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Review Article

Screening of tumor-targeting peptides for diagnosis and therapy through phage display technology

Zhiruo Song[1], Yufeng Zhang[1], Yuanjie Zhu[1,2], Mingming Zhang[2], Xinjian Liu[1,2*], Minjun Ji[1]

[1] *Department of Pathogen Biology, Nanjing Medical University, Nanjing, Jiangsu 211166, China;*
[2] *Key Laboratory of Antibody Techniques of National Health Commission, Nanjing Medical University, Nanjing, Jiangsu 211166, China.*

ABSTRACT

Phage display technology was introduced by G. Smith in 1985, which is highly effective in the selection of affinity peptides from a library containing billions of display peptides. The obtained peptides show potential efficacy in the development of further clinical applications, especially in tumor treatment. In this review, the basic principles, limits, developments of phage display technology and peptide libraries are introduced. Following that, the amino acid sequence of tumor target peptides for hematological and other systems are discussed. Finally, the application of target peptides in the design of imaging probes and the development of target peptide drugs for diagnosis and therapy are noted.

Keywords: tumor-targeting peptide, phage display technology, diagnosis, drug discovery

INTRODUCTION

Since the invention of phage display technology, a large number of phage peptide libraries have been constructed to screen for specific peptides. Peptides are substances formed by amino acids attached by peptide bonds, usually with a length of less than 50 amino acids[1]. So far, the application of peptides in tumor diagnosis and therapy has been widely accepted, involving both hematological and solid tumors. Tumor-targeting peptides, designed as carriers to transfer drugs to corresponding tumor cells, have been successfully applied to target therapy and as imaging agents[2], achieving precise medication delivery with few side effects. In this regard, for the special morphology of blood system tumors, target therapy is employed at an important stage, meanwhile, target peptides for solid tumors are constantly being studied. In this review, the development of phage display technology, the bio-panning process, and tumor target peptides, which are obtained from screening *in vitro* or *in vivo*, as well as the practical employment of these peptides are introduced.

PHAGE DISPLAY TECHNOLOGY

History and principle

In 1985, Smith first presented phage display technology by inserting a foreign gene into filamentous phage gene Ⅲ to produce a fusion protein for this specific ability[3]. In the process, pⅢ (the expression product of filamentous phage gene Ⅲ) function was not unduly disrupted by the exogenous sequence displayed on the phage coat protein. Since

*Corresponding to: Xinjian Liu, Department of Pathogen Biology, Nanjing Medical University, 101 Longmian Avenue, Nanjing, Jiangsu 211166, China. E-mail: liuxinjian@njmu.edu.cn.

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the exogenous sequence was shown on the viral surface[4], the phage carrying a peptide with a high affinity to the target could be picked up. The construction of a phage display library was described by Smith and Scott in 1993[5], in which a large number of peptide sequences could be presented by using degenerate synthetic oligonucleotides. To this aim, Winter and his colleagues employed filamentous phage to screen for antibodies as a typical application of phage display technology, enhancing this technology's practicality[6]. For their contribution in the field of phage display and its medical application, Smith and Winter were jointly awarded one-half of the Nobel Prize in Chemistry in 2018.

Construction of a phage peptide library

For inserting degenerate oligonucleotides into the phage genome, a random peptide library was obtained covering billions of peptide sequences[5]. M13 phage was the first kind of phage applied to display peptides, and subsequently, T7, T4, and λ phage were introduced as options[7].

M13 phage is a filamentous bacteriophage. Its infection process is initiated by the interaction between pⅢ at the end of the bacteriophage particle and *E. coli* F pili. The bacteriophages are assembled in *E. coli*, involving the synthesis of bacteria and phage proteins. Phages are secreted through the outer membrane of the host without lysing the host[8]. Subject to the correct expression of gene Ⅲ, the length of the cloned foreign peptide can be restricted ranges from 6 to 43 amino acids successfully *via* sustained studies. $(NNK)_n$ codon degeneracy is commonly used to produce random peptides. To generate the peptide libraries, the usual approach is to add an amber stop codon at the beginning of gene Ⅲ of the phage genome[9]. The design of $(NNK)_n$ is pivotal, where N represents an equimolar mixture of the deoxy-nucleotides (A, T, C, and G) and K represents an equal mixture of T and G[10].

