Research Article

Colorectal Cancer

Discovery of specific targeting ligands as the biomarkers for colorectal cancer

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Summary points

- Early colorectal cancer (CRC) detection and intervention improve patients' survival.
- Development of noninvasive tests for screening and diagnosis of CRC is limited by the lack of sensitive biomarkers.
- Peptidomimetic molecules are considered better cell surface-targeting agents than monoclonal antibodies. These smaller molecules can better penetrate large tumors; and are less likely to bind to the reticuloendothelial system. They are also easy to synthesize chemically.
- One-bead one-compound combinatorial strategy provides a powerful tool to detect specific ligand on the cancer cell's surface.
- Using one-bead one-compound combinatorial libraries, we isolated two ligands, CRC-6 and CRC-9, which bound specifically to HT-29 and DLD-1 colon cancer cell lines, but not to normal human colonic epithelial cells.
- Immunohistochemistry studies showed that fluorescent agents tagged to CRC-6 and CRC-9 were able to detect colon cancer cells grown on chamber slides at 1 uM after 30 min of incubation.
- *In vivo* studies showed that CRC-9 biotin–streptavidin–Cy5.5 was found to accumulate in nude mice implanted with subcutaneous HT-29 and DLD-1 xenografts.
- Further testing is underway to determine the binding profile of CRC-6 and CRC-9 to human colon cancer tissue as well as precancerous and benign lesions including ulcers, adenomas and polyps, and others.
- The binding of CRC-6 and CRC-9 to human CRC tissues can be determined by tagging the peptides with fluorescein dye to visualize tumors using fluorescent microscopy or epifluorescent endoscopy.

Aim: Targeted diagnosis and therapy for colorectal cancer (CRC) is limited by the lack of specific biomarkers. Our aim was to discover CRC-specific targeting ligands using a one-bead one-compound (OBOC) combinatorial library. **Method:** Samples of OBOC peptide libraries were color coded, mixed and incubated with commercially available human CRC cells (HT-29 and DLD-1). Libraries with compound beads that bound to CRC cells were selected for further screening. Compound beads that bound to both CRC cells were screened with human colonic epithelial cells to select beads that bound only to CRC cells but not to human colonic epithelial cells. Chemical structures of the positive peptides were determined by Edman chemistry. CRC-targeted imaging agents were developed by conjugation of CRC binding peptide with biotin through a hydrophilic linker and then complexed with streptavidin–Cy5.5. Immunohistochemistry studies were used to evaluate CRC detection efficacy. Targeting specificity was further tested with subcutaneous CRC xenografts in nude mice. **Results:** Two cyclic peptides, CRC-6 and CRC-9, composed of natural and unnatural amino acids, bind specifically to CRC cells with moderately high affinity and specificity. CRC-9 is able to detect CRC cells grown on chamber slides at the concentration of 1 μM after 30 min incubation. Tail vein injection of 1.8 nmol biotinylated peptide CRC-9, complexed with streptavidin–Cy5.5 (SA–Cy5.5), is able to target the subcutaneous CRC xenograft implants in nude mice. None of the two peptides showed cytotoxic effect on human blood cells, up to the concentration of 500 μM. **Conclusion:** CRC-9 has the potential to be developed as an effective biomarker for improving the management of CRC patients by enhancing the efficiency of detection and efficacy of targeting treatment.

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Colorectal cancer (CRC) is a significant health burden worldwide and is the second leading cause of cancer deaths in the USA. Despite development in diagnosis and treatment options, early detection and targeted therapy for CRC have been hindered by the lack of specific biomarkers [1,2]. Current studies focused primarily on arginine–glycine–aspartate (RGD)-based peptides that target the α5β1 integrin, but success was limited because of poor specificity [3–5].

Targeted therapy, whereby cancer cells are killed in the presence of normal tissue, is a major goal of cancer treatment. In the postgenomic era, clinical diagnosis and treatment regimen are becoming more dependent on the molecular characteristics of tumors. Furthermore, emphasis is now focused on the development of peptidic or peptidomimetic molecules, which are considered better cell surface-targeting agents than monoclonal antibodies because they are: smaller and therefore can better penetrate large tumors; less likely to bind to the reticuloendothelial system such as liver, spleen and bone marrow; and easy to synthesize chemically [6–8].

