

Targeting Tomoregulin for Radioimmunotherapy of Prostate Cancer

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Abstract

Radiotherapy is an effective approach for the treatment of local prostate cancer. However, once prostate cancer metastasizes, radiotherapy cannot be used due to the distribution of multiple metastases to lymph nodes and bones. In contrast, radioimmunotherapy should still be efficacious in metastatic prostate cancer as radioisotopes are brought to tumor cells by targeting antibodies. Here we identify and validate a prostate-expressed molecule, tomoregulin, as a target for radioimmunotherapy of prostate cancer. Tomoregulin is a transmembrane protein selectively expressed in the brain, prostate, and prostate cancer, but not expressed in other normal tissues. Immunohistochemical studies of tomoregulin protein in clinical samples show its location in the luminal epithelium of normal prostate, benign prostatic hyperplasia, and prostatic intraepithelial neoplasia. More importantly, the tomoregulin protein is expressed in primary prostate tumors and in their lymph node and bone metastases. The nature of tomoregulin as a transmembrane protein and its tissue-specific expression make tomoregulin an attractive target for radioimmunotherapy, in which tomoregulin-specific antibodies will deliver a radioisotope to prostate tumor cells and metastases. Indeed, biodistribution studies using a prostate tumor xenograft model showed that the ¹¹¹In-labeled anti-tomoregulin antibody 2H8 specifically recognizes tomoregulin protein *in vivo*, leading to a strong tumor-specific accumulation of the antibody. In efficacy studies, a single i.p. dose of 150 μ Ci (163 μ g) ⁹⁰Y-labeled 2H8 substantially inhibits the growth rate of established LNCaP human prostate tumor xenograft in nude mice but produces no overt toxicity despite cross-reactivity of 2H8 with mouse tomoregulin. Our data clearly validate tomoregulin as a target for radioimmunotherapy of prostate cancer. (Cancer Res 2005; 65(7): 2846-53)

Introduction

Prostate cancer continues to be the second leading cause of cancer deaths in men of the United States, with 30,000 deaths per year (see http://www.cancer.gov/docroot/STT/stt_0.asp). In the early stages of prostate cancer, tumor cells are usually localized to the prostate gland, so the organ-confined disease can be treated by surgical (radical prostatectomy) or physical (radiation therapy) means. In later stages, cancers often metastasize to the lymph nodes or bones and eventually become resistant to androgen ablation therapy (1). There is no cure for advanced metastatic androgen-independent prostate cancer.

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Radiation therapy such as external beam radiation therapy or internal radiation therapy (brachytherapy) has been successfully used in the treatment of clinically localized prostate cancer because prostate cancer cells are relatively radiosensitive (1). Conversely, distant metastases of prostate cancer cells escape local radiation, rendering radiation therapy useless at advanced stages of the disease. Radioimmunotherapy, in which a radiolabeled antibody specifically recognizes cancer cells, could be an appropriate treatment for metastatic prostate cancers because radiation is delivered to the metastatic cells expressing the target. Furthermore, metastatic prostate tumor cells tend to form small foci in the lymph nodes and bone marrow that are easily accessible to circulating antibodies (2).

For radioimmunotherapy, the target should be on the cell surface and abundantly and selectively expressed in disease tissues. To identify an appropriate target for radioimmunotherapy, we searched an expression database and found tomoregulin to be expressed on prostate and prostate cancer cells. In this study we verify the expression of tomoregulin on prostate cancer cells and show that tomoregulin is recognized by the specific antibody 2H8 *in vivo*, and that the administration of ⁹⁰Y-labeled 2H8 inhibits the growth of tumor xenografts (LNCaP) in nude mice.

Materials and Methods

Cell culture. The human prostate carcinoma cell lines LNCaP, PC-3, and DU 145 were obtained from American Type Culture Collection (Manassas, VA) and grown in RPMI 1640, supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin at 37°C in a humidified atmosphere of 5% CO₂ (3, 4). The Clone36 cell line was generated by stably transfecting PC-3 cells with the expression vector pcDNA3.1-tomoregulin. These cells were maintained in RPMI 1640, supplemented with 10% FBS and 400 μ g/mL G418.

Northern blot analysis. Northern blot analysis was done as previously described (5). Total RNA was isolated using the RNeasy kit (Qiagen, Valencia, CA) from cultured LNCaP, PC-3, DU 145, and PrEC cells according to the instructions of the vendor. Twelve-microgram samples of total RNA were denatured on a 1% denatured agarose gel and fractionated by electrophoresis and transferred to Hybond-N⁺ nylon membrane (Amersham, Piscataway, NJ). The bound RNA was immobilized by UV cross-linking and then hybridized with a random-primed ³³P-labeled tomoregulin probe at 70°C. To control for RNA sample loading and transfer efficiency, blots were also hybridized at 68°C with a ³³P-labeled human cDNA for actin.

Quantitative real-time reverse transcription-PCR. Expression of tomoregulin was measured by TaqMan assay using FAM dye on cDNA reversed transcribed from total RNAs. PCR reactions were carried out using an ABI Prism 7700 Sequencing Detection system (Perkin-Elmer Applied Biosystems, Foster City, CA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression as normalizer was measured using the Perkin-Elmer GAPDH kit.

Recombinant protein production. The extracellular domain of tomoregulin comprising amino acids 1 to 302 was expressed as a secretory protein in the baculovirus expression system. Briefly, the cDNA

encoding the extracellular domain was amplified with primers encoding for an N-terminal optimized Kozak sequence and a C-terminal 6-His tag which is preceded by two glycine residues and followed by a stop codon. The PCR product was restricted with the enzymes *Bam*HI and *Kpn*I and cloned downstream of the polyhedrin promoter into the baculovirus transfer vector pBBS250. Recombinant baculovirus was produced by cotransfection with BaculoGold DNA (BD Biosciences, Palo Alto, CA) and subsequent plaque isolation. High Five cells were infected at a multiplicity of infection of 3 and the cell culture supernatant was harvested after 72 hours. The protein was purified in a two-step procedure by Ni²⁺-chelating and size exclusion chromatography.

2H8 antibody. 2H8 antibody is a murine IgG1 monoclonal antibody that reacts with the follistatin domains of the extracellular region of tomoregulin protein. The antibody was recently described (6). A nonspecific control antibody, mouse IgG1, was purchased from BD Biosciences (Palo Alto, CA).

