Generation of PRL-3- and PRL-1-Specific Monoclonal Antibodies as Potential Diagnostic Markers for Cancer Metastases

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ABSTRACT

Purpose: The PRL-3 mRNA is consistently elevated in metastatic samples derived from colorectal cancers. We sought to generate a specific PRL-3 monoclonal antibody (mAb) that might serve as a potential diagnostic marker for colorectal cancer metastasis.

Experimental Design: PRL-3 is one of three members (PRL-1, PRL-2, and PRL-3) in a unique protein-tyrosine phosphatase family. Because the three PRLs are 76% to 87% identical in their amino acid sequences, it poses a great challenge to obtain mAbs that are specific for respective phosphatase of regenerating liver (PRL) but not for the other two in the family. We screened over 1,400 hybridoma clones to generate mAbs specific to each PRL member.

Results: We obtained two hybridoma clones specifically against PRL-3 and another two clones specifically against PRL-1. These antibodies had been evaluated by several critical tests to show their own specificities and applications. Most importantly, the PRL-3 mAbs were assessed on 282 human colorectal tissue samples (121 normal, 17 adenomas, and 144 adenocarcinomas). PRL-3 protein was detected in 11% of adenocarcinoma samples. The PRL-3- and PRL-1-specific mAbs were further examined on 204 human multiple cancer tissues. The differential expressions of PRL-3 and PRL-1 confirmed the mAbs’ specificity.

Conclusions: Using several approaches, we show that PRL-3- or PRL-1-specific mAbs react only to their respective antigen. The expression of PRL-3 in >10% of primary colorectal cancer samples indicates that PRL-3 may prime the metastatic process. These mAbs will be useful as markers in clinical diagnosis for assessing tumor aggressiveness.

INTRODUCTION

Metastasis is the most lethal pathologic aspect of cancer (1–3). Early treatment and prevention of cancer metastasis are extremely important. With early diagnosis, the cure rate is better than 90% in most cases. Unfortunately, because of a lack of early stage detection markers and treatment options for cancer patients, metastasis is usually not detected until the late stage of the disease. Few genes have yet been specifically linked to cancer metastasis. We know little about signaling pathways responsible for the metastatic process.

Recently, several reports implicated a group of oncogenic protein-tyrosine phosphatases (PTP), the phosphatase of regenerating liver (PRL) family, playing an important role in the progress of cancer metastasis. Increasing evidence suggests that PRL-1, PRL-2, and PRL-3 might take part individually in the process of cancer metastases. PRL-1 was expressed at high levels in proliferating cells and a number of human tumor cell lines, including HeLa cells (4). Overexpression of PRL-1 or PRL-2 in epithelial cells resulted in a transformed phenotype in culture and tumor growth in nude mice (5, 6). By PCR-based subtractive hybridization, PRL-2 was shown to be up-regulated in a prostate cancer cell line (LNCaP; ref. 7). More importantly, by gene expression profiling studies, among 144 up-regulated genes detected in metastatic colorectal liver samples, PRL-3 was the only gene specifically overexpressed in all 18 metastatic colorectal cancers examined (8). PRL-3 mRNA expression was elevated in nearly all metastatic lesions derived from colorectal cancers, regardless of the site of metastasis (liver, lung, brain, or ovary; ref. 9). By Northern hybridization analysis, PRL-3 was shown to be up-regulated in almost all 27 human liver carcinoma samples examined compared with normal liver samples (10). PRL-3 was also highly expressed in metastatic melanoma B16-BL6 cells but not in its less metastatic parental cell line (10). PRL-3 expression might also play a significant role in invasion and metastasis of gastric carcinoma (11). PRL-3 is a potential marker for liver metastasis of colorectal cancer and its level correlates negatively with prognosis of colorectal cancer patients (12). We discovered that PRL-1 and PRL-3 are prenylated and enriched on the plasma membrane (13). Overexpression of PRL-1 and PRL-3 in Chinese hamster ovary cells promoted cell migration, invasion and metastasis (14). Catalytic domain of PRL-3 PTP played an essential role in tumor metastasis (15). Together, these data suggest that the PRLs may be major players involved in oncogenic and metastatic processes. An excess of PRL phosphatase activity could be a general key alteration contributing to the acquisition of metastatic properties in tumor cells. The PRLs might provide new insights into metastasis and help us understand more about the process of cancer metastasis. Specific antibodies against each PRL member would be required for novel diagnostic, prognostic, or therapeutic approaches. Because the three members are 76% to 87% identical in their amino acid sequences (16), our polyclonal antibodies against PRL-3 generated in rabbit cross-react with all three members. We decided to overcome this problem by generating mouse monoclonal antibodies that are specific for individual PRLs.
As the levels of PRL-3 may reflect a cancer cell's menace (8, 17), PRL-3-specific antibodies may be a powerful and reliable marker in detecting metastatic tumor cells in colorectal cancer patients. PRL-1- and PRL-2-specific monoclonal antibodies might be similarly useful to detect other types of cancer metastases. The antibodies would also be helpful for oncologists in assessing tumor aggressiveness according to PRL expression level in tumors, which would indicate the risk of future distant metastases. The antibodies might help us to fish out PRLs’ cellular interacting partners and elucidate their working mechanism to log PRLs into gateways in current knowledge of tumor cell signaling.

