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Identification of High-affinity Novel Targeted Peptides for the Treatment of Human Ovarian Cancer

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Abstract

Background: To investigate the potential of the phage display-identified tumor cell-binding peptide as a biomarker of epithelial ovarian cancer using phage display technology.

Method: The Ph.D.-7 Phage Display Peptide Library was used to identify the specific conjugated phages with SKOV3 epithelial ovarian cancer cells, while Chinese hamster ovarycells formed the basis. After employing the rapid differential screening method invitro, the enzyme-linked immunosorbent assay (ELISA), DNA sequencing, immunohistochemistry, immunofluorescence, and the competitive inhibition test of synthetic peptides were used to determine the affinity and specificity of the phages with SKOV3 cells.

Results: Using biopanning, we screened the phages, showing a 3590-fold increase after the third round. A total of 61 titers of the phage were randomly selected for ELISA and 10 kinds of the phages with an optical density >0.5 were used for DNA sequencing. Clones of the phage TRRNIPN were derived from DNA sequencing based on ELISA, exhibiting both the brown granular phenomenon and green fluorescence. The specific targeted peptide TRRNIPN was incorporated in tumor cells through the competitive inhibition test.

Conclusion: The results of our study indicate that the phage display identified polypeptideTRRNIPN may be an effective biomarkerfor the early diagnosis and targeted therapy of ovarian cancer.

Keywords: Epithelial ovarian cancer; Phage display technology; Peptide; SKOV3 cells

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Introduction

Epithelial ovarian cancer is the seventh most common type of cancer in women and the eighth most common cause of death due to cancer worldwide. The 5-year survival rate of patients with this type of cancer is <45% [1]. In addition, the majority of patients with epithelial ovarian cancer are diagnosed at a late or metastatic stage of the disease [2]. Early diagnosis and targeted therapy are of great importance in the treatment of epithelial ovarian cancer, with limited progress achieved thus far [3-11]. At present, the most commonly used method for the diagnosis of ovarian cancer and monitoring of disease recurrence is the level of CA125 in the serum [12-14]. However, the level of CA125 is also elevated in patients with ovarian endometriosis and otherbenign diseases; thus, the specificity of this diagnostic approach is low [15]. Biological targeted technology is a valuable tool for the diagnosis and treatment of numerous diseases, [16] including epithelial ovarian cancer [17].

Peptide preparations can overcome the limitations of antibody preparations, providing an opportunity for the development of targeted peptide therapy. Therefore, research focused on the identification of peptides that can bind to receptors on cancer cells. In recent years, peptide libraries have been successfully used to identify short peptides that could be used for the early diagnosis and treatment of tumors [18-20]. Phage display is used to integrate the coding sequence of foreign polypeptides into the phage genome and display those on the phage surface in the form of fusion proteins [21]. Numerous studies in the fields of prevention and treatment of cancer have utilizedthis technology in the areas of molecular imaging diagnosis [22], the development of targeted drugs [23], antigen epitopes [24], and vaccines [25] etc. Based on the increasing popularity of phage display technology, this approach may be usefulin the screening of ovarian cancer targeted peptides, aimingto identify biomarkers for theearly diagnosis and targeted therapy of this disease [26].

Materials and Methods

Cell lines and cell culture

The human epithelial ovarian cancer cell line SKOV3 was obtained

from the Shanghai Academy of Sciences China (Shanghai, China). The cells were cultured in 1640 medium (Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA) and 1% penicillin streptomycin antibody. Chinese hamster ovary (CHO) and the human cervical cancer cell line Caski-obtained from the Shanghai Academy of Sciences-were cultured under identical conditions as previously reported [27].

