licr.org/SEREX.html.

[2] Abbreviations: BC, breast cancer; CC, colon cancer; HD, Hodgkins disease; GC, gastric cancer; LC, lung cancer; MEL, melanoma; OC, ovarian cancer; PC, prostate cancer; RC, renal cancer

Table 3. SEREX-defined breast cancer antigens: Antigens associated with autoimmune disease.

NY-BR-Antigen	Identity (Unigene Cluster)	SEREX Database ID Number[1] of Equivalent Isolate (TumorSource[2])	Autoimmune Disease Associations[3]	Reactivity with Normal Sera	Reactivity with Breast Cancer Sera
9	Ifn inducible autoantigen 16 (Hs.155530)		SLE	1/12	1/10
12	RNA pol. II 23kDa subunit (Hs.24301)		SLE, PSS	1/12	1/10
14	Proteasome activator subunit 3/ Ki nuclear autoantigen (Hs.152978)		SLE	1/12	1/10
28	Glutathione S-transferase theta –1 (Hs.77490)		AH	1/12	2/10
31	Ribosomal phosphoprotein P1 (Hs.177592)		SLE	5/25	2/25
39	DBI-related protein (Hs.15250)	244 (RC), 1328 (GC), 1031(BC)	AD	1/16	1/12
47	Dynamin 1-like protein (Hs.180628)		Sclero	0/20	1/20
58	Aldolase A (Hs.273415)	79 (LC)	RA	0/20	1/20
59	PARP (Hs.177766)	432 (CC), 698 (RC), 939 (BC)	SLE, SS, RA, PSS	1/18	1/20
64	Alanyl-tRNA synthetase (Hs.75102)	471 (RC)	PM	0/20	1/20
65	Nuclear matrix protein 238 (Hs.272822)		PM, AH	0/20	1/20
69	Centromere protein F (Hs.77204)	626 (PC), 1557 (EC), 809 (BC)	SLE, Sclero	8/20	1/20
91	U1 snRNP (Hs.180789)	24 (GC), 349 (CC), 1267 (RC), 751 (BC)	SLE, SS, RA, PM, Sclero	3/20	6/20
93	Human autoantigen (Hs.75682)		SS, SLE, Sclero, RA	0/20	1/20
97	Sjogren syndrome antigen A1 (Hs.1042)		SS, SLE	0/20	1/20
101	Vinculin (Hs.75350)	1288 (BC)	thrombocytopenia	0/20	1/20

[1]SEREX database ID number from http://www.licr.org/SEREX.html.

[2]Tumor tissue abbreviations: BC, breast cancer; CC, colon cancer; EC, esophageal cancer; GC, gastric cancer; LC, lung cancer; , no additional isolates; PC, prostate cancer; RC, renal cancer.
[3]Autoimmune disease abbreviations: AH, autoimmune hepatitis; AD, autoimmune diabetes; PM, polymyositis; PSS, Progressive Systemic Sclerosis; RA, Rheumatoid Arthritis; Sclero, scleroderma; SLE, systemic lupus erythematosus; SS, Sjogren's Syndrome.

Table 4. SEREX-defined breast cancer antigens: Antigens detected with sera from a single breast cancer patient[1].

NY-BR-Antigen	Identity/Similarities/Motifs (Unigene cluster)	NY-BR-Antigen	Identity/Similarities/Motifs (Unigene cluster)
Known Gene Products			
10	Desmoplakin I (Hs.74316)	**48**	Sorting nexin 6 (Hs.76127)
11	RING 3 gene product (Hs.75243)	**52**	Transcriptional activator SRCAP (Hs.87908)
19	CGI-149 protein (Hs.241266)	**54**	SOM172 gene product (Hs.100623)
21	KIAA0708 (Hs.117177)	**67**	TIS11D gene product (Hs.78909)
26	Modulator recognition factor (Hs.920)	**76**	MAGE A3 (Hs. 36978)
33	GSPT1 (Hs.2707)	**77**	SMRT corepressor (Hs.120980)
37	HRIHFB2157 gene product (Hs.4552)	**83**	KIAA0081 protein, (Hs.78871)
44	BMAL1e protein (Hs.74515)	**92**	Ribosomal protein L10 (Hs. 29797)
Novel Gene Products			
38	SCR repeat domain (not clustered)	**73**	Similar to coatomer proteins (Hs.217001)
46	None (not clustered)	**85**	Nuclear localization signals (Hs.105153)
49	Ankyrin repeat domain (Hs.55565)	**84**	Transmembrane domain (Hs.206196)
51	None (Hs.128685)	**99**	RNA splicing factors similarity (Hs.183438)

[1]Antigens reacted with sera from single breast cancer patients (1/25), but not with sera from normal individuals (0/25), colon cancer patients (0/19), lung cancer patients (0/15), ovarian cancer patients (0/15) or esophageal cancer patients (0/15), and no equivalent isolates of these antigens were found in the SEREX database.

Table 5. SEREX-defined breast cancer antigens: Antigens reactive with sera from breast cancer patients and patients with other forms of cancer[1].

