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Identification of Peptides Binding Specifically to Colon Cancer Cells through Phage Display Peptide Library Screening

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ABSTRACT

Objective: Colorectal cancer ranks among the most frequently occurring cancers. Addressing the diagnosis and treatment challenges requires the advancement of alternative approaches that can more precisely differentiate between cancerous and healthy cells.

Materials and Methods: We identified colon cancer cell surface specific 12-mer peptides using subtractive phage display technique. Four rounds of biopanning were conducted using the M13 bacteriophage PhD-12 peptide library with the human colon cancer cell line HCT-116. Subtractive selection involved the use of the human rectal cancer cell line SW837 and the healthy CCD112-CoN cell line. Binding levels of selected phage clones were tested by ELISA-HRP binding assays. Experimentally identified peptide sequences were synthesized by solid-phase chemistry. Peptides were labelled at the N-terminus with a fluorescent dye for visualization of cells by confocal microscopy.

Results: Peptide COL419 (Serine-Valine-Alanine-Histidine-Leucine-Serine-Proline-Histidine-Serine-Threonine-Threonine-Alanine; SVAHLSPHSTTA) can differentiate colon and rectal cancers from healthy cells. In addition, the COL-AA peptide (Histidine-Tyrosine-Proline-Threonine-Asparagine-Leucine-Histidine-Tyrosine-Proline-Threonine-Asparagine-Leucine; HYPTNLHYPTNL), which was designed from the amino acid composition analysis of the experimentally selected peptide sequences bound to colon cancer cells and neither rectal cancer cells nor healthy colorectal cells.

Conclusion: The peptides determined specifically target colon cancer cells. These findings enhance our understanding of the differences between these cancer types. The selected peptides have the potential for clinical application in targeted diagnosis and treatment of colon cancer.

Keywords: Colon cancer, peptides, phage display, rectal cancer, targeted approaches

INTRODUCTION

Globally, colorectal cancer (CRC) ranks as the third most prevalent type of cancer in terms of diagnosis and is the second major contributor to cancer-related mortality (1). CRC can have its beginnings in either the colon or rectum, and the terminology used to describe it varies depending on its point of origin, either as colon cancer or rectal cancer. Although colon and rectal cancers arise at similar anatomical sites, they are two different types of cancer with significant differences in pathogenesis. Rectal cancer has characteristics that should be treated separately because of its anatomical and biological differences. Therefore, in our study, rectal cancer cells as well as normal colon cells were used to identify colon cancer-specific peptides. This strategy provided a critical step in distinguishing results

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specific to colon cancer cells. Each cancer type has different risk factors, symptoms, and treatment options and needs to be treated as a separate type of cancer (2).

Conventional methods for diagnosing and treating CRC have proven inefficient, necessitating the development of novel techniques. Small peptides targeting tumors can improve treatment efficacy and reduce the side effects of traditional approaches. Compared to monoclonal antibodies and large protein ligands, peptides have several advantages, including smaller size, easier synthesis, and superior biocompatibility (3-5). Additionally, their affinity, charge, hydrophobicity, and stability can be chemically modified for optimal *in vivo* applications.

Phage display can be used for various targets, including cancer cells (6, 7). It has been used in numerous studies to select peptides/antibody fragments for various targets, thereby aiding in the assessment of molecular interactions. These include identifying cellular receptors (8), screening epitopes or mimotopes (9), and confirming peptides that target specific cells or tissues (10, 11). During selection, an array of peptide sequences is displayed on the tips of M13 phages, allowing attachment to the target. Non-specific peptides are eliminated, leaving M13 phages with high-affinity peptides that can be amplified. This method offers an alternative to conventional approaches, enabling the discovery of targeting molecules for diagnostic and therapeutic purposes (12, 13).

This study aimed to identify small peptides with the ability to effectively discriminate between colorectal cancer cells and healthy cells, as well as differentiate between colon and rectal cancer cells. To achieve this, HCT-116 (ATCC® CCL-247™) colon cancer cells, SW837 (ATCC® CCL-235™) rectal cancer cells, and CCD-112CoN (ATCC® CRL-1541™) normal human colon cells were employed. These cells were used in the subtraction biopanning process alongside a phage display peptide library to identify peptides that specifically bind to colon cancer cells. Although this study aimed to target colon and rectum cancers, the results obtained will also contribute to understanding the reasons for the difference between the two types of cancer. The outcomes have the potential to facilitate applications in the fields of diagnosis and treatment.

MATERIALS AND METHODS

Cell Lines and Cell Culture

Human colon cancer cells HCT-116 (ATCC® CCL-247™), human rectal cancer cells SW837 (ATCC® CCL-235™) and healthy colon cells CCD-112CoN (ATCC® CRL-1541™) were purchased from the American Type Culture Collection (ATCC). HCT-116 and CCD-112CoN cells were cultivated in Dulbecco's Modified Eagle Medium (DMEM; 10% fetal bovine serum, 2mM L-glutamine, 100 µg/mL penicillin and 100 µg/mL streptomycin). SW837 cells were initially cultured in Leibovitz's L-15 (ATCC® 30-2008™) medium before being transferred to DMEM medium. The cells were subcultured in plastic culture flasks (25 cm^2) and placed in an incubator (37°C and 5% CO_2 atmosphere). When the cells reached a subconfluent state, they were collected, and their total number was determined using a hemocytometer.

