RESEARCH ARTICLE

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Screening and Identification of Peptides Specifically Targeted to Gastric Cancer Cells from a Phage Display Peptide Library

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Abstract

Background: Gastric cancer is the second most common cancer among the malign cancer types. Inefficiency of traditional techniques both in diagnosis and therapy of the disease makes the development of alternative and novel techniques indispensable. As an alternative to traditional methods, tumor specific targeting small peptides can be used to increase the efficiency of the treatment and reduce the side effects related to traditional techniques. The aim of this study is screening and identification of individual peptides specifically targeted to human gastric cancer cells using a phage-displayed peptide library and designing specific peptide sequences by using experimentally-eluted peptide sequences. **Methods:** Here, MKN-45 human gastric cancer cells and HFE-145 human normal gastric epithelial cells were used as the target and control cells, respectively. 5 rounds of biopannning with a phage display 12-peptide library were applied following subtraction biopanning with HFE-145 control cells. The selected phage clones were established by enzyme-linked immunosorbent assay and immunofluorescence detection. We first obtain random phage clones after five biopanning rounds, determine the binding levels of each individual clone. Then, we analyze the frequencies of each amino acid in best binding clones to determine positively overexpressed amino acids for designing novel peptide sequences. **Results:** DE532 (VETSQYFRGTLS) phage clone was screened positive, showing specific binding on MKN-45 gastric cancer cells. DE-Obs (HNDLFPSWYHNY) peptide, which was designed by using amino acid frequencies of experimentally selected peptides in the 5th round of biopanning, showed specific binding in MKN-45 cells. **Conclusion:** Selection and characterization of individual clones may give us specifically binding peptides, but more importantly, data extracted from eluted phage clones may be used to design theoretical peptides with better binding properties than even experimentally selected ones. Both peptides, experimental and designed, may be potential candidates to be developed as useful diagnostic or therapeutic ligand molecules in gastric cancer research.

Keywords: Gastric cancer- phage display- targeted approach- subtraction biopanning

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Introduction

Gastric cancer is one of the most deadly cancer types in the world. It is diagnosed through endoscopic and histological examinations on biopsy sample (Jemal et al., 2011). However, in patients with asymptomatic gastric cancer, the diagnosis of this disease can be done only through noninvasive methods, and metastasis to other organs is not observed expect stomach. Therefore, higher mortality rates in patients with gastric cancer is justifiable. With respect to patient 'prognosis, surgery or chemotherapy is suggested as therapeutic intervention. However, chemotherapeutic treatment has some disadvantages such as low selectivity and drug resistance (Park and Kim, 2008; Rosati et al., 2009). Due to limitations in diagnosis and treatment steps, development of alternative methods is inevitable (Bai et al., 2007; Zhang et al., 2012).

As an alternative for traditional diagnostic and therapeutic methods, tumor-specific targeting peptides can be used to increase the efficiency of the treatment and reduce the side effects associated with traditional techniques (Borghouts et al., 2005; Qiu et al., 2007; Thayer, 2011; Sun et al., 2012).

Peptides have many advantages over monoclonal antibodies and large protein ligands, including small size, ease of synthesis, ease of tumor screening, and high biocompatibility (Borghouts et al., 2005; Qiu et al., 2007; Thayer, 2011; Sun et al., 2012). In addition, the affinity, charge, hydrophobicity, and stability of the peptide can be chemically modified. In this regard, the peptides may be optimized for use *in vivo*.

The peptides binding specifically to cancer cells can be identified by phage display method where M13

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filamentous phages are used most commonly (Kehoe and Kay, 2005). Different peptide sequences are expressed at the tail of M13 phages which can bind to the target. According to the binding specificity to the target, unspecific peptides are washed away and only high affinity peptide expressing phages are amplified (Sun et al., 2012; Thundimadathil, 2012). This technique, as an alternative to the conventional ones, permits the selection of targeting molecules as candidate ligand molecules for diagnostic and therapeutic purposes (Liang et al., 2006).

In this study, MKN-45 gastric cancer cells and HFE-145 normal human gastric mucous epithelial cells were used as the target and absorber cells, respectively, for subtraction biopanning with a 12-peptide phage display library to screen polypeptides that specifically bind to gastric cancer cells.