**MATERIALS AND METHODS**

**Alignment of Amino Acid Sequences of Mouse and Human Phosphatases of Regenerating Liver.** Amino acid sequences of mouse and human PRL-1 or PRL-2 are 100% identical, respectively, whereas mouse and human PRL-3 are 96% identical. We analyzed mouse PRL-1, PRL-2, PRL-3, and human PRL-3 amino acid sequences by using the DNASTAR and GeneDoc programs. Database searches were done using blast programs.

**Construction of Plasmids Encoding Mouse GST-PRL-1, GST-PRL-2, GST-PRL-3, and Human GST-PRL-3 Fusion Proteins.** We used a PCR-based approach to generate cDNA fragments using corresponding expression sequence–tagged clones as templates. The forward primer A (5'-CGGGATCCATGCTGCTGAAACCCGCAG-3') and the reverse primer B (5'-GTGAATTCTTATTGAATACAACAGTTG-3') were used to amplify mouse PRL-1 cDNA fragment. The forward primer C (5'-GTGGATCCATGAAACCCGCAGCTG-3') and the reverse primer D (5'-CGGAATTCCTTCTGAAACACACGACGTCG-3') were used to amplify mouse PRL-2 cDNA fragment. The forward primer E (5'-GGGGATCCATGGAGCGCATGAACCCGCAG-3') and the reverse primer F (5'-CTGAATTCGTATGGTATGGC-3') were used to amplify mouse PRL-3 cDNA fragment. The forward primer G (5'-GGGGATCCATGACGCGTAGTG-3') and the reverse primer H (5'-CTGAATTCTTACATAAAGCGAGCGAGGTC-3') were used to amplify human PRL-3 cDNA fragment. The four PCR products were gel purified and digested with EcoRI and BamHI and inserted (in frame) into the corresponding sites of the pGEX-KG vector (18).

**Preparation of GST-PRLs Fusion Proteins.** Each GST-PRL bacterial clone was inoculated in 5 mL of Luria-Bertani with 100 μg/mL ampicillin. The overnight culture was added to 500 mL of Luria-Bertani/ampicillin and grown until A600nm reached 0.6 to 0.8. Isopropyl-β-D-thiogalactopyranoside was added to the culture at 0.5 mmol/L/mL and the culture was shaken overnight at room temperature. The culture was centrifuged at 5,000 rpm for 10 minutes. The pellet was re-suspended in 25 mL GST extraction buffer [1 mg/mL lysozyme, 5 mmol/L DTT and 0.5 mmol/L phenylmethylsulfonyl fluoride in GST buffer: PBS, 50 mmol/L Tris (pH 8) and 0.5 mmol/L MgCl2]]. The lysate was incubated on ice for 15 minutes and sonicated for 3 minutes. The lysate was then centrifuged at 15,000 rpm for 30 minutes at 4°C. The supernatant was passed through a 0.45-μm filter. One milliliter of glutathione slurry (Pharmacia, Piscataway, NJ) was packed into a column, which was washed several times with PBS. The extract was incubated with the column at 4°C for 1 hour. The unbound extract was drained out and the column was washed with GST buffer thrice again. The GST fusion proteins were eluted with elution buffer [20 mmol/L of reduced glutathione, 100 mmol/L Tris-HCl (pH 8.0), and 120 mmol/L NaCl] and the fractions were collected and then analyzed by SDS-PAGE.

**Thrombin Cleavage of GST-PRLs.** To get pure PRLs antigens, GST-PRLs were bound with glutathione sepharose slurry (Pharmacia) in a 50-mL tube at 4°C for 2 hours. The beads were washed 3× 50 mL PBS and incubated with 1 mL of thrombin cleavage buffer [50 mmol/L Tris (pH 8), 150 mmol/L NaCl, 2.5 mmol/L CaCl2, and 0.1% β-mercaptoethanol]. Seventy microliters of thrombin 10 μg/mL (Sigma, St. Louis, MO) were added to 1.5 mL of beads. The reaction was transferred to a 2-mL tube and was incubated at room temperature with shaking for 1 hour. The beads were pelleted and the supernatant was transferred to a fresh tube with 1 mL of phenylmethylsulfonyl fluoride. The concentrations of the pure PRLs were then determined.