In vitro Panning

Subtractive biopanning was performed using HCT-116 colon cancer cells as targets, with SW837 rectum cancer cells and CCD-112CoN colon cells as absorbers. Cell cultures were maintained in DMEM containing 10% fetal bovine serum (FBS) at 37°C and 5% CO_2. *In vitro* screening followed kit manual guidelines with minor adaptations. At 70% confluence, cells were washed with phosphate-buffered saline (PBS), blocked with 2% bovine serum albumin (BSA) at 37°C for 1 h, and incubated with 1 x 10^{11} pfu phages. Unbound phages from CCD-112CoN cells were transferred to SW837 cells for a second subtraction step. The supernatant was then transferred to HCT-116 cells and incubated for 2 h. After washing with PBS with Tween 20, bound phages were eluted with glycine-HCl and neutralized with Tris-HCl. Some samples were used for microtitration, while the rest were amplified in *Escherichia coli* (*E. coli*) strain ER2738. Subsequent rounds increased the detergent and salt concentrations to 0.5%.

Phage Tittering

The selective enrichment of phages bound to HCT-116 cells was achieved after four biopanning rounds. Phage titers from each round were assessed using a blue-white plaque assay on tetracycline-enriched agar plates. The number of phages entering and obtained from each biopanning cycle was determined by blue-white colony screening. The initial biopanning cycle began with 1 x 10^{11} pfu/mL, and the same pfu count was obtained by amplifying the elution sample. This ensured a consistent number of phage clones in each cycle. Subsequent cycles started with 5 x 10^{10} pfu/mL for titer calculations. At the end of each cycle, the entering and recovered phages were counted, demonstrating decreasing phage diversity.

Cell-based Enzyme-Linked Immunosorbent Assay (ELISA)

For the cell-based ELISA, cells were cultured in 96-well plates until reaching 80-90% confluence and then blocked for 1 h with 2% BSA in PBS. Following three PBS washes, cells were fixed with 4% paraformaldehyde at room temperature for 15 min. After another three PBS washes, cells were blocked again with 2% BSA in PBS for 1 h at 37°C. Next, 1 × 10^{11} pfu of phages were added to each cell type and incubated at room temperature for 1 h. Thirty selected phage clones were assessed. Wells were washed four times with 0.2% Tween 20 in PBS. HRP-conjugated anti-M13 monoclonal antibody (1:3000 dilution) was added to each well, and plates were incubated at 37°C for 2 h. After three washes with PBS, tetramethylbenzidine (TMB) solution was added and incubated at room temperature for 30 min. The reaction was stopped by adding 2M H_2SO_4. Absorbance at 450 nm was measured using a microplate reader. Negative controls

included PBS and cells without phage clones. All experiments were conducted in triplicate.

Amplification and Sequencing of Selected Phages

5 phage clones selected in cell-based ELISA analysis were amplified and purified for DNA sequencing. ssDNA of the selected clones was isolated and used for sequencing (Medsantek, Istanbul, Turkiye) using the -96gIII primer (5'-CCCTCATAGTTAGCGTAACG-3').

Synthesis and Characterization of Peptides

Experimentally selected and theoretically designed peptides were synthesized by solid-phase peptide synthesis using the standard Fmoc/tBu strategy. The synthesis were carried out in a microwave-assisted fully automated peptide synthesizer at 0.1 mmol synthesis scale using Rink Amide resin (0.70 mmol/g). For the labeled peptides, the N-terminal amino group was linked with 5(6)-carboxyfluorescein in the presence of DIC/HOBt reagents. Subsequently, a mixture of trifluoroacetic acid (TFA)/H_2O/triisopropylsilane (TIS) (95:2.5:2.5 v/v) was used to detach the peptides from the resin for 30 min at 37°C. The peptides were then precipitated in cold diethyl ether, centrifuged at 4000 rpm for 5 min, and freeze-dried from water. The crude peptides were purified by reversed-phase high performance liquid chromatography (RP-HPLC, Agilent Technologies, 1260 Infinity) on a semipreparative Agilent VariTide RPC C18 column using gradient elution (A: 0.1% TFA in water; B: 0.1% TFA in acetonitrile/water, 80:20, v/v). Peptides were analyzed by liquid chromatography-mass spectrometry (LC-MS)/MS system (Agilent Technologies, 6420 Triple Quad). The mass spectra were recorded in positive ion mode in the 200–2000 m/z range.

Peptide-based Immunofluorescence Assay

Candidate peptides labeled with 5-(6)-carboxyfluorescein were used in a peptide-based immunofluorescence assay to validate their specific binding to HCT-116 colon cancer cells. HCT-116, SW837, and CCD-112CoN cells were cultured in DMEM at 37°C and seeded onto 4-well chamber slides at 1 x 10^4 cells per well until 70% confluence. The cells were washed with PBS, fixed with 4% paraformaldehyde for 15 min at room temperature, washed again, and blocked with 2% BSA in PBS for 30 min at 37°C. Labeled peptides (1 µM) were incubated with each cell type on a rocker platform for 90 min at room temperature. Samples were washed with PBS (0.1% Tween 20) and incubated with 4 ', 6-diamidino-2-phenylindole (DAPI) staining solution for 5 min. After two PBS rinses, confocal fluorescence microscopy was used for examination. The experiments were performed simultaneously with uniform microscope settings for all samples.