NY-BR-Antigen	Identity/Similarities/Motifs (Unigene cluster)	SEREX Database ID Number[2] of Equivalent Isolate (Tumor Source[3])	Seroreactivity				
			Breast Cancer	Colon Cancer	Lung Cancer	Ovarian Cancer	Esophageal Cancer
25	KIAA0854 protein, (Hs.30209)	128 (RC)	1/25	1/19	0/15	1/15	0/15
34	SHB adaptor protein (Hs.173752)		1/25	0/19	0/15	1/15	0/15
41	SNT-1 adaptor (Hs.251394)		1/25	0/19	1/15	0/15	0/15
45	Kinesin 2 (Hs.117977)	332 (GC), 96 (SM), 797 (RC)	2/25	0/19	0/15	0/15	0/15
53	LAGE-1[4] (Hs.167379)		4/25	1/19	2/15	1/15	2/15
55	Nucleosome assembly protein-1 (Hs.179662)	126 (RC), 1137 (MEL)	1/25	1/19	1/15	0/15	0/15
56	gC1Q binding protein (Hs.78614)		2/25	0/19	0/15	0/15	0/15
57	HER2 neu/erbB2 (Hs.173664)		2/25	0/19	0/15	0/15	0/15
62	Novel, kinesin similarity (Hs.278323)		2/25	0/19	0/15	0/15	0/15
63	Tumor protein D52 (Hs.2384)		2/25	0/19	0/15	0/15	0/15
72	MAGE A6 (Hs.198263)		1/25	0/19	0/15	1/15	0/15
75	Novel (Hs.5111)	1184 (MEL)	2/25	0/19	0/15	0/15	1/15
79	Tata element modulatory factor 1 (Hs.74985)	246 (GC)	1/25	0/19	0/15	0/15	0/15
94	Tumor protein p53 (Hs.1846)	33 (CC)	1/25	3/19	0/15	1/15	2/15
96	Novel, S/T kinase domain (Hs.4789)	296 (MEL)	2/25	0/19	0/15	0/15	0/15
98	MAGE D (Hs.4943)		3/25	0/19	1/15	0/15	0/15

[1]Antigens did not react with sera from normal individuals (0/25).
[2]SEREX database ID number from http://www.licr.org/SEREX.html.
[3]Abbreviations: BC, breast cancer; CC, colon cancer; GC, gastric cancer; MEL, melanoma; RC, renal cancer; SM, seminoma.
[4]Assays for LAGE-1 seroreactivity were performed using a recombinant phage expressing the highly homologous NY-ESO-1 protein.

Reactivity patterns of sera from normal individuals and cancer patients with SEREX-defined breast cancer antigens

To determine whether immune recognition of the isolated antigens was cancer-related, allogeneic sera samples obtained from 25 normal blood donors and 24 additional patients with breast cancer were tested for reactivity

against the panel of antigens using the plaque assay. Fifty-four of the 94 antigens (57%) had a serological profile that was not restricted to cancer patients, as evidenced by their reactivity with normal sera (Tables 1 and 2) or their known association with autoimmune disease (Tables 1 and 3). The remaining 40 antigens had a cancer-related serological profile, reacting only with sera from cancer patients (Tables 1, 4 and 5).

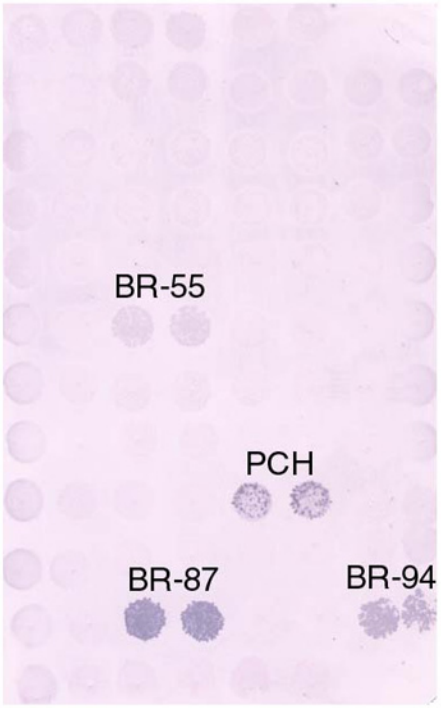

Figure 1. Nitrocellulose spot assay of duplicate bacteriophage clones expressing 44 different serologically-defined breast cancer antigens. Following incubation in a 1:200 dilution of serum from a colon cancer patient, the membrane was exposed to alkaline phosphatase-conjugated, Fc fragment-specific, goat anti-human IgG. A positive color reaction with 4-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate indicates seroreactivity with nucleosome assembly protein-1/NY-BR-55, NY-BR-87 and p53/BR-94, as well with the positive control antigen PINCH (PCH)/NY-CO-22.

