The Tumor Antigen Repertoire Identified in Tumor-Bearing Neu Transgenic Mice Predicts Human Tumor Antigens

Hailing Lu, Keith L. Knutson, Ekram Gad, and Mary L. Disis

Tumor Vaccine Group, Center for Translational Medicine in Women’s Health, University of Washington, Seattle, Washington

Abstract

FVB/N mice transgenic for nontransforming rat neu develop spontaneous breast cancers that are neu positive and estrogen receptor negative, mimicking premenopausal human breast cancer. These animals have been widely used as a model for immunobased therapies targeting HER-2/neu. In this study, we used serological analysis of recombinant cDNA expression libraries to characterize the antigenic repertoire of neu transgenic (neu-tg) mice and questioned the ability of this murine model to predict potential human tumor antigens. After screening 3 × 10^6 clones from 3 different cDNA libraries, 15 tumor antigens were identified, including cytokeratin 2-8, glutamyl-prolyl-tRNA synthetase, complement C3, galectin 8, and serine/threonine-rich protein kinase 1. Multiple proteins involved in the Rho/Rho-associated, coiled-coil–containing protein kinase (Rock) signal transduction pathway were found to be immunogenic, including Rock1, Rho/Rac guanine nucleotide exchange factor 2, and schistosoma mansoni adult worm antigen preparation 70. All of the identified antigens are self-proteins that are expressed in normal tissues in addition to breast tumors and the majority of the antigens are intracellular proteins. More than half of the mouse tumor antigens have human homologues that have been reported previously as tumor antigens. Finally, the tumor-specific antibody immunity and marked immune cell infiltration that was observed in mice with spontaneous tumors were not observed in mice with transplanted tumors. Our results indicate that neu-tg mice bearing spontaneous tumors develop humoral immunity to their tumors similar to cancer patients and that tumor antigens identified in transgenic mouse may predict immunogenic human homologues. (Cancer Res 2006; 66(19): 9754-61)

Introduction

The development of cancer vaccines and other immunobased therapeutics has been facilitated over the last several years by the identification of a multitude of tumor antigens. In fact, to date, >2,000 cancer-related immunogenic proteins have been identified (1). It is unknown, however, which of these proteins would potentially be "tumor rejection" antigens (i.e., those proteins, when targeted by the immune system, would mediate tumor destruction). Furthermore, it is unknown what factors or characteristics make a tumor-associated proteins immunogenic. We questioned whether endogenous tumor immunity in a transgenic mouse could mimic the antigenic repertoire in human cancers and potentially provide a model for addressing some of these questions. The neu transgenic (neu-tg) mouse is a model of neu-mediated, estrogen receptor (ER)–negative breast cancer that has significant biologic and pathologic similarity to human breast cancer (2–4).

We used the high-throughput screening method serological analysis of recombinant cDNA expression libraries (SEREX) to identify the tumor antigen repertoire in neu-tg mice. Pooled sera from transgenic mice bearing spontaneous tumors were used to screen three libraries, two made from syngeneic tumor cell lines and one from testsis. Approximately 3 million plaques were screened. Results showed that the majority of identified antigens were associated with immunogenic human homologues. The neu-tg mouse represents a useful model for tumor antigen discovery and immunotherapeutic testing, which may closely mirror human premenopausal breast cancer.

MATERIALS AND METHODS

Mice and tumor cell lines. Neu-tg mice [strain name, FVB/N-Tg(MMTV-neu)-202Md1] were obtained from Charles River Laboratory (Bar Harbor, ME) and bred under specific pathogen-free conditions at the University of Washington (Seattle, WA). The mice harbor nonmutated, nonactivated rat neu under control of the mouse mammary tumor virus (MMTV) promoter. For SEREX screening, serum samples were collected from animals bearing spontaneous tumors and control tumor-free female mice. Animal care and use was in accordance with institutional guidelines. Mouse mammary carcinoma (MMC) cells were derived from a spontaneous tumor in a neu-tg mouse. Antigen-negative variant (ANV) cells were derived from a rat neu-negative tumor that developed in a parental FVB mouse after partially rejecting an MMC implantation (5). For tumor implantation experiment, MMC cells were harvested using 2 mmol/L EDTA in PBS and washed before injection. Mice were inoculated with 1 × 10^3 MMC cells s.c. on the mid-dorsum with a 23-gauge needle. Tumors were measured every other day with Vernier calipers and tumor volume was calculated as the product of length × width × height × 0.5236. In vivo data are presented as mean ± SE.