RESULTS

Subtraction Biopanning and Subsequent Analysis of Peptide Enrichment From a 12-peptide Phage Display Library

In this study, a subtraction biopanning strategy was used to enrich positive phage clones from a 12-peptide phage display library. HCT-116 human colon cancer cells served as target cells, whereas SW837 human rectum cancer cells and CCD-112CoN human healthy colon cells served as absorber cells. The aim was to eliminate phages that bound non-specifically to absorber cells and enhance the selectivity of target cells (14).

The peptide library was initially applied to CCD-112CoN cells, and unbound phages were then incubated with SW837 cells. Unbound phage clones were subjected to conventional biopanning with HCT-116 cells. Successive rounds intensified washing conditions. Bound phages were recovered and amplified in *E. coli* ER2738 for subsequent rounds. In the fourth round, an additional subtraction phase with BSA was conducted to further reduce non-specific binding.

Enrichment analysis after four biopanning rounds showed a 140-fold increase in the number of phages recovered from HCT-116 cells (Table 1), indicating successful selective amplification of target-binding phages (15).

Table 1. Enrichment analysis of positive phage clones from 12-peptide phage library

Round of Screening	Input Phage (pfu/mL)	Output Phage (pfu/mL)	Recovery (%)
1	$1x10^{11}$	$1x10^{5}$	$1x10^{-6}$
2	$5x10^{10}$	$1.8x10^{5}$	$3.6x10^{-6}$
3	$5x10^{10}$	$4.5x10^{5}$	$9x10^{-6}$
4	$5x10^{10}$	$7x10^{6}$	$14x10^{-5}$

Table 2. Amino acid sequences of the 5 chosen phage clones for subsequent examination

Phage Clones	Peptide Sequence	MW	pI
COL401	VHMQRLIPDNVR	1477.75	9.58
COL409	TYPNTQTHLLNA	1372.5	6.4
COL411	YELPPYPPTKAH	1412.61	6.75
COL414	WPLRGHSNPTLY	1440.62	8.75
COL419	SVAHLSPHSTTA	1207.31	6.66

MW: Molecular wight ; pI: Isoelectric point

Primary Identification of Positive Phage Clones with Cell-based ELISA

After the fourth round of biopanning, 30 phage clones were randomly selected and assessed for binding selectivity using cell-based ELISA. Horseradish peroxidase (HRP)-conjugated anti-M13 phage antibody identified phage clones with affinity for HCT-116 colon cancer cells. Five clones (COL401, COL409, COL411, COL414, and COL419) were chosen for further analysis due to their high binding affinity (Table 2). Clones that exhibited at least 1.25-fold higher binding to colon cancer cells compared to healthy colon cells were selected as the threshold. The specificity of these clones was tested on CCD-112CoN normal colon and SW837 rectum cancer cells. Figure 1 shows the binding levels of each clone to both target and control cells. DNA sequencing identified the 12-mer peptide sequences responsible for binding. Among the five clones, those with minimal binding to CCD-112CoN cells were prioritized for further experiments. The M13KE phage control indicated non-specific binding to cancer and healthy cells. Notably, the COL419 clone exhibited higher binding levels on HCT-116 cells and lower binding on SW837 cells.

Table 3: Amino acid composition analysis of experimentally selected 5 best binders on the HCT-116 cell surface

Amino acid	PhD-12%	EO#	EO%	D%
Histidine, H	4.6	6	10	**117.4**
Tyrosine, Y	3.6	4	6.7	**85.2**
Proline, P	8.1	9	15	**85.2**
Threonine, T	7.8	7	11.7	**49.6**
Asparagine, N	4.5	4	6.7	**48.1**
Leucine, L	8.9	7	11.7	**31.1**
Alanine, A	7.4	4	6.7	-9.9
Arginine, R	5.7	3	5	-12.3
Glutamine, Q	3.9	2	3.3	-14.5
Valine, V	6.1	3	5	-18
Tryptophan, W	2.3	1	1.7	-27.5
Lysine, K	2.3	1	1.7	-27.5
Serine, S	11.2	4	6.7	-40.5
Methionine, M	3.1	1	1.7	-46.2
Glutamic acid, E	3.1	1	1.7	-46.2
Aspartic acid, D	4.6	1	1.7	-63.8
Glycine, G	5.8	1	1.7	-71.3
Phenylalanine, F	2.7	0	0	-100
Cysteine, C	1.5	0	0	-100

PhD-12%: PhD-12 peptide library amino acid composition percentages; EO#: experimentally observed/selected amino acid number in 5 selected binders; EO%; percentage of experimentally observed/selected amino acids; D%: percentage difference of experimentally expressed amino acid to library expression ratio.

Amino Acid Composition Analysis of Selected Peptide Sequences

To identify favored and disfavored amino acids in the selection process, we compared the frequency of each amino acid in the top 5 binding peptides from the cell-based ELISA experiments with the initial frequencies in the PhD-12 peptide library (Table 3). Amino acids that were more prevalent in the top binding peptides exhibited an affinity for target cell-specific binding (16). Table 3 shows that the positively expressed amino acids in the selected peptides for the HCT116 surface were histidine (H), tyrosine (Y), proline (P), threonine (T), asparagine (N), and leucine (L). Most of these amino acids have polar side groups; histidine is weakly basic at neutral pH, whereas Y, P, and L are hydrophobic, and N and L are hydrophilic.

