

Journal of Molecular Biomarkers & Diagnosis

Review Article

Open Access

Peptide Phage Display for Discovery of Novel Biomarkers for Imaging and Therapy of Cell Subpopulations in Ovarian Cancer

Mette Soendergaard¹, Jessica R Newton-Northup¹, Mark O Palmier¹ and Susan L Deutscher^{1,2*}

¹University of Missouri, Department of Biochemistry, Columbia, MO 65211 ²Harry S Truman Veterans Memorial Hospital, Columbia, MO, 65201

Abstract

Ovarian cancer is a very aggressive disease that is mostly asymptomatic at early onset. Approximately 85% of patients are diagnosed at late-stage disease, which greatly compromises full recovery. Standard detection methods include measurement of the ovarian cancer biomarker CA-125. However, CA-125 is associated with false positive diagnosis and is largely limited to late-stage disease. As a result, there is a great need to discover new biomarkers and develop novel detection and imaging methods for ovarian cancer. Patients with ovarian cancer often respond to initial chemotherapy but most will succumb to recurrent disease. Such poor prognosis is associated with a drug resistant subpopulation of cancer cells with stem-like properties known as cancer stem cells (CSC). Traditional chemotherapy fails to target CSC, and it is widely accepted that this process leads to the recurrence of more aggressive tumors. Therefore, it is essential to discover new ovarian CSC biomarkers and develop therapies that specifically target this subpopulation. Bacteriophage (phage) display technology allows identification of high affinity peptides by screening of peptide libraries against cellular targets. The large amount of unique peptides in a library facilitates high throughput selections both in vivo and in vitro. Here we discuss how phage display can be utilized to discover novel peptides with high binding affinity for normal ovarian cancer cells and ovarian CSC. Such peptides may be radiolabeled and employed in SPECT and PET imaging as well as in therapeutic settings. Further, both phage and phage display derived peptides can be employed in identification of targeted antigens and novel ovarian cancer biomarkers using mass spectrometry analysis. Such biomarkers may be utilized in diagnosis and in identification and selection of ovarian cancer subpopulations.

Keywords: Ovarian cancer; Phage display; Peptide; Cancer stem cells

Ovarian Cancer

Ovarian cancer is the fifth leading cause of cancer deaths in women and is the most lethal of the gynecological malignancies [1,2]. In 2011, it is estimated that 21,990 women in the US will be diagnosed with ovarian cancer and that 15,450 women will die from the disease [3]. Ovarian cancer is an aggressive disease that is characterized by a symptom free onset and early metastasis. Neoplastic cells rapidly invade surrounding tissues through the peritoneal fluid and metastasize predominantly as fluid containing ascites in the peritoneal cavity [4,5]. Approximately 85% of patients are diagnosed at late-stage disease, which leads to significantly decreased five-year survival rates of merely 30-45% [1,2]. Furthermore, ovarian cancer often develops resistance to therapy after initial platinum-based treatment, and even though most patients respond to chemotherapy, the majority relapse within 18 months and succumb to disease [1,6-8].

Ovarian cancer is a very heterogeneous disease that comprises three major types: epithelial, stromal and germ cell of which the former represents about 95% of diagnosed cases. Epithelial ovarian cancer can be further divided into eight subtypes: endometrioid, mucinous, serous, clear cell, transitional, squamous, undifferentiated and mixed epithelial that each exhibit different molecular and morphological characteristics [7,9-11]. In addition to the large diversity among ovarian cancer subtypes the largely asymptomatic early stages of the disease complicates diagnosis and treatment. At present, standard detection methods include measurement of the serum tumor-marker CA-125 (Mucin 16) as well as pelvic ultrasonography [12-14]. However, CA-125 levels are often negligible in early-stage disease and elevated in only 80% of advanced stage ovarian cancer. In addition, false positive CA-125 levels are common for a range of other conditions such as endometriosis, inflammatory disease and other cancers, and the method is, therefore, often not sufficient to be diagnostic. Ultrasonography of the pelvis is often used in combination with CA-125 levels to diagnose ovarian cancer, nevertheless, early stage tumors are difficult to detect using this method, and false-positives often arise due to benign cysts related to overactive ovaries [12-16]. Several attempts have been made to find novel serum tumor markers of early-stage ovarian cancer, including measurements of soluble epidermal growth factor receptor (sEGFR) [17,18], soluble cytokeratin 19 fragments [19], serum human kallikreins [20-23] and serum vascular endothelial growth factor (VEGF) [24,25]. However, most of these biomarkers are limited to advanced stage or metastatic disease and are, therefore, not sufficiently sensitive for early-stage ovarian cancer screening and diagnosis. For these reasons, it is necessary to develop new detection methods for both early- and advanced stage ovarian cancer.