Western blot analysis. Whole cell lysates in radioimmunoprecipitation assay buffer [150 mmol/L NaCl, 10 mmol/L Tris pH 7.2, 0.1% SDS, 1% Triton X-100, 1% deoxycholate, 5 mmol/L EDTA, protease inhibitors cocktail tablet (Roche Molecular Biochemicals, Mannheim, Germany)] containing 25 to 100 µg of protein were separated by 10% SDS-PAGE under nonreducing condition and transferred to nitrocellulose membrane. The blots were probed with 2H8 against tomoregulin and peroxidase-conjugated goat anti-mouse IgG antibody (Pierce, Rockford, IL) as secondary antibody. The signal was detected using the enhanced chemiluminescence detection system (Amersham).

Generation of antibody-chelator conjugates. All equipment used was rendered metal-free with 10 mmol/L EDTA followed by extensive rinsing with Chelex-treated MilliQ water. Buffers were prepared with reagents containing minimal trace metals and were treated with Chelex resin (Bio-Rad, Hercules, CA) to remove residual metals. Antibodies were concentrated to ~5 mg/mL by ultrafiltration and EDTA was added to 1 mmol/L and incubated for 1 hour to remove bound trace metals. Buffer exchange into 50 mmol/L sodium bicarbonate, 150 mmol/L NaCl, pH 8.5, was done using a G25 column (Pharmacia Desalt 26/10). Antibody-containing fractions were concentrated to 5 to 10 mg/mL. A stock solution of *p*-SCN-benzyl-DTPA was prepared in DMSO (100 mg/mL) and added to the antibody solution to a molar ratio of 50:1 (DTPA/antibody). The conjugation reaction was run overnight at room temperature. The reaction mixture was run on a G25 column to remove unreacted DTPA and to exchange buffer to 50 mmol/L sodium acetate, 150 mmol/L NaCl, pH 6.5. Total protein concentration of the final immunoconjugate was determined by bicinchoninic acid (BCA) assay (Pierce) and the antigen-binding activities of the immunoconjugates were determined by ELISA.

Radiolabeling of antibody-chelator conjugates. Radiolabeling of the antibody-chelator conjugates with ¹¹¹In or ⁹⁰Y was done via chelation of the radiometal to the DTPA moiety of the conjugate. Radioisotopes (Perkin-Elmer Lifesciences, Inc. Boston, MA) were first buffered by adding an equal volume of 100 mmol/L NH₃OAc (pH 5.5), then antibody-conjugate was added to a ratio of 1 mg antibody/mCi. The reaction was incubated for 1 hour, with mixing at room temperature, then EDTA was added to a final concentration of 1 mmol/L, to scavenge nonspecifically bound radioisotope for 15 minutes at room temperature. Radiolabeled antibody was separated from free radioisotope, and buffer exchanged into PBS, using a Pharmacia 26/10 Desalting column run at 2 mL/min, while collecting 1-mL fractions. Peak fractions were collected and sodium ascorbate was added at 1.0 mg/mL for ⁹⁰Y-labeled antibodies to serve as radioprotectant.

Activity of the ⁹⁰Y-radiocconjugates was measured by liquid scintillation counting, and a γ -counter was used for the ¹¹¹In-conjugates. Total protein concentration was determined using BCA assay (Pierce), and specific activities were calculated based on these results. Levels of free radioisotope and free DTPA were determined by TLC using a previously published method (7). The antigen binding activity of the radioconjugate was measured by ELISA or by radioimmunoassay. Typically, specific activities ranged from 0.25 to 1.0 mCi/mg. TLC results indicated that free ¹¹¹In and free DTPA levels were generally less than 3% and frequently less than 1% of the total activity. The DTPA conjugation and radiolabeling procedure did not affect immunoreactivity (by ELISA) of 2H8.

In vivo xenograft models. Male, athymic mice (nu/nu), 6 to 8 weeks old, were obtained from Simonsen (Gilroy, CA) and were injected with 1 × 10⁷ freshly trypsinized LNCaP cells (passage 31) in 200 µL mixed 1:1 with Matrigel (BD Biosciences, Bedford, MA) s.c. on the right dorsal flank. The mice were monitored for body weight and tumor volume for 4 to 6 weeks at which time palpable tumors became evident. Tumor volume (mm³) was estimated by caliper measurement in two perpendicular directions and calculated using the formula: (the shortest diameter)² × (the longest diameter) × 0.5. Mice with established tumors (50-500 mm³) were randomized into groups for the biodistribution study (*n* = 3/time point), the maximum tolerated dose study (*n* = 5/group), and the efficacy study (*n* = 15/group). Mice were maintained under germ-free conditions in facilities accredited by the American Association for Accreditation of Laboratory Animal Care. All experiments were conducted in accordance with the principles and procedures approved by Institutional Animal Care and Use Committee at Berlex Biosciences and according to National and International Standards.

Biodistribution study. Male nude mice bearing established LNCaP tumors (50-300 mm³) were selected for the time course study (at least three mice per time point). The specific antibody, 2H8, and the nonspecific control antibody, mouse IgG1, were radiolabeled with ¹¹¹In to a specific activity of 1.4 mCi/mg and administered i.v. by the lateral tail vein. At 4, 24, and 96 hours post injection, animals were euthanized. Tumor, blood, liver, kidney, and brain were collected and weighed, and radioactivity determined. The two variables calculated from the data, "percent of injected dose per gram" (%ID/g) and "tissue-to-blood ratio", quantified the biodistribution pattern. Tumor-specific accumulation is achieved when the tumor-to-blood ratio increases over time and increases to values greater than 1.

Dose ranging/maximum tolerated dose study. LNCaP tumor-bearing mice (50-500 mm³) were randomized (*n* = 5 per group) and treated i.p. with a single administration (45, 90, 128, or 170 µCi/mouse) of ⁹⁰Y-labeled 2H8 antibody. Treatment-related toxicity was evaluated by monitoring body weights and general clinical appearance. Tumor volume was monitored for 48 days after the start of treatment.

Efficacy study. LNCaP tumor-bearing mice (50-500 mm³) were randomly assigned to each of four treatment groups (*n* = 15/group). A single i.p. injection of 150 µCi of either ⁹⁰Y-labeled 2H8 or ⁹⁰Y-labeled mouse-IgG1 was given on day 1. Tumor growth was monitored until day 64. Controls included a no treatment group and a 2H8-treated group (which received a single injection of 160 µg of unlabeled 2H8). Mice in the no treatment group and the 2H8-treated group were euthanized on day 40 due to rapid growth of tumors. Mice in the ⁹⁰Y-labeled 2H8 and the ⁹⁰Y-labeled IgG groups were euthanized on day 67. Tumors were excised and weighed.