Based on the amino acid composition analysis of the 5 selected peptides for colon cancer cells, the peptide sequences to be synthesized were determined. The COL419 peptide sequence exhibited the highest binding level among the sequences selected after the fourth biopanning cycle. The COL-AA peptide was designed to contain positively selected amino acids compared to the initial expression rate. The COL-AA sequence included two repeats of H, Y, P, T, N, and L. Both the COL419 (SVAHLSPHSTTA) and COL-AA (HYPTNLHYPTNL) peptides were synthesized, purified, and conjugated with 5(6)-carboxyfluorescein.

Peptide Synthesis

5-(6)-carboxyfluorescein labelled peptides were synthesized and then purified by HPLC. Peptide bond can be detected at 214 nm and 5-(6)-carboxyfluorescein can be detected at 517 nm. Thus, we monitored HPLC with these two wavelengths. Chromatographic peaks that gave a signal at both wavelengths simultaneously were collected for further verification by mass spectrometry. The HPLC profiles of peptides COL-AA and COL419 are given in Figure 2 a, b, c and Figure 3 a, b, respectively.

Assessing the Targeting Affinity by Peptide-Based Immunofluorescence Assay

The peptide-based immunofluorescence assays provided valuable insights into the binding specificities of the synthesized COL419 and COL-AA peptides. The COL419 peptide exhibited strong binding to both HCT-116 colon cancer and SW837 rectal cancer cell lines (Figure 4). However, COL419 exhibited negligible binding to healthy CCD112-CoN colorectal cells. This suggests that COL419 can differentiate between cancerous (HCT-116 and SW837) and non-cancerous colorectal cells

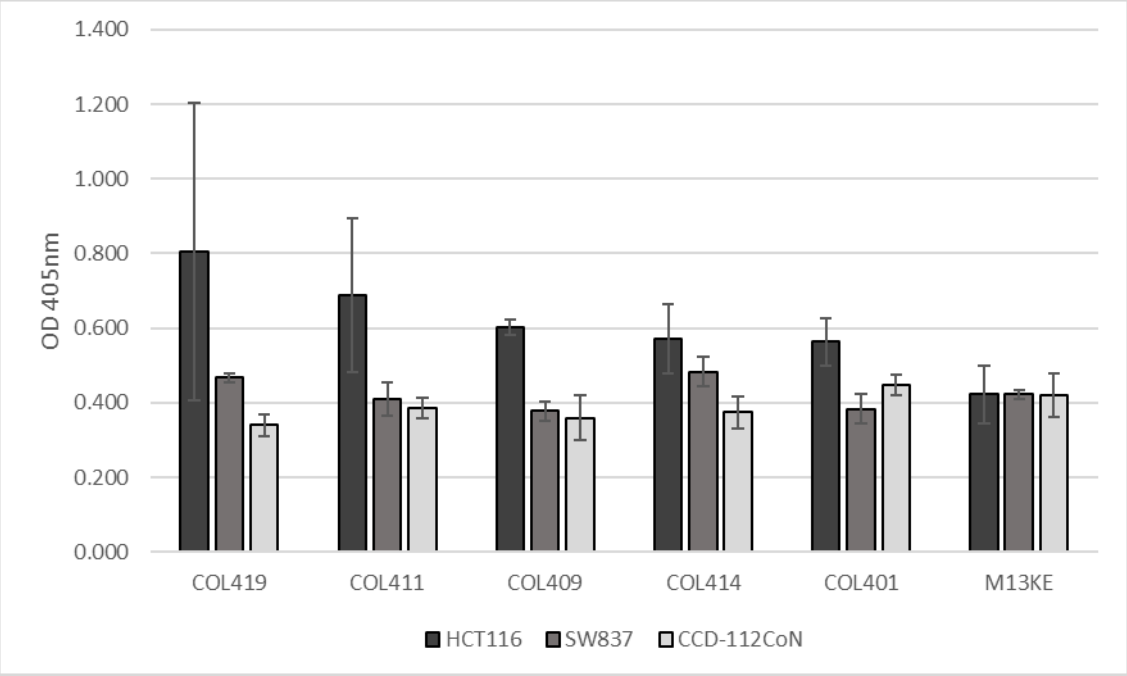

Figure 1. Cell-based ELISA results for 5 best phage clones on HCT-116, SW837 and CCD-112CoN cells. Binding levels of selected phage clones were tested on both target and absorber cells; COL419 had the best binding level among the clones. The M13KE phage was used as the control phage clone.

(CCD112-CoN), but does not distinguish between colon and rectal cancers. In contrast, the designed COL-AA peptide, comprised of two repeating units of histidine, tyrosine, proline, threonine, asparagine, and leucine amino acids (HYPTNL), bound specifically to colon cancer cells. Conversely, no binding was observed on the rectal cancer or healthy colorectal cells.

DISCUSSION

The subtraction biopanning strategy applied in this study enables the isolation of peptide ligands that interact exclusively with a given target cell by eliminating ligands that bind to non-target cells present in a sample (14). The progressive increase in the number of recovered phages over four rounds of biopanning demonstrated the successful enrichment of target-binding phages. This outcome underscores the utility of subtraction biopanning in enhancing the selectivity of phage clones, which is crucial for their application in targeted therapies and diagnostics.

The selection of 5 phage clones (COL401, COL409, COL411, COL414, and COL419) based on their strong binding affinity to HCT-116 colon cancer cells is a significant step in this research. These clones can serve as promising candidates for further development and applications in cancer diagnosis and treatment. Their ability to distinguish between cancer and normal cells highlights their potential for targeted therapies.