RNA extraction and construction of cDNA libraries. Poly(A)^+ RNA was isolated from MMC and ANV cells using RNAAqueous and Poly(A)Partist kit from Ambion (Austin, TX). cDNA expression libraries were constructed using a ZAP Express vector from Stratagene (La Jolla, CA) following the manufacturer’s instructions. The primary MMC and ANV library each contained ~1 × 10^6 recombinants and was amplified once before immunoscreening. To check the heterogeneity of the primary library, 20 plaques were randomly picked and PCR with T3 and T7 primer was done to validate the cDNA insert. The inserted ranged from 500 to 3,500 bp. A commercially available mouse testis cDNA library from Stratagene was also used for screening.

SEREX screening using murine sera. A total of 1 × 10^6 recombinant clones per library were screened with pooled sera from 10 tumor-bearing mice using the method as originally described by Sahin et al. (6) with some modifications. Briefly, 5 × 10^3 phage clones were plated with XL-Blue on NZY agar plates. After 4 hours of incubation at 37°C, isopropyl-L-thio-β-D-galactopyranoside (IPTG)–impregnated nitrocellulose membrane was
overlaid on the plates to induce protein expression. Nitrocellulose membranes were removed from the plates after an overnight incubation. The membrane was first washed in TBS with 0.05% Tween 20, blocked in TBS (20 mMol/L Tris-HCl and 150 mMol/L NaCl) with bovine serum albumin (BSA), and then incubated with 1:200 diluted sera (in TBS with 1% BSA and 0.05% sodium azide) overnight at room temperature. Preliminary experiments showed that mouse sera had nondetectable levels of anti-Schistosoma coi antibody most likely because of their controlled environment (data not shown), so no preabsorption with E. coli lysate was required. Instead, we did a pseudoscreening with lytic membrane to remove nonspecific binding. Alkaline phosphatase–conjugated goat anti-mouse antibody (diluted 1:2,000) was used as the secondary antibody. Nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate was used for color development. Positive clones that did not react to control sera from normal mice were purified to mononucleotides and converted to pBluescript phagemid by in vivo excision using XLORL cells and ExAssist helper phage (Stratagene). Plasmid DNA was prepared using a FastPlasmid kit (Eppendorf, Hamburg, Germany). The nucleotide sequences of the cDNA inserts were determined using Big Dye reaction and ABI Prism automated DNA sequencer. Blast was used to search for sequence homology.

**Crude lysate ELISA for the detection of tumor antigen–specific antibodies.** Crude lysates of bacteria expressing the protein of interest were used as a source of the specific antigens as described by Tureci et al. (7). A newly identified phage clone was excised in vivo to pBluescript phagemid allowing IPTG-inducible prokaryotic expression of insert. The XLORL bacteria were transformed with plasmid encoding the gene of interest or plasmid with no inserts (reference lysate) and grown in Luria-Bertani medium. Protein expression was induced with 2 mMol/L IPTG. After overnight culture at 37°C, the bacteria were spun down and resuspended in 2 mL PBS with protease inhibitor (Roche, Mannheim, Germany). Protein concentration was determined by bicinchoninic acid protein assay kit (Pierce, Rockford, IL). Bacterial lysate was frozen at −70°C until use in ELISA or Western blot. Ninety-six-well Immulon 4HBX microtiter plates (Dynex Technologies, Inc., Chantilly, VA) were coated with bacteria lysate (50 µg/mL diluted in carbonate buffer) overnight at 4°C. After blocking with 100 µL/well of PBS/1% BSA at room temperature on a rocker for 1 hour, plates were washed four times with PBS/0.05% Tween 20 and then 50 µL mouse sera (diluted 1:100) were added to each well and incubated at room temperature on a rocker for 2 hours. After serum incubation, plates were washed four times with PBS/Tween 20 and goat anti-mouse IgG-horseradish peroxidase conjugate (diluted 1:5,000; Zymed, San Francisco, CA) was added and incubated for 1 hour at room temperature on rocker. Following final PBS/Tween 20 wash, TMB developing reagent (Kirkegaard and Perry, Gaithersburg, MD) was added (75 µL/well) and reaction was then stopped with 75 µL/well 1 N HCl and read at absorbance of 450 nm. The absorbance of each serum dilution was calculated as the absorbance of the antigen lysate–coated wells minus the absorbance of reference lysate-coated wells. A response was defined as positive if the Δ4 was greater than the mean ± 2 SD of controls, which are negative for a particular antigen as determined by SEREX.