Reagents

The Ph.D.-7 Phage Display Peptide Library waspurchased from New England Biolabs (Ipswich, MA, USA). Thelibrary which contains approximately $1x10^{13}$ pfu/mL phageshad $1.28x10^{9}$ diverseunique peptide sequences has 70 copies. Mei Gu Bio Technology Co. Ltd. provided the *E. coli*. ER2738 and the FITC-labelled rabbit anti rat IgG antibody. Notably, the M13K07 phage (negative control phage) and the horseradish peroxidase (HRP)-conjugated anti-M13 antibody were purchased from Beijing Rong Chuang Da Science and Technology Ltd (Beijing, China).

Phage display peptide library and biopanning process

Preparation of the *E. coli*. ER2738 culture plate, as well as the digestion and collection of CHO and SKOV3 cells were initially performed. The cells were suspended in 1640 serum-free medium containing 1% bovine serum albumin. Subsequently, the number of cells was adjusted to $1x10^{7}$/mL. CHO cells (100 μL) and phage random seven peptide library (10 μL) were mixed at 4°C and incubated for 2 h (rotation: 30 rpm/min). Organic separation liquid (density of 1.03 g/mL) (200 μL)-consisting of dibutyl phthalate and cyclohexane (volume ratio: 9:1)-was added to the suspension of cells and phage and the mixture was centrifuged at 10,000 g for 10 min to isolate the unbound phages. Following centrifugation, the unbound phages remained in the water phase, which was incubated with the SKOV3 cells for 3 h. This process was followed by centrifugation as above. The precipitate was transferred to the ER2738 fluid at the logarithmic growth period and incubated for 30 min at 37°C to complete the phage resuscitation. In addition, the abundance of phageswas measured, amplified, and purified, while the number of phages after each round of washing and amplification was determined.The abundance of phages was amplified at 37°C. After amplification and purification, the phageswere used for the next round of biopanning. This method was repeated thriceto significantly increase thephage titer.

Extraction of phage DNA

A total of 61 blue plaques from the phage plate, which were dispersive and clear, were transferred to a centrifuge tube containing the dilution medium. Well-separated plaques were selected and cultured in centrifuge tubes at 37°C and centrifuged at 250 rpm/min for 4.5-5 h. Subsequently, the plaques were precipitated using PEG-8000/NaCl and re-centrifuged. The precipitate was suspended in iodide buffer and ethanol. Finally, the phage protein was removed through centrifugation and re-dissolved in TE buffer solution.

Enzyme-linked immunosorbent assay (ELISA)

The SKOV3 cells were inoculated in 96-well plates and fixed with paraformaldehyde (4%). The cells were repeatedly washed with phosphate-buffered saline (PBS) and sealing fluid was applied to seal the cells afterwards. A total of 61 phage clones were prepared and incubated with targeted cells which were inoculated in 96-well plates at 37°C. The PBS and M13K07 phages were used as negative controls. Subsequently, the clones were incubated for 30 min at room temperature with sealing liquid to remove non-specific cloning. After washing with PBS thrice, the HRP-conjugated anti-M13 antibody (diluted using liquid sealant at 1:6,000) was added (200 μL/well) and incubated for 1h at room temperature. After repeatedly washing with PBS, TMB color solution was added (200 μL/well), and the clones were incubated at room temperature for 20-60 min. The absorbance was measured using a microplate reader at 410 nm.

DNA sequencing and peptide synthesis

After DNA extraction, we selected the corresponding numbers of phage clones with an optical density >0.5 and high affinity after the ELISA experiment. Subsequently, the phage clones were sent to Shanghai Biotechnology for DNA sequencing. Analysis of the sequence data was obtained using BLAST and thep motif program Smooth chapter. Following this analysis, the peptide (TRRNIPN) and the unrelated control peptide (NNIPRTR) were biosynthesized by Shanghai Bio Technology Co. Ltd .

