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Identification of Mammary Tumour Homing Peptides by *In vivo* Biopanning using Phage Display Library

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Mammary tumour was induced by subcutaneous injections of LA7 cells (approximately 10^6 cells) into the mammary gland pad of 50 day old female Sprague-Dawley rats. Mammary tumour was observed at the site of injection of cells in sixteen out of thirty rats. The tumour was confirmed by physical examination and histopathology. We conducted four rounds of *in vivo* biopanning using M13 phage display library (7-mer) in mammary tumour bearing rats. The tumour binding phages were rescued and amplified by *E coli* ER2738. Then, sixty plaque-forming units (pfus) were selected randomly, each phage clone was amplified and DNA from individual clone was isolated. Afterwards pIII gene which contains the inserted peptide coding DNA sequence was confirmed by PCR and DNA sequencing. These sequences were analyzed by MegAlign (DNASTAR) software and identified consensus peptide motif 'SPPR'. This motif was predicted that, it is having mammary tumour homing ability and maybe useful for tumour targeted delivery of therapeutics and imaging agents.

Key words: Mammary tumour, LA7 cells, *In vivo* biopanning, Homing peptides, Motifs.

Cancer is one of the leading causes of death, with more than 1,300 people succumbing to cancer every day in India alone[1] and globally about 7.6 million human deaths every year[2]. Breast cancer is the second most important cause of cancer related death after lung cancer in humans[3] and in animals mammary tumours are one of the most common tumours in female dogs represents 70% of all tumours[4]. Presently cancer is treated by using combinations of conventional therapies like chemotherapy and radiotherapy. This classical regimen of cancer therapies resides with disadvantages like narrow therapeutic index. Recently, targeted delivery is gaining importance because it helps in effective localization of drugs to tumours leading to increased therapeutic index and reduced toxicity[5,6].

As normal cells transform into cancerous cells, their cell surface properties change, showing unique expression or over-expression of certain antigens and receptors. Monoclonal antibodies against these receptors are extensively used for targeting drug molecules specifically to tumours[7]. It faces number of challenges like discovery of novel cancer cell specific surface antigens, long development times, high production costs, manufacturing/quality control, conjugation of drugs and other cargos, solid tumour penetration and problem associated with internalization[8,9]. To overcome these problems, researchers are looking

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for alternative methods for targeted delivery. Recently homing peptides which selectively target specific organs and tumours are gaining importance as these peptides possess many advantages, such as easy to identify, small size, ease of synthesis and modification, tumour penetrating ability, and biocompatibility[5].

Over the past few years, many methods have been developed to identify the homing peptides. Phage display technology is a widely used method for the selection of peptides[10,11,12]. The most commonly phage display systems used to identify functional targeting peptides is fusion peptides displayed on the minor coat protein (pIII) of filamentous M13 bacteriophage[13,14]. This technology has been used to identify number of peptides for targeting specific tissue/organs including tumour blood vessels and cancer cells. Rodent models have been extensively used as experimental systems for the study of mammary cancer because in this species, the mammary gland is the source of hormone dependant neoplasms and similar in tumour histology of breast cancer[15,16,17]. But current methods for mammary tumour induction in rats for cancer research are not only time-consuming but also hazardous and expensive. So, a simple and cost-effective method was developed to induce solid mammary gland tumour in female Sprague-Dawley rats using LA7 cells[18,19].

In the present study, we sought to identify specific novel peptides or motifs for targeted delivery of drugs or imaging agents by *in vivo* biopanning method in mammary tumour of female Sprague-Dawley rats induced by LA7 cells.

MATERIALS AND METHODS

Experimental animals

Female Sprague-Dawley rats (n=30) of about 28-35 days of age were procured from Laboratory Animal Division, Central Drug Research Institute (CDRI), Lucknow. Rats were housed in polypropylene cages in the experimental animal house of Molecular Biology Lab, Division of Veterinary Biotechnology and acclimatized for 15 days before the start of experiment. The rats were provided with food and water *ad libitum* and housed in air conditioned room with controlled temperature and humidity and 12:12 h light:dark cycle. The experimentation was carried out as per the guidelines and approval of Institutional Animal Ethical Committee (IAEC) and Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

Cell culture

LA7 ratmammary tumour cell line (CRL-2283, ATCC) used in the present study was obtained from Dr Meena Kataria, Biochemistry Division, ICAR-IVRI, Izatnagar. These cells were grown and maintained in DMEM supplemented with 10% foetal bovine serum (FBS), 100 U/ml of penicillin, 100 mg/ml of streptomycin, 0.005mg/ml insulin and 50 nM dihydrocortisone at 37°C with 5% CO_2.