The biological process of T7 phage, as a lytic phage, is different from that of M13 phage[11]. Considering that the peptides expressed by filamentous phage need to be compatible with host cells, the diversity of its expressed peptide library is relatively limited. In contrast, lytic phage T7 is more dominant in the diversity of peptide libraries. M13 phage is the most widely used phage species in phage display technology. T7 phage is relatively focused on antibody screening, while T4 and λ phage have relatively few applications.

Bio-panning process

It is important to select and determine the targets before bio-panning. The molecular forms, such as the antibody, peptide, protein, and nucleic acid, were employed as the targets' type[12–13]. To overcome the difficulty of purification of cell surface markers, the whole cell is also chosen as the target[14].

Bio-panning is a bridge between phage display and protein screening. Before bio-panning, the phage peptide library should be constructed and amplified. The bio-panning process consists of adsorption, elution, and amplification. The adsorption refers to the incubation of the peptide library within the target. Before elution, firstly unbound phages should be removed through repeated washes. Following that, the specifically bound phages are recovered from eluent and amplified in *E.coli* to receive ample bound phages for the next round of bio-panning. A schematic diagram is presented below, showing the screening of the phage peptide library, resembling a circle (***Fig. 1***). After the last cycle of panning, a sufficient amount of target-binding phage clones could identify the affinity with the target by ELISA, while the corresponding nucleotide sequence of the peptide is obtained *via* DNA sequencing[15].

Protein can be screened *in vitro or in vivo*. *In vitro* screening is usually completed first, with molecules or whole cells as targets. *In vivo* screening which was first proposed in 1996[16] differs from *in vitro* screening in the bio-panning process. The increased operation appears when the phage peptide library needs to be introduced into model animals *via* systemic intravenous injection. Binding to the target completes within 1 –2 hours, and then, the model animals are perfused to remove unbound phages to improve the specificity[17]. Using tissues and organs as the targets, some peptides are selected even if the target molecules or their structural domains are unknown.

Limits and improvements of phage display technology

Unquestionably, phage display technology is a powerful methodology for the screening and identification of specific peptides binding to a designated target. However, false-positive target-unrelated peptides (TUPs) set an inevitable barrier, including selection-related and propagation-related TUPs[18]. Selection-related TUPs are related to the affinity of materials and reagents with phage peptides[19], while propagation-related TUPs (Pr-TUPs) are caused by the propagation advantage of some phage clones, meaning that some phages displaying Pr-TUPs grow faster in host cells. Phages show a faster growth rate, allowing them to exceed