**Analysis of mRNA expression in different tissue and cell lines by real-time reverse transcription-PCR.** Total RNA from normal mouse mammary tissue, fresh murine breast tumors, and breast cancer cell lines (MMC and ANV) were isolated using RNAAqueous kit. The integrity of RNA was tested using an Agilent BioAnalyzer (Foster City, CA). cDNA was generated from 5 µg RNA by SuperScript III reverse transcriptase (Invitrogen, San Diego, CA) with oligo(dT) as primers (Invitrogen, San Diego, CA) with oligo(dT) as primers (Applied Biosystems). mRNA expression level of tumor antigens was normalized to β-actin using the ΔCT method. Level of expression = 2−ΔCT, where ΔCT = CT antigen − CT actin, Ct is the cycle threshold at which the fluorescence signal crosses an arbitrary value.

**Flow cytometric analysis of tumor-infiltrating immune cells.** Immediately after tumor resection, the tumor was minced into small pieces and then immersed in 5 mL digestion mixture (RPMI 1640 with 10% fetal bovine serum, 50 µL of 1% collagenase, and 50 µL of 1% DNase, both are from Life Technologies, Carlsbad, CA). This mixture was incubated on rocker at room temperature for 30 minutes and then filtered through a 70-µm screen. The leukocytes were further enriched by Ficol. The resulting single-cell suspension was analyzed on a FC500 flow cytometer (Beckman Coulter, Fullerton, CA) after staining with the appropriate antibodies for 30 minutes at 4°C in the dark. All the antibodies (CD3-FITC, CD4-PE, CD8-PerCP, NK1.1-PE, CD19-FITC, and CD11c-PE) were purchased from BD Biosciences (San Diego, CA).

**Statistical analysis.** χ2 test was used to compare the frequency of antibody response between tumor group and control group. Student’s t test was used to compare the amount of infiltrating immune cells in spontaneous versus implanted tumor. SPSS version 13.0 for Windows (SPSS, Inc., Chicago, IL) was used for statistical analysis. P < 0.05 was considered to be statistically significant.

**Results**

Multiple immunogenic proteins, related to cancer progression, can be identified in tumor-bearing neu-tg mice. Immunoscreening of three libraries (~1 × 106 phages screened for each library) using sera from mice with spontaneous tumors yielded a total of 62 positive clones. Nucleotide sequence analysis of the cDNA inserts identified 15 different antigens (Table 1). The five mostly frequently identified antigens are the following: cytokeratin 2-8 (Krt2-8; represented by 15 overlapping clones), glutamyl-prolyl-tRNA synthetase (Eprs; 13 clones), complement C3 (C3; 9 clones), galectin 8 (Lgal8; 6 clones), and serine/threonine-rich protein kinase 1 (Srk1; 5 clones). The antigens are proteins of diverse functions, including structural proteins [Krt2-8, gelsolin (Gsn), and matrin 3 (Matr3)], signal transduction proteins [Rho-associated, coiled-coil–containing protein kinase (Rock) 1, Rho/Rac guanine nucleotide exchange factor 2 (Arhgef2), RAB3A-interacting protein (Rab3ip), and schistosoma mansoni adult worm antigen preparation 70 (Swap70)], and transcription factors [leucine zipper transcription factor-like protein 1 (Lzfl1) and Y box-binding protein (Yb1)]. The chromosomal location of the genes did not show any bias toward a specific chromosome (Table 1). In terms of cellular location, all Lgal8 and C3 are extracellular. All the other proteins are intracellular with five nuclear proteins [Yb1, ubiquitin-specific protease 7 (Usp7), Lzfl1, Matr3, and exportin 5 (Xpo5)]. Many of the antigenic proteins play an important role in tumorigenesis. For example, Rab3ip is a proto-oncogene that interacts with the cancer-testis antigen SSX2 (8). Gsn, an actin-severing protein, has tumor suppressor function and has been reported to be down-regulated in human ovarian and breast cancer (9–11). Three of the identified antigens, Rock1, Swap70, and Arhgef2, are involved in the Rho/Rac-Rock signal transduction pathway. Rock1, the effector of RhoA, regulates the organization of the actin cytoskeleton and is responsible for cell motility and cytokinesis (Fig. 1). Arhgef2 is a Rho/Rac guanine nucleotide exchange factor. Swap70 may also function as a guanine nucleotide exchange factor (12). Rho/Rock pathway plays an important role in tumor cell invasion and cancer metastasis (13–17).