The one-bead one-compound (OBOC) methodology was developed to rapidly identify affinity agents against a variety of biological targets [9]. This powerful combinatorial technique has been applied in the discovery of ligands against various biological targets, such as cancer cell-surface receptor [10–12], protein kinase substrates and inhibitors [13,14], protease substrates and inhibitors [15,16], artificial enzymes [17,18] and ligands for affinity column chromatography [19,20].

Targeted therapy needs specific recognition of the cancer cells and specific delivery of anticancer drugs to the tumor site. Nanotechnology is an emerging field that has shown great promise for the development of novel diagnostic, imaging and/or therapeutic agents for a variety of diseases, including cancers [21].

In this study, we employed OBOC combinatorial methodology to discover CRC specific targeting ligands in an attempt to develop a more efficacious and less toxic agent for CRC imaging as well as therapeutic application.

Materials & methods

Synthesis & screening of OBOC peptide libraries to identify CRC binding peptides

Linear and cyclic OBOC libraries were synthesized using a split-mix synthesis approach and standard Fmoc chemistry as previously described [9]. These libraries contained thousands and millions of compound beads, each carrying a peptide with distinct natural and unnatural amino acid sequence.

Two CRC cell lines HT-29 (ATCC® HTB-38™) and DLD-1 (ATCC® CCL-221™; Figure 1A & B) were used for these studies (ATCC, VA, USA). Individual OBOC libraries were labeled with oil-based organic dyes that were used as the coding tags (Figure 2A) [22]. In order to find suitable libraries for the study, small samples of each color-coded OBOC library were combined and screened concurrently against the live CRC cells in order to identify OBOC library that contained CRC binding compound beads. The OBOC library with CRC binding beads were selected for further large scale screening (Figure 2B).

Five OBOC libraries with CRC binding beads were further screened in a larger scale (75,000 beads/each OBOC library) against the CRC cells. The positive cell-bound beads were isolated and picked up with handheld micropipette under the microscope (indicated by the arrow in Figure 3). The selected compound beads with a full coating of CRC HT-29 cells were treated with 8 M guanidine hydrochloride to remove the binding cells and adsorbed protein. These positive beads were then screened with CRC DLD-1 cells. Compound beads that bound to both colon cancer cell lines were isolated for further studies.

Normal human colonic epithelial cells (HCEC), NCM 460 (ATCC, MD, USA) were grown in expanded M3:10 media (INCELL, TX, USA) supplemented with growth factor and insulin (Figure 4A). After harvesting by trypsin, these normal colonic cells were screened with the CRC cell binding beads identified above, for 8 h at 5% CO_2 and 50 rpm. The CRC binding beads that showed no binding to the colonic epithelial cells were isolated (Figure 4B, arrow). The chemical structure and amino acid sequence of the isolated peptide beads were determined by Edman chemistry.

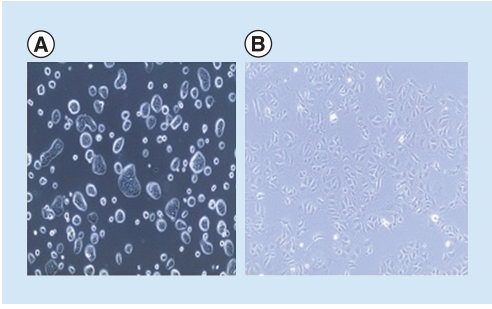

Figure 1. Cell lines used in this research. (A) Human colorectal cancer cell lines HT-29 and **(B)** DLD-1 were cultured in basic Dulbecco's modified Eagle media plus 10% fetal bovine serum and antibiotics.