Positive phage identification test

Immunohistochemistry: SKOV3, CHO, and Caski cells were cultured on cell slides at 37°C overnight, repeatedly washed with PBS, and treated with 4% polyformaldehyde. The bacteriophage TRRNIPN, M13K07, and PBS were then added, with PBS alone used as negative control. After treating with the closed liquid, the peptide was washed with PBS, and the unbound phage was removed. Diluted (1:100) HRP-conjugated anti-M13 antibody was added and the mixture was incubated for 2 h at 37°C. The antibody was subsequently washed, and the cell slides were placed on glass slides and colored with Fresh DAB solution at room temperature for 2-3 min. The DAB color rendering was monitored through microscopy. The residual DAB coloring solution was washed with PBS. Subsequently, hematoxylin was used to stain the nucleus. The cell slides were then subjected to dimethylbenzene transparency. A few minutes later, neutral balata was added to the transparent slide. Finally, the slides were observed under a microscope.

Immunofluorescence test

SKOV3, Caski and CHO cells were cultured on cell slides at 37°C overnight, washed with PBS, and treated with 4% polyoxymethylene. After the addition of phage, anti-M13 antibody (1:100 dilution) was added and the mixture was incubated for 2 h at 37°C. After washing with PBS to remove the primary antibody, FITC-labeled rabbit anti-mouse IgG antibody (1:300 dilution) was added and the mixture was incubated for 30 min in darkness. After repeated washing, the slides were stained with DAPI (1:5,000) for 5 min at room temperature, washed with PBS, and immediately observed using fluorescence microscopy.

Competitive inhibition of synthetic peptides

SKOV3 cells were digested, collected, and suspended in RPMI-1640 serum-free medium containing 1% bovine serum albumin. Subsequently, the cells were incubated with different concentrations (0,0.01,0.1,0.25,2.5,50, and 250 μmol/L) of polypeptide TRRNIPN and control peptide NNIPRTR at 4°C, followed by incubation with 10^{9} pfu phage TRRNIPN at 4°C for 2 h. The suspension and separation of organic liquid at 200 μL on the compound. After centrifugation (10,000g, 10 min, 4°C), the sediment which consisted of phage combined with targeted cells in the organic phase was transferred to the Colibacilli solution. The phage titer was measured after resuming treatment.

Results

Biopanning targeted phages using the phage peptide library

Initially, the partial phage-a non-specific bacteriophage that binds to CHO cells-was excluded. It could remove phage from normal cells and increase the probability of screening positive phages. After incubation with SKOV3cells, we initially obtained a specific targeted binding phage and calculated the titer of the phage using the LB/IPTG/X -gal plate.The results are shown in **Figure 1**. After three rounds of biopanning, the phage recovery (output/input) was significantly improved. The results showed that the number of phages after three rounds was 3,590-foldhigher than that observed after the first round (**Figure 1**).

ELISA and DNA sequencing

After three rounds of biopanning, we selected 61 relatively dispersed phages in the last round of panning plate, phage plaque size and clear , followed by amplification and purification of the phage plaques. The 61 phage clones, phage M13K07, and PBS were used to perform ELISA. The affinity of the 61 phages to targeted cells was determined (**Figure 2**). In view of the differences in affinity, we selected 10 phage clones with optical density >0.5 which have relatively high affinity for DNA sequencing. Subsequently, we processed and compared the data obtained from DNA sequencing using the BLAST and PMOTIF programs.DNA sequences were transformed into amino acid sequences, and the resultantsix amino acid sequences are shown in **Table 1**. However, four of the 10 examined phage clones showed identical DNA random sequences, meanwhile, while two phage clones had the other same random sequence of DNA. Thus, the phage clone was termed Phage 1.

From the data analysis and processing of DNA sequence (**Table 1**), we obtained the corresponding random sequences and compared them with the amino acid sequence codon conversion. A short peptide sequence containing seven amino acids was obtained as the target peptide andtermed peptide 1(TRRNIPN).

A.

Number	Input (pfu/ml)	Ouput (pfu/ml)	Recovery
1	2x10^11	7.8x10^5	3.9x10^-6
2	5.4 x10^10	4.2x10^8	7.8x10^-3
3	5.7x10^10	7.8x10^8	1.4x10^-2

B.