The 40 antigens having a cancer-related serological profile were further tested for reactivity with serum panels from colon, lung, ovarian and esophageal cancer patients using the spot assay shown in Figure 1, and the following seroreactivity patterns were defined: (a) antigens identified by serum from only a single breast cancer patient; (b) antigens that reacted exclusively with sera from 2 or more breast cancer patients; and (c) antigens that reacted with sera from patients having other forms of cancer in addition to breast cancer patients. Of the 40 antigens having a cancer-related serological profile, 24 antigens reacted with sera from only one breast cancer patient, either the autologous patient or a single allogeneic patient, and are listed in Table 4. Sixteen of the 40 antigens having a cancer-related serological profile reacted with sera from more than one cancer patient (breast cancer patients and/or patients with other tumor types), and are listed in Table 5. Four of these antigens [gC1Q/BR-56, Her2neu/BR-57, BR-62, and tumor protein D52 (TPD52)/BR-63] reacted with sera from more than one breast cancer patient, but not with sera from patients with other forms of cancer. The remaining antigens listed in Table 5 react with sera from both breast cancer patients and individuals with other forms of cancer. In terms of known tumor antigens, LAGE-1/BR-53, a cancer-testis antigen highly homologous to NY-ESO-1, was isolated in the current study. In accordance with results of previous serologic surveys (24), LAGE-1/NY-ESO-1 had the highest frequency of cancer-related seroreactivity; antibody responses were detected in patients with breast (4/20), colon (1/19), lung (2/15), ovarian (1/15) and esophageal cancer (2/15). Similarly, a cancer-related serological response was also detected against p53/BR-94 (1/25 breast cancer, 3/19 colon cancer, 1/15 ovarian cancer and 2/15 esophageal cancer patients), and Her2neu/BR-57 (2/25 breast cancers patients), confirming previous surveys of seroreactivity against these antigens (18, 26, 27). In addition, a new set of immunogenic

breast cancer antigens were defined in this study, including known proteins such as kinesin 2/BR-45, gC1Q binding protein/BR-56, TPD52/BR-63 and MAGE D/BR-98, as well as novel gene products such as NY-BR-62, NY-BR-75, and NY-BR-96.

Expression patterns of mRNA encoding serologically-defined breast cancer antigens in normal tissues