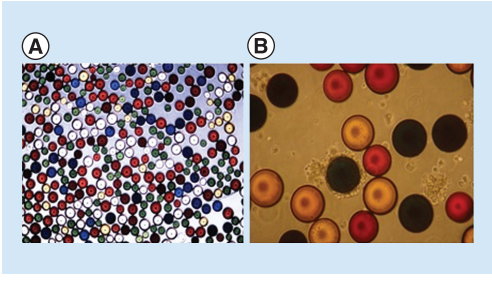

Figure 2. Image of rainbow beads library screening. (A) A mixture of rainbow-colored OBOC libraries, each color represented one OBOC library. **(B)** A CRC cell-binding bead was observed on a blue color bead from one of the OBOC library. CRC: Colorectal cancer; OBOC: One-bead one-compound.

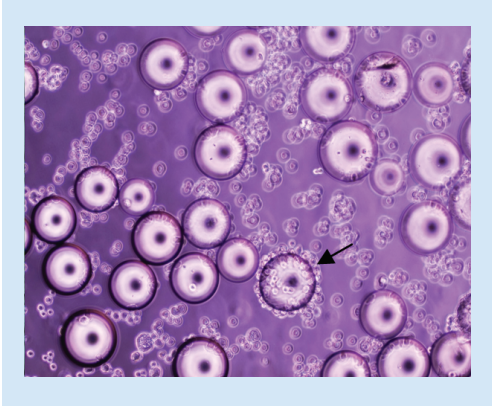

Figure 3. Beads screening. One-bead one-compound library in large scale screening with live colorectal cancer cell HT-29, arrow indicates positive colorectal cancer binding-bead.

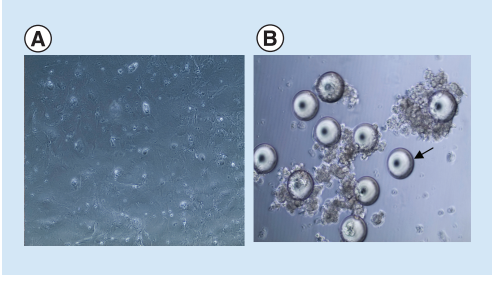

Figure 4. Negative screening against normal epithilial cells. (A) Micrograph of normal human colonic epithelial cells were cultured in media and **(B, arrow)** one colorectal cancer binding-bead showed no binding to normal colonic epithelial cell.

Evaluation of CRC binding affinity & specificity

The CRC binding peptides discovered from the above study were resynthesized in a large number of TentaGel beads and further evaluated with the two human CRC cell lines, HT-29 and DLD-1 in Dulbecco's modified Eagle medium with 10% FBS and normal HCEC in M3:10 media supplemented with growth factor and insulin for 1 h at 37°C and 50 r.p.m. After 1-h incubation, compound beads were inspected under the microscope.

Development & evaluation of CRC imaging agents using isolated CRC peptides

Of the 12 isolated specific CRC binding peptides, two high affinity peptides (CRC-6 and CRC-9, Supplementary Figure 1A & B) were selected for further studies. The CRC imaging agents were developed through conjugation of the peptide with biotin-hydrophilic linkers. Briefly, the peptides were synthesized on Rink amide resin (loading 0.59 mmol/g), using 1-hydroxybenzotriazole/1, 3-diisopropylcarbodiimide as coupling reagents. Threefold molar excess of Fmoc-protected amino acids to resin was used for coupling. Completion of coupling and Fmoc deprotection were monitored by the ninhydrin test. Cyclization was carried out using CLEAR-OX resin (Peptides Intl, KY, USA) and the crude cyclized peptide was purified using reverse phase HPLC. The purity and molecular weight of peptides were characterized using analytical HPLC and mass spectrometry, respectively [23–25].

Immunohistochemistry study was used to determine the binding affinity of the biotinylated CRC-6 and CRC-9 to CRC cells as well as normal HCEC. Cell suspensions of human CRC HT-29 (3.1×10^4), DLD-1 (4.5×10^4) and normal HCEC (2.1×10^4) were seeded separately on culture chamber slides (BD Falcon, CA, USA). At 70% fluency, cells were blocked with 5% bovine serum albumin. Biotinylated peptide CRC-9 at various concentrations were incubated with the CRC cells as well as normal HCEC cells for 30 min in Epilife media and then incubated with 1:400 dilution of Streptavidin-Alexa 488 (Invitrogen, NY, USA) in phosphate-buffered saline (PBS) for 30 min. One drop of Prolong Gold anti-fade reagent was added to each slide and the samples were examined under fluorescent microscopy.

