Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org

Original Research

Screening for novel peptides specifically binding to the surface of ectopic endometrium cells by phage display

Yan Linzhi, Cao Dan, Arhin Samuel Kofi, Wang Ledan, Li Wenju, Lv Jieqiang*

Department of Gynecology and Obstetrics, The Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University, Wenzhou, Zhejiang, 325027, P.R. China

Correspondence to: jieqianglu@126.com

Received July 10, 2018; **Accepted** August 9, 2018; **Published** August 30, 2018

Doi: http://dx.doi.org/10.14715/cmb/2018.64.11.7

Abstract: A 7-mer phage display library was employed to isolate novel peptides that specifically bind to ectopic endometrium in vitro. Phage display technology with biopanning and rapid analysis of selective interactive ligands between ectopic and eutopic endometrium cells was utilized. After 5 rounds of biopanning, 50 phage clones were randomly selected and analyzed by enzyme-linked immunosorbent assay and DNA sequencing. A peptide-competitive inhibition assay was performed to identify the affinity of positive phages toward ectopic endometrium cells. The most enriched polypeptide RTRLHTR showed higher affinity toward ectopic endometrium cells.The polypeptide RTRLHTR screened by phage display technology may offer a new direction for early diagnosis and treatment of endometriosis.

Key words: Ectopic endometrium cells; Phage display technology; Phage peptide library; Peptides.

Introduction

Endometriosis is a common gynecological benign disease in women of reproductive age. However, some of its biological behavior shows similarities to behavior of malignant tumors. In recent years, tumor-targeting therapy, specifically targeted drug therapy, has been one of the hot spots of tumor clinical treatment. Clinical treatment of endometriosis is currently insufficient due to the unavailability of a specific drug that can reach the lesion and effectively kill tumor cells; targeted therapy is an important means by which this problem can be solved. Phage display is a laboratory technique that can be used for high-throughput screening of tumor cell surface specific binding peptide (1). At present, it has been widely used in tumor antigen screening, monoclonal antibody preparation, biological vaccine, and drug development (2-7). This technique should thus be sufficient for the study of targeted therapies. The present study utilizes phage display to screen for novel peptides that specifically bind to endometriosis cells of the ectopic endometrium using a Ph.D.-7™ Phage Display Peptide Library. The affinity and specificity of the resulting positive phages were identified and the amino acid sequence of these peptides was analyzed. The results of the present study may assist with the diagnosis of endometriosis and the identified phages have the potential to be utilized as carriers for drug delivery.

Materials and Methods

A total of 10 premenopausal women with surgically and histologically diagnosed severe endometriosis stage III-IV (according to the revised American Fertility Society [ASF] classification) were included in this study. Fifty clones were randomly selected from each patient. All eutopic/ectopic endometrium specimens were obtained through dilation and curettage/laparoscopy cystectomy during the proliferative phase. They were divided into 2 groups: ectopic endometrium (n=10) and eutopic endometrium (n=10, from the same individuals in the group). The patients did not have hormonal treatments, such as GnRHa or sex steroids, for 6 months prior to surgery. The experiment was approved by Institutional Review Board (IRB) of the affiliated hospital of Wenzhou Medical University.

Cell isolation and culture

All tissues, including the matched ectopic and eutopic endometrial samples from each endometriosis patient, were collected under sterile conditions, irrigated with lactated Ringer's solution, and cut in half. One half was placed in sterilized ice-cold calcium- and magnesium-free Dulbecco's phosphate-buffered saline (PBS) and immediately stored in liquid nitrogen for cryopreservation. The other half was sent to the pathology laboratory for histopathological confirmation. After thawing, the tissues were rinsed, minced into small pieces, and digested with collagenase IV (1 mg/ml, Gibco, USA) for 1 h and with DNase I for 30 min (1 mg/ml, Gibco, USA) at 37°C with constant agitation. The tissue pieces were filtrated through sterile 100-mesh (150 μm) gauzes to remove debris and through a 400-mesh (38μm) wire sieve to separate endometrial stromal cells (ESCs). Following gentle centrifugation (800 ×*g* for 5 min), the supernatant was discarded and the cells were resuspended in DMEM/F-12 (Gibco, USA). The ESCs were placed in culture flasks and allowed to adhere for