Results

Tomoregulin mRNA is selectively expressed in the brain, prostate, and prostate cancer. A bioinformatics approach was used to search a commercial gene expression database (Incyte, Palo Alto, CA) for genes that are expressed in prostate/prostate cancer but not in essential normal tissues. One of the genes identified encodes tomoregulin (6, 8–10), which was expressed in only two tissue categories (male genitalia and nervous system; Table 1). Tomoregulin mRNA was found in 19 cDNA libraries derived from male genitalia. Eighteen of these libraries were generated from prostate tissues, of which 10 were derived from prostate tumors (data not shown). Importantly, tomoregulin expression was not detected in any of the 159 cDNA libraries generated from cells of the hemic and immune system (Table 1).

The predicted predominant species of tomoregulin mRNA is 2.2 kb (6), encoding a protein of 334 amino acids with a calculated molecular weight of about *M_r* 39,000 to 51,000 (6). Based on our homology analysis, tomoregulin is predicted to have a single transmembrane domain, two follistatin modules, and one epidermal growth factor (EGF)-like domain (data not shown).

Table 1. Tissue distribution of tomoregulin mRNA by mining Incyte gene expression database (LifeSeq Gold 5.1)

Tissue category	Positive libraries*	Abundance
Cardiovascular system	2/68	2
Connective tissue	0/47	0
Digestive system	0/148	0
Embryonic structures	1/21	3
Endocrine system	0/53	0
Exocrine glands	0/64	0
Genitalia, female	2/106	2
Genitalia, male	19/114	29
Germ cells	0/5	0
Hemic and immune system	0/159	0
Liver	1/35	1
Musculoskeletal system	2/47	3
Nervous system	25/198	31
Pancreas	0/24	0
Respiratory system	3/93	5
Sense organs	0/8	0
Skin	1/15	2
Stomatognathic system	0/10	0
Unclassified/mixed	2/13	4
Urinary tract	0/64	0
Totals	58/1,292	82

The two tissue categories expressing most abundant tomoregulin transcripts are highlighted in bold.

*Number of tomoregulin-positive libraries of libraries tested.

The expression of tomoregulin mRNA in a variety of human tissues was evaluated by quantitative real-time reverse transcription-PCR (RT-PCR) analysis (Taqman assay; Fig. 1). Consistent with the data in Table 1, Taqman analysis revealed the preferential expression of tomoregulin mRNA in the brain and prostate. Tomoregulin mRNA was not detected in normal tissues of breast, colon, heart, liver, lung, lymph node, pancreas, smooth muscle, spleen, stomach, or uterus. Nor was it detected in tumors of colon, liver, lung, ovary, rectum, stomach, or uterus (data not shown). Very low levels of tomoregulin mRNA were detected in kidney (<20% of prostate or brain) and minimal levels in testis. Among the prostate samples analyzed,

tomoregulin mRNA was detected in normal prostate and benign prostatic hyperplasia as well as in prostate tumor (Fig. 1). Moreover, expression levels of tomoregulin mRNA in these prostate specimens were much higher than those in the LNCaP human prostate cancer cell line.

Tomoregulin protein is detected in clinical samples of prostate tumors and prostate tumor metastases. Expression of tomoregulin protein in human tissues was determined by immunohistochemistry using the anti-tomoregulin mouse monoclonal antibody 2H8 (6). 2H8 binds specifically to human tomoregulin protein with high affinity ($K_D = 1.24$ nmol/L), as determined by BIAcore analysis (data not shown). It does not bind to its close homologue tomoregulin-1 or TMEFF-1 protein (ref. 8; data not shown).

To verify the tissue-specific expression of tomoregulin, 11 human normal tissues including prostate, brain, heart, lung, liver, pancreas, small intestine, kidney, spleen, skeletal muscle, and skin were evaluated by immunohistochemistry using 2H8. Mab2H8 was highly reactive with biopsies from normal prostate and produced intense staining in the glandular lumen. Specifically, the positive staining was distributed in the prostate luminal epithelial cells, but not in the basal cells. Some moderate staining was also seen in the brain (Fig. 2). In contrast, all of the other human tissues tested were negative for tomoregulin protein expression.

Expression of tomoregulin protein was also determined by immunohistochemistry using tumor tissue samples from prostate cancer patients. Overall, tomoregulin protein was strongly expressed in prostate primary tumors and their metastatic tissues. Intense positive staining was detected in prostate tumor tissues as well as in prostatic intraepithelial neoplasia, the precursor lesion of prostate carcinoma (Fig. 2). Adjacent normal tissues also showed some staining. Most importantly, tumor cells of the metastatic lesions in lymph node and bone showed very strong positive staining using anti-tomoregulin antibody 2H8 (Fig. 2). Therefore, the tomoregulin protein is highly expressed in both normal prostate and prostate tumor tissues, and it is also highly expressed in late-stage prostate carcinoma including metastatic tissues from lymph node and bone.

Expression of tomoregulin mRNA and protein in the LNCaP prostate cancer cell line model. We also investigated the expression of tomoregulin in cultured prostate cells to identify appropriate models for *in vivo* studies. Tomoregulin mRNA levels were first evaluated by Taqman analysis (data not shown). Among