SEREX-identified antigens are widely expressed intracellular self-proteins. Real-time reverse transcription-PCR (RT-PCR)

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was used to measure the mRNA expression level of the SEREX-identified antigens in different tissues to examine their expression profile. The expression level was evaluated in a panel of 10 normal tissues, including brain, eye, heart, kidney, liver, lung, spleen, testis, thymus, and uterus. All of the antigens were detected in normal and tumor tissues. Figure 2 shows the expression profile of the five most frequently identified antigens. Krt2-8 is the only antigen that shows relatively high level of expression in testis compared with other normal tissues (Fig. 2A). However, it is also expressed in the uterus, kidney, and liver. Srpk1, which was reported previously to have restricted expression in testis (18), was found to express in other tissue as well (Fig. 2E). In summary, none of the tumor-associated antigens (TAA) we found showed restricted expression as that of a cancer/testis antigen.

**SEREX-identified antigens have dysregulated expression in breast cancer tissue and cell line compared with normal mammary gland.** As tumor antigens may be overexpressed in cancer compared with normal tissue, we used real-time RT-PCR to evaluate the expression levels of identified antigens in fresh breast cancers (n = 7), normal mammary tissues (n = 5), and a neu-overexpressing cell line, MMC, derived from a spontaneous tumor in the neu-tg mouse (5). As shown in Fig. 3, the genes of some antigens are up-regulated in primary tumor and cell line (Krt2-8 and Matr3), whereas other genes (Gsn, C3, and Yb1) are down-regulated. Similar changes in expression of cytokeratin and Gsn have been reported for human cancer. Cytokeratin is up-regulated in breast cancer (19), especially in neu-overexpressing breast cancer (20). Cytokeratin was also overexpressed in hepatocarcinoma, cervical cancer, and renal cancer, similar tissues as we show here in the murine system (21–23). Gsn, as a tumor suppressor, is down-regulated significantly in human breast and ovarian cancers (9, 11, 19, 24).

**The majority of identified murine tumor antigens have immunogenic human homologues.** To examine if the mouse antigens we identified have immunogenic human homologues, we searched the human cancer immunome database (CIDB; http://www2.licr.org/CancerImmunomeDB/) and PubMed. We found that more than half of the mouse tumor antigens have immunogenic human homologues (Table 2). The relevant cancer types and SEREX clones are listed in Table 2. Two of the proteins involved in the metastatic pathway, Rock1 and Swap70, have been associated with breast and other types of cancer in humans. Furthermore, Rock1 and Srpk1, have been reported to play a role in tumor rejection in multiple myeloma patients (25). Of note, all of the top five most frequently identified antigens have immunogenic human homologues. The seropositivity to these antigens was evaluated in 42 sera from mice bearing spontaneous tumors and 40 sera from control non–tumor-bearing mice using crude lysate ELISA. As shown in the last two columns of Table 2, tumor-bearing mice had significantly higher frequency of antibodies to Krt2-8 and Rock1.