Induction of mammary tumour in female Sprague-Dawley rats using LA7 cells

For mammary tumour induction, the procedure used by Abbassalipourkabir *et al*.[18] and Munda *et al.*[19] was followed with minor laboratory modifications. The cells were grown upto 70-80% confluency and harvested using TVG solution (0.25% Trypsin, 0.02% EDTA and 0.05% Glucose in PBS). The cells were washed twice with sterile PBS by centrifugation. Finally, cells were pelleted by centrifugation at 2000xg for 10 min. The cell pellet was resuspended in 200 µl PBS. An aliquot of cells (10 µl) was stained with trypan blue and counted in Countess automated cell counter (Invitrogen) as per manufacturer instructions. Approximately 1×10^6 cells in 200 µl PBS were inoculated subcutaneously into mammary fat pads of rats.

Clinical and gross observations

Body weight of all the rats used in tumour induction experiment was measured at weekly interval. Animals were observed daily for clinical signs and symptoms and palpated twice a week for detection of any growth in the mammary glands. The visible tumour nodules were measured using vernier caliper at 3 days interval after first day of detection.

Histopathology

At the end of experiment, small portion of tumours were fixed in 10% neutral buffered formalin, processed to obtain 5 µm thick paraffin sections and stained with haematoxylin and eosin (H&E). Tumour sections were examined for histopathological changes.

Phage display library and culture of *E. coli* ER2738

The Ph.D-7 phage display peptide library kit and the *E. coli* host strain ER2738 purchased from New England Biolabs (Berverly, MA, USA) was used to screen the specific peptides homing to mammary tumour *in vivo*. This phage display library contains individual phage clones which displayed the random heptapeptide on minor coat protein pIII.

The *E.coli* host strain ER2738 (F+ strain with a rapid growth rate) was used for M13 phage propagation. It was plated on Luria-Bertani-tetracycline (LB-Tet) plates, and incubated at 37°C overnight, and then were inoculated into LB medium to achieve log-phase growth.

***In vivo* biopanning with PhD-7 phage display library**

Rats bearing mammary tumour (approximate size of 1-2 cm in diameter) were used for the *in vivo* biopanning experiments and all animal experiments were performed in a biosafety cabinet. Approximately, $2x10^{11}$ plague forming units (pfus) from Ph.D-7 library were taken in 200 µl of DMEM and injected intravenously in lateral tail vein of rat. After 30 min rats were sacrificed by cervical dislocation and immediately the tumour was harvested. Tumour was minced with scissor in ice-cold DMEM and then transferred to 15 ml centrifuge tube and washed three times with cold washing medium (1% BSA in DMEM) at 4000xg for 5 min.

Rescue of tumour binding phages by amplification

One ml of overnight grown culture of ER 2738 was added to tissue homogenate containing bound phages, mixed gently and allowed to stand for 30 min. Then this mixture was transferred to Erlenmeyer flask containing 49 ml of LB kept in an orbital shaker at 37°C for 4 h at 180 rpm. After 4 h, the culture was centrifuged at 4500xg for 10 min. The upper 80% of supernatant containing phages were precipitated in 1/6 volume of 20% PEG in 2.5 M NaCl at 4°C overnight. Next day, the PEG precipitated phage was recovered by centrifugation at 12,000xg for 15 min at 4°C. The pellet was re-suspended in 1 ml of TBS (Tris buffered saline pH 7.5) and centrifuged at 12,000xg for 5 min to pellet out residual cells. The supernatant was precipitated in 1/6 volume of 20% PEG in 2.5 M NaCl at 4°C for 60 min to precipitate phage. Then the content was centrifuged at 14,000xg for 10 min at 4°C, the supernatant was discarded and pellet was resuspended in 200 µl TBS.