DE532 (VETSQYFRGTLS) peptide was selected in the 5th round of biopanning and screened positive as the best binder among all experimentally selected clones. Additionally, amino acid frequencies of experimentally selected peptides in the 5th round of biopanning were used to determine over/under expressed amino acids during biopanning steps compared to amino acid frequencies of the starting phage display 12-peptide library. DE-Obs (HNDLFPSWYHNY) peptide was designed to include the overexpressed amino acids of the experimentally selected clones. Both of the peptides, DE532 and DE-Obs, were identified as specific targeting peptides for MKN-45 human gastric cancer cells. Both peptides can be potential candidates for further examination as gastric cancer specific ligand molecules.

Materials and Methods

Reagents

The Ph.D.-12TM phage display peptide library kit containing E.coli host strain ER2738 and M13KE control phage were purchased from New England BioLabs (Ipswich, MA, USA). The phage display library contained random peptides constructed at the N-terminus of the minor coat protein (pIII) of M13 phage. The titer of the library was $1x10^{13}$ pfu (plaque-forming units). Horseradish peroxidase (HRP)/anti-M13 monoclonal conjugate antibody was purchased from Abcam (United Kingdom. X-gal, IPTG and protease inhibitor were purchased from Merck (NJ, USA). QIAprep Spin M13 Kit was purchased from QIAGEN (CA, USA). Dulbecco's Modified Eagle Medium (DMEM), FBS (Fetal Bovine Serum), and trypsin were bought from Lonza (MD, USA). The sequencing primer was synthesized in Iontek (Istanbul, Turkey). Bacteria culture media, Bactotryptone, and Bacto-yeast extract were obtained from Merck (NJ, USA).

Cell lines and cell culture

MKN-45 human gastric cancer cells were obtained from American Type Culture Collection (ATCC). HFE-145 cells were primarily cultured by Prof. Hassan Ashktorab from Howard University. MKN-45 cell lines were cultured in DMEM supplemented with penicillin, streptomycin, and 10% fetal bovine serum. Then, they were subcultured in plastic 25-cm^2 culture flasks and incubated at 37 °C in 5% CO_2 atmosphere. Cells were harvested at subconfluence, and the total number of cells was counted using a hemocytometer.

In vitro panning

MKN-45 cells were selected as the target cells, and the normal stomach HFE-145 cells as the absorber cells for a whole-cell subtractive screening from a phage display 12-peptide library. Both cells were cultured in DMEM containing 10% FBS at 37°C in a humidified atmosphere with 5% CO_2. In vitro screening procedures were performed as described in the instruction manual of the kit (Ph.D.-12™ Phage Display Peptide Library Kit) with some modifications.

Briefly, when the HFE-145 cells reached ~70% confluency, the culture medium was removed. The cells were washed twice with PBS and blocked with PBS containing 1% bovine serum albumin (BSA) at 37°C for 1 h. Approximately, 1x1011 pfu phages was added and mixed gently with the blocked HFE-145 cells for 1 h at 37˚C. During this time, the MKN-45 cells were pre-washed and blocked in the same manner. Then, the supernatant containing unbound phages was transferred to blocked MKN45 containing wells and incubated for 2 h on a rocker platform at 37 ˚C. After four intensive washes of cells with PBS containing 0.2% Tween-20, bound phage were eluted from cells by incubation with 1 ml of elution buffer (0.2 M glycine–HCl, pH 2.2) for 15 min. The eluate was immediately neutralized by the addition of 200 µl of 1 M Tris–HCl buffer at pH 9.1. An aliquot of output samples was used for the microtitration, and the rest of the phage was amplified using E. coli ER2738 host cells for next rounds of biopanning according to standard protocols with modifications in washing buffer conditions. For each round of panning, 1x1011 pfu of collected phages was used and the detergent (Tween 20) and salt (NaCl) concentrations were increased in a stepwise manner to 0.5%.

Amplification and sequencing of the selected phages

After the fifth round of panning, 35 phage clones were selected, amplified, and purified. According to the instruction manual of the kit, ssDNA was extracted and sequenced by Genometri (Istanbul, Turkey) using the -96gIII primer (5'-CCCTCATAGTTAGCGTAACG-3'). Multiple sequence alignment were conducted using the CLUSTAL W programs to determine the groups of related peptides.