Confocal immunoassay of murine tumors also showed the presence of antibodies against Krt2-8, Gsn, C3, Yb1, and Usp7. Antibody titers were significantly increased in tumor-bearing mice compared with control mice (Fig. 4). In summary, none of the antigens we studied were restricted to tumor tissues, and nearly all of the murine tumor antigens we identified have immunogenic human homologues.

**Antibody immunity observed in mice with spontaneous tumors was not observed in mice with implanted tumors.** Because implanted tumors are more frequently used as a model system to study tumor immunity, we next questioned if the humoral immunity we observed in mice with spontaneous tumor could also be detected in mice with implanted tumors. Tumor-free neu-tg mice received 1 × 10^6 syngeneic tumor cells (MMC) implanted s.c. All of the neu-tg mice developed palpable tumors within 2 weeks after the injection. Serum was collected from neu-tg mice before and at 1 month after the tumor implantation to evaluate if new immunity developed associated with tumor growth. In contrast to neu-tg mice bearing spontaneous tumors, no mice bearing implanted tumors had evidence of increased antibody immunity.

### Table 1. Murine TAAs identified by SEREX

<table>
<thead>
<tr>
<th>Antigen</th>
<th>No. clones</th>
<th>Gene symbol</th>
<th>Chromosomal location</th>
<th>Subcellular location</th>
<th>Function</th>
<th>UniGene no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytokeratin 2-8</td>
<td>15</td>
<td>Krt2-8</td>
<td>15F3</td>
<td>Cytoplasm</td>
<td>Cytokinskeleton</td>
<td>5.38618</td>
</tr>
<tr>
<td>Glutamyl-prolyl-tRNA synthetase</td>
<td>13</td>
<td>Eprs</td>
<td>1H4</td>
<td>Cytoplasm</td>
<td>tRNA aminoacylation for protein translation</td>
<td>1.54511</td>
</tr>
<tr>
<td>Complement C3</td>
<td>9</td>
<td>C3</td>
<td>17E1</td>
<td>Extracellular</td>
<td>Complement activation</td>
<td>1.9131</td>
</tr>
<tr>
<td>Galectin 8</td>
<td>6</td>
<td>Lgals8</td>
<td>13A1</td>
<td>Extracellular</td>
<td>Cell adhesion, spreading</td>
<td>1.7186</td>
</tr>
<tr>
<td>Serine/threonine-rich protein kinase 1</td>
<td>5</td>
<td>Srpk1</td>
<td>17A3</td>
<td>Cytosol/nucleus</td>
<td>Regulate pre-mRNA splicing</td>
<td>1.5225</td>
</tr>
<tr>
<td>RAB3A-interacting protein</td>
<td>3</td>
<td>Rab3ip</td>
<td>10D2</td>
<td>Cytosol/nucleus</td>
<td>SSX2 interacting protein</td>
<td>3.36394</td>
</tr>
<tr>
<td>Rho-associated, coiled–containing protein kinase 1</td>
<td>2</td>
<td>Rock1</td>
<td>18A2</td>
<td>Cytoplasm</td>
<td>RhoA/Rock signaling pathway, cell differentiation, migration</td>
<td>6.710</td>