In vivo testing using CRC tumor xenografts in mice

Animal studies were done according to an approved protocol by the Institutional Animal Care and Use Committee (IACUC) of the University of California, Davis. 12 athymic nude mice (*nu*/*nu*), obtained from Harlan (CA, USA) at 5–6 weeks of age, were injected with a suspension of 6×10^6 tumor cells in 200 µl PBS. CRC HT-29 was implanted into the left flank and DLD-1 was injected into the right flank. When the induced CRC tumors reached a size of 0.5–1.0 cm in diameter or after 21–28 days, the tumor-bearing mice were subjected to *in vivo* and *ex vivo* imaging studies [26,27].

In vivo & *ex vivo* mouse optical imaging

The mice were anesthetized by an injection of 30 µl Nembutal (50 mg/ml) prior to optical imaging. Tetravalent CRC-9–biotin–streptavidin complex (1.8 nmol), prepared by mixing 7.2 nmol of biotinylated peptide with streptavidin–Cy5.5 in PBS overnight at 4°C, was injected into the mice *via* the tail vein. 12 hours after injection, images were acquired with a Kodak IS2000MM Image Station with excitation filter 625/20 band pass, emission filter 700WA/35 band pass and 150 W quartz halogen lamp light source set at maximum. The mice were then sacrificed and the organs were excised for *ex vivo* imaging. Data were collected and analyzed using the Kodak ID 3.6 software by mapping the region of interest on the images.

Evaluation of cytotoxicity of CRC-6 & CRC-9

The standard hemolytic assay was used to evaluate the potential toxicity of the CRC-6 and CRC-9. Briefly, the peptides CRC-6 and CRC-9 were synthesized in solution form and purified on C18 reversed-phase HPLC. The peptide structures were confirmed by matrix-assisted laser desorption/ionization time of flight mass spectrometry. The peptides were serially diluted in sterile saline and incubated with healthy human red blood cells (RBCs; 4×10^6 RBCs/ml) for 6 h, and absorbance was read by micro plate reader at 550 nm. Triton X-100 1% was used as 100% hemolytic-positive control. Red blood cells without added peptides were used as negative control [28,29].

Investigation of corresponding receptor involved CRC ligands

Before conducting the molecular receptor interaction study between CRC-9 and HT-29, we used flow cytometry to obtain evidence that the HT-29 cancer cells do express α3-integrin (data not shown). On the *in vitro* bead binding-blocking assay, a panel of anti-integrin monoclonal antibodies (anti-α1, α2, α3, α4, α5, α6, αV and β1) were incubated with HT-29 cancer cells prior to addition of CRC-9 compound beads. Marked cell binding inhibition was observed only with anti-α3 integrin antibody blocked HT-29 cells (Supplementary Figure 3A). The α3-integrin binding specificity was further confirmed by incubation of α3-transfected K562 cells and parent K562 cells with CRC-9 compound beads (Supplementary Figure 3B).

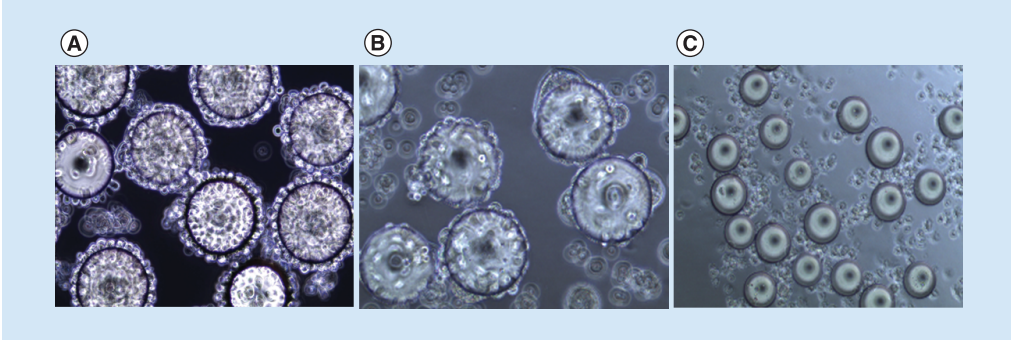

Figure 5. Different colorectal cancer cell lines bound to Tentagel beads with CRC-9 on the surface. (A) HT-29 and **(B)** DLD-1 bound to CRC-9 beads. **(C)** Normal human colonic epithelial cells did not bind to the CRC-9 compound beads.
CRC: Colorectal cancer.