Cell based enzyme-linked immunosorbent assay

MKN-45 cells and control HFE-145 cells were cultured in DMEM at 37°C and plated in 96-well plates ($5x10^4$ cells/well) until they adhered as a monolayer. The cells were washed three times with PBS and then fixed with 4% paraformaldehyde for 15 min at RT. After the fixation, the cells were washed three times with PBS again and blocked with PBS containing 1% BSA for 1 hour at 37°C. Next, 1×10^7 pfu of candidate phages were incubated separately with each cell type in triplicate at RT for 1 h. The wells were washed 4 times with PBS containing 0.2% Tween 20. In total, 75 µl of horseradish peroxidase

(HRP)-conjugated anti-M13 monoclonal antibody (1:400) was added to each well, and the plates were incubated at 37°C for 2 h. After washing three times with PBS, ABTS substrate solution was added to the wells and incubated at room temperature for 20 min. The reaction was terminated by the addition of 2M H_2SO_4. Subsequently, the plates were read on an automated ELISA plate reader at a wavelength of 405 nm. PBS and the cells without phage clones were used as negative controls. Three wells were used for each clone. All experiments were performed in triplicate.

Peptide Synthesis and labeling

The candidate peptides were synthesized and purified by Genoks/Ankara. FITC-conjugates were produced by the same company. The products were purified to a minimum purity of 95% by high-performance liquid chromatography (HPLC) and isolated by lyophilization.

Phage tittering

Specific enrichment of MKN-45 cell-bound phages was finished after five rounds of panning. The titers of the recovered phages from each round were calculated using a blue plaque-forming assay on agar plates containing tetracycline.

Peptide-based immunofluorescence assay

FITC-labelled candidate peptides were used for the peptide-based immunofluorescence assay to confirm the selective binding of the selected peptides to MKN-45 cells. MKN-45 cells and control HFE-145 cells were cultured in DMEM at 37°C and plated in 96-well plates ($1x10^4$ cells/well) until they reached ~70% confluency. The cells were washed three times with PBS and then fixed with 4% paraformaldehyde for 15 min at RT. After the fixation, the cells were washed three times with PBS again and blocked with PBS containing 1%BSA for 30 min at 37°C. For each candidate peptides, 75 µl FITC-labelled peptides (0.05M final concentration) were added on each cell type and incubated on a rocker platform for 1 h at 37°C. The wells were washed twice with PBS and incubated for 5 min with 75 µl DAPI staining solution. Then, the wells were washed twice with PBS and examined using fluorescence microscope. The experiments were done side by side on the same 96-well plate and the microscope settings was same for all the samples.

Results

Subtraction biopanning and enrichment analysis of 12-peptide phage display peptide library

MKN-45 human gastric cancer cells and HFE-145 human normal gastric epithelial cells were used as the target and control cells, respectively. First, 12-peptide phage display library was applied on HFE-145 cells which acted as absorber cells for subtraction biopanning. Then, unbound phage clones were incubated with MKN-45 cells for the regular biopanning steps. In each round, subtraction and regular biopanning steps were applied with increasing strength of washing conditions. In each round, the bound phages were rescued and amplified in E.coli ER2738 for the following round of biopanning. After five rounds of biopanning, the number of phages recovered from MKN-45 cells was increased gradually about 20 folds (Table 1), and positive candidate phage clones were enriched.

Primary identification of positive phage clones with C-ELISA

After the fifth round of panning, 35 phage clones were randomly selected and sequenced, binding selectivity of each clone was determined by using cellular ELISA. Phage clones binding to MKN45 cells (gray bars) and wild-type HFE-145 cells (black bars) were detected by the HRP-conjugated anti-M13 phage antibody (Figure 1). DE532 was selected for further analysis as the best binding clone on MKN45 cells with a ratio OD405nm of MKN45 cells to control cells which were more than 3. DE554 was

Table 1. Enrichment of Positive Phage Clones from 12-Peptide Phage Library

Round of Screening	Input Phage (pfu/ml)	Output Phage (pfu/ml)	Recovery (%)
1	$1x10^{10}$	$3x10^{6}$	$3x10^{-4}$
2	$1x10^{10}$	$1.6x10^{7}$	$1.6x10^{-3}$
3	$1x10^{10}$	$3.4x10^{7}$	$3.4x10^{-3}$
4	$1x10^{10}$	$4.2x10^{7}$	$4.2x10^{-3}$
5	$1x10^{10}$	$6.4x10^{7}$	$6.4x10^{-3}$