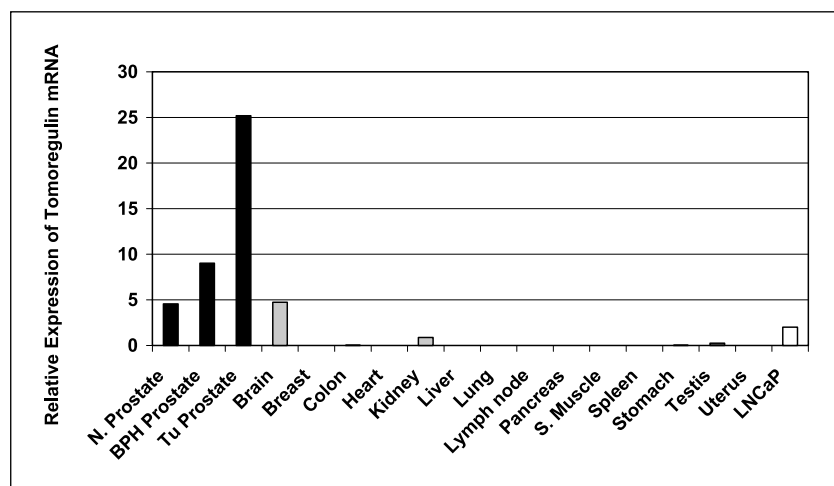
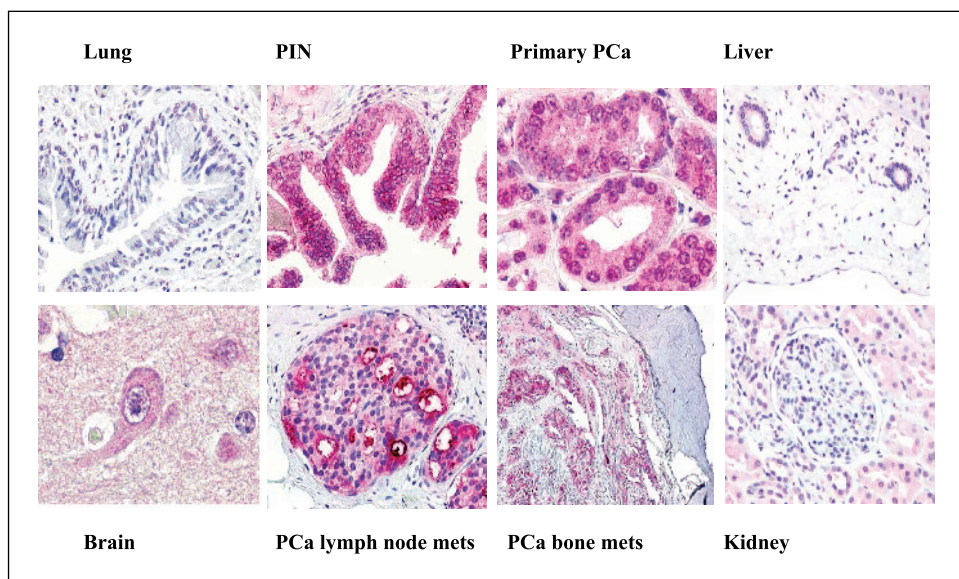


Figure 1. Real-time RT-PCR (Taqman) analysis of tomoregulin mRNA in human normal and tumor tissues. Prostate tumor sections were microdissected to generate samples containing over 80% tumor cells. Total RNAs from prostate tumor tissues were purified from clinical samples, and normal human tissue RNA was purchased from a commercial vendor (Biochain, Hayward, CA). Solid bars, normal prostate (*N. prostate*), benign prostatic hyperplasia (*BPH Prostate*), and prostate tumor (*Tu prostate*). LNCaP (open bar) is a human prostate cancer cell line. Grey bars, brain and other tissues. The expression of tomoregulin mRNA normalized to GAPDH mRNA is shown as relative expression of tomoregulin mRNA.

Figure 2. Immunohistochemical staining of clinical samples from prostate cancer patients using anti-tomoregulin antibody Mab2H8. The tomoregulin-positive cells are stained in red. *Top*, lung, prostatic intraepithelial neoplasia (PIN), primary prostate carcinoma, and liver. *Bottom*, brain, lymph node metastasis of prostate cancer, bone metastasis of prostate cancer, and kidney.



the five prostate cell cultures, including PrEC (primary culture of human normal prostatic epithelial cells), BPH1 (T-antigen-immortalized benign prostatic hyperplasia cells), and the human prostate cancer cell lines LNCaP, PC-3, and DU 145, only LNCaP cells express significant levels of tomoregulin mRNA (as shown in Fig. 1).

Northern blot analysis on several cell lines confirmed that tomoregulin mRNA is expressed in LNCaP cells (Fig. 3A). A single species of tomoregulin mRNA at 2.2 kb was detected in LNCaP, but not in PrEC, DU 145, and PC-3 cells.

The expression of tomoregulin protein in LNCaP was evaluated by Western blot analysis using antibody 2H8 (Fig. 3B). Cell lysates prepared from PC-3 (lane 1) and Clone36 (lane 2) were included as negative control and positive control, respectively. The Clone36

cell line was generated by stably transfecting PC-3 cells with the tomoregulin expression vector (Materials and Methods). As shown in Fig. 3B, PC-3 cells do not express tomoregulin (lane 1). Clone36 cells overexpress the tomoregulin protein tagged by V5-His (48 kDa; lane 2). The recombinant extracellular domain of tomoregulin protein was detected at 40 kDa (lane 3). The endogenous full-length tomoregulin protein was detected as a single band of 43 kDa in LNCaP (passage 31) cell lysates (lane 4). We noticed that the levels of tomoregulin protein decreased with increasing passage number in cultured LNCaP cells (passage 31 > passage 60; data not shown). Lanes 6 and 7 are lysates derived from Clone36 tumors and PC-3 tumors, respectively. Interestingly, LNCaP xenograft tumors (lanes 8-10) express much higher levels of tomoregulin protein than LNCaP cells in tissue culture (lane 4).