**Generation of Specific PRL-1 or PRL-3 Monoclonal Antibodies.** Hybridomas were generated using ClonaCell-HY Hybridoma Cloning Kit from Stemcell Technologies, Inc. (Vancouver, British Columbia, Canada; ref. 19). The procedures were followed according to the manufacturer's directions. Briefly, the following were done: (a) immunization of BALB/c mice with GST-mouse whole PRL-1, or PRL-3 fusion protein, respectively; (b) growth of BALB/c parental myeloma cells SP2/0; (c) preparation of BALB/c mice for spleenocytes from immunized mice; (d) fusion of spleenocytes with SP2/0 cells; and (e) selection and characterizations of the hybridoma clones. 

**Generation of Ascetic Fluids.** Hybridoma cells (5 × 10⁶) were suspended in 200 L of serum-free DMEM medium and injected with a 26-gauge needle into the peritoneal cavity. After 10 days, the mouse developed a large quantity of ascitic fluid, and the abdomen was greatly distended. The mouse was sacrificed and a small shallow was cut to open the abdominal cavity. The ascitic fluid was drawn with a 10-mL syringe fitted with an 18-gauge needle. The fluid was centrifuged at 200 × g for 10 minutes at 4°C. The supernatant fluid was collected and frozen at −70°C until further use.

**ELISA Assay.** A 96-well plate was rinsed with PBS thrice. PRL antigen stocks were made in carbonated buffer (pH 9.6). Solution (100 μL) containing an indicated amount of antigen was added to each well and incubated at 4°C for overnight to coat the antigen onto the plate. The plate was then blocked with 3% bovine serum albumin in PBS containing 0.05% Tween 20 for 30 minutes at 37°C. The plate was then washed thrice with PBS. Culture medium or monoclonal antibodies were added to each well and incubated for 40 minutes at 37°C. The secondary antibody, anti-mouse IgG conjugated with horseradish peroxidase (Pierce, Rockford, IL) diluted at 1:5,000 to 10,000 in PBS was added to each well and incubated at 37°C for 40 minutes. The plate was rinsed with PBS containing 0.05% Tween 20 thrice followed by three washes with sterile water. The substrate, 100 μL of Turbo-TM B (Pierce), was added to each well and incubated
for 10 minutes at room temperature. The reaction was stopped by adding 100 μL of concentrated H2SO4. Absorbance was measured at 450 nm (ELISA Reader DYNATECH MR7000).

**Western Blot Analysis.** The concentrations of PRL proteins were quantified by comparing them with sequentially diluted commercial bovine serum albumin (100 mg/mL) by SDS-PAGE and Coomassie blue staining. Indicated amount of each antigen was loaded onto SDS-PAGE gel and resolved by electrophoresis. The proteins were blotted onto a polyvinylidene difluoride membrane, which was first blocked with 5% skim milk in PBS/0.1% Tween 20 and probed with the appropriate monoclonal antibodies. The secondary anti-mouse horseradish peroxidase (1:1,000 dilutions) was used. The specific binding was detected using a chemiluminescence detection kit (Pierce). Cell lysates derived from stable cell lines 9, 20, and 36, respectively expressing myc-PRL-1, myc-PRL-2, or myc-PRL-3, (13) were analyzed. Amounts of protein were loaded with similar myc-signal to test monoclonal antibody (mAb)—specific binding to their respective expressing cell line. Mouse anti-myc antibody (9E10) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

**Confocal Microscopy.** The same stable cell lines (13) expressing myc-PRLs and the pool of cells stably expressing EGFP-PRL-3 (15) were seeded onto glass coverslips and grown for at least 24 hours. Cells were washed twice with PBSCM (PBS containing 1 mmol/L of MgCl2 and 1 mmol/L of CaCl2) and fixed in 2.7% paraformaldehyde for 20 minutes at room temperature. After three more washes with PBSCM, the cells were permeabilized for 15 minutes with 0.1% saponin in PBSCM. Cells were double-labeled with rabbit anti-PRLs together with either PRL-1 or PRL-3 mAb for 2 hours at room temperature. After the first antibodies incubation, the cells were washed thrice with PBSCM and incubated with anti-rabbit Texas red and anti-mouse FITC (Molecular Probes, Eugene, OR) for 2 hours. The slides were washed for four times with PBSCM and mounted onto a glass slide with one drop of antifade reagent in PBS glycerol (Biomedia Co., Foster City, CA). Confocal imaging was done using a Zeiss Axioshot fluorescence microscope with a scanning laser scanning (Zeiss LSM 510 image browser).

