Published in final edited form as:

Cancer Lett. 2008 December 8; 272(1): 122–132. doi:10.1016/j.canlet.2008.07.009.

A new TAG-72 cancer marker peptide identified by phage display

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Abstract

Radiolabeled peptides as markers of cancer targets have demonstrated their value in diagnostic imaging and radiotherapy. The 16 mer f88-4/Cys6 phage display library was applied to affinity purified TAG-72 and three consensus peptides were identified: VHHSTKLTHCCQNW (A2–13), GGVSCMQTSPVCENNL (A2–6) and TKRDCAQNYGCQKAI (A2–11). A2–13 and A2–6 phages showed the highest percent binding to LS-174T cells by flow cytometry that was 3-fold higher than a control phage. Fluorescence microscopy showed that both A2–6 and A2–13 phages bound to the LS-174T cell membrane. However, only the A2–6 phage demonstrated specificity by low binding to the TAG-72 negative cell HT-29. Furthermore, the synthesized A2–6 peptide demonstrated specific binding to LS-174T cells by flow cytometry and by immunohistochemical staining of xenograph tumor compared to normal colon. These data indicate that the A2–6 peptide is specific and selective for the TAG-72 cancer target.

Keywords

phage display; TAG-72; tumor imaging; peptide biomarker

1. Introduction

Clinical applications of radiolabeled peptides for cancer diagnostic imaging and therapy have been successful as evident by the numerous reports describing their use in imaging of tumor associated receptors such as the somatostatin analogs [1–4] and bombesin [5–10]. Because of their small size, peptides are likely to have more rapid and possibly more favorable pharmacokinetics than antibodies or their fragments. As a result, the identification and development of new peptides with specific targeting properties is an area of interest in the field of diagnostic cancer imaging. While a number of naturally occurring peptides are under investigation as diagnostic imaging agents, the use of phage display libraries and combinatorial technology offers the possibility of identifying novel peptides with improved binding affinities and selectivity for their target. Genetically modified bacteriophages that express randomized peptide sequences on their surface have been used to select peptides for targeting organs, tumor antigens, and tumor vasculature [11–13].

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The tumor-associated glycoprotein 72 (TAG-72) is a membrane protein complex of approximately 220 kD to 400 kD that is over expressed in a number of cancers, including colonic adenocarcinoma, invasive ductal carcinoma of the breast, non-small-cell lung carcinoma, epithelial ovarian carcinoma, as well as pancreatic and gastric esophageal cancers [14], with only trace levels found in histological sections of normal tissues [15,16]. TAG-72 is the target antigen for the B72.3 and CC49 antibodies used in the diagnostic imaging of both colorectal and ovarian cancers [17,18]. Since TAG-72 is present in an extensive number of cancers and has minimal occurrence in normal tissues, it is a reasonable and attractive target for the development of a diagnostic peptide.

The f88-4/Cys6 phage peptide library is comprised of 2.7×10^8 phages each expressing more than 150 copies of a 16 mer peptide containing a disulfide loop bridging six amino acids. Using this library, we previously identified three consensus peptides that demonstrated binding affinity to TAG-72 positive cells [19]. Of these three peptides, one with the sequence NPGTCKDKWIECLNG showed evidence of specific binding when presented as the radiolabeled phage to TAG-72 expressing tumor cells in culture and in excised tumor xenographs.

The goal of this investigation was to continue the identification of novel TAG-72 binding peptides by performing another phage display selection using the same library but with a longer final elution time. As before, the most promising peptides were identified while attached to their phage and used in binding studies with TAG-72 positive LS-174T colon cancer cells and the TAG-72 negative cell line HT-29 as control. Unlike our earlier study, in this investigation the most promising peptide was synthesized and evaluated as the free peptide.