Treatment of ovarian cancer most often includes cytoreductive surgery followed by a range of chemotherapeutic therapies dependent on disease stage [1,6]. Combinations of the drugs paclitaxel, carboplatin, cisplatin and cyclophosphamide are often used to treat both early and late-stage disease following surgery and most patients respond to this treatment. Nonetheless, the majority of patients relapse within 18 months with therapy resistant disease, which cause median

*Corresponding author: Susan L Deutscher, Ph.D., University of Missouri, Department of Biochemistry, Columbia, MO 65211, Tel: 573-882-2454; Fax: 573-884-4597; E-mail: deutschers@missouri.edu

Received September 30, 2011; Accepted November 15, 2011; Published November 19, 2011

Citation: Soendergaard M, Newton-Northup JR, Palmier MO, Deutscher SL (2011) Peptide Phage Display for Discovery of Novel Biomarkers for Imaging and Therapy of Cell Subpopulations in Ovarian Cancer. J Mol Biomark Diagn S2:004. doi:10.4172/2155-9929.S2-004

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survival times as low as 24 months post diagnosis [1,6-8,26]. Such poor prognosis seems to result from chemotherapy treatment that targets only the bulk of the tumor cells and fails to target the more aggressive cancer initiating cells (CIC) or cancer stem cells (CSC). This process most likely causes the occurrence of more aggressive tumors that are resistant to therapy (Figure 1) [27-29].

Ovarian Cancer Stem Cells

Cancer stem cells were first observed in acute myeloid leukemia [30] and have since been discovered in several solid tumors including breast, prostate, melanoma and ovarian cancer [31-34]. Ovarian CSC were first isolated by Bapat and co-workers (2005) from ascites in a patient with advanced disease. The cells were shown to display the stem cell surface markers CD44 (hyaluronic acid receptor) and CD117 (ckit) as well as the intracellular stem cell markers Nestin, Oct-4 and Nanog [31,35]. Later, ovarian CSC were found to express aldehyde dehydrogenase (ALDH) and the cell surface marker CD133 (prominin-1), [36,37]. The expression of these CSC biomarkers has been correlated with increased resistance to chemotherapeutic drugs. In fact, CD44 positive cells have been associated with resistance to the chemotherapeutic drugs carboplatin and paclitaxel [35], and CD133 expression has been correlated with resistance to cisplatin [37]. A subpopulation of cells expressing the biomarkers CD44 and CD117 from primary human ovarian tumors were shown to form floating spheroids in culture when grown under stem cell conditions (serum free, EGF, bFGF and insulin). The spheroids visually resembled spheroids found in ovarian cancer ascites [28,31] as well as cultured spheroids from breast and neural tissue stem cells [38,39]. Later CD133 and ALDH positive cells from ovarian epithelial carcinomas were also shown to form spheroids in culture and in addition cause larger and more rapid tumors to form compared to CD133 and ALDH negative cells [36]. The aggressiveness of CSC is also evident from their ability to initiate tumor formation. In fact, as few as 100 dissociated spheroid cells have been found to cause full establishment of tumors in mice, whereas up to 105 of unselected cells were unable to initiate tumor growth [40]. In addition, these cells were able to serial propagate and established heterogeneous tumors with original phenotype after several rounds of propagation. The chemoresistance of CSC is associated with expression of the membrane efflux transporter ABCG2 [27,41,42], which has been found to be upregulated in CSC from primary ovarian tumors and in both murine and human ovarian cancer cell lines [27,40,43]. Increased drug-efflux in CSC has been based on their ability to efflux the lipophillic dye Hoechst 33342 [27,28,44]. Side populations of mouse ovarian cancer cells exhibiting reduced Hoechst 33342 staining have been shown to increase tumorigenesis in nude mice [28]. A side population has also been observed in the human ovarian cancer cell line SK-OV-3, where approximately 10% of the cells showed reduced Hoechst 33342 staining [27]. Taking these results together it seems likely that the development of molecules that target CSC may hold the key to increase the therapeutic efficiency for ovarian cancer. So far most studies have focused on drug candidates that inhibit cellular signaling pathways [45], however, it may be necessary to target CSC cell surface biomarkers that are independent of the ABCG2 drug-efflux system. Cancer stem cell targeting radiolabeled peptides may provide an efficient method to eradicate the CSC subpopulation.