</tr>
<tr>
<td>Schistosoma mansoni adult worm antigen preparation 70</td>
<td>2</td>
<td>Swap70</td>
<td>7E3</td>
<td>Cytoplasm</td>
<td>Signal transduction, RacGEF</td>
<td>3.34144</td>
</tr>
<tr>
<td>Rho/Rac guanine nucleotide exchange factor 2</td>
<td>1</td>
<td>Arhgef2</td>
<td>3F1</td>
<td>Cytoplasm</td>
<td>Activation of RhoA/Rock pathway</td>
<td>2.93929</td>
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<tr>
<td>Gelsolin</td>
<td>1</td>
<td>Gsn</td>
<td>2B</td>
<td>Cytoplasm</td>
<td>Actin-binding protein, tumor suppressor</td>
<td>2.1109</td>
</tr>
<tr>
<td>Nuclease-sensitive element binding protein 1 (Y box-binding protein)</td>
<td>1</td>
<td>Nsepl (Yb1)</td>
<td>4D1</td>
<td>Nucleus</td>
<td>DNA-binding protein</td>
<td>2.58204</td>
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<tr>
<td>Ubiquitin-specific protease 7</td>
<td>1</td>
<td>Usp7</td>
<td>16A1</td>
<td>Nucleus</td>
<td>Deubiquitinating enzyme for p53 (39)</td>
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<tr>
<td>Leucine zipper transcription factor-like protein 1</td>
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<td>Lztfl1</td>
<td>9F</td>
<td>Nucleus</td>
<td>Transcription factor</td>
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<tr>
<td>Exportin 5</td>
<td>1</td>
<td>Xpo5</td>
<td>17C</td>
<td>Nucleus</td>
<td>Exports eEF1A via tRNA from nuclei</td>
<td>2.75039</td>
</tr>
<tr>
<td>Matrin 3</td>
<td>1</td>
<td>Matr3</td>
<td>18C</td>
<td>Nucleus</td>
<td>Nuclear protein</td>
<td>2.15034</td>
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Mm.154511
response to the most common antigens, cytokeratin, Eprs, Srpk1, C3, Yb1, and Rock1. Figure 4A and B shows the representative results on cytokeratin, which had increased serum antibodies in mice bearing spontaneous tumor but not in mice with implanted tumor. To further understand the difference between spontaneous and implanted tumors, we compared growth rates. The implanted tumor grew faster than spontaneous tumor (Fig. 4C). At 2 weeks after the first appearance of palpable tumors, the average size of a spontaneous tumor was significantly smaller than that of an implanted tumor (274.6 ± 49.2 versus 1,458.2 ± 102.6; P < 0.001). Furthermore, analysis of immune cell infiltrates in tumor showed that spontaneous tumor had significantly higher numbers of CD4 and CD8 T cells than implanted tumor [6.4 ± 4.9 versus 2.1 ± 0.8, CD4 T cells (P < 0.05); 4.8 ± 1.3 versus 1.1 ± 0.2, CD8 T cells (P < 0.05); Fig. 4D]. There was no difference for B cells (CD19^+), NK cells (NK1.1^+), or dendritic cells (CD11c^+).