Results

Of the 24 OBOC libraries screened, five libraries had fast and potent CRC cell binding beads and were selected for further large-scale screening. These included a focused X1 cyclic library, an RGD-based library and three linear and cyclic random libraries. As shown in Figure 2B, the blue color coded OBOC library has CRC binding beads. This OBOC library was selected for the large-scale screening.

Out of the five OBOC libraries studied, a total of 196 CRC cell binding beads were identified from approximately 750,000 beads belonging to the C-terminal X_1 cyclic focused library. The 196 CRC cell binding beads were then screened with normal HCEC. As shown in Figure 4B, the CRC binding beads have varying degree of binding to colonic epithelial cell and one nonbinding bead was identified (arrow). A total of 12 peptide beads were finally identified for their strong binding to CRC cells but no binding to normal colonic epithelial cells. The chemical structures and amino acid sequences were determined by Edman chemistry. Despite the small number of peptides, a motif was observed.

After resynthesis of the 12 peptides on a large number of beads, we re-evaluated the binding properties of these compounds using the two CRC cell lines as well as normal HCEC. Figure 5 showed that CRC-9 beads were completely coated with a monolayer of CRC cells after 30-min incubation in PBS with 1 mM MnCI2 (Figure 5A & B). These CRC cell binding beads do not bind to normal HCEC (Figure 5C). Six peptides were finally isolated for their strong binding to human CRC cells but no binding to normal HCEC. There was no cytotoxicity/hemolysis observed on human blood cells for any of the selected compounds tested up to a concentration of 500 μM (Supplementary Figure 2).

CRC imaging agents were developed by conjugating biotin at the carboxyl end via a hydrophilic linker to form a CRC-9–linker–lysine (biotin) complex. Initial immunohistochemistry studies revealed that biotinylated CRC-9 was able to detect CRC cells grown on the chamber slides at a concentration of 1 μM (Figure 6).

The *in vivo* targeting effect of CRC-9 was further shown in the nude mice implanted with subcutaneous CRC HT-29 and DLD-1 xenografts. The tetravalent imaging complex CRC-9 biotin–streptavidin–Cy5.5 was found to accumulate in the xenografts compared with the control groups injected with streptavidin–Cy5.5 only (Figure 7). Besides the high signal uptake seen in the xenografts, the liver and kidney were the organs showing noticeable accumulation of the imaging CRC-9 probe.

Discussion

As part of an NIH grant funded study, we used OBOC combinatory chemistry method to isolate peptide ligands that prevent bacterial adhesion as a means to coat biliary stents to prevent bacterial attachment and stent blockage [30–32]. However, the results were unsuccessful but our study led us to identify super-adhesive peptides which are ligands that bound strongly to bacteria. Based on this observation, we explored the possibility of using peptide ligands that bind specifically to special targets and in this case, colon cancer cells.

The OBOC combinatorial library method is useful for screening peptides from multiple libraries of peptide beads that possess ligands which bind to specific target cells. The special ligands can be used as biomarkers, and preliminary studies suggested that we have identified several biomarkers for colon cancer. The advantage of the