In a phage display biopanning experiment, the end-enriched pool contains clones with peptides that bind strongly to the target. Comparing the amino acid frequencies in this enriched pool with those in the original naive library can reveal key residues that are overrepresented and thus important for binding. Specifically, if certain amino acids are found with much higher frequency in the enriched pool, it indicates that these residues likely make critical contacts and contribute significantly to the interaction with the target (16).

The analysis of amino acid composition within the selected clones provided further insight into the binding specificity of the peptides. The abundance of serine and threonine residues may enhance binding through potential hydrogen bonding and hydrophilic interactions.

Aromatic sequences like histidine and tyrosine and repeating sequences may contribute to the specificity of the peptide. The COL-AA peptide is a sequence designed using experimentally selected peptide amino acid composition information, and the differential binding shown in the peptide-based immunofluorescence assay indicates that the amino acid composition information of experimentally selected peptides can be followed as a promising approach for the determination of target-specific peptides.

In the literature, several peptides have been reported as colorectal cancer-specific peptides identified by phage display, such as the CBP-DWS peptide (Aspartic acid-Tryptophan-Serine-Serine-Tryptophan-Valine-Tyrosine-Arginine-Aspartic-acid-Proline-Glutamine-Threonine; DWSSWVYRDPQT) (17), L20 peptide (Alanine-Asparagine-Leucine-Asparagine-Leucine-Tryptophan-

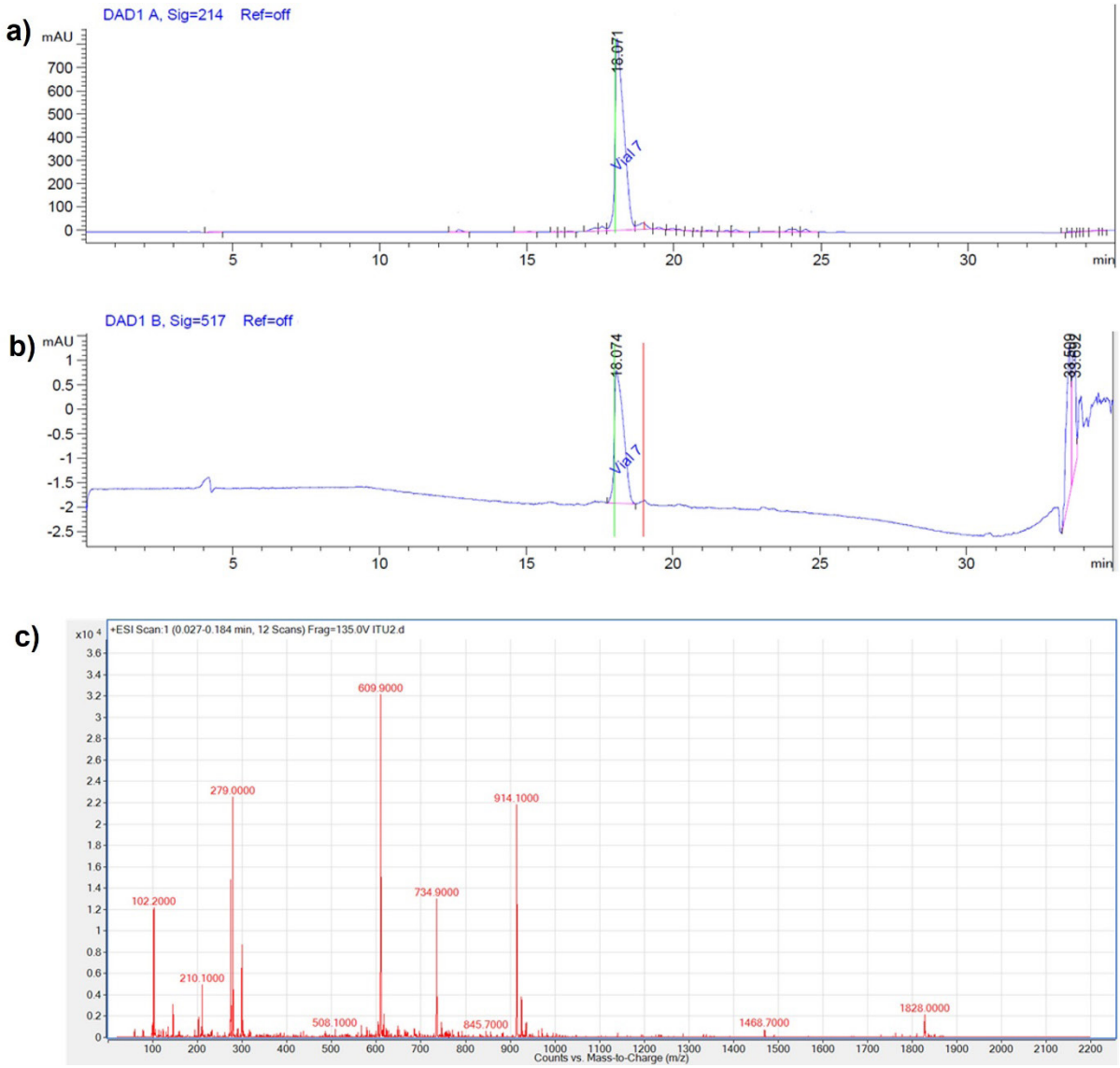

Figure 2. Reverse phase-HPLC profiles of 5-(6)-carboxyfluorescein labelled COL-AA at wavelengths of 214 and 517 nm. Solvent system; a) 0.1% TFA in water; b) 0.1% TFA in acetonitrile/water, 80:20, v/v; c) gradient 5-80%. LC-MS/MS analysis of 5-(6)-carboxyfluorescein labelled COL-AA peptide with molecular formula of ($C_{89}H_{107}N_{19}O_{24}$); m/z calculated for (M+H)+: 1826.777, found: 1828.0000; calculated for (M+2H)+: 913.892, found: 914.1000; calculated for (M+3H)+: 609.597, found: 609.9000.