Discussion

We comprehensively identified the immunogenic repertoire of the neu-tg mouse. After screening 3 cDNA libraries, we identified 15 tumor antigens with diverse biological functions, including structural proteins, transcription factors, and signal transduction molecules (Table 1). The majority of tumor antigens identified have immunogenic human homologues (Table 2), including Gsn, Rock1, Krt2-8, and Yb1, which have been reported to play important roles in tumorigenesis and metastasis in both human and animal models (9–11, 13–17, 21–25). The dysregulated expression of Krt2-8 and Gsn in breast tumor in neu-tg mice also mirrors the expression in cancer patients (9–11, 19, 20). The similarity between mouse and human tumor antigens indicate that neu-tg mice may be useful in modeling immunobased therapy for human breast cancer. Moreover, the majority of the tumor antigen repertoire is composed of intracellular proteins. Finally, data presented suggest that the generation of endogenous immunity in mice is associated only with spontaneous tumor development and not the more commonly used therapeutic model system, implanted tumors.

It has long been a matter of debate whether data collected in rodent models would be predictive of human disease response. Indeed, in animal models of tumor immunity, many of the tumor cell lines studied are chemically induced or inherently immunogenic, quite dissimilar to the human condition (26). In particular, the field of tumor immunology has suffered from a lack of success in translating successful murine therapeutic approaches into immunotherapeutics that have an effect in human malignancies. More recently, the ability to generate mice that are transgenic or knocked out for cancer-related genes has resulted in the development of rodent models that closely mimic human cancer from a pathologic standpoint (2, 27–29). Furthermore, a recent review by Roberts et al. (30) showed that transgenic and knockout mouse models could accurately predict some of the toxicities that have been seen in human clinical studies of anticancer agents, such as inhibitors of cyclooxygenase-2, vascular endothelial growth factor, KIT, and ER. Neu-tg mice are engineered to express nontransforming rat neu on an MMTV promoter and the breast cancers that occur in these mice mimic premenopausal breast cancer in women (2). Gene expression profiling of the mammary tumors in these mice shows that there are numerous similarities to human breast cancer (3). Results presented here show that the majority of tumor antigens identified in neu-tg mice have immunogenic human homologues. Thus, the neu-tg mouse may serve not only as a potential discovery tool for immunogenic human proteins but also as a model to evaluate the potential therapeutic efficacy of immunologic targeting of those proteins.
In humans, tumor antigens are characterized into different categories, including cancer/testis antigens, overexpressed proteins, differentiation antigens, and mutated proteins to name a few (31). As it is difficult to do comprehensive analyses of the immunologic repertoire in humans, it is unknown which types of proteins are most likely to elicit an immune response in cancer patients. Due to the homogeneity of the neu-tg mouse colony, exhaustive immunologic analyses of the antigenic repertoire can be done. Of note, not all mice developed immunity to their tumors, and not all animals developed immunity to the same antigens. Furthermore, many mice developed immunity to multiple antigens so the generation of immunity was heterogeneous despite the genetic similarity of the breast cancers arising in these animals. With one exception of finding a possible cancer/testis antigen, our studies show that the most common similar characteristic of all identified antigens was an intracellular location. Intracellular self-proteins are not normally recognized by the immune system due to immunologic ignorance. In disease conditions, such as cancer or other trauma, the proteins are made visible to the immune system due to tissue destruction. In addition, results presented here suggest that activation of metastatic pathways may make a protein immunogenic. The fact that 3 of the 15 identified antigens fall in the Rho/Rock signal transduction pathway support this hypothesis. Our results support the hypothesis that the immunogenicity of a

Figure 2. SEREX-identified antigens are widely expressed intracellular self-proteins. Real-time RT-PCR analyses measuring the mRNA expression levels of the top five most frequently identified TAAs. A, Krt2-8; B, Eprs; C, C3; D, Lgals8; E, Srpk1. MM1 to MM5, five mouse mammary specimen; T1 to T7, seven mouse tumor specimen from the neu-tg mice. The mRNA expression level of each TAA is normalized to h-actin.
given molecule depends on the context, in which it is presented than on its more or less restricted expression in certain tissues (32).

SEREX can identify not only new therapeutic targets but also new diagnostic markers (33). In the present study, we examined the presence of antibodies to SEREX-identified antigens in 42 sera from tumor-bearing animal and 40 controls. The results showed that tumor-bearing mice had significantly higher frequency of antibody responses to Krt2-8 and Rock1. Krt2-8 is a component of the intracellular cytoskeleton in cells of the single-layered sheet tissues. It was overexpressed in both tumor and a neu-positive cell line from neu-tg mice (Fig. 3). It is also reported to be overexpressed in cancer patients (19–23). Rock1 is the downstream effector of Rho and regulates actin polymerization and focal adhesion (Fig. 1). The Rho/Rock pathway plays an essential role in tumor metastasis (13–17). Whether cytokeratin or Rock1 could be potential new diagnostic markers needs more investigation. To examine if any of the antigens may be used as potential therapeutic targets, we evaluated the antibody response after successful immunomodulation, which may result in the development of new tumor-specific antibody immune responses that correlate with tumor regressions. We treated neu-tg mice with a reagent to deplete T regulatory cells, an anti-CD25 immunotoxin, and the spontaneous tumors