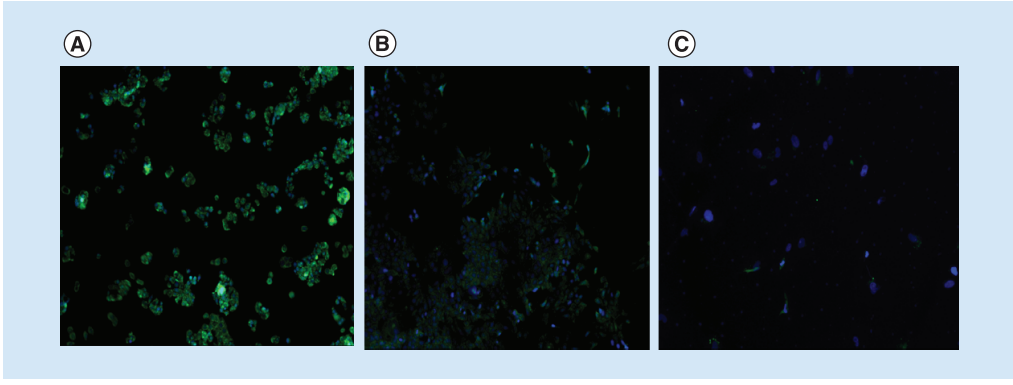

Figure 6. CRC-9 stained to different colorectal cancer cell lines. (A) Biotinylated CRC-9 stained to colon cancer cells HT-29 and **(B)** colon cancer cells DLD-1, **(C)** but not to normal human colonic epithelial cell at a concentration of 1 μM. CRC-9 peptide bound more strongly (green fluorescence) to HT-29 than DLD-1.
CRC: Colorectal cancer.

OBOC method is that a large number of random peptides with different amino acid sequences can be generated easily for testing. The actual chemical nature and configuration of these peptides may not be known prior to analysis. With specific attachment, index beads with positive ligands can be isolated and the peptide sequence analyzed using Edman chemistry to give the exact nature of the peptide(s). Once the amino acids sequence is known, such peptide(s) can easily be synthesized in a large quantity for further testing against a larger target cell population. The discovery of peptide such as CRC-9 in our current study has offered a potential avenue for specific diagnosis and target therapy for colon cancer.

In order to make this potential biomarker useful in the clinical setting, further testing on safety and application is necessary. Preliminary studies showed that the peptide(s) were not cytotoxic and did not cause hemolysis of RBCs. They also did not interact with colonic epithelial cells making them target-specific to colon cancer. Although specific, the affinity of the peptide to different colon cancer cell lines did vary as there was more binding to HCT-29 compared with the DLD-1 CRC cells.

Preliminary studies by creating xenografts of CRC tumor in an animal model offered further testing of the specificity of the CRC-9 peptide as a biomarker for CRC. The binding to the xenograft in the animal model suggested that this specific binding of CRC-9 can be used to explore the effects on targeted delivery of drugs. Binding these peptides to optical compounds can also improve the early diagnosis of CRC on imaging. Another potential advantage of these specific peptide ligands is that they can be conjugated to nanomicelles loaded with anticancer drugs for targeted therapy. The initial success in animal models suggested that this new technology has the potential to be translated into novel and effective therapeutic agents for treatment of human colon cancer.

Recently, Lam *et al.* developed several novel nanocarriers for the delivery of paclitaxel (PTX) and other hydrophobic anticancer drugs [33,34]. Using a reversible polyethylene glycol (PEG)-based disulfide cross-linked micelle derivatized with cholic acid (PEG^{5k}-Cys_4-CA_8 telodendrimers), hydrophobic drugs were encapsulated in the micelle core, and triggered for release at the tumor site and inside the cancer cells with high reductive potential [23]. Furthermore, such micelles, when conjugated to ovarian cancer binding ligands (discovered through OBOC library screening) and loaded with PTX, exhibit superior antitumor activity and lower systemic toxicity profile in nude mice bearing ovarian cancer tumor xenografts, when compared with equivalent doses of nontargeted PTX nanoparticles, as well as clinical PTX formulation (Taxol) [35].

At this stage of exploratory study, we only tested the peptide ligand against colon cancer cell lines. If CRC-9 is proved specific to human CRC, It is possible to tag these peptides with fluorescein dye which if subsequently bound to colon cancer cells would highlight such changes in the target tumors on fluorescent microscopy or with special epifluorescent endoscopy [36]. Another potential application of this biomarker could be in the early diagnosis of dysplastic or premalignant changes in patients with chronic inflammatory bowel disease. This may help in the detection of early cancer changes in potential dysplastic conditions.