Threonine-Asparticacid-Tyrosine-Isoleucine-Arginine-Tryptophan; ANLNLWTDYIRW) (18), disulfide-constrained RPMrel peptide (Cysteine-Proline-Isoleucine-Glutamicacid-Aspartic acid-Arginine-Proline-Methionine-Cysteine;CPIEDRPMC) (19), cyclic peptide TCP-1 (Cysteine-Threonine-Proline-Serine-Proline-Phenylalanine-Serine-Histidine-Cysteine; CTPSPFSHC) (5), RKOpep peptide (Cysteine-Proline-Lysine-Serine-Asparagine-Asparagine-Glycine-Valine-Cysteine; CPKSNNGVC) (20), CCBP1 peptide (Histidine-Alanine-Methionine-Arginine-Alaine-Glutamine-Proline; HAMRAQP) (21) and CP15 peptide (Valine-Histidine-Leucine-Glycine-Tyrosine-Alanine-Threonine; VHLGYAT) (22). Although the specificity of the peptides identified in these studies has been demonstrated for different colorectal cancer cell lines and tissue samples, the specificity of the peptides to differentiate colon and rectal cancer cells has not been studied. Among these peptides, the CBP-DWS, L20, CCBP1, and CP15 peptides are enriched in aromatic residues like tryptophan and tyrosine, which likely facilitate binding through pi-pi stacking and hydrophobic interactions RPMrel and TCP-1 peptides contain high proportions of charged residues glutamate, aspartate and histidine that promote electrostatic interactions with cancer cell receptors. The asparagine content of the RKOpep peptide may confer affinity through its hydrophilic and hydrogen bonding nature.

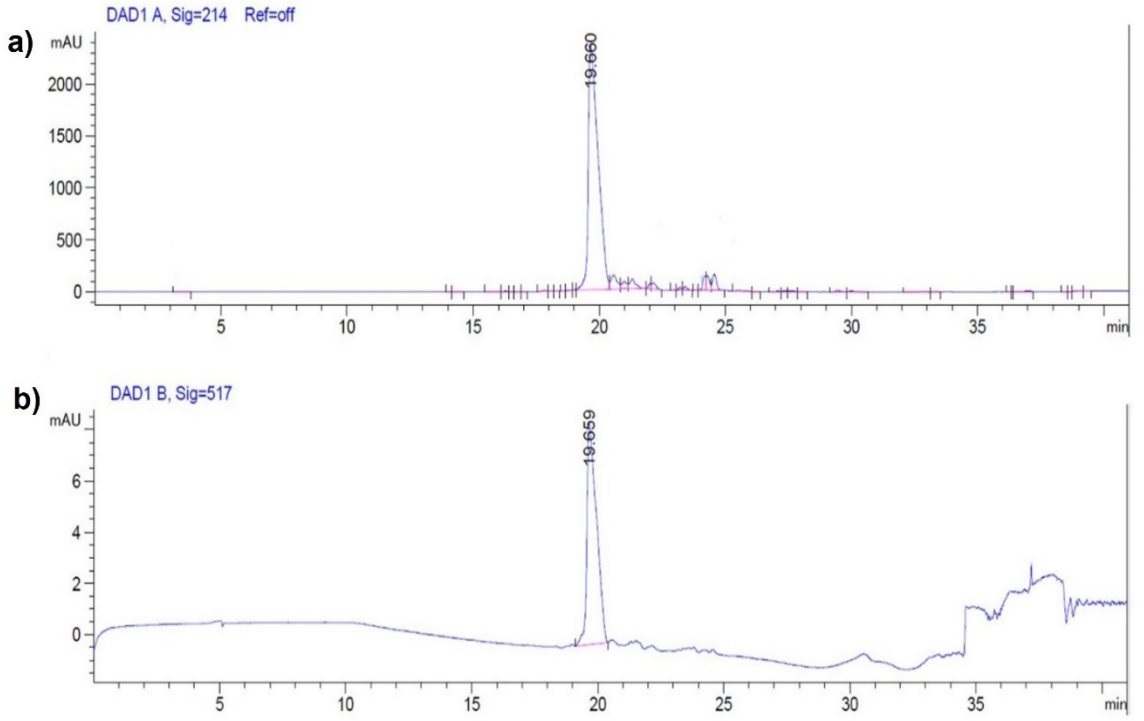

Figure 3. Reverse phase HPLC profiles of 5-(6)-carboxyfluorescein labelled COL419.
Solvent system; a) 0.1% TFA in water; b) 0.1% TFA in acetonitrile/water (80:20, v/v; gradient 5-80%).