**Table 2. Murine TAAs that have immunogenic human homologues**

<table>
<thead>
<tr>
<th>Mouse TAA</th>
<th>Human homologue</th>
<th>Cancer types</th>
<th>References</th>
<th>SEREX clone in CIDB</th>
<th>Murine serum reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Krt2-8</td>
<td>KRT2-8</td>
<td>Ovarian, colon, renal</td>
<td>C&gt;IDB (40)</td>
<td>MO-ova-122</td>
<td>15/42 5/40 1</td>
</tr>
<tr>
<td>Eprs</td>
<td>EPRS</td>
<td>Breast, colon, gastric</td>
<td>(41)</td>
<td>N/A</td>
<td>7/42 2/40</td>
</tr>
<tr>
<td>C3</td>
<td>C3</td>
<td>Head, neck</td>
<td>CIDB</td>
<td>AU-HN-4</td>
<td>14/42 6/40</td>
</tr>
<tr>
<td>Lgals8</td>
<td>LGALS8</td>
<td>Prostate</td>
<td>(42)</td>
<td>N/A</td>
<td>14/42 13/40</td>
</tr>
<tr>
<td>Srpk1</td>
<td>SRPK1</td>
<td>Breast, leukemia</td>
<td>CIDB (25, 43)</td>
<td>NGO-BR-51</td>
<td>7/42 3/40</td>
</tr>
<tr>
<td>Nsep1 (Yb1)</td>
<td>NSEP1 (YB1)</td>
<td>Breast, ovarian</td>
<td>CIDB (44)</td>
<td>HOM-Ts-PMR2-12, HOM-TSOv2-35</td>
<td>4/42 0/40</td>
</tr>
</tbody>
</table>

1Human CIDB (http://www2.licr.org/CancerImmunomeDB/).
1P < 0.05, χ2 test.
regressed. Both anti-neu antibody and T-cell immunity was augmented after the treatment. We did ELISA using pretumor and post-tumor regression sera and found that antibody immunity to one of the SEREX-identified antigens, Srpk1, increased post-tumor regression (34). It is interesting that patients with relapsed myeloma developed new antibody immunity to Srpk1 after complete tumor regression was induced with donor lymphocytes infusions (25).

Tumor implants have long been used to evaluate immunobased strategies in rodent models. Our studies show that there are significant differences in the immune responses that occur endogenously in animals that spontaneously developed tumors versus received s.c. tumors implants. A humoral immune response was only increased in transgenic mice that developed spontaneous tumors. Moreover, the magnitude and phenotype of infiltrating cells were significantly different between spontaneous and implanted tumors. Several factors could contribute to this finding. First, the growth rate of implanted tumors is accelerated, which may impede the development of immunity. Second, the tumor implants are in a foreign anatomic site, which may affect the ability of initiating an immune response. Furthermore, implanted tumors generally do not have associated stromal elements, which are critical in the generation of immunity (35, 36). The cell line (MMC) used for the implanted tumor was derived from a spontaneous tumor in neu-tg mice. Previous work in our laboratory has shown that antigen loss variant may develop from this cell line when implanted in mice (37, 38). Therefore, we examined the rat neu expression in the dissected implanted tumor. The expression of rat neu remained high in those tumors (data not shown), indicating the absence of antibody response was not due to antigen loss. Data presented here would support the use of animal models, which develop spontaneous tumors as an experimental system that more closely resembles endogenous immunity occurring in human tumors (26).

High-throughput methods, such as SEREX and other proteomic techniques, have resulted in the identification of a multitude of immunogenic targets. The critical block in exploiting these targets for therapeutic use to affect human disease is determining which proteins have clinical importance (i.e., tumor rejection antigens). Data presented here suggest that, at least for ER-negative breast cancer, the neu-tg mouse may be an ideal model for prioritization of therapeutic immunologic targets due to the high degree of homology with the immunologic signature of human cancers.

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References

Figure 4. Antibody immunity observed in mice with spontaneous tumor was not observed in mice with implanted tumor. A, representative ELISA result showing the antibody response to Krt2-8 in sera from mice with spontaneous tumor (●; n = 42) and control mice (○; n = 40). JA for each individual sample. Solid line, mean ± 2 SDs of 10 serum samples that were tested to be negative for the Krt2-8 antibody by SEREX. B, antibody response to Krt2-8 in sera collected from neu-tg mice before and after tumor implantation (n = 26). C, growth curve of spontaneous (●) and implanted (▲) tumors. Points, mean of 10 mice; bars, SE. X axis, days after the first appearance of palpable tumors. D, percentage of tumor-infiltrating immune cells as determined by flow cytometric analyses. Slashed columns, cells in spontaneous tumors; solid columns, cells in implanted tumors. *, (P < 0.05), a significant difference between the spontaneous and implanted tumors.
The Tumor Antigen Repertoire Identified in Tumor-Bearing Neu Transgenic Mice Predicts Human Tumor Antigens

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