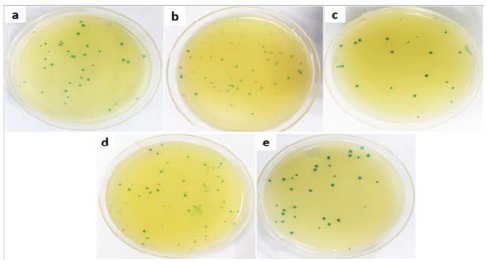

Figure 1: a. Specific enrichment of SKOV3 cell-bound phages using an initial input of 10^{11} pfu; **b.** The distribution of blue plaque and moderate number of plates were selected for counting: (a) The first round of washing titer with the dilution of 10^4 times, (b) The first-round amplification titer with the dilution of 10^9 times; **c.** The second round of washing titer with the dilution of 10^7 times; **d.** The second-round amplification titer with the dilution of 10^9 times e. The third round of washing titer with the dilution of 10^7 times.

Immunohistochemistry staining

In this study, the obtained corresponding phage 1(TRRNIPN) was incubated with SKOV3, Caski, and CHO cells. Immunochemical staining with M13K07 was performed, using PBS as control. This resulted in the attachment of peptide 1 to the experimental phage targeted ovarian cancer cells. Only the positive phage and cells exhibited the brown granular phenomenon occurring after staining, as shown in **Figure 3**.

Immunofluorescence

The identified bacteriophages were incubated with three kinds of cells (as in the immunohistochemical staining experiment), and subsequently incubated with fluorescent-labeled antibodies. The results showed that only the combination of SKOV3 cells and targeted phages produced green fluorescence under blue light excitation, as shown in **Figure 4**.

Competitive inhibition of synthetic peptides

Under the same conditions, there is competition between the

Table 1. Biopanning specific targeted peptide (amino acid sequence from DNA sequence).

Phage number	Polypeptide number	Amino acid sequence	Frequency of occurrence
Z5/8/47/56	ZP1	TRRNIPN	4
Z4/24	ZP2	SRRRITL	2
Z51	ZP3	IRNIPRR	1
Z58	ZP4	NHLSRRP	1
Z42	ZP5	MQPRILT	1
Z54	ZP6	RMRTIQR	1

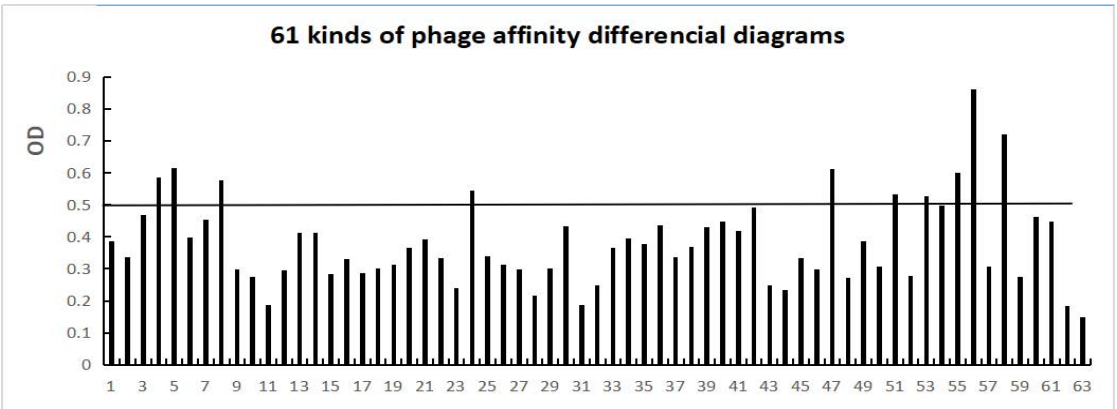

Figure 2: The affinity of 61 kinds of phage clones (1-61), the contrast phage (62) and PBS negative control (63) to the targeted cells. The results shown indicate that the affinity of (62) and (63) was significantly lower than that observed for the 61 kinds of phage clones (1-61). According to the results, 10 kinds of phage clones were selected for DNA sequencing (above the horizontal line).