**Immunoprecipitation.** PRL-1 and PRL-3 ascitic fluids (1:200 dilutions) were used to immunoprecipitate the lysates derived from abovementioned stable myc-PRL-1- and myc-PRL-3-expressing cell lines (13). We followed the procedure provided by Transduction Laboratories (San Jose, CA). Briefly, cells expressing myc-PRL-1 or myc-PRL-3 were lysed with immunoprecipitation buffer [1% Triton X-100, 150 mmol/L NaCl, 10 mmol/L Tris (pH 7.4), 1 mmol/L phenylmethylsulfonyl fluoride, and 0.5% NP40]. After cleaning cell debris by centrifugation, the lysates were immunoprecipitated with PRL-3 (clones 318 and 223) or PRL-1 (clone 269) mAbs followed by rabbit anti-mouse IgG and protein A-Sepharose beads. The immunoprecipitates were resolved via SDS-PAGE and transferred to polyvinylidene difluoride membranes. After blocking, filters were probed with mouse anti-myc antibody to assess respective immunoprecipitation protein by a chemiluminescence detection kit (Pierce).

**Immunohistochemistry.** We investigated PRL-3 expression on human colorectal cancer tissue arrays T8235790D (BioChain Institute, Inc., Hayward, CA), TS-4205-05, and TS43050702 (BioGenex, San Ramon, CA). We assessed PRL-3 and PRL-1 tissue-specific expression patterns on human multiple cancer tissue arrays TS42040704 and TS43040303 (BioGenex). We used Dako EnVision Systems K 1395 (Dako, Carpinteria, CA) to perform immunohistochemistry experiments. The formalin-fixed, paraffin-embedded slides were dewaxed in fresh xylene for 5 minutes. The step was repeated once more. The slides were subjected to rehydration by going through sequential 100%, 95%, 80%, and 75% ethanol, PBS (2 minutes for each change) followed by antigen retrieval with 200 μL (2.5 mg/mL) pepsin (EK000-10K, BioGenex) on each slide in 37°C water bath for 5 minutes. The slides were transferred to PBS with 2% glycine for 2 minutes and to PBS with 1% H2O2 in the dark for 5 minutes. The slides were washed in PBS for several times and treated in PBS with 0.1% Tween 20, 0.1% TX-100, and 0.1% saponin (Merek, Beeeston, United Kingdom) for 20 minutes at room temperature. Each slide was blocked in 300 μL PBS with 10% goat serum, 1% bovine serum albumin (Sigma), and 0.1% saponin for 2 hours at room temperature. The excess blocking solution was wiped off. The mAbs were diluted as described in Table 1 with blocking buffer. Appropriate mAb (150 μL) was added to each slide and incubated at room temperature for 3 hours, and at 4°C for overnight. Next day, the slide was washed in PBS containing 0.05% Tween 20, 0.05% TX-100 several times with gently shaking. The slide was incubated with labeled polymer-horseradish peroxidase or polymer-AP for 2 hours. The washing steps were repeated; 200 μL of substrate-chromogen solution (1 mL buffer + 20 μL 3,3′-diaminobenzidine for polymer-horseradish peroxidase or Fast-Red Chromogen for polymer-AP) were applied to each slide for 10 to 20 minutes in the dark. The washing steps were repeated. The results were analyzed under microscopy.

**RESULTS**

**Establishment of Two PRL-1-Specific Hybridomas (269 and 29).** The alignment of amino acid sequences among mouse PRL-1, PRL-2, PRL-3, and human PRL-3 is shown (Fig. 1). The challenge is to obtain hybridoma clones secreting mAbs specific against respective PRL but not cross-react with other members in the family. We used ClonaCell-HY Hybridoma Cloning Kit (described in Materials and Methods) to make these hybridomas. After fusing spleenocytes derived from mouse immunized with

<table>
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Abbreviations: WB, Western blot; IF, immunofluorescence; IP, immunoprecipitation; IHC, immunohistochemistry.
PRL-1m : HAGMMPAPVEYVRMRFL1THNFTNAILKREATLYGVTIVRVCATTITLVEKEGIHLWDFPGAGPSNQIVDWSLWKLRFV : 93

PRL-2m : HAGMMPAPVEYVRMRFL1THNFTNAILKREATLYGVTIVRVCATTITLVEKEGIHLWDFPGAGPSNQIVDWSLWKLRFV : 90

PRL-3m : HAGMMPAPVEYVRMRFL1THNFTNAILKREATLYGVTIVRVCATTITLVEKEGIHLWDFPGAGPSNQIVDWSLWKLRFV : 93

R3-H : HAGMMPAPVEYVRMRFL1THNFTNAILKREATLYGVTIVRVCATTITLVEKEGIHLWDFPGAGPSNQIVDWSLWKLRFV : 93

PRL-1m : ETPGCCTAVICHCAVLGAPVVALALTGBKNMYEDAVQFGIRQPRGASPNSQQLYLEKRYPRRLRFLGDSNGHNNCTQ : 173
PRL-2m : ETPGCCTAVICHCAVLGAPVVALALTGBKNMYEDAVQFGIRQPRGASPNSQQLYLEKRYPRRLRFLGDSNGHNNCTQ : 173
PRL-3m : ETPGCCTAVICHCAVLGAPVVALALTGBKNMYEDAVQFGIRQPRGASPNSQQLYLEKRYPRRLRFLGDSNGHNNCTQ : 173
R3-H : ETPGCCTAVICHCAVLGAPVVALALTGBKNMYEDAVQFGIRQPRGASPNSQQLYLEKRYPRRLRFLGDSNGHNNCTQ : 173