2. Material and methods

2.1. Reagents and cell lines

The LS-174T and HT-29 cell lines, both human colon adenocarcinomas, were obtained from the American Type Culture Collection (Rockville, MD). The LS-174T cells were cultured in Eagle's minimal essential medium (MEM) with nonessential amino acids, supplemented with 10% fetal bovine serum (FBS) while the HT-29 cells were cultured in McCoy's 5a medium modified with 1.5 mM L-glutamine, adjusted to contain 2.2 g/l sodium bicarbonate, and supplemented with 10% FBS. The phage display library, f88-4/Cys6, was provided by George Smith, Ph.D. (University of Missouri). The primer used for sequencing was: 5'-AGT AGC AGA AGC CTG AAG A-3' (Qiagen, Alameda, CA). The partially purified TAG-72, rabbit anti-fd bacteriophage IgG, R-phycoerythrin (PE) conjugated goat anti-rabbit F(ab')₂, mouse monoclonal anti-biotin antibody and PE conjugated sheep anti-mouse IgG F(ab')₂ were from Sigma Aldrich (St. Louis, MO). The B72.3 monoclonal antibody was provided by the NCI Biological Resource Branch Preclinical Repository (Rockville, MD). Alexa Fluor 633 carboxylic acid succinimidyl ester was from Invitrogen (Eugene, OR). The anti-mouse IgG horseradish peroxidase (HRP)-linked antibody was from Cell Signaling Technology (Danvers, MA). The sulfo-NHS-LC-biotin was from Pierce Biotechnology (Rockford, IL). All other chemicals were from standard suppliers and were used as received. The ^{99m}Tc-pertechnetate was eluted from a ⁹⁹Mo-^{99m}Tc generator (Perkin-Elmer, Billerica, MA).

2.2. Selection of TAG-72 specific phage clones

The phage clone selection was similar to that described previously using partially purified TAG-72 that was purified further on a B72.3 antibody affinity column [19]. The fractions containing TAG-72 were pooled and diluted to 0.67 µg protein in 0.4 ml of 0.1 M sodium bicarbonate buffer pH 8.5 for coating one well surface of a 6-well polystyrene plate (Corning, NY). Two other samples were prepared for coating two additional wells. One of these samples

was from the first wash of the B72.3 affinity column, containing 65 µg protein per ml, and was considered TAG-72-free. The second was 0.5% BSA. Both of these samples were prepared in 0.4 ml of 0.1 M sodium bicarbonate buffer, pH 8.5. These two additional samples were used to select against phages with affinity for surfaces other than TAG-72 prior to incubation of the library in the purified TAG-72 well. The remaining three wells were left empty. Three identical plates were prepared, one plate for each cycle of selection. To begin one selection cycle, the three coated wells of one plate were washed with 50 mM Tris containing 150 mM sodium chloride and 0.5% Tween-20 (TBS/Tween), using a volume sufficient to fill the well. The wells were then blocked with 0.1 M sodium bicarbonate buffer containing 0.5% BSA. The phage library, containing about 2×10^8 phages in 400 µl of TBS/Tween and 0.1% BSA, was added first to the well coated with the TAG-72 free eluant from the affinity column. After incubation for 20 min the library was transferred and incubated for 30 min in the BSA coated well. Thereafter, the library was transferred to the final well containing the purified TAG-72 and incubated with gentle mixing for 1 hr at room temperature. After extensive washing of this well with TBS/Tween, the tightly bound phages were eluted by incubation for 10 min in 0.1 M glycine buffer pH 2.2. The eluted phage sample was neutralized with 50 µl Tris/HCl (pH 9.1) and amplified by infecting *E. coli* host strain K91BluKan. The amplified phage were recovered and then titered following standard procedures [20] before serving as starting material for the next round of selection. The depleted phage pool was subjected to two additional cycles of selection, in the identical fashion except in the second cycle, a second aliquot of glycine was added after the first glycine was removed, and the well left for an additional 2 days at room temperature to elute high affinity binders. Eluants from the second and third cycles were amplified and titered as before.