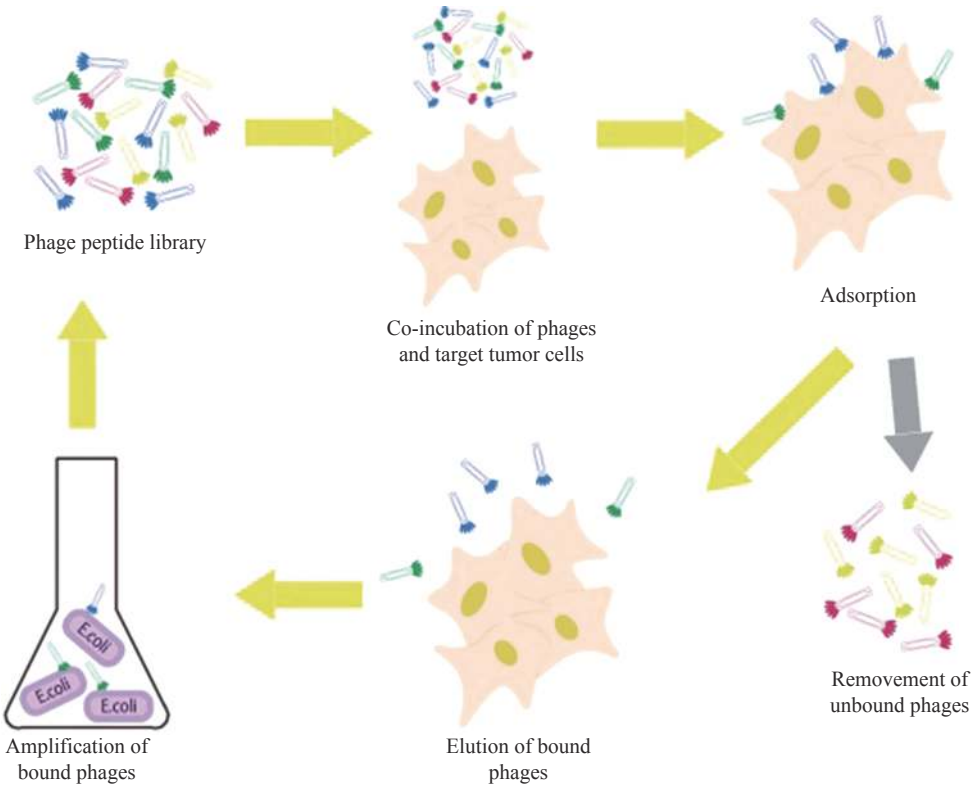

***Fig. 1* The general strategy of phage bio-panning targeting tumor cells.** When the phage peptide library is constructed, phages and target tumor cells incubate to realize adsorption of specifically bound phages. After removement of unbound phages, bound phages can be eluted to be amplified to be the raw material of phage peptide library used in the next round panning.

other clones in the library and are more likely to be discovered[20]. Negative bio-panning is a new method proposed to inhibit the enrichment of TUPs, *via* depleting the library[18]. Moreover, SAROTUP (Scanner and Reporter of Target-Unrelated Peptides) is a powerful tool to rapidly identify TUPs in phage display data[21].

Considering the interference coming from the phages' non-specific binding and steric hindrance caused by blocking agents, researchers introduced two modified methods. One was designed to omit the initial blocking step and added operations at the final stage to the unblocked vessel surface, so as to remove non-specifically bound peptides. The other was the method where phages bind to targets in the liquid phase, and the complexes were fixed on the surface of the vessel[22]. Through the above approaches, certain sites on the target are prevented from being blocked by BSA, thereby the chance of contact between the target and the phage peptides is increased.

Cyclic peptides have more advantageous properties compared with ordinary small peptides, working with higher affinity and selectivity to the target, as well as having a more stable and easily modified structure[23]. Based on these characteristics, the phage cyclic peptide library has become a new tool to find more valuable drugs. By establishing disulfide bonds to cyclize the peptide chains, phage cyclic peptide libraries have been used in the development of streptavidin ligands, and commercial phage cyclic peptide libraries are available[24]. Cyclized peptides are not only efficient in the screening of tumor-targeted peptides but also could be an important tool for screening in the future.

SPECIFIC PEPTIDES BINDING TO TUMOR CELLS

With these developments, phage display technology has been widely employed for screening tumor affinity peptides. Tumor affinity peptides can be selected from millions of various peptides, which are applied in tumor diagnosis and targeted therapy.

The peptide WSLGYTG was found to specifically bind to T lymphocytes *via* CD3ε expressed on its surface[25]. CD3ε is the subunit of the CD3 complex uniting with T cell receptors, which plays the role in antigen recognition and T cell activation[26]. Because of this, it has potential applied prospects in the treatment of T lymphocyte tumors.

Colorectal cancer has been the third most commonly diagnosed cancer[27] and the second leading

cause of cancer death around the world[28]. The peptide, CBP-DWS (DWSSWVYRDPQT), was confirmed showing high affinity for COLO320HSR (human colon cell line) rather than NCM460 (the normal human intestinal epithelial cell line) by phage display technology[29]. Ferreira *et al.* found that the peptide sequence CPKSNNGVC can bind to the colorectal cancer cell line RKO with high specificity and affinity, and is considered as a candidate for targeted therapy of colorectal cancer. Moreover, this peptide was verified able to bond with other human colorectal cancer cells including HCT-15, HCT-116, and Caco-2[30]. The two peptides mentioned above show specific affinities for colorectal cancer cells, but not normal cells. The peptide (pHCT74) observed also has a specific affinity for colorectal cancer cells, and could be applied to carry tumor drugs by binding to liposomes, performing the potential application for target drug delivery for colorectal cancer treatment[31].