Bacteriophage Display

Bacteriophage (phage) display technology was first developed by Dr. George Smith in 1985 [46]. The technology involves the expression of combinatorial peptide libraries on filamentous phage coat proteins. Every phage in a library expresses a unique peptide, and this diversity can be used to screen phage libraries against *in vitro* or *in vivo* targets Page 2 of 8

[46-48]. A typical phage display library contains up to 10⁹ different phage clones, each displaying a random type of peptide. The large number of different peptides in a library makes phage display a high throughput method for affinity selections. The best characterized of the filamentous phage is the Ff class, which include the M13, fd and f1 viruses that are structurally very similar and 98% identical at the DNA level. Structurally, the Ff class of phage resembles a flexible rod, which measures $\sim 0.9 \ \mu m$ in length and $\sim 65 \ \text{\AA}$ in diameter. The Ff genome is approximately 6.4 kb large (ssDNA) and encodes 11 proteins of which five are structural. Two of these are coat protein III (cpIII) and coat protein VIII (cpVIII). Both are surface exposed and are, for this reason, used to display the foreign peptides on the phage surface [46-48]. One of the most common phage display vector systems is the fUSE5 vector, which displays up to five copies of the peptides on cpIII. Another commonly used phage display vector is the f88-4 vector system, which displays several hundred copies of the peptide on cpVIII. Experimentally, the phage display library is most often screened against an antigen of interest using several rounds of affinity selection, elution and amplification. The amplification step is relatively straight forward in that the Ff class of phage infects gram-negative bacteria, such as E. coli, and uses the bacterial machinery to produce progeny phage, which are released without lysis through the bacterial plasma membrane [46-51,52,2,53]. Phage display technology has been employed to discover novel peptides that bind cancer cells. For example, RGD-peptides have been developed that target the tumor vasculature by binding to $\alpha\nu\beta$ 3-integrin (vitronectin receptor) [54,55]. The SGRSA peptide has been found to have high binding affinity to urokinase plasminogen activator (uPA) [56] and the peptide CGNSNPKSC to bind to gastric cancer endothelium [57]. Our laboratory has developed a number of peptides that target cancer cells. Among these are the peptide KCCYSL that binds to the ErbB-2 (HER2/neu) receptor [58,59], which is a member of the EGF receptor family and is upregulated in both ovarian and breast carcinoma. The HER2/neu oncogene is overexpressed in approximately 15-30% of ovarian carcinomas and is associated with an increased risk of progression and death, especially among women diagnosed with stage I and II ovarian carcinoma [60,61]. In addition, the peptides IAGLATPGWSHWLAL and ANTPCGPYTHDCPVKR were selected for binding to the prostate carcinoma cell line PC-3





[62,63] and galectin-3, respectively [64-67]. Furthermore, a number of peptides have been developed that bind to the Thomsen-Friedenreich (TF) carbohydrate antigen, which is present on approximately 90% of human carcinoma cells and is involved in cell adhesion and migration [68-71]. Most of these tumor targeting peptides have been developed to function as imaging and/or therapeutic agents of human cancers. Radiolabeling of tumor targeting peptides provides an effective method of eradicating cancer cells as well as imaging tumors *in vivo* using technologies such as single photon emission computed tomography (SPECT) and positron emission tomography (PET).