From the titration plates, 25 to 29 well-isolated phage colonies, each representing a single clone, were removed randomly and were prepared for DNA sequencing as described previously. 19 Sequencing was performed with the primer: 5'-AGT AGC AGA AGC CTG AAG A-3' using an automated DNA sequencer (Applied Biosystems 3100, Foster City, CA) to identify common peptide sequences among the clones and thus the extent of consensus.

2.3. Conjugation of phages

Three phages expressing consensus peptides and a non consensus phage as control were amplified as before and purified by precipitation with PEG/NaCl (16.7%/3.3 M) added at one sixth the total volume. After 1 hr on ice, the precipitated phages were recovered by centrifugation at 13,000 rpm for 10 min, and the phage pellet was resuspended in 100 µl D-PBS. Each phage was then radiolabeled with ^{99m}Tc after conjugation with S-acetyl NHS-MAG₃ following methods standard in this laboratory [21,22]. Radiochemical purity was determined by both instant thin-layer chromatography (ITLC) with acetone as the solvent (ITLC-SG; Gelman) and by paper chromatography (Whatman no. 1; VWR) with saline as the solvent. Both radiolabeled phage and colloids remain at the origin in both systems, whereas labeled pertechnetate, tartrate, and MAG₃ migrate in saline and only pertechnetate migrates in acetone. The chromatography strips were cut into 1 cm sections, and the radioactivity was determined in a gamma well counter (Cobra II Auto-Gamma; Packard Instrument Co.). Each phage was also conjugated with a fluorophore for analysis by flow cytometry. Briefly, Alexa Fluor 633 N-hydroxy succinimidyl ester was prepared in 1-methyl-2-pyrrolidinone, anhydrous (Sigma) at 1 mg per ml and 10 µl was added to 5×10^{12} phage transducing unit (TU) in 40 µl Dulbecco's PBS (D-PBS) to which was added 50 µl of 0.1 M sodium bicarbonate buffer, pH 8.5, and the sample was set on ice. After 1 hr the phages were separated from unbound fluorophore by precipitation as before. After 1 hr on ice, the precipitated phages were recovered by centrifugation at 13,000 rpm for 10 min, and the phage pellet was resuspended in 100 µl D-PBS. The precipitation was repeated a second time and the final Alexa Fluor 633-phage pellet was suspended in 150 µl D-PBS.

2.4. TAG-72 expression by western blot analysis

Hand et al [23] has reported on the variable expression of the TAG-72 in LS-174T cells resulting from differences in culture conditions. A western blot analysis was performed to confirm the TAG-72 expression in the LS-174T cells grown for this report, and to verify the absence of TAG-72 in the negative control cell, HT-29. About 10^6 cells of each type were lysed in buffer containing 150 μ l 100 mM Tris pH 8.0, 5 mM EDTA, 5% NP40, and protease inhibitor cocktail (1 tablet per 50 ml) (Boehringer Mannheim, Germany) by incubation on ice for 30 min. Cell lysates were spun at 12,000 rpm for 10 min at 4°C, and the protein in the supernatant was quantified using the micro BCA protein assay kit (Pierce Biotechnology, Rockford, IL). Samples containing 100 μ g protein were mixed with Laemmli SDS-sample buffer (Boston Bioproducts, Boston, MA) and set in a 100°C heating block for 5 min before separation on a 4–15% gradient SDS-polyacrylamide gel (Bio-Rad, Hercules, CA). The protein was then transferred onto a nitrocellulose (NC) membrane (Bio-Rad, Hercules, CA) along with kaleidoscope pre-stained standards (Bio-Rad, Hercules, CA) and a biotinylated protein ladder (Cell Signaling Technology, Danvers, MA). After the transfer, the NC membrane was blocked with 5% nonfat dry milk in TBS/0.1% Tween-20 (TBST) for 1 hr and then incubated with the mouse anti-TAG-72 monoclonal antibody B72.3 using a 1:2000 dilution of the 5.8 mg/ml stock. Gamma tubulin expression served as a housekeeping protein for the two cell lines, and was detected with a mouse anti-gamma tubulin monoclonal antibody (Sigma) diluted 1:3000. The relative intensity of the gamma tubulin bands for the two cell types was used to evaluate the amount of each protein loaded. After an overnight incubation at 4°C the NC membrane was washed 3 times, each for 5 min in TBST at room temperature. Thereafter the secondary antibody, HRP-linked anti-mouse IgG diluted 1:2000, was added and the membrane was incubated for 1 hr at room temperature, and washed again 3 times for 5 min each with TBST. After incubation of the membrane for 1 min, the protein was visualized with enhanced chemiluminescent (ECL) substrate (Amersham Biosciences, Buckinghamshire, UK) and subsequent exposure to Amersham Hyperfilm ECL (Amersham Biosciences, Buckinghamshire, UK) for about 60 sec.