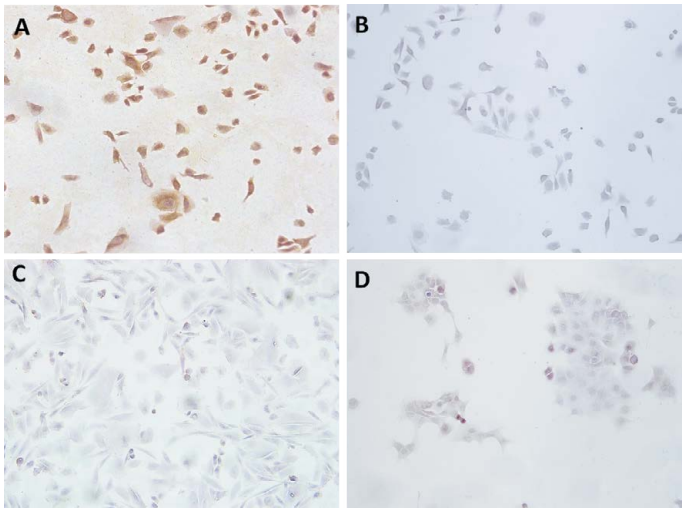

Figure 3: The immunohistochemistry pictures of the combination of phages and cells under 20x magnification microscopy. **a:** Following the reaction of SKOV3 cells and phage 1, the basic morphology of cells and brown granules were observed. **b:** Cell morphology and nuclear structure can be clearly observed when SKOV3 cells and phage M13K07 are combined; however, there are no visible brown granules. **c:** The combination of CHO cells and phage 1 produced results consistent with those observed in B. **d:** The result of the combination of Caski cells and phage M13K07 was also characterized by cell morphology and absence of brown granules.

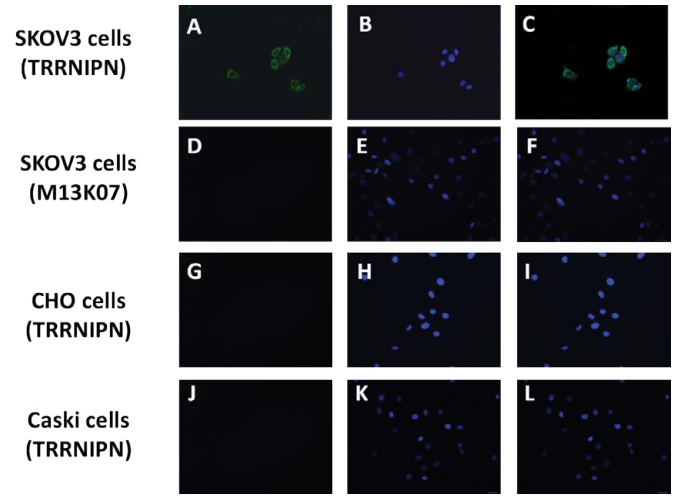

Figure 4: The fluorescence picture of the combination of cells and phages.After staining, the positive phage 1 combined with the cell to presents green fluorescence, while the nucleus presents blue fluorescence under the ultraviolet light; **(a-c):** Following the reaction of SKOV3 cells and phage 1, green fluorescence was clearly observed under x20 magnification fluorescence microscopy, around the blue fluorescent nucleus; **(d-f):** SKOV3 cells with the negative phage M13K07 did not exhibit green fluorescence and the nuclear structure was observed under ultraviolet light; (**g-i):** When CHO cells (negative control) reacted with phage 1, only strong blue fluorescence was observed representing the nuclear structure; (**j-l):** When Caski cells (negative control) reacted with phage 1, only weak nuclear structure was observed through fluorescence microscopy.