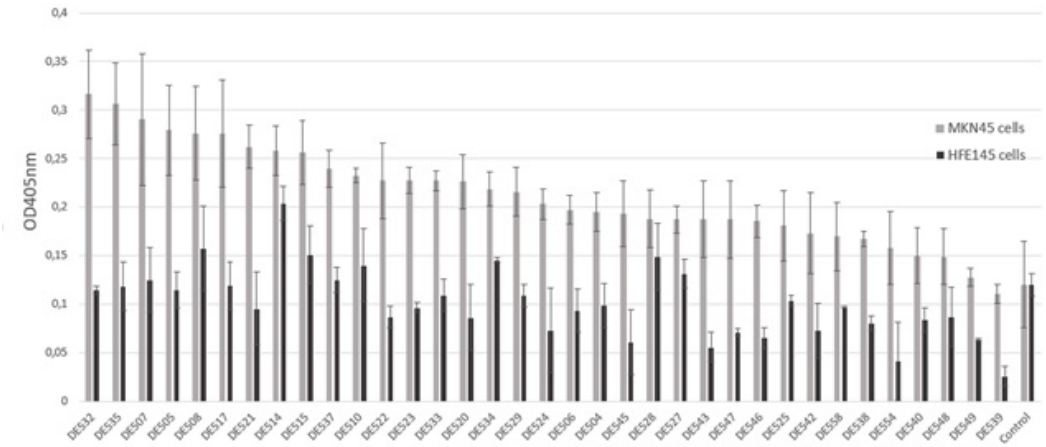

Figure 1. ELISA Results for Phage Clones Binding to Both MKN45 Cells and HFE145 Control Cells

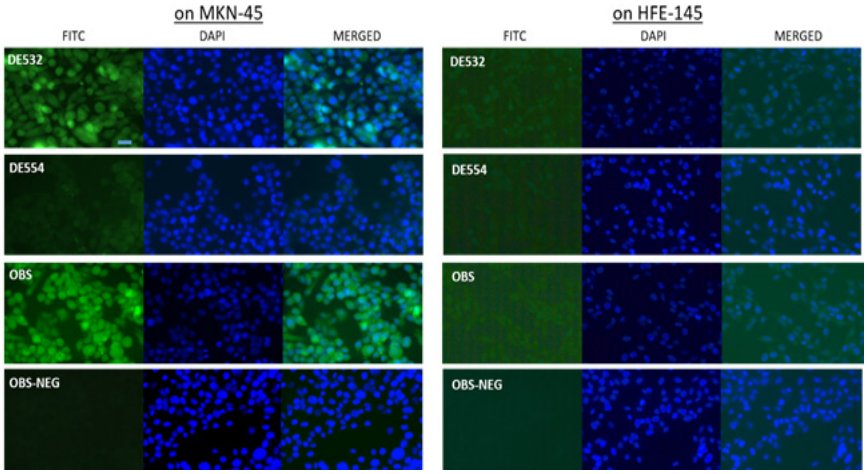

Figure 2. The FITC-Conjugated DE532, DE554, DE-Obs and DE-Obs-Neg Peptides were Incubated with MKN-45 Cells (left) and HFE-145 Cells (Right). The cells were observed under a fluorescence microscope.

Table 2. Amino Acid Sequences of Selected 21 Phage Clones for Further Analysis

Phage Clones	Peptide Sequence	Phage Clones	Peptide Sequence	Phage Clones	Peptide Sequence
DE532	VETSQYFRGTLS	DE514	SSMAPTRSLFVI	DE520	GSAPLLTVDTSK
DE535	RYSGLPDIPEPY	DE515	STTHNPDHSLYR	DE534	FGSSSTYPLGNW
DE507	GPIVIYNAPHTN	DE537	LTGDHSYQIRGA	DE529	TTNSWHPHNRVL
DE505	SDGDISLRWYVS	DE510	ASTPMFSPSHSM	DE524	SVLEPLVNVTHW
DE508	SQPWDDSTNRRV	DE522	VHHASSSFAVHP	DE506	LPHMGAFNLPLQ
DE517	NLGDNPLHRDHI	DE523	GLNNTNYFGVPM	DE504	SQAQYLHLYPYQ
DE521	NVSRFLAQPLLP	DE533	KSIVPSYLDHMQ	DE545	TTENADLSSRWS