2.5. Phage binding analyses

To compare the TAG-72 binding of the consensus phage clones, LS-174T cells were grown to 80% confluence and were suspended in culture medium with 1% fetal bovine serum (FBS). In triplicate, 2×10^5 cells in 100 μ l were distributed into each well of a 48-well plate, and 1×10^{12} TU of each consensus phage along with a non consensus phage was added. After the plate was incubated for 2 hr with rocking at room temperature, samples were transferred to microfuge tubes and washed 3 times with 0.2 ml ice-cold D-PBS with 3% BSA (PBSB) to remove unbound phage. Bound phage were detected by indirect flow cytometry by addition of rabbit anti-fd bacteriophage antibody diluted 1:5000 with PBSB. One hour later the cells were washed as above, and the secondary antibody, PE-conjugated goat anti-rabbit F(ab')₂, diluted 1:500 with PBSB was added and incubated for a further 30 min in the dark. Following 3 washes with 0.2 ml ice-cold PBSB, the cells were fixed by addition of 0.5 to 1.0 ml cold 0.5% paraformaldehyde solution and stored at 4°C in the dark. The cells were analyzed for PE fluorescence (emission at 575 nm) using a FACSCalibur (Becton Dickinson, Franklin Lakes, NJ). Background fluorescence was measured on a set of cells treated with the PE-conjugated goat anti-rabbit F(ab')₂ alone.

Direct flow cytometry was also used to analyze selectivity, specificity and kinetics of cell binding of the two consensus phage that demonstrated the best binding. The Alexa Fluor 633 conjugated phages (1×10^{12} TU) were incubated with LS-174T and HT-29 cells for 2 hr with rocking at room temperature and after 3 washes with 0.2 ml ice-cold PBSB to remove unbound phages, the cells were fixed with 0.5% paraformaldehyde solution and stored at 4°C in the dark prior to analysis. To investigate the kinetics of cell binding, 2×10^5 LS-174T cells were

incubated as before with Alexa Fluor 633 conjugated phages (1×10^{12} TU) from 10 min to 3 hr while specific binding was measured by identical incubations but with increasing amounts of their respective unlabeled phage from 10^4 to 10^{12} TU.

Cell binding of phage was also performed after labeling with ^{99m}Tc . LS-174T cells (1×10^5) were seeded into 12-well plates with 2 ml of media and, after reaching 80–90% confluence in 24 hr at 37°C , the media was removed and replaced with 1 ml of fresh media (with 1% FBS) containing one of the ^{99m}Tc -labeled phage (1×10^{12} TU). Unbound phages were removed after 1 hr at 37°C by washing the cells 3 times with ice-cold D-PBS. The cells were then released from the plate with 0.5 ml of 0.2 M sodium hydroxide containing 1% sodium dodecyl sulfate (SDS) and cell associated radioactivity was measured in a gamma well counter.