Fig. 1 Alignment of amino acid sequences of mouse PRLs (PRL-1m, PRL-2m, and PRL-3m) with human PRL-3 (R3-H). The amino acid sequences of human PRL-1 and PRL-2 were not shown because they are 100% identical to the mouse proteins. The alignment shows that the three mouse PRLs and human PRL-3 share high homologies in their protein sequences. Arrows, positions where the residues are different between mouse and human PRL-3.

GST-mouse whole PRL-1 with SP2/0 myeloma cells, 807 surviving hybridoma clones were isolated and grown up. All clones were initially tested for PRL-1 binding by ELISA. Seventy clones showed good reactions with PRL-1 but most of them also reacted with its relatives PRL-2 and PRL-3. Only 21 clones were shown to be specific for PRL-1. Among them, we selected clones 17, 28, 29, 195, 207, 269, 497, and 515 for further analysis. Clones 269 and 29 were eventually deemed the best (Table 2) as they can specifically detect PRL-1 in several applications.

Establishment of Two PRL-3-Specific Hybridomas (318 and 223). The same procedure was used to develop PRL-3-specific antibodies. Mice were immunized by GST-mouse whole PRL-3. Clones (n = 601) were isolated and grown up. Again, all clones were initially tested for PRL-3 binding by ELISA. Sixty-two clones showed good reactivity with the PRL-3 but most of them also reacted with its relatives PRL-1 and PRL-2. Only 23 of 62 clones preferentially detected PRL-3. Among them, five clones (clones 4, 89, 223, 324, and 318) were further characterized. Clones 318 and 223 were finally chosen (Table 2) as the best PRL-3 clones because they reacted with both mouse and human PRL-3 with high specificity. These two clones were further tested for other analytic procedure.

Characterization of PRL-1 and PRL-3 Monoclonal Antibodies. To assess the antibodies for their potential uses in basic and clinical applications, we used various approaches to show that these antibodies can be used in several applications (Table 1). (a) By ELISA, PRL-1 mAbs clone 269 or 29 bind specifically to 10 ng pure PRL-1 antigen but not to 100 ng each of pure PRL-2, PRL-3, or GST proteins (Fig. 2A). The PRL-3 mAbs clone 318 or 223 bind specifically to 10 ng each of mouse PRL-3 or human GST-PRL-3 antigen but not to 100 ng each of pure PRL-1, PRL-2 or GST protein (Fig. 2B). By Western blots, a careful calculation for the loading of GST-PRL proteins was done several times on Coomassie blue-stained gels by comparing with standard concentrations from commercial bovine serum albumin products. The concentrations of GST-PRLs were determined. Using 10 ng of GST-PRL-1 and 20 times excess (200 ng each) of GST-PRL-2 or GST-PRL-3 on the blot, the PRL-1 antibodies reacted with PRL-1 at least four times more efficiently as judged by the intensity of the signal. The result suggests that the PRL-1 antibodies reacted with PRL-1 at least 80 (20 x 4 = 80) times more efficiently as compared with PRL-2 and PRL-3 (Fig. 3A). Similarly, using 10 ng each of pure mouse PRL-3 or human GST-PRL-3, together with 20 times excess (200 ng each) of pure PRL-1 or PRL-2 on the blots, the PRL-3 antibodies reacted with PRL-3 at least 10 times more efficiently as judged by the intensity of the signal, which suggests that the PRL-3 antibodies reacted with PRL-3 proteins at least 200 times (20 x 10 = 200) more efficiently compared with PRL-1 and PRL-2 (Fig. 3B). Because mouse and human PRL-3 differ by six amino acids (Fig. 1), the data obtained from ELISA (Fig. 2B) and Western blot (Fig. 3A and B) is important to show that the two PRL-3 mAbs reacted with mouse and human PRL-3 with a similar efficiency. This means that the antibodies are against a conserved epitope in PRL-3 between human and mouse. The PRL-1 and PRL-3 mAbs were further assessed for their specificities on cell lysates derived from stable Chinese hamster ovary cell lines (13) respectively expressing myc-PRL-1, myc-PRL-2, and myc-PRL-3. We loaded cell lysates with similar myc signal intensity as control (Fig. 3C, bottom); thesame sets of filters were probed with respective mAbs. PRL-1 mAb 269 reacted only with myc-PRL-1 but not myc-PRL-2 or myc-PRL-3 (Fig. 3C, top), whereas PRL-3 mAb 223 (Fig. 3C, upper middle) and mAb 318 (Fig. 3C, lower middle) reacted only with myc-PRL-3 but not with myc-PRL-1 or myc-PRL-2. (c) By indirect immunofluorescence, the four monoclonal antibodies did well with desired specificities in experiments using the same stable Chinese hamster ovary cell lines expressing myc-PRL-1, myc-PRL-2, and myc-PRL-3, respectively (13). We double-labeled the cells with rabbit anti-PRLs (react with all three PRLs) and with PRL-1 clone 269 to show that the mAb reacted specifically with myc-PRL-1 expressing cells but not with myc-PRL-2, myc-PRL-3-, or GFP-PRL-3-expressing cells (Fig. 4A). PRL-1 clone 29 had the similar property (data not shown). PRL-3 clone 318 reacted specifically with myc-PRL-3 or GFP-PRL-3 expressing cells.