Phage Titration

Ten fold serial dilutions of phage were prepared in TBS buffer (10^{-1} to 10^{-16} dilution) and 10 µl of diluted phages were added to 200 µl *E. coli* culture at mid-log phase (OD_{600}~ 0.4-0.6) and incubated at room temperature for 5 min. The infected cells were transferred to culture tubes containing top agar (42°C), mixed briefly and poured immediately onto a pre-warmed LB/IPTG/Xgal plate to spread top agar evenly. The plates were allowed to cool for 5 min. Once solidified, plates were inverted and incubated overnight at 37°C. Numbers of plaques were counted on plates using formula:

Pfu/ml= no. of plaques × dilution factor × 100

In the similar pattern further three rounds of biopanning was conducted in tumour bearing rats to enrich mammary tumour homing peptides. After four rounds of selection, individual blue plaques were selected randomly and amplified. Single stranded DNA was isolated from each phage clone by phenol chloroform method. Phage DNA containing displayed peptide nucleotide sequence was amplified and sequenced using primers (Forward primer- 5' TTCGCAATTCCTTTAGTGGTA 3' and Reverse primer- 5' GCGGGGTTTTGCTCAGTAC 3').

Analysis of tumour specific peptides

The phage displayed peptide sequences were translated and aligned to find the consensus sequence using the DNASTAR and BioEdit software. The repository for phage derived peptide database, MimoDB (http://immunet.cn/mimodb) was examined to check if the peptide sequences identified in this study have any other targets. The presence of any target unrelated peptides were examined by using Sarotup software (http://immunet.cn/sarotup/). The peptide sequences were aligned by 'clustalW' method using MegAlign (DNASTAR) software to identify the common motifs.

RESULTS AND DISCUSSION

The results of the present study indicated that injection of LA7 cells subcutaneously into

mammary fat pads is a very simple technique and mammary tumour was developed in less than 15 days in female Sprague-Dawley rats (53.3%) suggesting the utility of the model for rapid induction of mammary tumours. All the mammary tumours harvested showed solid consistency with 1-3 cm in diameter (Fig. 1). Further tumour development was microscopically confirmed by histopathological examination of H&E stained tissue sections, which showed population of pleomorphic epithelial cells containing large nuclei with enlarged nucleoli, along with focal areas of necrosis. We also noticed the infiltration of these cells into surrounding connective tissues (Fig. 2). The similar observation was also reported by Munda *et al.*[19]

Screening phage display libraries against specific target tissues is a direct and fast method

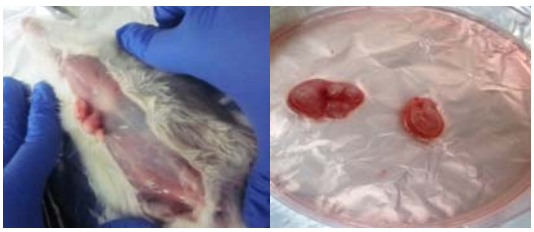

Fig. 1. A. Mammary tumour developed at the site of injection of LA7 cells. B. post operative mammary tumour tissue

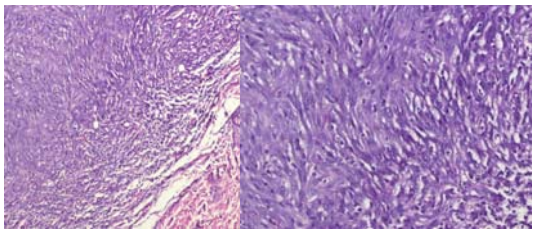

Fig. 2. Representative photomicrographs of rat mammary tumour sections induced by LA7 cells.