Table 3. Percent Change of Experimentally Selected Amino Acids of 21 Phage Clones Compared to Starting PhD-12 Phage Library Amino Acid Expression Rates. Exp-Obs: Experimentally selected observed amino acids. (↑: Increase in expression; ↓: Decrease in expression. * www.neb.com for amino acid frequencies of PhD-12 peptide library)

Amino acids	Total count	Percent of Exp-Obs (%)	Percent of PhD-12 library (%)*	Percent change
Y	13	5.2	3.6	44.4 ↑
N	16	6.3	4.5	40 ↑
H	16	6.3	4.6	36.9 ↑
S	35	13.9	11.2	24.1 ↑
F	8	3.2	2.7	18.5 ↑
P	23	9.1	8.1	12.3 ↑
L	25	9.9	8.9	11.2 ↑
D	12	4.8	4.6	4.3 ↑
W	6	2.4	2.3	4.3 ↑
V	15	6	6.1	-1.6 ↓
Q	9	3.6	3.9	-7.7 ↓
T	18	7.1	7.8	-8.8 ↓
G	13	5.2	5.8	-10.3 ↓
R	12	4.8	5.7	-15.8 ↓
A	11	4.4	7.4	-40.5 ↓
I	8	1.8	3.4	-47 ↓
E	4	1.6	3.1	-48.3 ↓
M	6	1.3	3.1	-58 ↓
K	2	0.4	2.3	-82.6 ↓
C	0	0	1.5	-100 ↓

selected as one of the weak binders for the following immunofluorescence assay.

Positively selected-observed amino acids

To determine the overall positively selected amino acid frequency, 21 phage clones with higher ELISA readings were selected (Table 2). Here, the frequencies of each amino acid selected in the fifth round were compared with the observed amino acid frequencies of the starting PhD-12 peptide library to determine the positively/ negatively selected amino acids (Table 3). According to the observed amino acid frequencies, Y, N, H, S, F, P, L, D, and W were positively selected amino acids; whereas, C, K, M, E, I, A, R, G, T, Q, and V were negatively selected.

Assessing the targeting affinity by peptide based immunofluorescence assay

Experimentally selected DE532 (VETSQYFRGTLS) and DE554 (LFGASGLAGFSL) peptides were first synthesized and commercially purified, and FITC conjugates were formed. Similarly, 2 theoretical peptide sequences, DE-Obs and DE-Obs-Neg, were determined according to the positively and negatively expressed amino acid frequencies, respectively. DE-Obs peptide sequence included the most positively selected amino acids to form the peptide as Nter-HNDLFPSWYHNY- Cter. On the other hand, DE-Obs-Neg peptide included the least expressed amino acids to form the peptide as Nter- AEQKTIAEQKTI- Cter.

Binding specificities of the FITC-labelled synthesized

peptides were assessed using MKN-45 and HFE-145 cells as target and control cells, respectively (Figure 2). On MKN-45 target cells, DE532 showed better affinity than DE554 which was consistent with c-ELISA assay results. DE-Obs was the best binder and DE-Obs-Neg showed no binding as expected. On HFE-145 control cells, none of the peptides showed observable binding affinities.

Discussion

The phage display technique has been widely used as an important tool for drug discovery and the research of biological molecules since it was first introduced by Smith (1985). Different targets, including specific organs, tumors, cell lines, and proteins have been used successfully to select short peptides specific for desired targets. The screening procedure may involve a specified target molecule with prior knowledge of the molecular structure or whole cells, tumors or tissues can be directly used as target without any prior knowledge of specific binding sites. Here, we selected the 12-mer peptide DE532 using the PhD-12 library and designed DE-Obs peptide according to amino acid frequencies of strong binding peptides selected from 5th biopanning round. These peptides exhibited specific binding to MKN-45 human gastric cancer cells in culture.