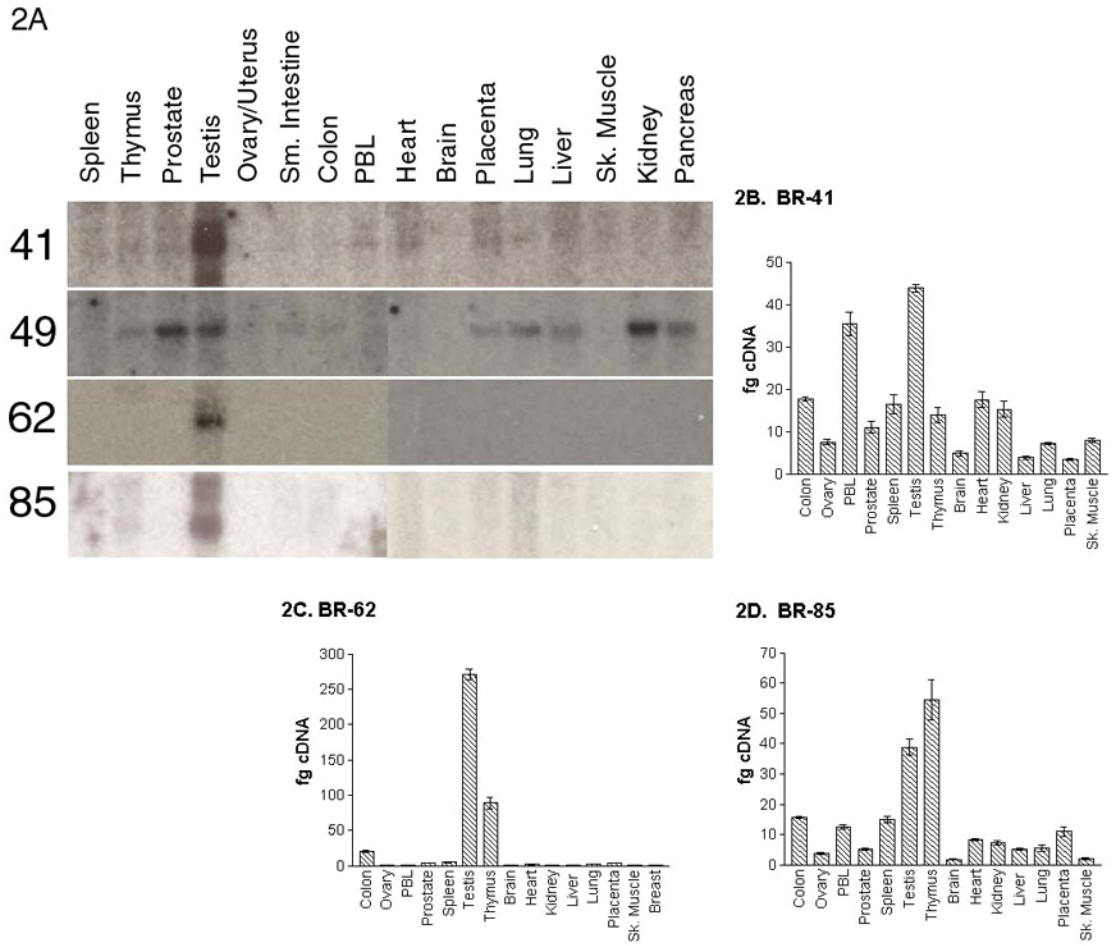

Figure 2. mRNA expression pattern of four antigens associated with a cancer related serological response. (A) Northern blot analysis of NY-BR-41, -49, -62 and -85 mRNA. Testis restricted expression was observed for NY-BR-41, -62 and -85, while NY-BR-49 was expressed in 10/16 normal tissues. (B) Real-time RT-PCR analysis of the level of NY-BR-41 mRNA expression (fg of homologous cDNA ± SEM). Results show low level ubiquitous expression. Highest levels of NY-BR-41 mRNA were detected in testis (43.8 ± 0.83 fg) and peripheral blood leukocytes (35.4 ± 2.65 fg), while lower levels (less than 18.0 fg) were detected in the remaining 12 normal tissues. (C) Real-time RT-PCR analysis of mRNA encoding NY-BR-62 showing low level ubiquitous expression (fg of homologous cDNA ± SEM). Highest levels of NY-BR-62 mRNA expression were detected in testis (270.7 ± 7.13 fg), thymus (88.8 ± 7.89 fg), and colon (20.2 ± 1.40 fg), while low levels (less 6.0 fg) were observed in 11 other normal tissues. (D) Real-time RT-PCR analysis of mRNA encoding NY-BR-85 showing low level ubiquitous expression (fg of homologous cDNA ± SEM). Expression of NY-BR-85 mRNA was detected in thymus (54.5 ± 6.70 fg), testis (38.8 ± 2.61 fg), colon (15.7 ± 0.38 fg), spleen (15.0 ± 1.04 fg) and peripheral blood leukocytes (12.5 ± 0.72 fg), while lower levels (less 10 fg) were detected in each of the remaining 9 normal tissues.

A preliminary *in silico* mRNA expression profile of all gene products identified in this study was based on the tissue distribution of expressed sequence tags (ESTs) in the human EST database. Products with no EST matches or those having EST matches limited to tumor tissue, fetal tissue or a single normal tissue were further

examined by Northern blotting and RT-PCR. Gene products with restricted EST profiles include the three well-characterized cancer-testis antigens, MAGE A3/BR-76, MAGE A6/ BR-72 and LAGE-1/BR-53, which are expressed exclusively in normal testis and a range of different tumor types (13), and 4 putative tissue restricted antigens, including a known gene product, SNT-1/BR-41, and 3 novel proteins, BR-49, -62 and -85. All seven of these antigens showed a cancer-related serological profile.

As shown in Figure 2A, the mRNA expression profiles of BR-41, -49, -62 and -85 were examined in Northern blots of non-normalized mRNA preparations derived from 16 different human tissues. Expression of SNT-1/BR-41, BR-62 and BR-85 was restricted to testis, appearing as a 3.2 kb mRNA transcript, a 4.0 kb mRNA transcript, and 2.4 kb and 3.2 kb mRNA transcripts, respectively. Expression of BR-49 was widespread, appearing as a 4.2 kb hybridization signal in 10 of 16 normal tissues (absent in spleen, ovary, PBL, heart, brain and skeletal muscle). The mRNA expression patterns of BR-41, -49, -62 and -85 were also analyzed by conventional RT-PCR, and transcripts for these 4 gene products were found in all normal tissues tested (lung, testis, small intestine, breast, liver, and placenta).

The detection of BR-41, -62 and -85 mRNA in normal tissues by RT-PCR but not by Northern blotting indicates low level ubiquitous expression. To examine this further, real-time RT-PCR was used to quantify mRNA expression in a panel of normalized cDNA preparations from 15 different human tissues. PCR amplification of cDNA coding for BR-41, -62 and -85 yielded distinct values for cycle threshold (Ct), which were compared to the Ct values obtained from a set of homologous cDNA standards of known concentration. As shown in Figure 2B, the highest levels of BR-41 mRNA expression in the normalized cDNA panel were detected in testis (equivalent to 43.8 fg of cDNA) and peripheral blood leukocytes (equivalent to 35.4 fg of cDNA), and lower levels (less than 18.0 fg of cDNA) in each of the remaining 12 normal tissues. Since cDNA from normal breast tissue was not included in this commercially obtained cDNA panel, the concentration of BR-41 mRNA in normal mammary gland was calculated relative to normal testis, and determined to be approximated 170 times higher in normal breast tissue compared to testis, and was equivalent 7300 fg of cDNA. The highest levels of BR-62 mRNA expression were detected in normal testis (271 fg of cDNA), thymus (89 fg of cDNA), and colon (20 fg of cDNA), with lower levels (less 6 fg of cDNA) detected in each of the remaining 11 normal tissues. The concentration of BR-62 mRNA in normal breast was equivalent to 0.8 fg of cDNA. The highest levels of BR-85 mRNA expression were detected in normal thymus (54 fg of cDNA) and testis (39 fg of cDNA), while lower levels (less than 16 fg of cDNA) were detected in each of the remaining 12 normal tissues. The concentration of BR-85 mRNA in normal breast was equivalent to 8.0 fg of cDNA.