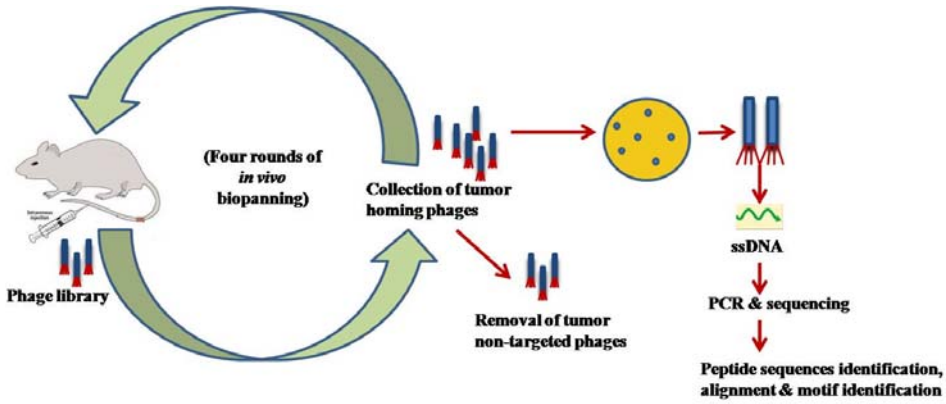

Fig. 3. Schematic representation of four rounds of *in vivo* biopanning in mammary tumour bearing rats

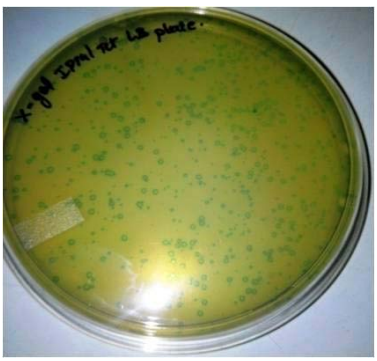

Fig. 4. LB/Tet/Xgal/IPTG plate showing blue colonies (plaques) of phage grown on α-Complementing host *E. coli* ER2738

to identify novel peptide sequences with tissue/organ specificity[20]. *In vivo* biopanning allow selection of peptides that interact with tissues and cells in their native microenvironment[21]. This approach was used to identify peptides that exhibit specific homing ability to various organs[22,23], tumour vasculature[24] and tumour lymphatics[25]. In past few years many tumour specific homing peptides were identified by using *in vitro*/*in vivo* phage biopanning strategies against human lung cancer cells[26], esophageal cancer[27], gastric cancer[28], breast cancer cell[29], human brain metastatic breast cancer[30], ovarian cancer[31] etc.

In the present study, four rounds of *in vivo* biopanning were conducted by injecting 7 mer phage display library in LA7 cells-induced mammary tumour bearing rats (Fig. 3). After each round of biopanning, population of the phage attached to mammary tumour tissue was rescued in host *E. coli* cells, amplified, purified and titrated by plaque assay (Fig. 4). Phage titer before biopanning, after biopanning and amplification is depicted in table 1. The phages homing to tumour was enriched after a successive round of biopanning was indicated by gradual increasing in output of pfus which bound to tumours.

After four rounds of biopanning 60 plaques from LA7 cell induced mammary tumour specific plaques were randomly selected, amplified and single stranded phage DNA was isolated. The 401 bp targeted region of phage DNA flanking the cloning site (Fig. 5) was amplified and the amplicons were subjected to DNA sequencing and analyzed using DNASTAR and BioEdit software.

All sixty peptide sequences were analysed in mimotope database (MimoDB 2.0) to check if the peptide sequences identified in this study have any other targets[32] and SAROTUP software (target unrelated peptide scanner) to

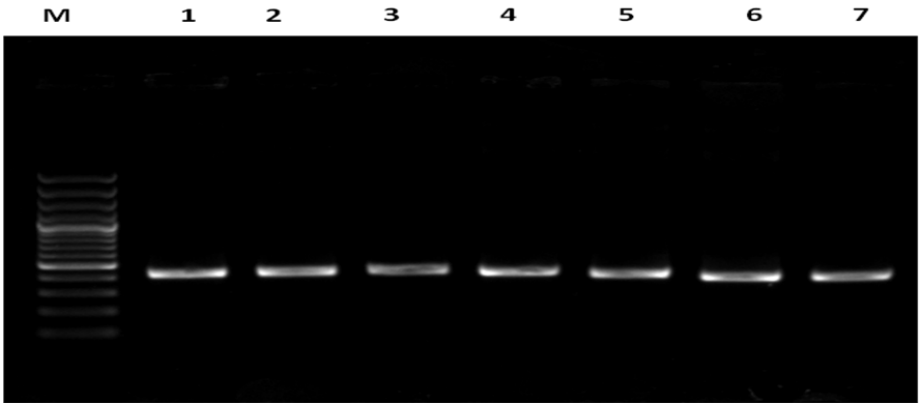

Fig. 5. Agarose gel electrophoresis of amplified ssDNA of phage showing 401 bp PCR products. Lane M: 100bp DNA ladder, lane 1-7 shows representative amplicons from 60 plaques