New Ovarian Cancer Cell Targeting Peptides

In order to efficiently select ovarian targeting peptides with high affinity for a tumor and desirable pharmacokinetics, it is important to initially pre-clear the phage display library from peptide motifs that bind to normal vasculature. This may be accomplished by intravenously injecting non-tumor bearing mice with an appropriate volume of > 10¹⁴ virions/mL of phage library followed by a 15 minute incubation time. Mice are then sacrificed, the blood harvested and unbound phage amplified and purified for further rounds of selection. For in vivo selections against ovarian carcinoma, our laboratory has used SCID mice carrying SK-OV-3 human carcinoma cell xenografted tumors. After injection of pre-cleared phage library tumor bound phage may be eluted after excision by using detergents. Amplified and purified phage may then be used for further rounds of in vivo/ex vivo selections [53,62,72]. Selected phage clones may be additionally analyzed for their binding affinity by micropanning experiments in which phage are incubated with different carcinoma and normal cell lines. Phage binding affinity may be evaluated by comparing the number of infectious units (TU/mL) between the cancerous and normal cell lines. In order to analyze the binding affinities of peptides outside of the phage environment, biotinylated or radiolabeled peptides may be synthesized and used in in vitro/in vivo binding studies [62,73].

In vivo selection of ovarian carcinoma specific peptides can be an inefficient procedure. One potential barrier is the presence of many tissue types within the tumor, endothelial cells, connective tissue, etc. Determination/selection of the targeted tissue for each selected peptide can be difficult when the *in vivo* milieu is so complex. Thus, an additional round of ex vivo selection may be added to try and select peptides that bind directly to ovarian carcinoma tumor cells. For this purpose MACS® technology may be utilized to separate ovarian carcinoma tumor cells from undesired tissue types. In order to avoid non-specific binding of phage, the phage may first be selected negatively against the MACS column and streptavidin labeled magnetic beads. Cells from excised human ovarian carcinoma tumors from xenografted mice may then be labeled with a mixture of biotinylated antibodies against known ovarian cancer biomarkers and then bound to streptavidin magnetic beads. Cells can then be loaded onto a MACS separation column and incubated with phage from previous selection rounds. Bound phage can be eluted from cells using detergents and subsequently amplified and purified. A good candidate for a known ovarian cancer biomarker includes the epithelial cell adhesion molecule (EpCAm; CD326), which is overexpressed in a variety of carcinomas, as well as normal epithelial tissues. Overexpression is present in ~70% of ovarian carcinomas and is significantly related to a decreased overall survival [74]. Another known ovarian cancer marker is CA-125 (Mucin 16) that is an ovarian cancer-associated antigen, and is, as described above, used as a serum biomarker for ovarian cancer. CA-125 is the extracellular domain (ECD) of the cell surface protein MUC16 and levels of the protein are known to increase with disease stage [75]. ErbB-2 (HER2/neu) is a member of the EGF receptor family and is also overexpressed in both ovarian and breast carcinoma. The HER2/ neu oncogene is overexpressed in approximately 15-30% of ovarian carcinomas and is associated with an increased risk of progression and death, especially among women diagnosed with stage I and II ovarian carcinoma, which makes it an interesting cell surface biomarker for ovarian cancer [60,61].

New Ovarian Cancer Stem Cell (CSC) Targeting Peptides

Selection of phage display derived peptides with high binding affinity for ovarian CSC is complicated by the fact that CSC only represents a small percentage of the entire tumor mass [28,31]. Thus CSC must initially be selected and separated from the remaining regular cancer cells. This may be done, as described above, utilizing MACS[®] technology using antibodies against known ovarian CSC biomarkers such as CD44, CD117 and CD133 [31,35-37]. Alternatively, CSC can be selected by growing ovarian cancer cells in stem cell appropriate medium [35,43]. Successful separation of CSC may be visualized by formation of spheroids in culture and staining with antibodies against ovarian CSC biomarkers. It may be advisable to further select CSC using flow cytometry cell sorting using antibodies against the known ovarian CSC biomarkers [28,43]. Further, normal cancer cells may be separated during this process and used for negative selections.