30 min. The adherent cells were cultured as a monolayer in flasks with DMEM/F-12 containing 10% fetal calf serum (Hyclone, Logan, UT, USA) and 20 mM HEPES in a humidified atmosphere of 5% CO_2 at 37°C. The purity of the ESCs was detected by immunocytochemistry with the anti-vimentin antibody (diluted 1:100, Zhongshan Golden Bridge Inc., Beijing, China) and the anti-cytokeratin 19 antibody (diluted 1:100, Zhongshan Golden Bridge Inc., Beijing, China), following the method described above.

Phage display biopanning procedures

Firstly, the ESCs were resuspended in DMEM and the number of cells was adjusted to $1x10^7$/ml. Subsequently, 100 µl control cells (eutopic endometrium) were transferred to an Eppendorf tube and 10 µl of the Ph.D.-7 Phage Display Peptide Library (New England BioLabs, Inc.Ipswich, MA, USA) was added, which initially contained $2x10^{11}$ plaque forming units (pfu). The cells were incubated at 4°C for 2 h. A total of 200 µl organic solvent was added to the tube, which consisted of 180 µl dibutyl phthalate (DBP) and 20 µl cyclohexane (Beijing Yiqiangsheng Technology Co., Ltd., Beijing, China). The tube was subsequently centrifuged at 10,000 ×*g* for 10 min. Following centrifugation, the soluble fluid upper layer was pipetted into a fresh tube containing endometriosis cells (ectopic endometrium) and was incubated at 4°C for 3 h. The precipitate was transferred to a fresh tube, and 200 µl Luria-Bertani (LB) with *E. coli* ER2738 (mid-log phase) (Biovector Co., Ltd., Beijing, China) was added and incubated at 37°C for 30 min. Subsequently, the phage was titrated and amplified according to the manufacturer's instructions. Finally, 5 rounds of *in vitro* reiterative biopanning were performed.

ELISA

Following 5 rounds of biopanning, 50 blue plaques were randomly selected and were individually added to *E. coli* ER2738 cultures for amplification and titration. The identified phage clones with different peptide sequences were demonstrated by ELISA in triplicate surveys. The ESCs (ectopic and eutopic) were plated in 96-well plates at a density of 10^4 cells/well. Following 1 h of incubation at 37°C, the selected positive phage clones (10^{10} pfu/well), M13K07 phage (Sangon Biotech Co., Ltd., Shanghai, China) and PBS were added individually to the cells and incubated at 37°C for 2 h. Subsequently, the cells were washed 3 times with PBS and cultured at 37°C for 2 h in the presence of anti-M13 mouse monoclonal antibody (dilution, 1:6,000 Sangon Biotech Co., Ltd., Shanghai, China). Subsequently, the plates were washed and HRP-conjugated goat anti-mouse antibody (dilution, 1:4,000 Sangon Biotech Co., Ltd., Shanghai, China) was added. Following 2 h of incubation, the plates were washed and 200 µl fresh substrate solution (3,3',5,5'-tetrameth-ylbenzidine; Sigma-Aldrich China, Inc., Shanghai, China) was added to each well and the absorbance values at 450 nm were recorded using a plate reader.

DNA sequencing

A total of 13 phage clones were selected if their optical density OD_{450} was >0.5 and the single stranded DNA from the positive phages was purified using an M13 purification kit (Beijing Sunny Instruments Co., Ltd., Beijing, China) according to the manufacturer's protocol. The samples were sent to Sangon Biotech Co., Ltd. for sequencing, and the sequences were analyzed using Vector NTI Advance® soft-ware (version 10.3; Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Immunohistochemical staining

The biotin-RTRLHTR was added to endometriosis, cervical epithelioid cancer, ovarian epithelioid cancer, and normal endometrium samples, which were obtained from the Department of Gynecology and Obstetrics, The Second Affiliated Hospital & Yuying Children's Hospital of Wenzhou Medical University (Wenzhou, China). Following 30 min of incubation at room temperature, the tissue samples were sequentially incubated with anti-M13 mouse monoclonal antibody (dilution, 1:300) for 1 h followed by incubation with the HRP-conjugated goat anti-mouse antibody (dilution, 1:100). A total of 1 h later, 3,3'-diamino-benzidine solution was added and the samples were visualized using a light microscope.