2.6. Fluorescence microscopy

Fluorescence microscopy was used to confirm that the selected phages were binding to TAG-72 positive cells. LS-174T cells (2×10^3 per well) were grown overnight in an 8-well chamber slide (Nalge Nunc Inc., Rochester, NY). Thereafter phages were added at 1×10^{12} TU per well, and incubated overnight at 37°C . The cells were then rinsed 3 times with D-PBS and fixed with 3.7% paraformaldehyde for 10 min, followed by 3 washes with D-PBS, and blocked with D-PBS containing 3% BSA for 30 min at room temperature. The fixed cells were then incubated with the rabbit anti-fd-bacteriophage antibody (dilution 1:5000) for 2 hr at room temperature, washed in D-PBS with 0.5% BSA 3 times, and then incubated with the PE-conjugated goat anti-rabbit F(ab')₂ diluted 1:500 for 1 hr at room temperature. Finally, the excess antibody was removed by 3 washes in D-PBS and the cells were viewed on an Olympus IX 70 (Olympus America, Inc., NY) standard wide field fluorescence inverted microscope at $200\times$ using an excitation filter at 460 – 490 nm and emission filter at 515 – 550 nm.

2.7. Free peptide binding analyses

As described below, the peptide (A2–6) expressed by the phage that demonstrated superior binding to LS-174 cells was synthesized commercially (New England Peptide LLC, Gardner, MA) in its native L configuration with an amino hexyl linker on the terminal amine for conjugation and was provided by the company with a 97% purity as determined by C18 HPLC.

Binding of the free A2–6 peptide was evaluated by flow cytometry and histology. For both studies the peptide was biotinylated by adding 0.5 mg of the peptide, at 2.5 mg/ml in phosphate buffer pH 6.5, to sulfo-NHS-LC-biotin as a dry powder, at a biotin to peptide molar ratio of 20:1. Biotinylation of the peptide was confirmed by reversed phase C-18 HPLC (Vydac column, The Nest Group, Southborough, MA) on a Waters system (Waters, Milford, MA). All analyses were performed with a linear gradient from 10% to 90% acetonitrile in 0.08% TFA over 45 min at a flow rate of 1 ml/min.

The biotinylated peptide was used without purification since free excess biotin would be removed in the washing steps prior to both flow cytometry and histology. For flow cytometry, the biotinylated peptide was incubated with LS-174T and HT-29 cells at increasing concentrations from 10^{-7} to 2×10^{-4} mol/l for 1 hr with rocking at room temperature. After 3 washes, a mouse anti-biotin antibody (1 hr) followed by PE-anti-mouse antibody (30 min) was used to detect the peptide bound to cells.

To establish binding of the A2–6 peptide to solid tumor, LS-174T tumor xenographs were grown in nude mice (Taconic Farms, Germantown, NY) with 1×10^6 LS-174T cells administered subcutaneously in one thigh. After 14 days, tumor and normal colon to serve as normal control tissue were removed and fixed in 10% buffered formalin for 24 hr and then embedded in paraffin. Paraffin sections were cut to 5 μm and deparaffinized by 2 washes of

xylene for 10 min each, then in 100% ethanol twice for 2 min each. Endogenous peroxidase activity was blocked by incubation for 10 min in 3% peroxide. After rinsing in PBS, the slides were heated in a microwave at 90°C for 10 min in Retrieval A solution (BD Biosciences), and then cooled to room temperature.

After block for 1 hr with blocking reagent (Vector Laboratories, Burlingame, CA), one set of LS-174T tumor slides and one set of normal colon slides were incubated for 1 hr with the mouse anti-TAG-72 antibody B72.3 (dilution of 1:500 of 5 mg/ml stock). The bound B72.3 antibody was detected by incubating the slides with HRP-conjugated anti-mouse antibody (dilution of 1:200) for 30 min. An identical set of tumor and colon slides were also incubated with the biotinylated A2-6 peptide for 1 hr, then with conjugated streptavidin (dilution of 1:100) for 30 min (Vector Laboratories, Burlingame, CA). All samples were counter stained with hematoxylin (blue stain) to delineate cell structure.

Statistical analysis—The student's t-test was used to test for significance where indicated.