Table 2 Summary of PRL-1 and PRL-3 mAbs generation

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<td>Original</td>
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<td>601</td>
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but not with myc-PRL-1- or myc-PRL-2-expressing cells (Fig. 4B). Similar results were obtained using PRL-3 mAb 223 (data not shown). (d) By immunoprecipitation, using cell lysates derived from stable cell lines expressing myc-PRL-1 or myc-PRL-3, the PRL-1 mAb 269 and PRL-3 mAbs 318 or 223 are able to specifically immunoprecipitate their respective PRL but not cross-immunoprecipitation with other PRL members (data not shown). The fact that the PRL-3 antibodies reacted with GST-human PRL-3 (MW: 45 kDa) with slightly less reactivity than the mouse PRL-3 (MW: 20 kDa) is due to their different protein sizes. The molecule numbers in 10 ng of pure mouse PRL-3 are almost doubled comparing with in 10 ng of human GST-PRL-3.

**PRL-3 Monoclonal Antibodies Were Assessed on Mouse and Human Samples Using Immunohistochemistry:** PRL-3 Protein Was Expressed in ~11% of Human Colorectal Cancer Samples. To apply the mAbs to animal research, we tested PRL-3 mAbs on mouse tissue expressing PRL-3. Mouse lung carrying EGFP-PRL-3 tumors (15) was cut at 10-μm thicknesses at −18°C. The cryosections were stained with PRL-3 mAbs 318 or 223, and then anti-mouse conjugated with Texas Red. The positive signals were consistent with EGFP expression (Fig. 5A). To verify that the PRL-3 mAbs are useful in clinical applications, we did immunohistochemistry on human colorectal tissue samples to examine PRL-3 expression levels. PRL-3 mAbs were tested on array TS-4205-05, which contains 21 normal and 29 diseased human colorectal samples. Three of 29 (10.3%) colorectal cancer specimens were PRL-3 positive. Samples A6 and E8 showed PRL-3 at high levels (Fig. 5B), whereas sample B4 at low level (data not shown). Array TS43050702 contains 70 normal, 17 adenomas, and 85 adenocarcinomas. We detected nine (D10, D16, F6, F14, G8, G10, H15, H16, and I13) of 85 adenocarcinoma samples that were PRL-3 positive. Clones 223 and 318 showed similar results. Altogether, we had examined 121 normal, 17 adenomas, and 144 adenocarcinomas human colorectal samples. PRL-3 protein was detected in 16 of 144 adenocarcinoma samples (11%). We concluded here that the PRL-3 mAbs could react with PRL-3 protein in both mouse and human tissues and could be used in both fresh-frozen and formalin-fixed, paraffin-embedded samples.

**Immunohistochemistry Analyses with PRL-1 and PRL-3 Monoclonal Antibodies on Respective Phosphatase of Regenerating Liver Stable Expressing Cell Lines and on Human Multiple Cancer Tissue Arrays.** To exclude the possibility of mAbs cross-reactivity with other PRL members in the family, we did immunohistochemistry to show their specific