Competitive inhibition assay

The ESCs (ectopic endometrium) were plated into 96-well plates at a density of 10^4 cells/well. Following 1 h of incubation at 37°C, the PBS series diluted synthetic polypeptide RTRLHTR with different concentrations (0, 0.1, 1, 10, 100, and 1000 nM) were added individually to the cells and incubated at 4°C for 1 h. Then, 10 µl of selected P_5 clones was added (2×10^{11} pfu). The cells were incubated at 4°C for 1 h. Subsequently, the cells were washed 3 times with PBS and cultured at 37°C for 2 h in the presence of anti-M13 mouse monoclonal antibody (dilution, 1:6,000). Subsequently, the plates were washed and HRP-conjugated goat anti-mouse antibody (dilution, 1:4,000) was added. Following 2 h of incubation, the plates were washed and 200 µl fresh substrate solution (3,3',5,5'-tetrameth-ylbenzidine) was added to each well and the absorbance values at 450 nm were recorded using a plate reader. The non-specificity of phage encoding in the peptide library was negative, and the experiment was repeated three times. The SAS system (SAS Institute Inc., Cary, NC) with analyses of variance was used for statistical analyses.

Results

Phage display biopanning

In the present study, a 7-mer phage display library was employed to screen the peptides with binding specificity to endometrial cells. During the biopanning processes, the recovered phage number (10^6 pfu/mL) in Parts 1, 2, 3, 4, and 5 of the study were 0.9±0.2, 3.3±0.6, 8.2±1.2, 14.2±1.2, and 26.9±1.9, respectively ($P<0.05$). The fifth biopanning recovery rate was 30-fold more than Part 1 of the study.

ELISA

Following 5 rounds of biopanning, 50 plaques were randomly selected and analyzed using ELISA in triplicate surveys. As demonstrated in Fig.1, the OD_{450} value of 13 clones was >0.5. The M13K07 phage and PBS were used as negative controls. The OD_{450} values of

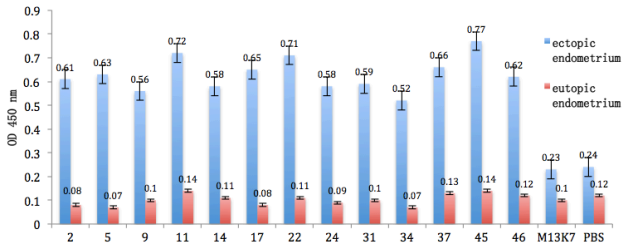

Figure 1. Comparison of selected individual phage clones binding to ectopic endometrium and eutopic endometrium (P<0.05).

these 13 clones reacted with endometriosis were 0.07-0.14 in comparison to those of the controls, which were significantly different (Fig.1).

DNA sequencing

After the ELISA, the OD_{450} value of 13 clones was >0.5 and these phase clones (P_2, P_5, P_9, P_{11}, P_{14}, P_{17}, P_{22}, P_{24}, P_{31}, P_{34}, P_{37}, P_{45}, P_{46}) were sequenced. As demonstrated in Table 1, 6 distinct sequences from the 13 clones were obtained and the most frequent DNA sequence was CGCACCCGCCTGCATACCCGC, with a corresponding amino acid sequence of RTRLHTR. # Error bar indicates standard error.