3. Results

3.1. TAG-72 expression by western blot analysis

Fig. 1 shows the results of a western blot analysis of LS-174T and HT-29 cells. The equal intensity of the gamma tubulin bands, among the two cell lines demonstrates that both lanes were loaded with an equivalent amount of protein. The figure shows that the LS-174T cells (left lane) used in this investigation express the TAG-72 glycoprotein at a reasonable level, while the HT-29 cells (right lane), also a human colon adenocarcinoma, show no evidence of TAG-72, and are therefore an appropriate negative control cells.

3.2. Selection of TAG-72 specific phage clones

After the second cycle of selection, twenty-five well isolated clones were randomly selected for DNA sequencing. From the sequence data three sets of identical peptides were identified: GGVSCMQTSPVCENNL (phage A2-6), VVHSC TKLTHCCQNW (phage A2-13), and TKRD CSAQNYGCQKAI (phage A2-11) expressed in 24%, 44%, and 8% of the 25 clones selected, respectively. After the third cycle, 29 clones were sequenced and of these the A2-13 phage was found in 52% of the clones and the A2-6 phage in 24%. The A2-11 phage was not present.

3.3. Phage binding analyses

Flow cytometry was performed with the TAG-72 positive LS-174T cells using the anti-fd bacteriophage antibody and PE-conjugated secondary antibody to evaluate phage binding. Fig. 2a shows binding of the A2-6 (left panel) and A2-13 phages (middle panel) to LS-174T cells as indicated by the increase in fluorescence intensity (shift to the right) and much lower binding of the non-consensus control phage (right panel). Superimposed on the figure are results obtained in identical binding study of the same cells but treated only with the PE-conjugated second antibody alone (black lines). Phages A2-6 and A2-13 showed signal shifts of 52% and 55%, respectively, compared to the non-consensus control phage at 16% indicating high affinity binding of A2-6 and A2-13 phages to LS-174T cells.

The phages were labeled with ^{99m}Tc using MAG3 as chelator. Following purification, greater than 90% of the label remained at the origin on ITLC and paper chromatography as evidence of radiochemical purity (data not shown). The fact that we demonstrate specific binding shows that the activity at the origin is not colloid.

As shown in Fig. 2b, similar relative binding was observed for the ^{99m}Tc -labeled A2-6 and A2-13 phages and the control phage to the LS-174T cells as was observed by flow cytometry (Fig. 2a). Error bars are barely visible.

The binding kinetics of Alexa Fluor 633 conjugated A2-6 and A2-13 phages to LS-174T cells over 3 hr was evaluated by flow cytometry and as shown in Fig. 3a, both phages showed a time-dependent increase in binding. However, The A2-6 phage reached a maximum at 120 min, while phage A2-13 showed a continual increase in percent bound through the 180 min incubation.

To evaluate specific binding, both selected phages conjugated with Alexa Fluor 633 (1×10^{12} TU) were incubated with LS-174T cells in the presence of unconjugated phage added from 10^4 to 10^{12} TU. By flow cytometry analysis, at 10^{12} TU, binding of A2-6 phage to cells was reduced significantly by 20% ($P < 0.03$) compared to a statistically insignificant 15% for the A2-13 ($P > 0.07$) after subtraction of background fluorescence (data not shown).

Flow cytometry was also used with the TAG-72 negative HT-29 cells to further confirm that these two consensus phages were binding selectively. The results shown in Fig. 3b were obtained after 1 hr of incubation with 10^{12} TU phage. Only the A2-6 phage showed significantly higher binding to LS-174T cells compared to control HT-29 cells ($P < 0.01$). There was no significant difference in the binding of the A2-13 and the non-consensus control phage to both cell types.

3.4. Fluorescence microscopy

Fluorescence microscopy was also used to confirm that the selected phages were binding to TAG-72 positive cells. The A2-6 and A2-13 phages and control phage were incubated overnight at 37°C with LS-174T cells and were then washed and fixed. The phages were visualized with the rabbit anti-fd-bacteriophage IgG followed by the PE-goat anti-rabbit antibody. Fig. 4 presents representative regions of both the bright field (BF) and fluorescent field (PE). Positive staining was shown only for both the A2-6 and A2-13 phages but not for the control phage. Furthermore, the fluorescence distribution in the case of the A2-6 phage is more uniform around the entire membrane with evidence of more concentrated patches. In contrast the A2-13 phage shows the label is concentrated at a cellular pole.