Even though the nature of CSC prevents selection of phage display derived peptides in vivo, pre-clearing of the phage display library may still be performed in non-tumor bearing mice as described above. In the early stages of selections, it is also important to consider the tumor microenvironment in that CSC comprise only a small part of the tumor bulk [28,31]. Thus in order to ensure specific binding, the library may be further pre-cleared against normal ovarian cancer cells before selecting for binding to CSC. Such a selection should be done ex vivo using cultured normal tumor tissue, and may be performed by utilizing MACS® technology as previously described. However, if the normal cancer cells have already been separated from CSC using flow cytometry, it will be sufficient to use non-labeled cells in suspension. Experimentally, it may be difficult to obtain large numbers of ovarian CSC, and it can be essential to cultivate CSC in appropriate stem cell medium [28,31,35,43] after separation from the tumor bulk. Cultured ovarian CSC form three dimensional spheroids in stem cell medium and it may be necessary to dissociate the cells to a singlecell suspension for the phage display selection. For the selection, the MACS® technology may be employed by labeling ovarian CSC with antibodies against the known biomarkers CD44, CD117 and CD133 [31,35-37]. Alternatively, CSC in suspension or grown on plates can be used instead. It is important to note that cells grown under such conditions must be tested for the presence of ovarian CSC biomarkers before selection in order to ensure that cells have not differentiated. Furthermore, it is imperative to use a large number (> 10¹³ virions) of phage in the first selection rounds in order to guarantee high diversity of phage clones [76]. After initial rounds the amount of phage may be lowered to increase selection stringency. As described above, selected phage clones may be further analyzed for their binding affinity by micropanning experiments in which phage are incubated with normal ovarian cancer cells and ovarian CSC. Once high binding clones have been identified, biotinylated or radiolabeled peptides can be synthesized and analyzed for their tumor targeting abilities [62,73]. Peptides with high binding affinity for CSC will most likely not be applicable as imaging agents due to the low percentage of CSC in a tumor. However, the chemotherapeutic abilities of such radiolabeled peptides can be evaluated in in vivo therapy studies using xenografted mice [77-80].

Page 3 of 8

Most likely, radiolabeled peptides for CSC tumor therapy must be combined with other forms of chemotherapy, such as more traditional platinum based drugs, in order to eradicate all cells in a tumor [81]. Thus, in a mouse therapy study it may be necessary to compare the therapeutic effects of drugs such as carboplatin and paclitaxel, which are standard in current ovarian cancer treatment, the effects of the radiolabeled peptide as well as a combined approach.

Radiolabeled Peptides for Tumor Imaging and Therapy

While antibodies and their fragments are by far the most used cancer targeting imaging and therapeutic agents [82], peptides exhibit better biodistribution properties. Antibodies often cause drug resistance, have long biodistribution times and clear through the hepatobiliary system, whereas peptides show low immunogenicity, rapid blood-clearance and are excreted in the urine [83-87]. High kidney uptake has, however, been observed with peptides, which poses a problem in regard to tumor imaging near the kidney and with toxicity caused by accumulation of therapeutic radiolabeled peptides [82,86-88]. Thus lowering renal uptake is important and may be done by changing parts of the peptide sequence, trying different radionuclides and chelates or by co-administration of lysine or arginine [86,87,89].

Radiolabeling of tumor targeting peptides affords a proficient way of imaging in vivo using technologies such as single photon emission computed tomography (SPECT) and positron emission tomography (PET). SPECT was one of the first imaging modalities used clinically and is today widely employed in cancer imaging. Both 99mTc [90] (6 h half-life) and ¹¹¹In (2.8 day half-life) are frequently used in SPECT [91]. PET is an emerging imaging technique that offers certain advantages compared to SPECT in regard to sensitivity as well as quantitation [92]. The β^+ -emitting tracer fluorine-18 2-deoxy-2-fluoro-D-glucose (18F-FDG) is a commonly used PET tracer for imaging processes with increased glucose metabolism [93]. However, the uptake of ¹⁸F-FDG is not increased in all cells and has not shown great promise in early stage ovarian cancer diagnosis [94]. Therefore, alternative PET tracers are being developed that target antigens on cancer cells [92,95]. Octreotide is an eight amino acid cyclized peptide that has been successfully developed for imaging of somatostatin receptor positive tumors in humans when labeled with 111In- diethylenetriaminepentaacetic acid (DTPA) [96]. The α -Melanocyte Stimulating Hocmone (α -MSH) analog has been conjugated with the chelator 1,4,7,10-tetraazacyclodecane-1,4,7,10-tetraacetic acid (DOTA) and labeled with 64Cu, 86Y and ⁶⁸Ga for PET imaging of melanoma [97,98]. Our laboratory has identified peptides that bind ovarian, breast, and prostate tumors [59,63,64,73,99,100]. The phage display selected peptide KCCYSL against ErbB-2 has been radiolabeled with ¹¹¹In and used for SPECT/ CT imaging of human MDA-MB-435 breast and OV-CAR-3 and SK-OV-3 ovarian xenografted tumors (Figure 2) [58,59,73,99].