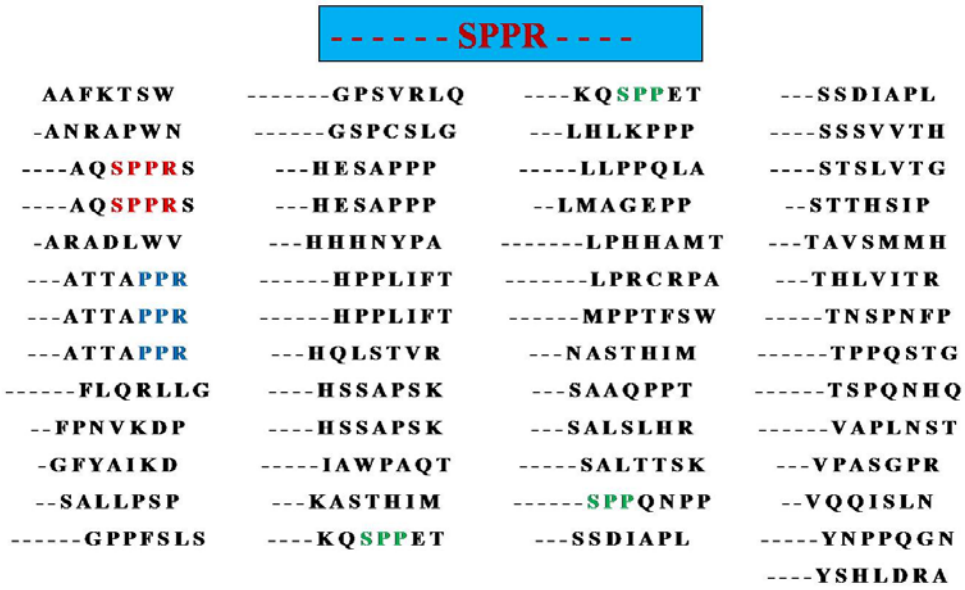

Fig. 6. Alignment of mammary tumour specific peptides and identification of common motif by clustalW method using DNASTAR software

Table 1. LA7 cells induced mammary tumour specific phage titre before and after biopanning (recovery and amplification)

Biopanning	Initial phage dose injected(pfus)	Phage titreafter recovery and amplification
Round 1	2×10^{11}	20×10^{12}
Round 2	2×10^{11}	38×10^{12}
Round 3	2×10^{11}	12×10^{14}
Round 4	2×10^{11}	78×10^{14}

check presence of any target unrelated peptides[33]. The result indicated the peptide 'GETRAPL' which repeated three times out of sixty peptides, is a target unrelated peptide, which found to bind target non-specifically and it was also reported previously[32,34]. Remaining peptides were aligned by clustalW method using MegAlign (DNASTAR) software to identify the common motifs. The alignment report of ClustalW indicated the 'SPPR' is a common motif and appearing several times in many tumour specific peptide sequences (Fig. 6). Similarly many tripeptide motifs which are having affinities towards specific organs and tumours were reported previously[23,35,36]. We anticipate that 'SPPR' motif may have high affinity and specificity to mammary tumour and useful for imaging detection and targeted drug delivery to tumours.

In conclusion, the results obtained indicated that the induction of mammary tumour by LA7 cells in female Sprague-Dawley rats can be used as a simple and rapid method to develop mammary tumour model. *In vivo* biopanning using M13 phage display library is a simple and fast method to identify homing peptides and identified novel tumour homing peptide motif (SPPR) might be useful for targeted drug delivery and imaging agents specifically to mammary tumours.