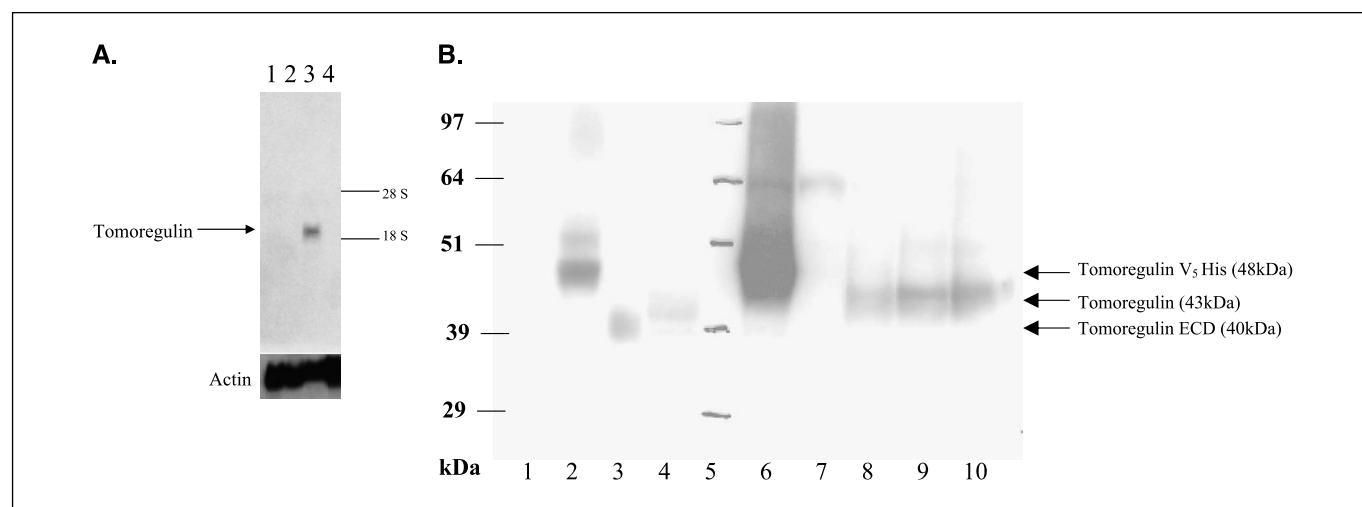


Figure 3. Tomoregulin mRNA and protein expression in the LNCaP human prostate cancer cell line. *A*, Northern blot analysis of tomoregulin mRNA in human prostate normal epithelia cells PrEC (lane 1) and the human prostate cancer cell lines DU 145 (lane 2), LNCaP (lane 3), and PC-3 (lane 4). Total RNAs (12 μ g per lane) were fractionated by a 1% agarose/formaldehyde gel and then transferred to a nylon membrane. The blot was hybridized with a tomoregulin-specific probe. Hybridization with actin serves as a control for equal loading. *B*, Western blot analysis of tomoregulin protein expression in cultured cells and xenograft tumors. Lane 1, PC-3 cell lysates (25 μ g); lane 2, Clone36 cell lysates (25 μ g); lane 3, recombinant extracellular domain of tomoregulin (5 ng); lane 4, LNCaP cell lysates (100 μ g); lane 5, MW size standard; lane 6, Clone36 xenograft tumor lysates (70 μ g); lane 7, PC-3 xenograft tumor lysates (70 μ g); lanes 8 to 10, LNCaP xenograft tumor lysates (70 μ g).

In view of these data, we have selected the LNCaP tumor xenograft model for *in vivo* studies.

¹¹¹In-labeled tomoregulin antibody shows tumor-specific accumulation in a xenograft model of human prostate cancer.

To determine whether the tomoregulin antibody 2H8 binds *in vivo* to tumor tissues expressing tomoregulin, biodistribution studies were done with systemically delivered radiolabeled 2H8 antibody. The anti-tomoregulin antibody, 2H8, or an irrelevant, isotype-matched control antibody, mouse IgG1, was labeled with ¹¹¹In, each to a specific activity of ~1.4 mCi/mg and administered i.v. to mice bearing LNCaP s.c. tumors. At various time points ($t = 4, 24,$ and 96 hours) following the injection, organ-bound radioactivity was determined in blood, tumor, liver, kidney, and brain. Both 2H8 and IgG control were cleared from the circulation in a similar fashion (Fig. 4A), however, the amount of IgG control retained in tumors did not change. Thus, 2H8, but not IgG1, showed a tumor-specific and time-dependent accumulation. At 96 hours after injection, the tumor retention of the ¹¹¹In-labeled 2H8 in LNCaP tumors reached nearly 25% injected dose per gram of tissue (Fig. 4A). The very high tumor-to-blood ratio of 4:1 (Fig. 4B) further reinforces the targeting specificity of the antibody. There was no specific 2H8 accumulation in liver and kidney, highly vascularized tissues that do not express tomoregulin protein. Although 2H8 cross-reacts with the mouse tomoregulin protein (data not shown) and mouse tomoregulin is expressed in mouse brain (8), no specific accumulation of 2H8 was observed in mouse brain, most likely due to the inability of antibody to penetrate the blood-brain barrier. The same tumor localization data of 2H8 were reproduced in a second biodistribution study using nude mice s.c. implanted with Clone36 cells (data not shown).

Radioimmunotherapy with ⁹⁰Y-labeled tomoregulin antibody inhibits growth of LNCaP tumors. To evaluate the efficacy of tomoregulin-based radioimmunotherapy, 2H8 was radiolabeled with the β -emitting isotope ⁹⁰Y ($t_{1/2} = 64$ hours), and administered i.p. to nude, male, tumor-bearing mice. First, the potential toxicity from radiolabeled antibody was investigated in a dose-ranging study. LNCaP tumor-bearing mice ($n = 5/\text{group}$) were given a single i.p. injection of 45, 90, 128, or 170 μCi of ⁹⁰Y-labeled 2H8. The mice were monitored for body weight and general clinical appearance. Evidence of toxicity from body weight loss was not observed and there were no animal deaths. Thus, all dose levels were tolerated. Interestingly, a dose-dependent antitumor effect was observed at doses $\geq 45 \mu\text{Ci}$ (Fig. 5A). Next, mice bearing LNCaP tumors of 50 to 500 mm^3 were given a single i.p. injection of 150 μCi of ⁹⁰Y-labeled 2H8, 150 μCi of ⁹⁰Y-labeled IgG1, or unlabeled 2H8 ($n = 15/\text{group}$). Tumor growth was monitored until >30% of the tumors in a treatment group exceeded 1,000 mm^3 . Radioimmunotherapy with both ⁹⁰Y-labeled 2H8 and ⁹⁰Y-labeled IgG1 at 150 μCi was well tolerated and resulted in significant efficacy (Fig. 5B and C). However, the antitumor effect of ⁹⁰Y-labeled 2H8 treatment persisted much longer, resulting in a statistically significant difference in the tumor sizes between the ⁹⁰Y-labeled 2H8-treated group and the ⁹⁰Y-labeled IgG1-treated group. This result was confirmed by the tumor weights taken at necropsy on day 67 ($P < 0.05$; Fig. 5C).

All xenografts showed the same histology regardless of treatment group (data not shown). There was no increase in macrophages, etc. observed in tumors of animals treated with antibodies. Yet there was a significant difference in average viable weight (199.7 μg for ⁹⁰Y-labeled 2H8-treated group versus 342.9 μg for ⁹⁰Y-labeled IgG1-treated group).

