Journal of Animal and Veterinary Advances 11 (12): 2166-2175, 2012
ISSN: 1680-5593

Screening and Identification of Polypeptides Specifically Binding to Hepatoma Carcinoma Cells

[1]Hong Cao, [1]Xiaofeng Tian, [2]Yuanjing Xu, [1]Xudong Wang, [2]Wulin Tian,
[2]Ten Wei, [1]Xuedong Fang and [2]Guanghong Xie
[1]The Second Hospital of Jilin University, 130041 Changchun, China
[2]College of Animal Science and Veterinary Medicine, Jilin University, 130062 Changchun, China

Abstract: To screen specific polypeptides binding to hepatoma carcinoma cells by using C7C™ Phage Display Peptide Library and carry out biological identification on their binding capacity. HL-7702 were used as the negative subtractive cells and HepG2 were used to screen as the target cells, 60 positive phage clones were randomly selected and cell-based ELISA was carried out to identify their binding activities to HepG2 cells. The positive clones were subjected to sequencing analysis, the polypeptides were synthesized, immunochemical and immunofluorescence analysis were also carried out for further identification. After four rounds of screening, significant enriching of phages appeared on the target cells HepG2; ELISA and immunochemical analysis were carried out, 18 positive phage clones with high binding capacity to hepatoma carcinoma cells were obtained from the randomly selected 60 phage clones, after sequencing and immunofluorescence identification, two polypeptides were confirmed having high affinity and their amino acid sequences had no homology.

Key words: Hepatoma carcinoma cells, targeted therapy, polypeptide, amino acid, liver cancer

INTRODUCTION

Hepatic Cellular Cancer (HCC) (which is abbreviated as liver cancer) is one of the most common malignant tumors in the world, showed insidious onset, rapid progression, early metastasis, high malignancy and poor prognosis and most of the cases are confirmed as middle and advanced stages when they were discovered (El-Serag and Rudolph, 2007). Its morbidity has continuously increased in recent years and it has shown an increasing tendency in young people. It is mainly treated by operations, chemotherapy and radiotherapy but most of the chemotherapeutics effects are non-selective which are widely distributed in the body, the toxic and side effects on normal tissues and organs under the therapeutic dosages are significant, though the pathogenetic conditions of patients can be transiently alleviated, the therapeutic efficacy is not satisfactory and >95% of the liver cancer patients fail in their treatments (Du *et al*., 2006; Hu *et al*., 2006). It is required to increase the selectivity of drugs to tumors and reduce their accumulation at non-target positions for improving the therapeutic efficacy of anti-tumor drugs which can not only reduce their toxic and side effects on non-target positions but also reduce the therapeutic dosage and the frequency of drug administration and improve the pharmacodynamic action. Therefore, it is urgent to find a kind of innovative and breakthrough method for early diagnosis and treatments.

Drug targeting therapy on tumor cells has always been the hotspot for investigations on tumors (Midorikawa *et al*., 2010; Thomas, 2009). Some specific receptors show high expression levels on the surface of tumor cells (Thomas, 2009) and they can selectively bind to certain ligand. The targeted therapy on tumor cells can be realizing by utilizing the specificity for the binding between receptors and ligands in the mean time the damages on normal cells can be reduced.

Screening for polypeptide ligands specifically binding to tumor cells by using phage display random polypeptide library is a kind of new and highly efficient screening technique that is developed in recent years. Since, the discovery of phage display random peptide library in 1990 (Scott and Smith, 1990), it has been rapidly and widely used (Witt *et al*., 2009; Kelly *et al*., 2008; Kumar *et al*., 2007). It can display the expressed polypeptides or proteins in the form of fusion protein on the surface of phage by fusing the gene encoding the exogenous polypeptide and the gene encoding phage coat protein and their independent spatial configuration and biological activity are maintained (Sidhu *et al*., 2003; Ferrer *et al*., 1999). This technique connects phenotypes

Corresponding Author: Guanghong Xie, College of Animal Science and Veterinary Medicine, Jilin University, 130062 Changchun, China

with genotypes, combines recognition of corresponding target molecules with the capacity for re-amplification and the high-affinity polypeptides specifically binding to target molecules can be obtained only by several rounds of enriching and screening process absorption-elusion-amplification. Furthermore, the peptides have high biological activities and are less prone to immunological rejection or the side effects (Yang *et al*., 2008) so, the screened polypeptides from the phage random peptide library can be effectively modified to further improve their clinical values for applications. As a kind of simple and efficient screening tool, phage random peptide library has been widely used for screening and development of anti-tumor drugs and markers for tumor diagnosis (Du *et al*., 2006; Chen *et al*., 2009; Wu *et al*., 2010; Staquicini *et al*., 2010; Pameijer *et al*., 2006) which provided the technical platform for targeted therapy and molecular imaging for tumors (Shukla and Krag, 2005).