Expression levels of mRNA encoding serologically-defined breast cancer antigens in normal and malignant breast tissue

The mRNA expression levels of ten antigens associated with a cancer-related serological response (Table 5) and/or showing differential mRNA expression (Figure 2) were measured in 10 breast cancer specimens and normal breast tissue by real-time RT-PCR. The relative level of mRNA expression in breast cancer compared to normal breast tissue was calculated on the basis of differences in normalized Ct values between the 2 tissues. Overexpression was defined as an mRNA expression level that is 3 times higher in tumor compared to normal breast tissue. As shown in Table 6, mRNA transcripts encoding 6/10 antigens, BR-56, -57, -62, -63, -85 and -98, were overexpressed in at least 1 breast cancer specimen. Four of these 10 antigens were frequently overexpressed in breast cancer, including Her2neu/BR-57 (3/10 cases), BR-62 (6/10 cases), TPD52/BR-63 (5/10 cases), and BR-85 (9/10 cases). Exceptionally high levels of mRNA expression in breast cancer were associated with Her2neu/BR-57 (107 times that of normal breast in patient # 7), BR-62 (53 times that of normal breast in patient # 8) and TPD52/BR-63 (132 times that of normal breast in patient # 8). In contrast, expression of SNT-1/BR-41 mRNA was consistently lower in breast cancer relative to normal breast tissue, with apparent downregulated expression (defined as an expression level of less than 20% of normal breast) occurring in 7/10 cases. The remaining products, kinesin 2/BR-45, BR-75 and BR-96, had similar mRNA expression levels in tumor and normal breast tissue.

The relationship between upregulation of gene expression and serological reactivity was examined in 8 breast cancer patients whose tumors and sera were available for typing (informative cases, patients # 1, 3, 5, 6, 7, 8, 9, 10). Serum antibodies against BR-56, -57, -62, -63, -75 and -85 were detected in breast cancer patient #10. In this case, mRNA transcripts encoding 4/6 of these antigens were overexpressed in the autologous tumor (BR-56, -62, -63 and -85). None of the other cases showed seroreactivity against the amplified/overexpressed SEREX-defined antigens.

Table 6. Quantitative analysis of mRNA encoding SEREX-defined breast cancer antigens in normal and malignant breast tissue: Relation between mRNA level and immunogenicity.

Antigen	Ratio of mRNA in breast cancer to mRNA in normal breast in patient # ('+' indicates serum antibody reactivity[1])									
	1	2	3	4	5	6	7	8	9	10[2]
BR-41	0.01	0.19	0.04	0.26	0.38	0.11	0.10	0.86	0.03	0.07
BR-45/Kinesin 2	0.50	0.35	0.41	0.25	0.34	0.27	0.43	0.85	0.23	0.57
BR-56/gC1Q	0.66	0.49	0.22	0.83	0.40	0.44	0.69	0.77	0.44	3.71 (+)
BR-57/Her2neu	0.30	1.17	2.48	2.18	1.43	65.49	107.39	5.91	0.44	1.81 (+)
BR-62	1.21	1.65	0.26	2.20	5.19	3.80	4.22	53.44	15.49	12.21 (+)
BR-63/TP D52	1.44	4.08	0.91	7.36	6.26	2.08	2.88	132.51	1.67	5.33 (+)
BR-75	2.27	0.34	0.48	0.59	0.43	0.34	0.37	0.78	0.36	1.06 (+)
BR-85	5.55	6.28	1.15	6.65	3.17	4.98	5.91	11.42	10.34	11.34 (+)
BR-96	0.53	1.09	0.45	0.65	0.39	0.33	0.56	0.81	0.31	1.26
BR-98/MAGE D	0.16	2.10	0.58	2.55	0.73 (+)	0.58	0.67 (+)	12.67	1.44	0.60

[1]Serum reactivity of patients # 1, 3, 5, 6, 7, 8, 9, and 10 assessed in the plaque assay.
[2]Patient # 10 referred to as allogeneic-1 in Table 1.

Discussion

Knowledge regarding the structure, expression pattern, and immunogenicity of serologically-defined cancer antigens is critical in assessing their relevance to cancer and their therapeutic and diagnostic potential. Given the large number of tumor antigens defined by SEREX analysis, a strategy needed to be developed that could identify antigens of the greatest interest in the context of cancer. Although mutational changes or cancer-restricted expression would be properties of obvious importance, these turn out to be characteristics that are rarely associated with SEREX defined antigens. For this reason, we have chosen to focus our attention on the frequency of antibody responses to the antigens in normal individuals and cancer patients, selecting those antigens with cancer-related serological profiles for detailed study. In terms of allogeneic serum reactivity, 54 of the 94 antigens identified in the current study had a serological profile that was not restricted to cancer patients. Although this provides presumptive evidence that these antigens are unrelated to cancer, they should not be dismissed without further study since some of these antigens have functional properties associated with tumor growth, such as angiogenesis [e.g. vascular endothelial cell growth factor/BR-35 (28)], anti-apoptosis [e.g. defender against death protein-1/BR-43 (29)], and regulation of cell proliferation [e.g. Zuotin related factor 1/BR-13 (30)]. The remaining 40 antigens were characterized by a cancer-related serological profile, reacting only with sera from cancer patients and not with sera from normal individuals. These include known tumor antigens such as LAGE-1/BR-53 (31), Her2neu/BR-57 (26), MAGE-A6/BR-72 (32), MAGE-A3/BR-76 (33) and p53/BR-94 (27), as well as several transcriptional regulators such as RING 3/BR-11 (34), modulator recognition factor/BR-26 (35), SNT-1 adaptor protein/BR-41 (36), Snf2-related CBP activator protein/BR-52 (37), SMRT corepressor/BR-77 (38) and TATA modulatory factor 1/BR-79 (39). With regard to novel antigens identified in this study, 11 were associated with a cancer-related serological profile, and their significance in the context of cancer is currently being investigated.