Competitive inhibition assay

To verify whether the polypeptide isolated from the phage retained its specific affinity toward endometriosis cells and whether the polypeptide and its corresponding phage were competitive with the same binding site, we carried out a study on the competition inhibition of peptides. We found that the binding capacity of the phage clone P_5 for endometriosis cells was reduced in a peptide dose-dependent pattern after pretreatment with the synthetic polypeptide RTRLHTR. In contrast, the control peptide revealed no specific binding activity and had overall poor binding activity. Their OD_{450} values toward the polypeptide RTRLHTR in different concentrations (0, 0.1, 1, 10, 100, and 1000 nmol/L) were 0.64±0.04, 0.59±0.05, 0.54±0.03, 0.46±0.05, 0.38±0.02, and 0.29±0.03, respectively (P<0.05; Fig.2). A RTRLHTR concentration of 1000 nmol/L demonstrated inhibition of 50% of the total binding activity, whereas the control peptide had no such effect (P=not significant; Fig.2). Furthermore, peptide concentrations over 10 nmol/L resulted in significant differences between polypeptide RTRLHTR and controls. Lower concentrations (0, 0.1, 1 nmol/L) resulted in a nonsignificant difference.

Immunohistochemical staining results

Immunohistochemistry was utilized to verify whether biotin-RTRLHTR demonstrated binding specificity. The biotin-RTRLHTR was found to bind specifically to endometriosis cells; the binding efficiencies of the biotin-RTRLHTR toward endometriosis, cervical epithelioid cancer, ovarian epithelioid cancer, and normal endometrium cells were 1.32±0.05, 0.66±0.08, 0.49±0.06, and 0.23±0.04, respectively (P<0.05).

Discussion

Endometriosis is morphologically benign, but has similar characteristics to malignant tumors in its clinical behavior. Therefore, high recurrence rate is a major hindrance to its clinical treatment. Studies show that surgical treatment is an effective means of endometriosis treatment (8) However, complications of this avenue of treatment, such as cystectomy and electrocauterization, often result in loss of the ovary cortex, damage of ovarian function, and correlate with high recurrence rates. Some hormone stimulants, antagonists, and regulators (GnRHa, danazole, oral contraceptives, mifepristone) also have therapeutic effects on endometriosis; currently, GnRHa is the most effective drug (9). Nonetheless, the effects of these pharmacotherapeutic interventions should not be considered as transient, conservative, or preoperative managements (10). In addition to therapeutic effects, a variety of side effects including osteoporosis, vaginal dryness, decreased libido, hot flashes, irregular vaginal bleeding, low back pain, and weight gain are associated with these drugs and cause a decrease in patient compliance. This phenomenon is attributed to the fact that these hormone treatments are not selectively applied to ectopic endometrium cells.

Tumor targeting therapy is a popular approach to tumor therapy that utilizes highly specific tumor material as a carrier to deliver anti-tumor drugs to the tumor site (11-14). Small molecule polypeptides are ideal vectors for anti-tumor targeting drugs; due to their simple structure, ease of preparation, strong penetrating ability, and lack of immunogenicity (15). Phage display technology is considered to be a powerful and high-throughput bio-

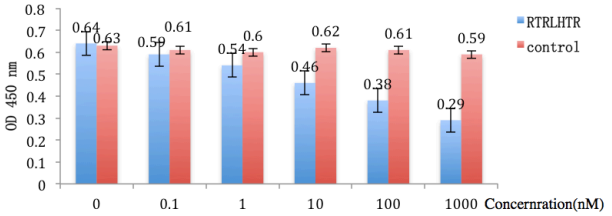

Figure 2. Competitive inhibition of phage clone binding to ectopic endometrium by synthetic peptides.

Table 1. DNA sequences of the 13 clones.

Clones	DNA sequences	Amino Acid sequence	Frequency
P_5 P_9 P_{17} P_{34} P_{46}	CGCACCCGCCTGCATACCCGC	RTRLHTR	5
P_{11} P_{14} P_{31}	CGCCCGCGCAAAATTAGCCGC	RPRKISR	3
P_2 P_{24}	CTGCGCCTGCGCAACACCCGC	LRLRNTR	2
P_{22}	CGCCATCTGCGCCATCGCATT	RHLRHRI	1
P_{37}	CCGATTCGCACCAACCGCAAA	PIRTNRK	1
P_{45}	CGCCCGACCATTCCGACCAAA	RPRIPTK	1

logical technique for screening for tumor cell surface specific binding peptides. Antibodies or polypeptide ligands with molecular affinity to the target can be easily and quickly selected for. However, the greatest advantage of phage display is its direct linkage between phage phenotype and its encapsulated genotype (16), which allows one to proceed without knowing the structural information of the target molecule in advance. Through DNA sequencing of positive phage clones enriched after several rounds of selection, one can indirectly deduce the exogenous peptides presented from the sequence of amino acids. Thus, phage display is capable of serving as a promising new method for the diagnosis and treatment of endometriosis. It has been recently used to isolate and identify high-affinity peptides with surfaces of different tumor cells serving as the targets in endometrial carcinoma (17), ovarian cancer (18), and cervical cancer (19). These peptides have potential application for early diagnosis of tumors, anti-tumor metastasis, and tumor targeting therapy.