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**Fig. 2** PRL-1 and PRL-3 mAbs are specific against their respective antigen by ELISA. A, to assess the specificity of PRL-1 antibodies, a 96-well plate was coated with indicated antigens. The ascitic fluids from PRL-1 clone 269 or clone 29 displayed ~10 or 8 times, respectively, more reactivity to 10 ng of GST-PRL-1 (GST-R1) than to 10 times excesses (100 ng each) of GST-PRL-2 (GST-R2), GST-PRL-3 (GST-R3), GST-human PRL-3 (GST-HR3), or pure GST antigens. B, ascitic fluids from PRL-3 clone 318 and clone 223 displayed ~8 times more reactivity to 10 ng each of pure mouse PRL-3 (R3) and GST-human PRL-3 (GST-HR3) than to 10 times excess of its relatives, pure mouse PRL-1 (R1) and pure mouse PRL-2 (R2) antigens (100 ng each). The fact that the PRL-3 antibodies reacted with GST-human PRL-3 (MW: 45 kDa) with slightly less reactivity than the mouse PRL-3 (MW: 20 kDa) is due to their different protein sizes. The molecule numbers in 10 ng of pure mouse PRL-3 are almost doubled comparing with in 10 ng of human GST-PRL-3.
reactivity. The stable cell lines (13) respectively expressing myc-PRL-1, myc-PRL-2, or myc-PRL-3 were used to examine the mAbs’ specific reactivity. The results clearly showed that these mAbs displayed desired specificity (Fig. 6A). In addition, we used these mAbs to examine their respective protein expression on human multiple cancer tissue array TS43040303, which contains 204 elements of cancer and premalignant lesions. Two adjacent slides were subjected to label with PRL-1 or PRL-3 mAb, respectively. Among the 204 samples, we detected 10 PRL-1-positive samples (B10, C20, D19, E17, E20, E21, I3, I15, I17, and J18) and 21 PRL-3-positive samples (B19, B20, B21, C18, D2, D4, D7, D8, E5, E6, E7, E10, E17, E20, E21, F3, G6, G9, G13, G15, and G19). Only three cases (E17, E20, and E21) showed PRL-3 and PRL-1 double positive. The data here suggest that the mAbs did detect only their respective PRLs in human samples. The results of five representative samples are shown in Fig. 6B. As shown, PRL-3 mAb 223 detected specifically to some squamous epithelium in these cervix carcinomas (D2 and D7), whereas PRL-1 mAb did not exhibit any reactivity with these samples. One the other hand, PRL-1 mAb 269 revealed positive signals in epithelium renal carcinoma (D19) and in ovary lymphoma (B10), whereas PRL-3 mAb 223 did not show any specific labeling in these samples, suggesting these cancer patients expressed PRL-1 and PRL-3 differently. Sample E21 (skin melanoma) exhibited strongly reactivity to both PRL-1 and PRL-3 mAbs, suggesting that both PRL-1 and PRL-3 are expressed in the same sample.

**DISCUSSION**

PRL-3 mRNA has been reported to be consistently and specifically up-regulated in all the liver metastases derived from colorectal cancer patients by Dr. Bert Vogelstein’s team (8). Recently, the group also showed that PRL-3 mRNA expression was elevated in nearly all metastatic lesions derived from colorectal cancers, regardless of the sites of metastasis (liver, lung, brain, or ovary). In contrast, no PRL-3 mRNA expression was detected in normal colon, nonmetastatic primary cancers, or metastatic lesions derived from noncolon cancers (pancreas, stomach, or esophagus; ref. 9). The PRL-3-specific antibodies are thus critical to extend these studies from mRNA to protein levels during oncogenesis and metastasis. The mAbs may be useful for clinical diagnosis of colorectal metastatic cancer specimens.

The hallmark of PRL-3 mRNA up-regulation in all examined colorectal metastatic samples is of particular interest as no gene has been reported previously to be specifically associated with colon cancer metastasis. These striking discoveries suggest that PRL-3 may play an important role in the process of metastasis. It is reasonable to hypothesize that PRL-1 and PRL-2 (the same family members) may be similarly associated with other types of cancer progression. Based on these exciting findings, we immediately undertook the task of generating monoclonal antibodies specifically against each PRL. With these specific antibodies in hand, we hope to gain a better understanding into the molecular basis of metastasis.
and to learn more about this lethal process. Furthermore, the antibodies may give us a direct diagnostic indication in the stages of cancers. We previously had generated rabbit PRLs antibodies that failed in their specificities because these three members in the family share high homology in their protein sequences, resulting in cross-reactivity. To overcome this extremely tough problem, it was necessary to make monoclonal antibodies against each member. Culturing and selecting from >1,000 clones with careful analyses, we have successfully generated two specific PRL-1 mAbs and two PRL-3 mAbs. These clones could be used in several applications in both basic research and clinical studies. The up-regulated PRL-3 expression in ~11% (16 of 144 cases) of primary colorectal tumors might indicate their high risk for future distant metastases. If paired metastatic-primary samples could be obtained from same individuals, the antibodies might allow us to elucidate the relatedness of metastatic risk with PRL-3 expression levels. It would be intriguing if we could follow-up the small fraction of PRL-3-positive primary cancers. We would be able to see whether they have higher chance to give rise to metastases. Our PRL-3 and PRL-1 mAbs will provide tools for these studies. The mAbs would offer oncologists as predictive biomolecular markers in early diagnosis to treat the cancer patients earlier and reduce the risk of future cancer metastases. We hope our efforts in generating these mAbs could be useful to clinic. The discovery of specific up-regulation of PRL-3 mRNA in colorectal cancers suggests that the PRL-3 antibody might be an important key to unlock the secrete door of the colon cancer metastasis. PRL-1 mAb might be similarly important to other type of cancer.