In this study, we applied five rounds of screening to select 12-mer peptides from phage display peptide library against human gastric cancer MKN-45 cell lines. HFE-145 human normal gastric epithelial cells were applied as control group for subtraction biopanning. After the fifth round of selection, 35 phage clones were chosen randomly and binding affinities of each clone was assessed by using c-ELISA to identify the positive clones with better affinities. The clones were sorted in descending order from strong to weak binders. DE532 clone was identified as the best binder among the chosen phage clones and selected for further analysis. Although DE532 was chosen as the best one, none of the phage clones such as DE532 dominated the screening rounds. So, we decided to follow a combinatorial strategy and selected the top 20 phage clones to design theoretical peptides according to the expression levels of each amino acids. This strategy had already been followed in the literature for designing inorganic binding peptides from phage display selected quartz binding peptides (Oren et al., 2007). Here, we simply compared the frequencies of each amino acid from 20 selected best binding phage clones with the observed amino acid frequencies of the starting PhD-peptide library. DE-Obs (HNDLFPSWYHNY) and DE-Obs-Neg (AEQKTIAEQKTI) peptides were designed to include over-expressed and under-expressed amino acids, respectively.

DE532 (Experimental Strong), DE554 (Experimental Weak), DE-Obs (Designed Strong), and DE-Obs-Neg (Designed Weak) peptides were synthesized and labeled with FITC for immunofluorescence microscopy binding experiments. MKN-45 cells and HFE-145 cells were used as target and control cells, respectively. The best binding level among the four peptides against MKN-45 target cells was achieved with DE-Obs peptide, while DE532 was also a strong binder as expected from c-ELISA results. DE554 was binding weakly on the target cells; however, DE-Obs-Neg gave no shining at all. All of the peptides were noticeably binded weakly against HFE-145 control cells.

Here, we presented an example of combining experimental and theoretical procedures to select specific peptides binding on gastric cancer cells. Phage display is a very useful technique for the selection of target specific peptides; however, the experimental limitations may not let to obtain the best binding peptides. In case of no dominating peptides, overall amino acid characteristics of selected clones may be useful to design theoretical peptides, including overexpressed amino acids. The order and repetition number of each amino acid in the designed peptide will be determining in the binding strength of the final peptide. We placed the most overexpressed amino acids randomly in the peptide. Best ones were repeated and placed at N-terminal and C-terminal ends.

Comparing over and under expressed amino acids, we realized that both of the peptides, DE-Obs and DE-Obs-Neg, include acidic/basic, hydrophobic/hydrophilic or charged/uncharged residues. DE-Obs has mostly hydrophobic charateristics with a pI value of 6.5 while DE-Obs-Neg has more hydrophilic with a pI value of 7.04. DE-Obs has very poor water solubility while DE-Obs-Neg has good water solubility. Accordingly, hydrophobic character and poor water solubility might be effective on the binding affinity.

Another difference is the selection of especially aromatic amino acids such as H, Y, W, and F, as overexpressed residues which may be the reason of strong binding on MKN-45 cells. DE-Obs peptide includes 6 aromatic residues while DE-Obs-Neg has no aromatic residues.

When we compared DE532 and DE554, we did not observe the similar features with designed peptides. Both of them had poor water solubility and no net charge at neutral pH. For further analysis, different orientations of amino acids with different properties can be tried to achieve better binding properties. Thus, the importance of position of each amino acid within the peptide is suggested to be explored. Furthermore, any identified 3-4 amino acid long motif sequences may be used when designing the final peptide. This research was performed only at cellular level in an in vitro environment. To verify the binding affinity of the peptides on the tissue level is needed as the next step .

In conclusion, 5 rounds of biopanning with a phage display 12-peptide library were applied to select peptides specific for MKN-45 human gastric cancer cells. Among the selected phage clones, DE532 (VETSQYFRGTLS) was identified as the best binding peptide with relatively less affinity against HFE-145 human gastric epithelial cells. To improve the efficiency of biopanning process, amino acid frequencies of 20 phage clones with better binding levels were analyzed and DE-Obs (HNDLFPSWYHNY) peptide was designed to include overexpressed amino acids. Both peptides are the potential candidates to be developed further as ligand molecules for diagnostic and therapeutic purposes against gastric cancer. Here, we

simply extracted the data from experimentally selected phage clones to design theoretical peptides with better binding affinities.

Compliance with Ethical Standards
Conflict of interest

The authors, Deniz Sahin, Sevket Onur Taflan, Gizem Yartas, Hassan Ashktorab and Duane T Smoot, declare that they have no conflict of interest.

Statement on the welfare of animals

This article does not contain any studies with human participants or animals performed by any of the authors.

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