surface peptide and phage in ovarian cancer cells. Hence, the peptide and cell-binding may reduce phage binding on the cellular level. Therefore, we diluted the synthesized peptide to different concentrations and performed a phage-binding analysis. The results showed that in the absence of polypeptide, the phage titer was 100%. Interestingly, with the increasing concentration of polypeptide, the phage titer was decreased due to competition (**Figure 5**). Using two-way factorial ANOVA, we found that the differences on the inhibition of peptide 1 and control peptide (NNPIRTR) in six groups (except for the group without peptide) after treatment with the same concentration were statistically significant (P<0.01) (**Figure 5a**). In addition, treatment with the peptide 1 under different concentrations demonstrated statistical significance (P<0.01) broadly by using remarkable difference analysis and relevant accounting method (**Figure 5b**). In other words, peptide 1 exhibited specificbinding to tumor cells, and the effect of this combination was related to different concentrations .

Discussion

In recent years, the early diagnosis and tumor-oriented therapy of epithelial ovarian cancer have attracted considerable attention in clinical oncology. There are specific antigens on the surface of solid tumor cells; however, the specific antigens of many tumors remain unknown or difficult to detect. Studies have shown that peptides may assist in the early diagnosis of ovarian cancer and prognosis of chemotherapy. For example, the growth differentiation factor 15 (GDF15) can predict response to platinum during first-line chemotherapy, as a complementary diagnostic serum biomarker to CA125 in epithelial ovarian cancer. However, in these studies, it was not possible todetermine whether GDF15 is an independent predictor of response to first-line chemotherapy in patients with epithelial ovarian cancer. Moreover, it has been shown that the level of GDF15 may be increaseddue to various factors, such as resistance to chemotherapy, etc. [28]. Therefore, there is an unmet need for the identification of biomarkers in this setting.

Phage display technology can rapidly screen antibodies or polypeptide ligands with high affinity to target molecules. Its greatest advantage is the combination of genotype and phenotype [29]. Therefore, it is not necessary to determine the structure of target molecules in advance. By sequencing the genes cloned through affinity screening positive bacteriophage , we can indirectly determine the amino acid sequence of the presented foreign polypeptides. Inpreviousstudies, phage display technology was successfully applied to screen polypeptides that can specifically bind to the surface of different cancer cells, such as breast cancer cells [30], liver cancer cells [31], and lung cancer cells [32]. These cell surface-binding peptides may be

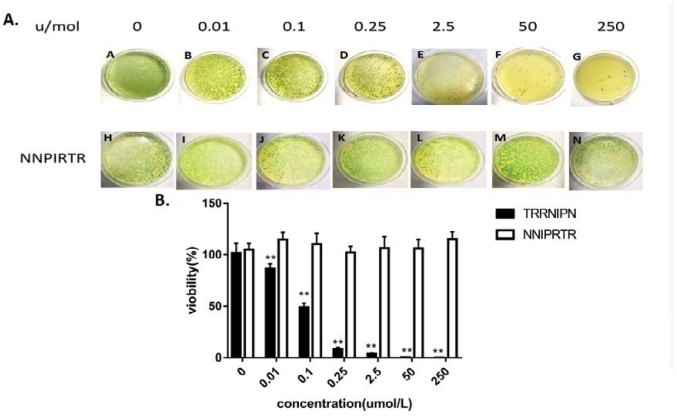

Figure 5: The phage titer was determined under different concentrations of polypeptides; **A: (a and h)**: No peptide added; (**b-g):** The concentration of the peptide 1 added with SKOV3 cells was 0.01 u/mol, 0.1 u/mol, 0.25 u/mol, 2.5 u/mol, 50 u/mol, and 250 u/mol; (**i-n):** The concentration of the control peptide (NNPIRTR) added with SKOV3 cells was 0.01 u/mol, 0.1 u/mol, 0.25 u/mol, 2.5 u/mol, 50 u/mol, 250 u/mol. The phage titer was decreased in response to the increasing concentration of the polypeptide (TRRNIPN). In contrast, the control peptide (NNPIRTR) did not exert a similar effect; **B:** When the concentration of peptide 1 was increased, the titer of the phage was decreased by the fold line map. In contrast, the phage titer did not decline significantly after treatment with the control peptide. The bars represent mean±standard deviation.**P<0.0vs. NNPIRTR (if not specially indicated).