Sahin *et al.* performed MKN-45 human gastric cancer cells as target cells and HFE-145 human normal gastric epithelial cells as the control group. DE532 phage clone with amino acid sequence (VETSQYFRGTLS) represented a specific affinity with MKN-45 gastric cancer cells. Furthermore, a more valuable peptide, DE-Obs (HNDLFPSWYHNY), which was designed on the base of the peptide sequence accepted in the 5th round of bio-panning, showed specific binding on MKN-45 cells[32].

For females, breast cancer is the most common cause of cancer mortality, followed by colorectal cancer[33]. A positive peptide (GYSASRSTIPGK) was found by Liu F *et al.* through several rounds of bio-panning[34]. Meanwhile, to achieve precise treatment in breast cancer, X. Qu *et al.* used phage display technology for *in vivo* screening to obtain the required tumor homing peptide and coupled the peptide (AREYGTRFSLIGGYR) with gold nanorods to enhance the killing effect on breast cancer cells[35].

Phage peptide library technology makes it convenient to screen for tumor affinity peptides and provides important technical support for the discovery of more affinity peptides, which might be applied to the diagnosis and treatment of clinical tumors in the future. Based on previous experiments[36], our team is focusing on cyclin-dependent kinase 9 (CDK9) as the target, which plays its signal transduction role in the proliferation of cervical cancer cells.

APPLYING PEPTIDES IN CLINICAL DIAGNOSIS AND TREATMENT

Researches on tumor-specific binding peptides are ongoing, while obtained peptides are coupled with fluorescent or radioactive substances to achieve imaging for the early diagnosis of tumors. Meanwhile, tumor-specific binding peptides provide a direction for tumor drug development. Usually, peptides act as carriers to achieve targeted drug delivery, or the affinity peptide itself has an inhibitory function for tumors and therefore has potential as an anti-tumor drug.

Target peptides and molecular fluorescence imaging of tumors

Molecular fluorescence imaging makes tumor-specific or up-regulated proteins visible, records tumor tissue in real-time in high resolution, and monitors the tumor microenvironment[37], which can be applied to tumor diagnosis, treatment, and drug development[38]. The efficient clinical application of molecular fluorescence imaging depends on the research and development of molecular probes.

Bladder cancer cell-specific peptides screened *in vivo* by phage display technology have shown a favorable foreground since the establishment of bladder cancer-specific bimodal imaging probes. An *in vitro* air-pouch bladder cancer model suitable for probe infusion was designed to assess the effect of photoacoustic imaging (PAI) diagnosis and near-infrared (NIR) imaging-guided resection for bladder cancer under the indication of the probe. The study of PLSWT7-DMI indicates it as a suitably selective and specific probe for the diagnosis and resection of bladder cancer, reducing the recurrence rate of bladder cancer[39]. Similarly, phage display technology has also been applied to the development of new targeting agents for the imaging of different breast cancer subtypes. Nuclear imaging traces of specific biomarkers of breast cancer enable the detection of changes in marker expression during disease development, which recognized human epidermal growth factor receptor 2 (HER2), estrogen receptor (ER), or progesterone (PR) as detection targets, to guide clinicians in choosing a personalized treatment plan for the best treatment results[40]. To combat kidney tumors overexpressing KIM-1, the high-affinity peptide CNWMINKEC was identified by M. E. Haque. The progress of CNWMINKEC peptide binding to 769-P cells was specifically mediated by KIM-1, proven by co-localization and competition analysis using the anti-KIM-1 antibody. Peptide CNWMINKEC is non-cytotoxic to cells and is stable in serum up to 24 hours, indicated as a useful probe for imaging and diagnosing tumors with high expression of KIM-1 *in vivo*[41].