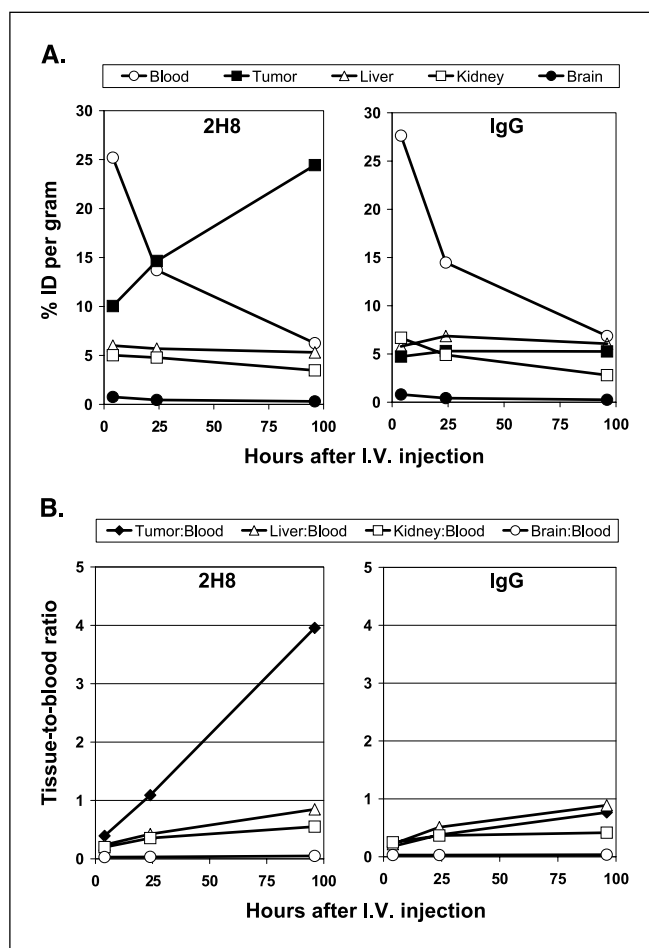


Figure 4. Biodistribution profiles of ¹¹¹In-labeled tomoregulin antibody, 2H8, and a nonspecific control antibody, mouse-IgG1. DTPA-conjugated tomoregulin antibody (2H8) and an irrelevant, isotype-matched control (IgG) were radiolabeled with ¹¹¹In to similar specific activity and injected i.v. to LNCaP tumor-bearing mice. Mice were euthanized at 4, 24, and 96 hours after a single i.v. injection and the radioactivity and wet weight of tumor, blood, liver, kidney, and brain were assessed. The calculated percent of injected dose per gram of tissue (%ID/g; A) and the tumor/blood ratios (B) show that 2H8 accumulates at high levels in tumors, whereas accumulation of the control IgG1 is almost nonexistent. Points, average of at least three mice per time point.

Our results clearly show that the anti-tomoregulin antibody 2H8 specifically interacts with tomoregulin *in vivo*, leading to specific accumulation in the LNCaP tumors. Furthermore, radioimmunotherapy using ⁹⁰Y-labeled 2H8 leads to a significant growth inhibition of established human tumor xenografts in nude mice, whereas indications of overt toxicity were not observed.

Discussion

Tomoregulin represents an attractive target for radioimmunotherapy of prostate cancer for the following reasons:

(a) As shown in our study tomoregulin is abundantly expressed in the prostate and prostate cancers, as well as in their lymph node and bone metastases. Other investigators have reported similar findings (11–13). Glynn-Jones et al. (11) analyzed 85 clinical samples by Taqman analysis and showed that tomoregulin mRNA is significantly overexpressed in prostate carcinomas when compared with their benign counterpart. Furthermore, there also seems to be a direct correlation between increasing tomoregulin

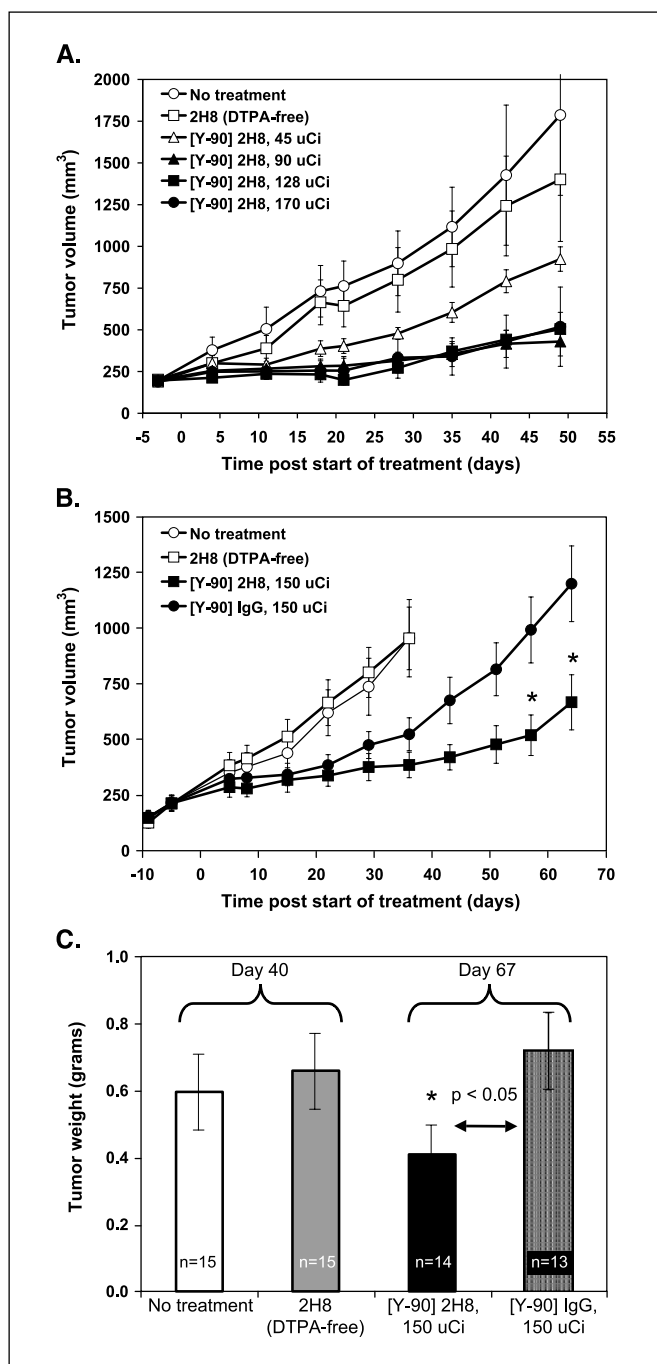


Figure 5. Antitumor effect of the ⁹⁰Y-labeled tomoregulin antibody, 2H8, against established LNCaP tumors. A, dose ranging study. DTPA-conjugated tomoregulin antibody (2H8) was radiolabeled with ⁹⁰Y and administered i.p. at 45, 90, 128, and 170 μ Ci/mouse to mice bearing LNCaP tumors (50-350 mm³). A group treated with 2H8 (not DTPA conjugated, not radiolabeled) and a group without any treatment were included. No overt toxicity (animal deaths or excessive body weight loss) was observed in any of the groups. Points, group average tumor volume ($n = 5$ /group); bars, SE. B and C, efficacy study. On day 0, mice with tumors of 50 to 500 mm³ in volume were distributed in treatment and control groups ($n = 15$ /group). ⁹⁰Y-labeled 2H8 and ⁹⁰Y-labeled IgG1 with the same approximate specific activity were administered as a single i.p. dose of 150 μ Ci. A group treated with 2H8 (not DTPA conjugated, not radiolabeled) and a group without any treatment were included. The mice were monitored for tumor growth (tumor volume) over time (B) and for final tumor wet weight (C). Asterisks indicate where group average tumor volume or tumor wet weight between ⁹⁰Y-2H8 and ⁹⁰Y-IgG was significantly different ($P < 0.05$). Mice receiving no treatment or unlabeled 2H8 were euthanized on day 40 due to tumor burden. Mice treated with ⁹⁰Y-labeled antibodies were euthanized on day 67. Columns, group average (mean); bars, SE.