involved in early diagnosis, anti-metastasis, and invasion of cancer. Moreover, they may have clinical value in tumor-oriented therapy.

In the study, the phages were bound to normal ovarian epithelial cellsandit couldfilter out thephages that were non-specific to the targets. The phages, which could not attach to the normal cells, were mixed with tumor cells to screen the phage clones with tumor-specificcells. In addition, the X-gal blue spot screening method was used to display the specific binding of phagesto SKOV3 so that the natural phages were eliminated to some extent. The result of this analysis is expressed by the determination of the titer after biopanning process. After the third round of subtractive screening, the phage clones were obviously enriched (**Figure 1**). In comparison with the first round,the titer after the third round demonstrateda 3,590-fold increase. Therefore, the specific small molecule on the tumor surface has the potential of binding to high-affinity specific phagesobtainedvia high-throughput screening and purification.

After biopanning, well-separated plaques were selected to ensure that each plaque contained a single DNA sequence. The phage DNA was completely suspended in iodide buffer and ethanol during extraction to precipitate the single-stranded phage DNA for most of the phage protein to be in the solution .

Through the amplification and purification of the phage plaques, we obtained purified phage liquid. Wedetermined the affinity of 61 phage clones to targeted cells using ELISA experiment, and the phage clones demonstrating the highest affinity were selected for DNA sequencing (**Figure 2**).

We identified the most repetitive target peptide-peptide 1 (TRRNIPN)-via ELISA and DNA sequencing (**Table 1**), confirmed by immunohistochemistry (**Figure 3**) and immunofluorescence (Figure 4). The results showed that only TRRNIPN incubated with SKOV3 cells displayed brown granules and fluorescent. In contrast, there were no positive results obtained with other phages and control cells. Moreover, the competitive inhibition test results (Figure 5) showed that polypeptides could specifically bind to SKOV3 epithelial ovarian cancer cells and inhibit their expression and biological characteristics. In contrast, the negative control peptide did not exhibit any activity in the ovarian cancer cells. The statistical analysis indicated that there were different inhibited viabilities between peptide 1 and the control peptide. Six of the seven groups under the same concentrations demonstrated statistically significant differences ($P<0.01$). Furthermore, peptide 1 demonstrated statistically significantdifferences under different concentrations ($P<0.01$). The results indicated that TRRNIPN has the potential to become a targeted drug,aiming at the inhibition of ovarian cancerprogression.

The present findings strongly suggest that TRRNIPN has high specificity and high affinity for SKOV3 ovarian cancer cells, indicating that this peptide may be a favorableoptions for the diagnosis and treatment of ovarian cancer. In addition, it may play a vital role as a carrier to binding fluorescent substances or drugs.

This study is the first to investigate TRRNIPN. This peptide does not belong to any type of protein due to itsdistinct amino acid sequence. It is a novel kind of small molecule which possesses binding specificity for tumor cells, but not for normal cells.

TRRNIPNwas shown to specifically bind to tumor cells and demonstrate specific fluorescence in vitro. However, the affinity and specificity of this peptidein vivoremains to be determined by future studies.

Conclusion

In conclusion, the present study investigated a novel small molecular peptide termed TRRNIPN, which showed specific binding properties for ovarian cancer cells SKOV3 and may inhibit the progression of tumor cells *in vitro*. Therefore, this peptide may be a good biological target in the early diagnosis and therapy of ovarian cancer.

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