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REFERENCES

1. Cancer Statisitics. National Cancer Registry Programme. 2015; Indian Council of Medical Research, India.
2. Jemal, A., Bray, F., Center, M.M., Ferlay, J., Ward, E., Forman, D. Global cancer statistics. *CA-Cancer J. Clin.,* 2011; **60**: 69–90.
3. Siegel, R., Ma, J., Zou, Z., Jemal, A. Cancer statistics, 2014. *CA-Cancer J. Clin.,* 2014; **64**: 9–29.
4. Merlo, D.F., Rossi, L., Pellegrino, C., Ceppi, M., Cardellino, U., Capurro, C., Ratto, A., Sambucco, P.L., Sestito, V., Tanara, G., Bocchini, V. Cancer incidence in pet dogs: findings of the Animal Tumor Registry of Genoa, Italy. *J Vet Intern Med.,* 2008; **22**(4): 976-84.
5. Thundimadathil, J. Cancer treatment using peptides: Current therapies and future prospects. *J. amino acid.,* 2012; 2012: 967347.
6. Orza, A., Casciano, D., Biris, A. Nanomaterials for targeted drug delivery to cancer stem cells. *Drug Metab Rev.,* 2014; **46**(2): 191-206.
7. Scott, A.M., Wolchok, J.D., Old, L.J. Antibody therapy of cancer. *Nat Rev Cancer.,* 2012; **12**(4): 278-87.
8. Firer, M. A. Antibody-drug conjugates in cancer therapy filling in the potholes that lie ahead. *OA Cancer.,* 2013; **1**(1): 8-13.
9. Firer, M. A., Gellerman, G. Targeted drug delivery for cancer therapy: the other side of antibodies. *J Hematol Oncol.,* 2012; **5**: 70-85.
10. Colwill, K., Gräslund, S.A. roadmap to generate renewable protein binders to the human proteome. *Nat. Methods.,* 2011; **8**: 551–8.
11. Liu, B.A., Engelmann, B.W., Nash, P.D. High-throughput analysis of peptide-binding modules. *Proteomics.,* 2012; **12**: 1527–46.
12. Rentero, I., Heinis, C. Screening of large molecule diversities by phage display. *Chimia (Aarau).,* 2011; **65**: 843–5.
13. Smith, G.P. Filamentous fusion phage: novel

expression vectors that display cloned antigens on the virion surface. *Science.*, 1985; **228**: 1315–7.