3.5. Free peptide binding analyses

The C18 HPLC analysis of the A2-6 peptide showed a major peak at 14.3 min that shifted completely after biotinylation to 16.25 min indicating complete biotinylation of the peptide (data not shown). The biotinylated peptide was incubated with LS-174T and HT-29 cells at different concentrations for 1 hr and binding detected by flow cytometry using a mouse anti-biotin antibody followed by a PE-anti-mouse antibody. Fig. 5 demonstrates that the biotinylated A2-6 peptide showed a significant increase in percent binding with an increase in peptide concentration but only to the TAG72 expressing LS-174T cells, indicating the selective binding of biotinylated A2-6 peptide to LS-174T cells.

Immunohistochemical staining demonstrated that the A2-6 peptide bound to LS-174T solid tumor xenographs. Slides with sections of LS-174T solid tumor and normal mouse colon (as control) were stained for TAG-72 using the B72.3 antibody as a control and the biotinylated A2-6 peptide. As shown in Fig. 6 positive staining was observed for the B72.3 antibody (brown stain), as expected, indicating that TAG-72 is highly expressed in this tumor (top left). Normal colon tissue (negative control) shows no TAG72 expression (top right). Similarly, after incubation with the biotinylated A2-6 peptide positive staining (brown) was observed with the

LS-174T tumor (bottom left), and no positive stain (bottom right) was observed with the normal colon tissue.

4. Discussion

The TAG-72 glycoprotein is a recognized tumor marker present in a number of cancers. A radiolabeled peptide against TAG-72 could potentially exhibit tumor binding properties comparable to that of the B72.3 and CC49 antibodies used in the clinic but with more favorable pharmacokinetic properties. Our long term objective is to identify a number of phage with specificity to TAG-72 and from this group identify those peptides with the best binding properties and pharmacokinetics. In a previous publication [19] using the same library, we identified a phage clone (Clone A) expressing the peptide, NPGTCKDKWEICLLNGG, that demonstrated binding to TAG-72. Characterization and evaluation of the binding properties of Clone A was performed by analysis of the ^{99m}Tc -labeled phage with cells in culture, solid tumor cubes, and in a mouse tumor model. These studies demonstrated specificity of binding for Clone A by showing saturation with increasing phage with cells in culture, and by reduced accumulation of labeled B72.3 antibody to solid tumor cubes with increasing phage. In the present investigation, the same phage library was again used along with a similar selection strategy, but with an extended elution time in the second cycle. In addition, potential binding phages were evaluated with ^{99m}Tc -labeling, flow cytometry and fluorescence microscopy.

Possibly because of the longer elution time used in this compared to our first investigation, the two consensus phages A2-6 and A2-13 were found after only two cycles of selection and these two were the only consensus phage peptides found after the third cycle of selection. Therefore these two phages were characterized further for evidence of specific binding.

While both phages showed higher binding to the TAG-72 positive LS-174T cells by both flow cytometry and radioactivity cell counting of labeled phages compared to a control phage as shown in Fig. 2a and 2b, considerable differences in binding kinetics and selectivity was observed between these two phages. As shown in Fig. 3a, the A2-6 phage showed evidence of saturation and therefore specific binding in contrast to the A2-13 phage that showed a continuous increase in binding through 180 min. As additional evidence of specific binding for the A2-6 phage, when in competition with an equal number of unlabeled phages, flow cytometry analysis showed greater reduction in the amount of A2-6 phage bound than the A2-13 phage by 20% versus 15%, (data not shown). In addition, as shown in Fig. 4, by direct fluorescence microscopy of LS-174T cells, the A2-6 phage showed binding around the entire cell membrane while the A2-13 phage showed the fluorescence restricted to an area at one end. For both phages the fluorescence of Fig. 4 is restricted to the cell surface with no evidence for internalization. Finally, further evidence for specificity and selectivity of the A2-6 phage was obtained using the HT-29 cells, as demonstrated by western blot analysis to be TAG-72 negative. As shown in Fig. 3b, while the A2-13 phage showed similar binding to both the LS-174T and negative HT-29 cell, the A2-6 phage showed about five times greater binding to the LS-174T cells than the HT-29 cells. Binding to the non-consensus control phage was similar for both cells. These results show that while both phages are most likely binding to a cell surface receptor, it is most probably TAG-72 only in the case of the A2-6 phage.