The discovery of a potential biomarker needs more work to identify the binding mechanism. Isolating the particular peptide that binds to CRC cells is only the first step. Further studies will be conducted to determine the nature or configuration of the surface receptor that binds the peptide ligand. Current reported studies have focused

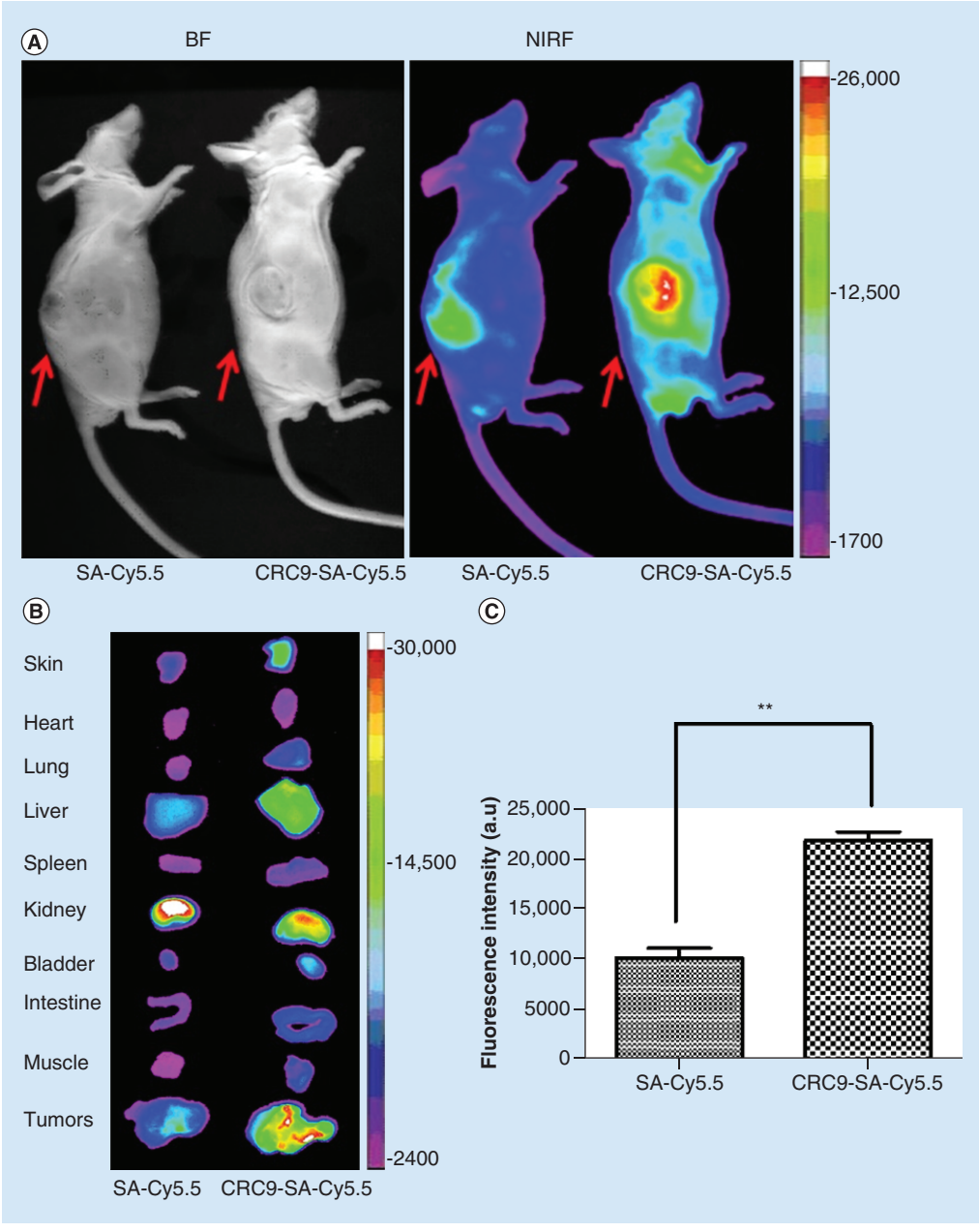

Figure 7. ***In vivo*** **and** ***ex vivo*** **near-IR fluorescent imaging of nude mice bearing HT-29 xenografts after injection of streptavidin–Cy5.5-biotinylated CRC-9. (A)** Bright-field image of nude mice implanted with HT-29 xenografts (left panel). 24 h after injection with streptavidin–Cy5.5-biotinylated CRC-9 complex or streptavidin–Cy5.5 alone (control) *via* the tail vein, preferential uptake of streptavidin–Cy5.5 in the HT-29 tumors was noted in mice given the streptavidin–Cy5.5-biotinylated CRC-9 complex (right panel). **(B)** *Ex vivo* images were conducted. Compared with the control with the injection of streptavidin–Cy5.5 only, CRC-9 showed 2.17-fold higher uptake, kidneys' uptake was high in both the mice. **(C)** The quantification of NIRF images indicated that subcutaneous xenografts had higher uptakes of CRC-9–biotin/SA–Cy5.5 compared with that of the biotin/SA–Cy5.5 ($p < 0.05$). Relative fluorescence uptake by tumor was quantified in arbitary units.
BF: Bright field; NIRF: Near infra-red fluorescence.

primarily on RGD-based peptides that target the α5β1 integrin [3–5], but the binding is not specific. Available test kits will be used to help identify the nature of the receptor and to determine the specificity of this binding [23].

Work is currently underway in our laboratory to evaluate the binding profile of CRC-9 and CRC-6 to cancer tissues obtained directly from primary human CRC tumors as well as a series of benign and precancerous lesions

(such as ulcer, adenoma, polyp and etc.). If it is proved to bind strongly to a significant portion of clinical CRC cancer specimens, it will have great translational potential. First, CRC-9 based optical probe can be developed as a simple noninvasive method for early detection of CRC. This test can potentially be performed by primary care providers. Second, CRC-9 can be developed into a PET imaging probe for detection of both local and metastatic diseases, making it a valuable tool for staging workup. Third, it can also be used as a vehicle