Whereas many cancer therapeutic agents function by binding and inhibiting receptors or other molecules involved in the progression of cancer, radiolabeling of peptides provides a method to target and eradicate cancer cells independent of peptide function and intracellular signaling pathways. Labeling peptides with β -particle emitting radioisotopes are being used for targeted tumor radiotherapy and offer advantages in regard to varying degrees of energy emission. Highenergy β -emitters such as ⁹⁰Y (2.7 day half-life) are appropriate for the treatment of large tumor burdens, whereas medium and lower-energy β -emitters, such as ¹⁷⁷Lu (6.7 day half-life) may be more suitable for treating smaller tumors, residual tumor or metastatic deposits found in ovarian cancer [101,102]. One example of a radiolabeled peptide being developed for tumor therapy is the α -MSH peptide analog, CCMSH, which targets melanoma cells. The α -MSH analog has been conjugated with the chelator DOTA and labeled with ²¹²Pb and used for melanoma therapy studies in mice. The treatment showed significantly increased survival rates in which 45% of the mice receiving the highest dose of radiation survived the study disease-free [78,79].

These results demonstrate that radiolabeled peptides offer great promise as both cancer imaging and therapeutic agents. Peptides that target early stage ovarian cancer cells could be developed into diagnostic imaging/therapeutic agents. Furthermore, peptides may be developed that target tumor subpopulations such as ovarian CSC.

Identification of Antigens Targeted by Selected Phage Displayed Peptides

Both phage and peptides can be used in formats that allow identification of targeted antigens. Previous studies have employed cross-linking of phage or selected peptides to antigens as a means to identify peptide targets [103,104]. Kelly *et al.* have employed photolinker and biotin labeled phage to bind and capture target antigens on the cell surface. After binding and cross-linking, cell lysates were incubated with streptavidin beads. Antigens were eluted by reversing the cross-link and subsequently used for SDS-PAGE followed by tryptic digest and mass spectrometry analysis [104]. However, these techniques can suffer from lack of specificity due to the use of multiple cross-linkers and the large size of filamentous phage. In addition, phage are known to aggregate and exhibit high non-specific binding to cells, which may result in identification of numerous irrelevant proteins via mass spectrometry.

Alternatively, uniquely designed immobilized fusion proteins may offer a powerful means of isolating specific targets of phage display selected peptide sequences. For example a recombinant fusion protein containing a phage display selected peptide can be developed to aid the antigen capture process. Such a fusion protein may contain an N-terminal protein, such as thioredoxin, which acts as a soluble fusion partner linked to a matrix specific binder and a series of protease cleavage sites. Our laboratory has adapted a thioredoxin fusion protein that remains soluble in *E. coli* cytosol and can be purified, during immobilization on S-protein sepharose (Figure 3). Thioredoxin will be removed by cleavage with a protease, leaving the phage display peptide still bound to the S-protein sepharose. The immobilized



Figure 2: SPECT imaging of ¹¹¹In-DOTA(GSG)-KCCYSL ErbB-2 targeting peptide in MDA-MB-435 breast and OV-CAR-3 ovarian tumor bearing SCID mice. MDA-MB-435 breast and OVCAR ovarian tumor xenografted SCID mice were injected in the tail vein with 11.1 MBq of ¹¹¹In-DOTA(GSG)-KCCYSL peptide or ¹¹¹In-DOTA)GSG)-KYLCSC scrambled peptide and imaged by microSPECT/CT one hour later.



peptide complex will then be accessible for binding to its target protein. The phage display derived peptide and its target antigen may then be released from the S-protein sepharose by cleavage with a second protease. Once bound target proteins from cell lysates have been obtained they may be analyzed by 2D gel electrophoresis and identified by proteomic methodologies.

Acknowledgements

This work was supported by a Ment Review Award form the Veterans Administration (SLD).

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Page 5 of 8

Page 6 of 8

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Page 7 of 8