In conformity with previous results, a small subset of antigens that show a cancer-related serological profile are characterized by differential gene expression; these include the CT antigens, LAGE-1/BR-53, MAGE-6/BR-72 and MAGE-3/BR-76, and two novel antigens, BR-62 and BR-85. The BR-62 cDNA encodes a 312 amino acid protein which contains a bZIP transcription factor domain and is 48% identical to Xklp2, a kinesin-like protein required for mitotic centrosome separation in *Xenopus laevis* (40). The BR-85 cDNA encodes a 278 amino acid protein containing two bipartite nuclear localization signals and lacking any similarity with proteins in the existing databases. Although Northern blots indicated that BR-62 and BR-85 expression is restricted to testis, quantitative RT-PCR analysis of these antigens showed ubiquitous, low level expression in several normal tissues. In tumor tissue, BR-62 mRNA was found to be overexpressed in 60% of breast cancer specimens at levels that were approximately 4-50 times higher than normal breast tissue, while BR-85 mRNA was overexpressed in 90% of breast cancer specimens at levels that were approximately 3-11 times higher than normal breast tissue. Transcripts encoding 4 other antigens with cancer related-serological profiles, Her2neu/ BR-57, TPD52/BR-63, gC1Q/BR-56 and MAGE D/BR-98, were overexpressed in 30%, 50%, 10% and 10% of the breast cancer specimens, respectively. As bulk tumor and normal tissue were used in these studies, the precise cell type responsible for overexpression is not known and requires detailed investigation. TPD52/BR-63, which has been reported to be overexpressed in breast (41) and lung cancer (42), was found to be immunogenic in 2/25 breast cancer patients. This protein is thought to be involved in calcium-mediated signal transduction and the regulation of cell proliferation (41), suggesting that its upregulation is of possible etiologic significance. BR-56/gC1q binding protein, which was found to be immunogenic in 2/25 breast cancer patients, is a multifunctional protein that interacts with the globular domain of complement component C1q at the cell surface (43), and is a component of the pre-mRNA splicing factor, SF2 (44). Unpublished reports (GenBank entries) concerning MAGE D/BR-98 indicate that it is overexpressed in breast and hepatocellular cancer. MAGE D/BR-98 was associated with a serological response in patients with breast (3/25) and lung cancer (1/15). Patients with serological reactivity toward MAGE D lacked detectable antibodies against MAGE A3 and MAGE A6, indicating that isolation of MAGE D/BR-98 by SEREX is not a result of cross-reacting antibodies to other MAGE antigens. Although MAGE D/BR-98 appears to be a member of the MAGE gene family, it is ubiquitously expressed (contrary to the cancer/testis expression pattern of other MAGE genes) and does not contain any of the CTL-defined epitopes previously described for members of this antigen family (45). In contrast to SEREX-defined gene products showing overexpression in breast cancer, expression of SNT-1/BR-41 appears to be markedly downregulated in 70% of breast cancer specimens relative to normal breast tissue. SNT-1 /BR-41 is a membrane-associated adaptor protein that undergoes rapid tyrosine phosphorylation following stimulation with growth factors and interacts with the Ras guanine nucleotide exchange factor, SOS. Given its potential role in signal transduction pathways (36), downregulated expression of SNT-1/BR-41 may have significance in relation to cancer. In this regard, how does a protein having reduced levels in cancer induce an immune response? One possibility is that the immune system has the capacity to respond to downregulated as well as upregulated (overexpressed/amplified) epitopes. The SEREX-defined "3p" antigens, NY-LU-12 (23), NY-REN-9/ LUCA-15 (19), NY-REN-10/gene 21(19) and NY-BR-79/TATA modulatory factor-1, represent other examples of an immune response to gene products that are deleted or downregulated in cancer. These antigens map to the putative tumor suppressor gene locus on 3p21, a region often deleted (downregulated) in small cell lung cancer (46), breast cancer (47) and renal cancer (48).

Brass and colleagues (20) have reported a striking example of several overexpressed/amplified gene products associated with antibody responses in patients with lung cancer and suggested that this upregulated expression was the basis for the immunogenicity of these antigens. One patient in our breast cancer series (patient #10) also had a strong association between overexpression and immune response, with an antibody response to 4 of 6 overexpressed gene products in the autologous tumor. However, other patients failed to mount an immune response to overexpressed products in their tumors, indicating that overexpression is not an invariable prelude to an antibody response.