for delivery of nanocarriers with encapsulated anticancer drugs. Fourth, CRC-9 can be used to decorate nanoporphyrin and used as a highly effective photosensitizer for phototherapy of CRC cancers since most of CRC cancers are endoscopically accessible. Finally, CRC-9 is composed of natural, unnatural amino acids and small molecules. It is expected to resist proteolytic degradation and be stable for *in vivo* application. Therefore, CRC-9 is a peptide ligand that has great translational potential in tumor-specific imaging and chemotherapy drug delivery to the tumor sites while sparing the normal tissues. If CRC-6 and CRC-9 are not specific enough to target human CRC, optimization of identified ligands will be performed after the structure–activity relationship study. The highly focused OBOC combinatorial libraries will be designed with motif reserved or biased and then screened under higher stringency conditions by lowering the bead surface substitution, shorter incubation time or by adding soluble competing receptor specific antagonist against CRC, so that ligands with higher affinity and higher specificity against CRC can be identified.

Conclusion & future perspective

Using combinatorial strategy, we identified specific targets against CRC through screening OBOC libraries. *In vitro* studies show that CRC-9 is able to detect different CRC cell lines grown on the chamber slides at the concentration of 1 μm. *In vivo* studies demonstrated that CRC-9 is able to specifically accumulate in the CRC subcutaneous tumors. Therefore, CRC-9 has a potential to be developed as an effective biomarker for detecting the CRC.

At this stage of exploratory study, we only tested the peptide ligand against colon cancer cell lines and nude mice bearing CRC xenografts. If CRC-9 is proved specific to human CRC tissues, it is possible to tag these peptides with fluorescein dye to visualize tumors on fluorescent microscope or with special epifluorescent endoscopy [36].

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/suppl/10.2217/crc-2017-0017

Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations.

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•• **Introduced the development of rainbow beads assay to select suitable OBOC library for further studies.**

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●● **Described fluorescence endoscope could be applied for clinical setting.**