Based on these positive findings on TAG-72 specific binding, the A2-6 peptide was synthesized and biotinylated. The free peptide, like the phage bound peptide, again showed specificity and selectivity by flow cytometry with binding to LS-174T cells relative to the HT-29 cells as shown in Fig. 5. Additionally, histological staining of LS-174T tumor xenographs incubated with the biotinylated A2-6 peptide followed with HRP-streptavidin, showed staining consistent with positive binding as shown in Fig. 6. In summary, of the three consensus phage peptides identified in this study by phage display, only the A2-6 phage and

its free peptide were shown to bind specifically to TAG-72 positive cell. Future studies of the radiolabeled A2–6 peptide will be conducted in tumor bearing mice to evaluate pharmacokinetics and imaging properties

Acknowledgements

We express our appreciation to the NCI Biological Resource Branch Preclinical Repository (Rockville, MD) for providing the B72.3 monoclonal antibody and to Dr. Sue Deutcher (University of Missouri, Columbia) for providing the phage library from the Dr. George Smith's laboratory. Partial funding was provided by the NIH under CA111606.

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Fig. 1. Western blot analysis of TAG-72 expression in LS-174T (left) and HT-29 (right) colon adenocarcinoma cells. The gamma-tubulin serves as an internal loading control.

Fig. 2. Phage binding by flow cytometry and radiolabeling analyses. (a) Flow cytometry of phages (gray line) A2-6 (left), A2-13 (middle) and non-consensus control phage (right) following incubation with LS-174T cells. The relative numbers of cells are shown as a function of fluorescence intensity. The background fluorescence (black line) is with the secondary antibody alone. (b) Histogram showing the percent of radioactivity bound to LS-174T cells for 99mTc-labeled A2-6 phage, A2-13 phage, and a non consensus control phage. Mean of $n = 3$ with SD is shown, error bars are present but small.

Fig. 3.

Phage binding kinetics, specificity and selectivity by flow cytometry analyses. (a) Percent of A2-6 phage (solid) and A2-13 phage (dashed) binding to LS-174T cells from 10 min to 180 min. Mean of $n=3$ with SD is shown. (b) Percent binding of A2-6, A2-13 and a non consensus control phage to LS-174T (TAG-72 positive) and HT-29 (TAG-72 negative) cells. Mean of $n=3$ with SD is shown.

Fig. 4. Fluorescence microscopy of LS-174T cells incubated with a non consensus control phage (top row) or with A2-13 phage (middle row) or A2-6 phage (bottom row). Left panels show the bright field (BF) and right panels show PE fluorescence (Magnification $\times 200$). Results shown are representative of the field.

Fig. 5.

Flow cytometry analysis of the free peptide as a function of concentration. Shown is the percent binding for the biotinylated A2-6 peptide at concentrations from 10^{-7} to 2×10^{-4} mol/l to LS-174T (solid) and HT-29 (dashed). Mean of $n=3$ with SD is shown.

Fig. 6. Immunohistochemical staining of formalin fixed, paraffin embedded LS-174T xenographs and normal colon. The tumor sections (left panels) show positive staining (brown) for both the B72.3 antibody (top), as positive control, and the biotinylated A2-6 peptide (bottom). Whereas the normal colon (right panels) shows only the hematoxylin counter stain (blue) and is negative for both B72.3 (top) and peptide (bottom) (Magnification $\times 400$).