In this study, utilization of this combination of biopanning and rapid analysis of selected ligands allowed for screening for endometriosis cell-specific binding peptides improved the efficiency of screening by effectively reducing the nonspecific combination (20). We performed 5 rounds of differential screening, and found phage concentration increased from 0.9±0.2 to 26.9±1.9. The fifth biopanning recovery rate was 30-fold more than Part 1 of the study, suggesting that the conjugated phage was enriched. After 5 rounds of biopanning, 50 plaques were randomly selected to be used for an ELISA test and DNA sequencing. This resulted in a total of 13 phages (P_2, P_5, P_9, P_{11}, P_{14}, P_{17}, P_{22}, P_{24}, P_{31}, P_{34}, P_{37}, P_{45}, P_{46}) containing 6 unique amino acid sequences. The DNA sequence was found to be enriched by the fragment CGCACCCGCCTGCATACCCGC, which has the corresponding amino acid sequence RTRLHTR. This suggests that the polypeptide encoded by the insertion sequence may target endometriosis. Based on these results, the amino acid sequence RTRLHTR displays the most potential research value. Therefore, we proceeded to identify both the affinity and specificity of the polypeptide RTRLHTR to endometriosis after its isolation from phage capsid proteins. A synthetic polypeptide RTRLHTR was used to identify the binding specificity of polypeptide RTRLHTR for endometriosis cells using a combination of cellular immunohistochemistry and a competitive inhibition assay. We found that the binding capacity of the phage clone P_5 for endometriosis cells was reduced in peptide a dose-dependent manner following pretreatment of the synthetic polypeptide RTRLHTR. This indicates that a polypeptide removed from its source phage will retain its specific affinity toward endometriosis cells. The results of immunohistochemical staining demonstrated that the polypeptide RTRLHTR was specific to and had a high affinity for ectopic endometrial cells, but was not specific to other tumor cells or normal endometrial cells. We speculate that using a combination of 2 or more of these peptides is expected to result in a superior targeting effect for treatment of endometriosis.

To summarize, phage display technology was employed to screen for and identify a new peptide sequence. The resulting polypeptide RTRLHTR showed a high affinity for endometriosis cells and is a promising candidate for use in drug therapies that target endometrial cells. This technique could potentially be used in conjunction with liposome-based hormone therapy and improve upon the specificity and effectiveness of hormone therapy used in treatment of endometriosis. While there are still many problems to be considered, including the improvement of affinity, the study of pharmacokinetics, and the application of peptides in living animals, we expect this combination to result in the inhibition of ectopic endometrial cell growth without causing systemic side effects.

Acknowledgements

The study was supported by grants from the Zhejiang Provincal Natural Science Foundation (no.LY16H040011), and Wenzhou Technology Bureau,Wenzhou,China (no.2014S0272 and no.2015Y0372).

Authors' contributions

YL, CD, and **LJ** designed the protocol and concept for the experiment. **CD, ASK, WL,** and **LW** performed the experiment. **YL, CD,** and **ASK** drafted the manuscript. All authors contributed to the final revision of the manuscript. All authors have read and approved the final manuscript for publication.

Competing interests

The authors declare no competing interests.

References

1. Henry KA, Arbabi Ghahroudi M, Scott JK. Beyond phage display: Non-traditional applications of the filamentous bacteriophage as a vaccine carrier, therapeutic biologic, and bioconjugation scaffold. Front Microbiol 2015; 6:755.

2. Brown KC. Peptidic tumor targeting agents: the road from phage display peptide selections to clinical applications. Curr Pharm Des 2010; 16(9):1040-54.