In addition to quantitative changes in gene expression (overexpression/amplification, underexpression/loss) being involved in the immunogenicity of SEREX-defined antigens, examples of qualitative changes eliciting an antibody response, although uncommon, are also known. These include aberrant expression of tissue restricted gene products, e.g., CT antigens, and structural changes, e.g., mutation, abnormal splice variants, and altered reading frames. With regard to the latter, the novel antigen, NY-BR-87, has two possible reading frames of 202 amino acids or 205 amino acids. Given the immunogenicity of BR-87, it would be important to determine whether expression of an alternative BR-87 reading frame in cancer cells can elicit an immune response. Immunogenic alternative reading frames have been described for tumor antigens such as NY-ESO-1 (49) and RU2AS (50).

Serological analysis with purified recombinant proteins representing both reading frames of BR-87, as well as the development of monoclonal antibodies to the two putative proteins and their subsequent use in immunohistochemistry will clarify this possibility. One of the more perplexing aspects of SEREX analysis is how rarely mutated gene products are isolated. Products of mutational events may in fact be the immunogenic stimulus for initiating a humoral immune response, but the resulting antibody reacts predominately with non-mutated protein sequences. This appears to be the case for the immunogenicity of p53 in patients with cancer (51), although it is possible that overexpression plays a role in antibody responses to p53, since mutations in p53 increase its half life and leads to p53 accumulation in tumor cells. A consequence of the possible predilection of the antibody response to focus on non-mutated sequences in mutated gene products is the likelihood that the non-mutated gene counterpart will be isolated by SEREX, derived from a non-mutated allele in the cancer cell, or from normal admixed cells in the tumor mass. To exclude this latter possibility, isolated tumor cell populations or tumor cell lines should be employed as SEREX targets. Clearly, much more attention needs to be given to the possibility that mutation is a primary reason for the immunogenicity of SEREX-defined antigens, and approaches such as single-strand conformation polymorphism, dideoxy fingerprinting, or direct sequence analysis of multiple clones (both cDNA and genomic) need to be included in the analysis of these gene products. Antigens that map to loci implicated in human cancer are prime candidates for further mutational analysis, and include BR-79/TATA modulatory factor-1, a coactivator of the androgen receptor (39), which maps to 3p21.3, a site of minimal deletion in breast (47), renal (48) and non-small cell lung cancer (46), as well as BR-81, which maps to chromosome 6q23.3 (D6S311-D6S440), a site of minimal deletion in breast (52), ovarian (53) and cervical cancers (54).

Abbreviations

Ct, cycle threshold; CT, cancer/testis; SEREX, serological expression cloning

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Materials and methods

Construction of cDNA libraries

Tumor samples from breast cancer patients 184 (invasive ductal carcinoma), 297 (invasive ductal carcinoma), 257 (invasive ductal carcinoma), and 263 (invasive pleomorphic lobular carcinoma) were obtained as surgical specimens and used in the construction of cDNA libraries. A cDNA library was also prepared from the SK-BR-3

breast cancer cell line (ATCC HTB-30). Total RNA was prepared by the guanidinium thiocyanate method and purified to poly(A)$^+$ RNA using the Dynabeads mRNA purification Kit (Dynal, Lake Success, NY). Manufacturer's protocols were used for cDNA synthesis and ligation into appropriate vectors. Two bacteriophage expression vectors were utilized; the lambda ZAP vector (Stratagene, La Jolla, CA) was used for cDNA derived from the tumor of patient 184 and the SK-BR-3 cell line, and the lambda TRIPLX vector (Clontech laboratories Inc., Palo Alto, CA) was used for cDNA derived from the tumors of patients 297, 257 and 263. Following *in vitro* packaging, libraries containing 1-2 x 10^6 primary recombinants were obtained. cDNA libraries were not amplified prior to immunoscreening.

Immunoscreening

Sera were obtained from individuals undergoing diagnostic or therapeutic procedures. To remove antibodies reactive with vector-related antigens, sera (1:10 dilution) were absorbed by passage through columns containing Sepharose 4B coupled to lysates of *E. coli* Y1090 and bacteriophage-infected *E. coli* BNN97 (5 Prime 3 Prime, Inc., Boulder, Co.), followed by a 15 hour incubation with nitrocellulose filters precoated with proteins derived from *E. coli* and *E.coli*/phage lysates (mock experimental membranes). Library screenings were performed as previously described (18, 19) using preadsorbed patient sera at a dilution of 1:200. A total of 5-6 x 10^5 recombinants were screened per cDNA library. Serum reactive phage clones were converted to plasmid forms by *in vivo* excision. Plasmid DNA was sequenced at the Cornell University DNA Service (Ithaca, NY) using an ABI Prism automated DNA sequencer (Perkin Elmer, Foster City, CA).