expression levels and high tumor grade, suggesting that tomoregulin is associated with disease progression and androgen independence (11). Importantly, in an extensive study with 74 clinical prostate tumor samples, tomoregulin protein was detected by immunohistochemistry in 74% of primary prostate cancers and 42% of metastatic lesions (13). Heterogeneity of tomoregulin expression in clinical samples was noticed in our study and other studies. Samples within one tumor grade show some heterogeneity in their levels of tomoregulin mRNA expression (11). In addition, heterogeneity of tomoregulin protein expression within one sample was also observed (13). Such heterogeneity of tomoregulin expression in prostate tumors represents a challenge for antibody-based approaches of cancer therapy because only those cells which present sufficiently high levels of the target protein on their surface will be affected. However, it can be overcome by the radioimmunotherapy due to the intrinsic "bystander" killing effect of the radiation emitted by the radioisotopes accumulated in the tumor tissue.

(b) Tomoregulin is not expressed in most normal tissues except for the prostate and brain. This limited distribution in normal tissues should minimize the target-driven undesirable side effects of radioimmunotherapy. Potential toxicity in normal prostate is not a serious concern because patients with metastatic prostate cancer usually had prostatectomy for the primary tumors. Expression of tomoregulin mRNA and protein in the brain has been reported (8, 13). However, tomoregulin expression in brain is also not a serious concern because antibodies cannot penetrate the blood-brain barrier. This hypothesis is supported by the lack of antibody uptake into the brain in our biodistribution study (Fig. 4), although 2H8 cross-reacts with mouse tomoregulin protein (data not shown). The expression of tomoregulin mRNA in kidney that we detected in Taqman studies could not be verified by other methods. Multiple tissue Northern blots did not reveal kidney expression (data not shown), in line with the data by Glynne-Jones et al. (11). Therefore, the low signal in kidney detected in the Taqman study (Fig. 1) may be due to the ultrasensitive nature of Taqman assay. This assumption is supported by our immunohistochemical analysis showing little staining of human kidney by the 2H8 antibody and also by our biodistribution study showing no uptake of 2H8 in mouse kidney (Fig. 4). Because tomoregulin protein is detected in normal prostate, benign prostatic hyperplasia, prostatic intraepithelial neoplasia, and prostate tumors, it seems to be a prostate-specific differentiating antigen.

(c) Finally, the feasibility of targeting tomoregulin by radioimmunotherapy is warranted by the fact that tomoregulin is a plasma membrane protein and easily accessible to circulating radiolabeled antibodies. Fluorescence-activated cell sorting using 2H8 showed that tomoregulin protein is expressed on the cell surface of LNCaP and tomoregulin-transfected Clone36 cells but not in PC-3 parental cells (data not shown). Afar et al. (13) also showed that tomoregulin is located at the cell surface and is rapidly internalized at 37°C on antibody binding. It is important to note that tomoregulin is not a secreted protein like prostate-specific antigen and prostatic acid phosphatase. Targeting these secreted proteins may be problematic due to the potential binding capacity of circulating protein to the antibody (2).

The function of tomoregulin is currently unclear. It was reported that tomoregulin increases the survival of neurons from hippocampus and midbrain (8). Furthermore, the presence of the follistatin domains in tomoregulin may suggest that tomoregulin has a growth promoting function similar to Follistatin, which was

shown to be an antagonist of activin-mediated growth inhibition and seems to be overexpressed in melanoma and liver tumors (14, 15). However, tomoregulin exhibited antiproliferative activity when ectopically overexpressed in PC-3 and DU 145 cells (16). In our experiments, we were unable to detect any antiproliferative effect when tomoregulin was overexpressed in PC-3 cells. Furthermore, the addition of recombinant extracellular domain of tomoregulin to LNCaP or PC-3 cells did not alter the proliferative rate of these cells (data not shown) as these cells do not express the erbB-4/HER-4 receptor (6, 17).

For our biodistribution study (Fig. 4), ^{111}In was used to monitor accumulation of the antibodies in various tissues. ^{111}In -labeled 2H8 showed a time-dependent accumulation in tumor tissues expressing either the endogenous tomoregulin protein (LNCaP, Fig. 4) or the transfected tomoregulin protein (Clone36 model, data not shown). No antibody uptake was observed in mouse brain presumably due to exclusion of antibodies by the blood-brain barrier. Mouse liver and kidney had no uptake, although they are well vascularized. Although LNCaP tumors and Clone36 tumors have different degrees of vascularization, 2H8 showed similar biodistribution profiles in these two tumor models, suggesting that the targeting of 2H8 *in vivo* is determined by tomoregulin expression. In both models, the maximum tumor-specific accumulation of 2H8 reached 25% of the injected dose. This is comparable to biodistribution results using an anti-prostate-specific membrane antigen antibody (J591) in the same LNCaP model (18).

The efficacy of an antibody-drug conjugate targeting tomoregulin has been explored in animal studies (13). The tomoregulin-targeting antibody-drug conjugate showed excellent efficacy in both LNCaP and CWR-22 xenografts. However, the heterogeneity of tomoregulin protein expression observed in clinical specimens may not be reflected in these xenograft models. We used a radioimmunotherapy approach because this approach has the advantage of an intrinsic "bystander" effect that could overcome the heterogeneity of tomoregulin expression in prostate tumor tissues. Radiation emitted by radioisotopes such as ^{90}Y , a pure β -emitter, can penetrate the tissue by 5 to 10 mm and thereby can affect even tumor cells that do not express tomoregulin.

We showed the efficacy of the radioimmunotherapy approach. A single treatment with ^{90}Y -labeled 2H8 inhibited the growth of established human tumor xenografts in nude mice without causing overt toxicity (Fig. 5). In contrast, the unconjugated 2H8 given as a single injection of 163 μg had no effect on tumor growth. This is supported by *in vitro* experiments in which incubation of 2H8 with LNCaP cells did not affect cell proliferation (data not shown). This may indicate that (a) tomoregulin does not signal or its signaling is not important for LNCaP cell proliferation, and/or (b) tomoregulin

signaling was not blocked by 2H8. Both ^{90}Y -labeled 2H8 and ^{90}Y -labeled IgG control caused a tumor growth inhibition. However, the specific targeting of 2H8 led to a significantly greater efficacy of ^{90}Y -labeled 2H8 than ^{90}Y -labeled IgG in both tumor volume ($P < 0.05$) and tumor weight (50% reduction). The nonspecific effect of radiolabeled mouse IgG in tumor-bearing mice has been reported (19) and is likely due to the slow clearance of mouse IgG in mouse.