3. Oyama T, Rombel IT, Samli KN, Zhou X, Brown KC. Isolation of multiple cell-binding ligands from different phage displayed-peptide libraries. Biosens Bioelectron 2006; 21(10):1867-75.

4. Takakusagi Y, Takakusagi K, Sugawara F, Sakaguchi K.. Use of phage display technology for the determination of the targets for small-molecule therapeutics. Expert Opin Drug Discov 2010; 5(4):361-89.

5. Ueberberg S, Schneider S. Phage library-screening: a powerful approach for generation of targeting-agents specific for normal pancreatic islet-cells and islet-cell carcinoma in vivo. Regul Pept 2010; 160(1-3):1-8.

6. Zhang J, Li H, Wang X, Qi H,Miao X, Zhang T et al. Phage-derived fully human antibody scFv fragment directed against human vascular endothelial growth factor receptor 2 blocked its interaction with VEGF. Biotechnol Prog 2012; 28(4):981-9.

7. Schüller C, Wiebe JC, Pegel A, Kramer K, Skerra A,Hock B. A system for repertoire cloning and phage display of murine and leporid antibody fragments. J AOAC Int 2010; 93(1):66-79.

8. Mu BR, Zhang ZY, Xu HM. Correlative factors analysis and effect of pelvic pain associated with in endometriosis after conservative surgery. Chin J Obstet Gynecol 2014; 09:681-4.

9. Lang JH, Leng JH, Wang ZH. Consensus on the complementary application of gonadotropin-releasing hormone agonist in endometriosis and uterine fibroids. Chin J Obstet Gynecol 2017; 02:77-81.

10. Bian ML, Huang SH, Deng WH, Song J,Liang Z, Zhang Y et al.

Randomized, blind, parallel-controlled and multiple-centre clinical trial on the effectiveness and safety of leuprolide acetate in the treatment of endometriosis. Chin J Obstet Gynecol 2015; 3:188-93.
11. Velasco Velázquez M, Xolalpa W, Pestell RG. The potential to target CCL5/CCR5 in breast cancer. Expert Opin Ther Targets 2014; 18:1265-75.
12. Arnaiz B, Madrigal-Estebas L, Todryk S, James TC, Doherty DG, Bond U. A novel method to identify and characterise peptide mimotopes of heat shock protein 70-associated antigens. J Immune Based Ther Vaccines 2016; 4:2.
13. Wang J, Wang XW, Wang YW, Ma DX, Wang P. Paclitaxel-octreotide conjugates inhibit growth of human small cell lung cancer cells. Journal of Shandong University (medical edition) 2011; 49(3):27-32.
14. Zhang XY, Sun LX, Rong Y, Ran YL,Yang ZH. Targeting therapy of lung cancer using functional monoclonal antibody 40E11. Chin J Cancer Prev Treat 2012; 19(6):401-5.
15. Bastien JI, McNeill KA, Fine HA. Molecular characterizations of glioblastoma, targeted therapy, and clinical results to date. Cancer 2015; 121:502-16.
16. Kaplan G, Gershoni JM. A general insert label for peptide display on chimeric filamentous bacteriophages. Anal Biochem 2012; 420(1):68-72.
17. Lu L, Zhong XY, Wang ZN, Yan YL,Bin DU . Selection and analysis of targeted cell-binding mimicry peptides in endometrial adenocarcinoma cell line through random peptide phage display. Chin J Pathophysiol 2005; 06:1136-8.
18. Li WJ, Wang LD, Lv JQ. Screening metastasis related peptides binding to the surface of ovarian tumor cells by phage display. J Wenzhou Med Univ 2014; 44(02):127-33.
19. He XJ, Guo CX, Yang C, Liang XQ. Screening and identification of the peptides specifically binding to cervical cancer HeLa cell. China Oncology 2012; 22(03):173-6.
20. Wang LD, Hu Y, Li WJ, Wang F, Lu X, Han X et al. Identification of a peptide specifically targeting ovarian cancer by screening a phage display peptide library. Oncol Lett 2016; 11:4022-6.