In addition to targeting tumor cells, and considering

the significance of tumor angiogenesis in tumorigenesis, some receptors in the tumor vasculature have been chosen as targets for design imaging probes to optimize tumor diagnosis and treatment[42]. The extra domain B splice variant (EDB), which is selectively expressed in the tumor vasculature, was also considered as a target for imaging and treatment of cancer tissues, and some selected specific ligands for EDB have been delivered through phage display technology[43]. As a small molecule protein, it is expected to be used for further probe development.

The targeting polypeptide molecule can be used as a carrier molecule combined with the image marker to participate in probe synthesis. However, a large amount of probe development is in either the research or clinical trial stage, so more research is still needed to make the peptide molecular probes required for early tumor diagnosis and treatment evaluation to become a reality.

Target peptides and drug development

Target peptide molecules also show great prospects for drug development. One way is to use it as a carrier molecule, coupled with a drug molecule, then to deliver it to the targeted lesion site. Another way is to focus on screening peptides with ideal biological functions to provide more possibilities for tumor treatment. Below, currently known tumor-targeting peptides obtained from the phage peptide library are introduced, whose classification method is based on their respective targets.

HER2/neu

HER2 is overexpressed in about 30% of human breast cancers. HER2/neu stimulates the Phosphatidylinositol-3 kinase-AKT-NF-κB pathway to activate the mitogenic cyclin D1/Cdk4-Rb-E2F pathway. Further research found that HER2/neu-derived peptide GP2 can be combined with nanoparticles to develop drugs for breast cancer with HER2/neu overexpression[44].

CD44

CD44 is a single-chain glycoprotein, containing 19 exons in the human body. The middle 9 exons are alternatively spliced and assembled with the other ten exons, which are referred to as CD44 variant isoforms (CD44v)[45]. CD44 shows function in the regulation of cells' microenvironment, *via* binding to hyaluronic acid (HA), chondroitin, collagen, fibronectin, and other extracellular matrix components as a receptor, while simultaneously existing as a cofactor for growth factors and cytokines. CD44 binding to HA assists the gathering of Versican (VCAN), a chondroitin sulfate proteoglycan, and enhances the aggressiveness of cancer cells[46]. CD44, especially CD44v, has been proven as a marker of cancer stem cells (CSC), participating in gene expression of cell-matrix adhesion, cell proliferation, migration, and differentiation[45]. CD44 is highly expressed in several cancers, including pancreatic cancer, breast cancer, and gastric cancer. Current research is focusing on the establishment of a synthetic phage library and screening at the peptide or cell level to obtain substances with a high affinity to CD44, which are expected to block CD44-mediated signal pathways and become a potential drug for tumor treatment[47]. A CD44-specific peptide, RP-1, exhibits capacities for assisting the diagnosis and prognosis assessment of gastric cancer[48].

CD56

In addition to being widely expressed on the surface of natural killer cells, CD56 also appears in multiple tumors, such as NK/T cell lymphoma, leukemia, and small cell lung cancer (SCLC)[49]. Researchers used the T7 phage library to exploit a new peptide that binds to CD56, calling it 'Natein'. *Via* the process of staining, the combination of 'Natein' and NK/T lymphocyte tumors showed the potential to act as a CD56 binding peptide instead of CD56 antibody for $CD56^+$ tumor cell isolation and cancer therapy[50].

Fibroblast growth factor (FGF)

Fibroblast growth factor regulates growth, migration, and differentiation of multiple cell types, *via* signaling through its receptors, FGFR1, FGFR2, FGFR3, or FGFR4[51]. The FGF/FGFR network is disturbed in some tumors, due to oncogenic fusions, activating mutations, and gene amplification[52]. Thus, the targeting inhibitors of FGF or its receptors are the focus of tumor therapy in basic or preclinical studies. Target peptide VLWLKNR has a significant inhibitory effect on fibroblast growth factor 3 (FGF3), which arrests the cell cycle at G0/G1 phase by inhibiting cyclin D1, and inhibits FGF3-induced extracellular signal-regulated kinase 1/2 and Akt activated kinase, considered as a promising potential drug in tumor treatment[53].