Our data might have clinical implications, given the fact that increased expression of tomoregulin mRNA is found in tumor tissues with advanced tumor grade and that the presence of the tomoregulin protein in metastases was detected by immunohistochemistry. In addition, the high tumor-specific accumulation of 2H8 and the efficacy of ^{90}Y -labeled 2H8 without overt toxicity make tomoregulin-targeted radioimmunotherapy an attractive approach for the treatment of advanced prostate cancer. A recent phase I clinical trial of radioimmunotherapy on androgen-independent prostate cancer has been reported using ^{90}Y -labeled anti-prostate-specific membrane antigen humanized antibody J591 (20). Seventeen of 19 patients (89%) with bone lesions and only 9 of 13 patients (69%) with soft tissue lesions were accurately targeted by J591. Furthermore, signs of efficacy were only observed at doses at or above the maximal tolerated dose and, thus, an increased therapeutic window will be needed. A radioimmunotherapy targeting tomoregulin may offer a better therapeutic window. In addition, our expression database indicates that a subpopulation of patients express tomoregulin but not anti-prostate-specific membrane antigen, suggesting that patients who cannot be targeted by J591 could benefit from tomoregulin-targeted radioimmunotherapy.

In summary, our work has established that tomoregulin is a good target for radioimmunotherapy of prostate cancer because it shows restricted expression in normal tissues and overexpression in prostate cancer and metastases. Our *in vivo* data show that the radiolabeled tomoregulin antibody localized to the tumor site, and that its strong tumor-specific accumulation resulted in significant growth inhibition of established LNCaP tumors, but produced no overt toxicity.

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References

- Raghavan D, Skinner E. Genitourinary cancer in the elderly. *Semin Oncol* 2004;31:249-63.
- Small EJ. Monoclonal antibody therapy for prostate cancer: finally a reality? *J Clin Oncol* 2004;22:2515-6.
- Zhao XY, Malloy PJ, Krishnan AV, et al. Glucocorticoids can promote androgen-independent growth of prostate cancer cells through a mutated androgen receptor. *Nat Med* 2000;6:703-6.
- Krishnan AV, Zhao XY, Swami S, et al. A glucocorticoid-responsive mutant androgen receptor exhibits unique ligand specificity: therapeutic implications for androgen-independent prostate cancer. *Endocrinology* 2002;5:1889-900.
- Zhao XY, Ly LH, Peehl DM, Feldman D. Induction of androgen receptor by $1\alpha,25$ -dihydroxyvitamin D₃ and 9-*cis* retinoic acid in LNCaP human prostate cancer cells. *Endocrinology* 1999;140:1205-12.
- Uchida T, Wada K, Akamatsu T, et al. A novel epidermal growth factor-like molecule containing two follistatin modules stimulates tyrosine phosphorylation of erbB-4 in MKN28 gastric cancer cells. *Biochem Biophys Res Commun* 1999;266:593-602.
- Nikula TK, Curcio MJ, Brechbiel MW, Gansow OA, Finn RD, Scheinberg DA. A rapid, single vessel method for preparation of clinical grade ligand conjugated monoclonal antibodies. *Nucl Med Biol* 1995;22:387-90.
- Horie M, Mitsumoto Y, Kyushiki H, et al. Identification and characterization of TMEFF2, a novel survival factor for hippocampal and mesencephalic neurons. *Genomics* 2000;67:146-52.
- Young J, Biden KG, Simms LA, et al. HPP1: a transmembrane protein-encoding gene commonly methylated in colorectal polyps and cancers. *Proc Natl Acad Sci U S A* 2001;98:265-70.
- Liang G, Robertson KD, Talmadge C, Sumegi J, Jones PA. The gene for a novel transmembrane protein containing epidermal growth factor and follistatin

- domains is frequently hypermethylated in human tumor cells. *Cancer Res* 2000;60:4907-12.
11. Glynne-Jones E, Harper ME, Seery LT, et al. TENB2, a proteoglycan identified in prostate cancer that is associated with disease progression and androgen independence. *Int J Cancer* 2001;94:178-84.
12. Mohler JL, Morris TL, Ford OH III, Alvey RF, Sakamoto C, Gregory CW. Identification of differentially expressed genes associated with androgen-independent growth of prostate cancer. *Prostate* 2002;51:247-55.
13. Afar DE, Bhaskar V, Ibsen E, et al. Preclinical validation of anti-TMEFF2-auristatin E-conjugated antibodies in the treatment of prostate cancer. *Mol Cancer Ther* 2004;3:921-32.
14. Stove C, Vanrobaeys F, Devreese B, Van Beeumen J, Mareel M, Bracke M. Melanoma cells secrete follistatin, an antagonist of activin-mediated growth inhibition. *Oncogene* 2004 Jul 8;23:5330-9.
15. Rossmannith W, Chabicovsky M, Grasl-Kraupp B, Peter B, Schausberger E, Schulte-Hermann R. Follistatin overexpression in rodent liver tumors: a possible mechanism to overcome activin growth control. *Mol Carcinog* 2002 Sep;35:1-5.
16. Gery S, Sawyers CL, Agus DB, Said JW, Koeffler HP. TMEFF2 is an androgen-regulated gene exhibiting antiproliferative effects in prostate cancer cells. *Oncogene* 2002;21:4739-46.
17. Grasso AW, Wen D, Miller CM, Rhim JS, Pretlow TG, Kung HJ. ErbB kinases and NDF signaling in human prostate cancer cells. *Oncogene* 1997 Nov 27;15:2705-16.
18. McDevitt MR, Ma D, Lai LT, et al. Tumor therapy with targeted atomic nanogenerators. *Science* 2001;294:1537-40.
19. Shih LB, Xuan H, Anipot R, Stein R, Goldenberg DM. *In vitro* and *in vivo* reactivity of an internalizing antibody, RS7, with human breast cancer. *Can Res* 1995; 55:5857-63.
20. Milowsky MI, Nanus DM, Kostakoglu L, Vallabhajosula S, Goldsmith SJ, Bander NH. Phase I trial of ⁹⁰Y-labeled anti-prostate-specific membrane antigen monoclonal antibody J591 for androgen-independent prostate cancer. *J Clin Oncol* 2004;22:2522-31.