14. Krumpe, L.R.H., Mori, T. Potential of phage-displayed peptide library technology to identify functional targeting peptides. *Expert Opin Drug Discov*., 2007; **2**(4): 525-42.
15. Masso-Welch, P.A., Darcy, K.M., Stangle-Castor, N.C., Ip, M.M. A developmental atlas of rat mammary gland histology. *J Mammary Gland Biol Neoplasia*., 2000; **5**(2): 165-85.
16. Russo, J., Russo, I.H. Atlas and histologic classification of tumors of the rat mammary gland. *J Mammary Gland Biol Neoplasia*. 2000; **5**(2): 187-200.
17. Saminathan, M., Rai, R.B., Dhama, K., Ranganath, G.J., Murugesan, V., Kannan, K., Pavulraj, S., Gopalakrishnan, A., Suresh, C. Histopathology and Immunohistochemical Expression of N-Methyl-N-Nitrosourea (NMU) Induced Mammary Tumours in Sprague-Dawley Rats. *Asian J Anim Vet Adv.*, 2014; **9**(10): 621-40.
18. Abbasalipourkabir, R., Dehghan, A., Salehzadeh, A., Shamsabadi, F., Abdullah. R. Induction of mammary gland tumor in female Sprague-Dawley rats with LA7 cells. *Afr J Biotechnol.*, 2010; **9**(28): 4491-8.
19. Munda, L.R., Rawat, S., Thakuria, D., Regon, M., Kurade, N.P., Kataria, M., Sharma, A.K. and Tiwari, A.K. Pathology of LA7 cell induced tumours in Sprague Dawley rats. *Indian J. Vet. Pathol.*, 2014; **38**(1): 43-8.
20. Rasmussen, U.B., Schreiber, V., Schultz, H., Mischler, F., Schughart, K. Tumor cell-targeting by phage-displayed peptides. *Cancer Gene Therapy.*, 2002; **9**: 606-12.
21. Work, L.M., Nicol, C.G., Denby L. Baker, A.H. In vivo biopanning: A methodological Approach to identifying novel targeting ligands for delivery of biological agents to the vasculature. *Methods Mol Med.*, 2004; **108**: 395-413.
22. Pasqualini, R., Ruoslahti, E. Organ targeting in vivo using phage display peptide libraries. *Nature.*, 1996, **380**: 364–66.
23. Kolonin, M.G., Sun, J., Do, K.A., Vidal, C.I., Ji, Y., Baggerly, K.A., Pasqualini, R., Arap, W. Synchronous selection of homing peptides for multiple tissues by in vivo phage display. *FASEB J.*, 2006; **20**(7): 979-81.
24. Arap, W., Pasqualini, R., Ruoslahti, E. Cancer treatment by targeted drug delivery to tumor vasculature in a mouse model. *Science.*, 1998; **279**: 377–80.
25. Laakkonen, P., Porkka, K., Hoffman, J.A., Ruoslahti, E. A tumor-homing peptide with a targeting specificity related to lymphatic vessels, *Nat. Med.*, 2002; **8**: 751–5.
26. Lee, K.J., Lee, J.H., Chung, H.K., Choi, J., Park, J., Park, S.S., Ju, E.J., Park, J., Shin, S.H., Park, H.J., Ko, E.J., Suh, N., Kim, I., Hwang, J.J., Song, S.Y., Jeong, S.Y., Choi, E.K. Novel peptides functionally targeting in vivo human lung cancer discovered by in vivo peptide displayed phage screening. *Amino Acids.*, 2015; **47**(2): 281-9.
27. Zhang, Z.F., Shan, X., Wang, Y.X., Wang, W., Feng, S.Y., Cui, Y.B. Screening and selection of peptides specific for esophageal cancer cells from a phage display peptide library. *J Cardiothorac Surg.*, 2014; **9**: 76-82.
28. Zhang, W.J., Sui, Y.X., Budha, A., Zheng, J.B., Sun, X.J., Hou, Y.C., Wang, T.D., Lu, S.Y. Affinity peptide developed by phage display selection for targeting gastric cancer. *World J Gastroenterol.*, 2012; **18**(17): 2053-60.
29. Lu, R.M., Chen, M.S., Chang, D.K., Chiu, C.Y., Lin, W.C., Yan, S.L., Wang, Y.P., Kuo, Y.S., Yeh, C.Y., Lo, A., Wu, H.C. Targeted drug delivery systems mediated by a novel Peptide in breast cancer therapy and imaging. *PLoS One*. 2013; **8**(6): e66128.
30. Fu, B., Zhang, Y., Long, W., Zhang, A., Zhang, Y., An, Y., Miao, F., Nie, F., Li, M., He, Y., Zhang, J., Teng, G. Identification and characterization of a novel phage display-derived peptide with affinity for human brain metastatic breast cancer. *Biotechnol Lett.*, 2014; **36**(11): 2291-301.
31. Soendergaard, M., Newton-Northup, J.R., Deutscher, S.L. In vivo phage display selection of an ovarian cancer targeting peptide for SPECT/CT imaging. *Am J Nucl Med Mol Imaging.*, 2014; **4**(6): 561-70.
32. Huang, J., Ru, B., Zhu, P., Nie, F., Yang, J., Wang, X., Dai, P., Lin, H., Guo, F.B., Rao, N. MimoDB 2.0: a mimotope database and beyond. *Nucleic Acids Res.*, 2012; 40(Database issue): D271-7.
33. Huang, J., Ru, B., Li, S., Lin, H., Guo, F.B. SAROTUP: scanner and reporter of target-unrelated peptides. *J Biomed Biotechnol.*, 2010; 2010: 101932.
34. Hoen, P.A., Jirka, S.M., Ten Broeke, B R., Schultes, E.A., Aguilera, B., Pang, K.H., Heemskerk, H., Aartsma-Rus, A., van Ommen,

G.J., Dunnen, J.T. Phage display screening without repetitious selection rounds. *Anal. Biochem.* 2012; **421**: 622–31.

35. Koivunen, E., Arap, W., Rajotte, D., Lahdenranta, J., Pasqualini, R. Identification of receptor ligands with phage display peptide libraries. *J Nucl Med.*, 1999; **40**(5): 883-8.

36. Ulises, H.C., Tatiana, G., Karlen, G., Guillermo, M.H., Juan, X.C., Carlos, E. Peptide sequences identified by phage display are immunodominant functional motifs of Pet and Pic serineproteases secreted by Escherichia coli and Shigella flexneri. *Peptides.*, 2009; **30**(12): 2127-35.