Vascular endothelial growth factor (VEGF)

VEGF is an important class of angiogenic factors involved in the generation of vascular endothelial cells[54]. During various tumors, there is a phenomenon whereby the expression of VEGF is up-

regulated with VEGF-mediated signaling pathways, regulating tumor angiogenesis and participating in tumorigenesis[55]. Therefore, VEGF is regarded as an important target for tumor therapy, by the direct or indirect inhibition of VEGF-mediated signaling pathways. Taking into account the role of VEGF, S. Zuo *et al.* designed T4 recombinant phages, which expressed the extracellular domain of VEGFR2. *In vivo* experiments confirmed that the T4-VEGFR2 phages could decrease microvascular density and inhibit tumor growth, by binding to VEGF and suppressing its downstream signal transduction mediated by VEGFR2 in murine models of Lewis lung carcinoma and colon carcinoma[56]. The VEGF-Fc segment fusion protein was taken as the target in another study and a novel peptide was obtained, namely HRTTKQRHTALH. This peptide was found to inhibit endothelial cell proliferation *in vitro* and *in vivo* in a dose-dependent fashion and may become a potential drug for inhibiting angiogenesis[57].

G-protein-coupled receptor (GPR55)

G-protein-coupled receptor (GPR55), whose ligand is L-a-lysophosphatidylinositol (LPI)[58], widely presents in human tissues and participates in the pathogenesis of cancer[59]. After inhibition of GPR55 by drugs or other methods, the cell cycle was arrested by activation of mitogen-activated protein kinase (MAPK) signaling and protein levels of ribonucleotide reductases were reduced, to inhibit the proliferation of pancreatic cancer cells[60]. LPI-GPR55 complexes promoted breast cancer cell migration *via* two signaling pathways - HBXIP/p-ERK1/2/Capn4 and MLCK/MLC[61]. Small molecule libraries have traditionally been used to identify potential drugs, however, new methods including the design of phage cyclic peptide libraries have been developed for drug discovery. Cyclic peptides with inherent advantages in structure display the high affinity and stability[62]. Researchers selected a library of CX7C cyclic-peptide to screen an allosteric modulator of the G-protein-coupled receptor GPR55, namely CCKNSPTLC, which stimulates GPR55 endocytosis and inhibits GPR55-mediated proliferation of EHEB and DeFew B-lymphoblastoid cell lines[63].

SUMMARY AND PERSPECTIVES

Phage display technology has played a powerful role in the screening of affinity peptides. The screening of high-affinity peptides for tumor cells provides a drug basis for the early diagnosis and targeted therapy of tumors. The phage peptide library is limited by the established conditions of the technology itself, and the length of the peptides. The final peptides obtained by screening are affected by the experimental process and the biological characteristics of the phage. When certain screened peptides bind to corresponding target molecules or cancer cells, there is the possibility of binding to other tissues or molecules. The safety of probes or drugs based on peptides still need to be considered. In existing experimental studies, most of the peptides obtained by screening are used as targeting carriers to design probes or deliver tumor suppressor drugs. Only a few experimental peptide results show tumor-suppressive effects. The acquisition of peptides with high affinity and specificity is the most ideal experimental result. Given the higher affinity of cyclic peptides and the advantages of biological configuration, the establishment of phage cyclic peptide libraries has become a new advantageous development direction.

In recent years, the phage peptide library has been widely used in the development of new anti-tumor drugs, pre-cancerous molecular imaging diagnosis, and treatment research. When cells are used as targets, the exploration of their specific target molecules and downstream pathways can be envisaged, and more mysteries in the pathogenesis of cancers should be elucidated. In the future, more peptides might be found and further modified, to achieve both wider and more efficient clinical applications. Phage peptide library technology will be employed much more widely in the field of cancer prevention, diagnosis, and treatment.

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