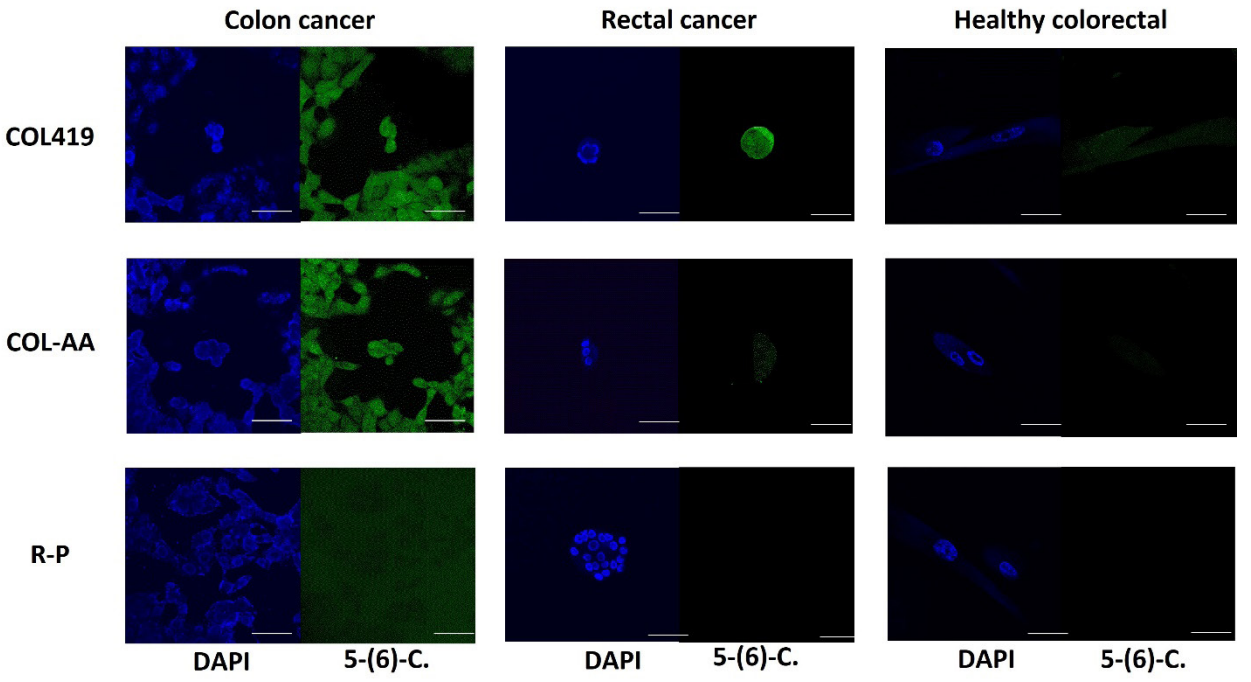

Figure 4. Confocal fluorescence binding assays for COL419 and COL-AA peptides. The degree of binding of colon-specific COL419 and COL-AA peptides to HCT-116 colon cancer, SW837 rectal cancer, and CCD-112 healthy colorectal cells was demonstrated using DAPI and 5-(6)-carboxyfluorescein stain. (R-P: Random peptide selected from phage library.) Scale bars: 50 µm.

On the other hand, COL419 peptide is enriched in serine, threonine, and alanine residues. Their hydroxyl groups can form hydrogen bonds, whereas alanine provides hydrophobic contacts. COL-AA possesses a repetitive sequence with proline, asparagine, leucine, and tyrosine. Proline induces rigidity, whereas the others enable hydrophobic and hydrogen bonding. The COL419 and COL-AA peptides identified in this study exhibited significant binding specificity toward colon cancer cells. This further corroborates the efficacy of phage display technology as a means of isolating tumor-specific peptides. Related studies have demonstrated the isolation of peptides directed against different cancer antigens through phage display libraries (23, 24). These findings underscore the potential of small peptides in cancer targeting and drug delivery applications.

Overall, both experimentally selected and theoretically designed peptides successfully differentiated colorectal cancer cells from healthy cells. Further optimization of the peptide sequences could potentially achieve subtype-specific recognition between colon and rectal cancers.

CONCLUSION

The subtraction biopanning strategy employed in this study successfully enriched positive phage clones that specifically bind to HCT-116 cells. Through a series of rounds of biopanning and selective amplification, five phage clones were identified as best binders. Further analysis of their amino acid composition and binding specificity revealed the presence of specific amino acids and their selective expression in binding peptides called experimentally selected COL419 and theoretically designed COL-AA peptides. The synthesized peptides exhibited targeted binding to HCT-116 cells in immunofluorescence assays. Both the COL419 and COL-AA peptides can differentiate colon and rectal cancer cells from healthy cells. The COL-AA peptide can differentiate colon cancer cells from both rectal cancer cells and normal healthy colorectal cells. These findings contribute to our understanding of peptide-target interactions and provide potential avenues for developing targeted approaches for colon cancer research and therapy. The COL419 and COL-AA peptides identified in this study have considerable potential for future applications in cancer targeting and drug delivery systems. These peptides can be employed to facilitate targeted delivery of therapeutic agents to tumor sites owing to their cancer cell-specific binding ability. The identified peptide sequences can be further optimized and used as targeting ligands in drug delivery systems or diagnostic agents specifically designed for HCT-116 colon cancer cells. By targeting these specific cells, it may be possible to enhance the efficacy and reduce the off-target effects of therapeutic interventions.

Ethics Committee Approval: Ethics committee approval is not required due to the use of commercial cell lines.