Two assays were employed to determine allogeneic serum reactivity. In the case of allogeneic sera derived from breast cancer patients and normal individuals, a previously described plaque assay was used (18, 19). Briefly, 1500 plaque-forming units (pfu) of monoclonal phage encoding individual serologically-defined breast cancer antigens were mixed with an equal number of negative control phage (phage without cDNA inserts) and used to infect exponentially growing *E. coli* XL-1 Blue MRF' (Stratagene). Following a 15 hour amplification phase, plaque-derived proteins were transferred to 47 mm nitrocellulose membrane discs and tested for reactivity with individual serum samples (1:200 dilution) as previously described (18, 19). Subsequent screening of serum samples from patients with other forms of cancer (lung, colon, ovarian, esophageal) was carried out using a modification of the plaque assay, termed a spot assay. In this method, 80 x 120 mm nitrocellulose membranes were precoated with a film of NZY/0.7% Agarose/2.5 mM IPTG and placed on a reservoir layer of NZY/0.7% Agarose in a 86 x 128 mm Omni Tray (Nalge Nunc International Corp., Naperville, IL). Approximately 1.0 x 10^5 pfu of monoclonal phage encoding individual serologically-defined breast cancer antigens, in a volume of 20 µl, were mixed with 20 µl of exponentially growing *E. coli* XL-1 Blue MRF and spotted (0.7µl aliquots) on the precoated nitrocellulose membranes. Membranes were incubated for 15 hours at 37°C. A total of 46 different serologically-defined breast cancer antigens were spotted in duplicate per nitrocellulose membrane. The agarose film was then removed from the membrane and the filters were processed for reactivity with individual serum samples (1:200 dilution) as previously described (18, 19). Serum reactivity detected by the spot assay was verified in the plaque assay. Both assays appear to have comparable specificity and sensitivity.

Northern blot analysis and standard RT-PCR

Northern blots containing normal tissue poly(A)$^+$ RNA (2 µg/lane) were obtained from Clontech, laboratories Inc. (Palo Alto, CA). Random-primed ^{32}P-labeled probes consisting of 300-600 bp PCR products from coding sequences of selected seroreactive cDNA clones were hybridized for 1.5 hours in Expresshyb (Clontech) at 68°C, and washed at high stringency (2 times, 30 min each, with 0.1X SSC/0.1% SDS at 65°C). The resultant Northern blots were developed with Biomax MS autoradiography films (Eastman Kodak Co., Rochester, NY).

The mRNA expression pattern of a selected set of serologically-defined breast cancer antigens was also determined by standard RT-PCR, using a panel of normal tissue RNA from lung, testis, small intestine, breast, liver, and placenta (Clontech). cDNA preparations used as templates in the RT-PCR reactions were synthesized using MuLV reverse transcriptase as described previously (19). As a control for genomic DNA contamination, all cDNA synthesis reactions were set up in duplicate with additional samples lacking reverse transcriptase. The cDNA was then amplified by PCR (30 cycles), using gene-specific primers (Gibco BRL, Grand Island, NY) and AmpliTaq Gold DNA polymerase (PE Biosystems), as described (19).

Real-time quantitative RT-PCR

Total RNA from 10 different breast cancer specimens was prepared by the guanidinium thiocyanate method. Total RNA from normal breast consisted of a pool of RNA from 2 healthy individuals (Clontech). RNA (1 µg) was reverse transcribed into cDNA using the TaqMan EZ RT-PCR kit (PE Biosystems, Foster City CA). As a control for genomic DNA contamination, all cDNA synthesis reactions were set up in duplicate with additional samples lacking reverse transcriptase. Reagents were purchased from PE Biosystems, except where noted. Multiplex PCR reactions were prepared using 2.5 µl of cDNA diluted in TaqMan Universal PCR Master Mix supplemented with Vic-labeled (PE Biosystems proprietary dye) human beta-glucuronidase endogenous control probe/primer mix, 200 nM 6-carboxy-fluorescein (Fam)-labeled gene-specific TaqMan probe, and a predetermined, optimum concentration of the corresponding gene-specific forward and reverse primers (300-900 nM). Triplicate PCR reactions were prepared for each cDNA sample. PCR consisted of 40 cycles of 95°C denaturation (15 seconds) and 60°C annealing/extension (60 seconds). Thermal cycling and fluorescent monitoring were performed using an ABI 7700 sequence analyzer (PE Biosystems). The point at which the PCR product is first detected above a fixed threshold, termed cycle threshold (Ct), was determined, and normalized against the Ct value of the endogenous control product (DELTACt = Ct Fam - Ct Vic). The quantity of gene-specific transcripts present in the breast cancer cDNA samples relative to normal breast tissue was calculated by comparing the normalized cycle thresholds in tumor with non-malignant breast tissue (DELTADELTACt = DELTACt of tumor - DELTACt of normal breast), and determining the relative concentration (Relative Concentration = $2^{-DELTADELTACt}$).

In addition, a selected set of Fam-labeled gene-specific TaqMan probe/primer combinations were used to amplify a panel of 14 normal tissue cDNA preparations which had been normalized against 6 housekeeping genes and purported to be virtually free of genomic DNA (Clontech). The relative abundance of gene-specific transcripts in normal tissues was determined by comparison with a standard curve generated from the Ct values of known concentrations of plasmid DNA containing the relevant gene. Since cDNA derived from normal breast tissue was not included in the commercially obtained panel, a cDNA preparation derived from normal breast tissue (see above) was normalized according to the Ct values for beta-glucuronidase, and the concentration of gene-specific transcripts in normal breast tissue was also calculated relative to its expression in testis using the formula described above.

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