Fig. 4 PRL-1 and PRL-3 mAbs react specifically to their respective antigens by indirect immunofluorescence. Cells from three stable cell lines: 9, 20, and 36 expressing myc-PRL-1, myc-PRL-2, or myc-PRL-3, respectively (13), and cells from stable pool expressing GFP-PRL-3 (14) were seeded onto coverslips for at least 24 hours. Indirect immunofluorescence was performed with double labeling using two primary antibodies: rabbit anti-PRL (reacting with all three PRLs) and PRL-1 (clone 269) or PRL-3 mAb (clone 318), and with two secondary antibodies: anti-rabbit-Texas Red and anti-mouse-FITC. A, PRL-1 269 mAb specifically reacted with stable cell line 9 expressing myc-PRL-1 but not with 20 expressing myc-PRL-2, 36 expressing myc-PRL-3, or stable pool expressing GFP-PRL-3 (single label with mAb and anti-mouse Texas Red). B, PRL-3 318 mAb specifically reacted with stable cell line 36 expressing myc-PRL-3 as well as stable pool expressing GFP-PRL-3 but react neither with 9 expressing myc-PRL-1 nor 20 expressing myc-PRL-2. Bar, 15 μm.
The specificity of PRL-3 and PRL-1 mAbs was further assessed on human multiple cancer samples and showed the mAbs were indeed displayed their specific reactivity. PRL-3 expression was detected in squamous epithelium in some cervix carcinoma cases, whereas PRL-1 was detected in a few brain and bone cases. The clear segregation of PRL-3 and PRL-1 expression in human multiple cancer tissues excluded the possibilities of cross-reactivity. All PRL-3 and PRL-1 signals that we detected in cancer cells thus far were localized mainly to the cytoplasm and cell membrane, suggesting that they play a role in signal transduction. Although PRL-3 represents a valuable candidate for metastasis-tailored therapies (20), the cellular function of its role in cancer remained elusive. The mAbs might reveal new clues for elucidating PRLs biological functions in tumor progression. The antibodies would also help us to fish out their potential interacting partners. It is envisioned that these PRL antibodies will help us in dissecting steps involved in transitions from primary tumors to metastatic lesions in their clinical setups.

Lastly, we would like to emphasize one of the important features, which makes PRL-3 so attractive is that it is specifically switched on during metastasis which makes drug target possible.

Fig. 5 Clones 318 and 223 mAbs detect PRL-3 protein expression in mouse and human samples by immunohistochemistry and indirect immunofluorescence. The experiments were performed using PRL-3 antibodies on sections derived from fresh-frozen mouse lung carrying EGFP-PRL-3 tumors. A, an overview of EGFP-PRL-3 tumor lung (a); PRL-3 mAb (clone 318) was used to detect EGFP-PRL-3 tumor by immunohistochemistry with DAB chromogen (b); cryosection of EGFP-PRL-3 lung showed EGFP tumor (c, white arrows); same section was labeled with clone 318, followed by anti-mouse IgG conjugated with Texas Red (d); merged image formed by c and d (e). B, 50 formalin-fixed and paraffin-embedded normal and disease human colon samples (TS-4205-05) were assessed for PRL-3 protein expression. The PRL-3 protein was revealed by stained with PRL-3 clone 223 specific antibody (clone 318 revealed similar results, data not shown) using Dako EnVision System with 3,3′-diaminobenzidine chromogen. Selected sections from array TS-4205-05: adenocarcinoma (A6′ and A6); adenocarcinoma (E8′ and E8); please note that A6′ and E8′ (without first antibody) were used as negative controls for A6 and E8, respectively. Normal mucosa, PRL-3 undetectable (D3); adenocarcinoma, PRL-3 undetectable (B10). Results of selected sections from array T8235790D: adenocarcinoma, low PRL-3 expression (A9); adenocarcinoma, strong PRL-3 expression (F1); and adenocarcinoma, PRL-3 negative (F9). Magnification, ×200.
whereas most of tumor suppressor genes are switched off which make drug targets impossible. Inhibitors for excess of these phosphatases would be one of the hopes for thwarting cancer metastasis. Our monoclonal antibodies will facilitate these drugs development.

ACKNOWLEDGMENTS

We thank Drs. Catherine J. Pallen, W.J. Hong, B.L. Tang, and X.J. Yin for their critical reading of the article; Drs. J.H. Lu, S.K. Lim, and Tham Mac Lan Jill for their technical discussions for generation of monoclonal antibodies; Dr. Sathivel Ponniah and Mohamad Sharudin Bin for immunization of BALB/c mice; and Dr. Graeme Wistow from NIH for the gift of human PRL-3 expression sequence–tagged clone.

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