Peer-review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study – D.S., Z.K., B.E., B.A., Y.H.; Data Acquisition – D.S., Z.K., B.E., B.A., Y.H., E.S.; Data Analysis/Interpretation - D.S., Z.K., B.E., B.A., Y.H.; Drafting Manuscript – D.S., Z.K., B.E., B.A., Y.H., E.S.; Critical Revision of Manuscript – D.Ş., Z.K.; Final Approval and Accountability – D.S., Z.K., B.E., B.A., Y.H., E.S.

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REFERENCES

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 2021; 71: 209-49.
2. Paschke S, Jafarov S, Staib L, Kreuser ED, Maulbecker-Armstrong C, Roitman M, et al. Are colon and rectal cancer two different tumor entities? A proposal to abandon the term colorectal cancer. Int J Mol Sci 2018; 19(9): 2577.
3. Dmitrieva MD, Voitova AA, Dymova MA, Richter VA, Kuligina EV. Tumor-targeting peptides search strategy for the delivery of therapeutic and diagnostic molecules to tumor cells. Int J Mol Sci 2020; 22(1): 314.
4. Raucher D. Tumor targeting peptides: novel therapeutic strategies in glioblastoma. Curr Opin Pharmacol 2019; 47: 14-19.
5. Liu R, Li X, Xiao W, Lam KS. Tumor-targeting peptides from combinatorial libraries. Adv Drug Deliv Rev 2017; 110: 13-37.
6. Kehoe JW, Kay BK. Filamentous phage display in the new millennium. Chem rev 2005; 105(11); 4056-72.
7. Zhang ZF, Shan X, Wang YX, Wang W, Feng SY, Cui YB. Screening and selection of peptides specific for esophageal cancer cells from a phage display peptide library. J Cardiothorac Surg 2014; 9: 1-7.
8. Lü X, Yao M, Zhang JM, Yang J, Lei YF, Huang XJ, Yin W. Identification of peptides that bind hepatitis C virus envelope protein E2 and inhibit viral cellular entry from a phage-display peptide library. Int J Mol Med 2014; 33(5): 1312-8.
9. Pérez-Martínez D, Infante YC, Ramírez BS, Rojas G. Domain-level epitope mapping of polyclonal antibodies against HER-1 and HER-2 receptors using phage display technology. Sci Rep 2022; 12(1): 12268.
10. Sahin D, Taflan SO, Yartas G, Ashktorab H, Smoot DT. Screening and identification of peptides specifically targeted to gastric cancer cells from a phage display peptide library. APJCP 2018; 19(4): 927.
11. Rojas G, Tundidor Y, Infante YC. High throughput functional epitope mapping: revisiting phage display platform to scan target antigen surface. MAbs 2014; 6(6): 1368-76.
12. Sun J, Zhang C, Liu G, Liu H, Zhou C, Lu Y, Li X. A novel mouse CD133 binding-peptide screened by phage display inhibits cancer cell motility in vitro. Clin Exp Met 2012; 29: 185-96.
13. Thundimadathil J. Cancer treatment using peptides: current therapies and future prospects. J Amino Acids 2012; 2012: 967347.

14. Zhang D, Huang J, Li W, Zhang Z, Zhu M, Feng Y, He S. Screening and identification of a CD44v6 specific peptide using improved phage display for gastric cancer targeting. Ann Transl Med 2020; 8: 21.
15. Panagides N, Zacchi LF, De Souza MJ, Morales RAV, Karnowski A, Liddament MT, et al. Evaluation of phage display biopanning strategies for the selection of anti-cell surface receptor antibodies. Int J Mol Sci 2022; 23(15): 8470.
16. Freund NT, Enshell-Seijffers D, Gershoni JM. Phage display selection, analysis, and prediction of B cell epitopes. Curr Protoc Immunol 2009; 86(1): 9-8.
17. Hou L, Zhu D, Liang Y, Tian X, Li L, Wang P, Meng X. Identification of a specific peptide binding to colon cancer cells from a phage-displayed peptide library. Br J Cancer 2018; 118(1): 79-87.
18. Kwak MH, Yi G, Yang SM, Choe Y, Choi S, Lee HS, Park JM. A dodecapeptide selected by phage display as a potential theranostic probe for colon cancers. Transl Oncol 2020; 13(9): 100798.
19. Kelly KA, Jones DA. Isolation of a colon tumor specific binding peptide using phage display selection. Neoplasia 2003; 5(5): 437-44.
20. Ferreira D, Silva AP, Nobrega FL, Martins IM, Barbosa-Matos C, Granja S, et al. Rational identification of a colorectal cancer targeting peptide through phage display. Sci Rep 2019; 9(1): 3958.
21. Bakhshinejad B, Sadeghizadeh M. Identification of a novel colon adenocarcinoma cell targeting peptide using phage display library biopanning. Biotechnol Appl Biochem 2022; 69(6): 2753-65.
22. Zhang Y, Chen J, Zhang Y, Pan Y, Zhao J, Ren L, et al. A novel PEGylation of chitosan nanoparticles for gene delivery. Biotechnol Appl Bioc 2007; 46(4): 197-204.
23. Yu X, Yan J, Chen X, Wei J, Yu L, Liu F, et al. Identification of a peptide binding to cancer antigen Kita Kyushu lung cancer antigen 1 from a phage-display library. Cancer Science 2021; 112(10): 4335-45.
24. Bakhshinejad B, Sadeghizadeh M. Identification of a novel colon adenocarcinoma cell targeting peptide using phage display library biopanning. Biotechnol Appl Biochem 2022; 69(6): 2753-65.