The present study aimed at searching for the polypeptides with high affinity to hepatoma carcinoma cells by using C7C™ Phage Display Peptide Library for whole-cell subtractive screening in HepG2 cells which provided research basis for further detection on early diagnosis of liver cancer and drug-targeting therapy.

MATERIALS AND METHODS

C7C™ Phage Display Peptide Library (Ph.D-C7C™ Phage Display Peptide Library) was purchased from New England Biolabs Company, the titer of the library was 1.5×10^{13} pfu mL^{-1} and the complexity was 2.7×10^{9}; HRP/anti-M13 antibody (#27-9421-01, 1:5000 dilution as the working concentration) was purchased from New England Biolabs Company; anti-FITC antibody was purchased from Molecular Probes, Invitrogen, California; DAB kit, Mayor's hematoxylin was purchased from Sigma-Aldrich, America; Fetal Bovine Serum (FBS) and RPMI1640 were purchased from GiBCO, USA; OPD, IPTG, Xgal and other reagents were all purchased from Sigma-Aldrich, America.

Subtractive screening by using phage peptide library: Human hepatoma carcinoma cell HepG2 and human normal hepatic cell HL-7702 were incubated with RPMI-1640 containing 10% FBS at 37°C and 5% CO_2 and the cells were inoculated into the culture dishes of (60×15) mm^2 in a density of 10^5 cells/dish when the cells grew to 80–90%. The HepG2 cells were firstly subjected to serum-free culture for 1 h before the screening then the blocking solution (RPMI-1640 + 0.5% BSA) was added for blocking at 37°C for 1 h, the phage peptide library was added (the titer was about 1.5×10^{11} pfu) to the cells incubated at 37°C for 1 h, the culture solution was discarded after the cells were kept on ice for 5 min; subsequently the cells were rinsed with pre-cooled PBS containing 0.1% BSA at 4°C for three times after the precipitation was rinsed with TBST (0.05%) for six times, 1 mL glycine buffer (pH 2.2) was used for elution and Tris-HCl (pH 9.1) was used for neutralization after 10 min. The phages binding to the cells were collected and the cells were fully digested with 0.25% trypsin, the precipitation was collected and 2 mL l% Triton X-100 was added for lysis. The supernatant was collected by centrifugation and the internalized phages were collected by using the same elution method and the collected phages were added to the HL-7702 cells subjected to the same blocking treatment the cells were incubated at 37°C for 1 h, the supernatant was collected and the phages for the first round of screening were obtained, they were used to infect ER2738, the titer was determined and they were amplified for the screening for the next round. In the screenings for the second, third and fourth rounds, the concentrations of the rinsing solution Tween-20 were successively increased to 0.2, 0.4 and 0.8%, the incubation durations with HepG2 were successively reduced to 45, 30 and 15 min and the frequency of rinsing were increased to 8, 10 and 12 times, respectively while the conditions and the procedures were all the same to those in the first round.

Phage monocloning: The phage elution from the fourth round was used to directly infect *E. coli* ER2738 in the plates. The overnight cultures of ER2738 were diluted in 1:100 and inoculated in LB medium and they were divided into small vials of 1 mL. A blue negative colony was randomly selected and inoculated into the culture tube of 1 mL as mentioned above and it was incubated at 37°C on a shaker for 4.5 h and totally 60 positive phage clones were selected. The cultures were transferred into micro centrifuge tubes and centrifuged for 30 sec, the supernatant was transferred into fresh tubes and centrifuged again. About 80% of the supernatant was transferred into fresh tubes and the amplified phages can be used for purification of sequencing template and ELISA detection.

ELISA identification of phage monoclones: HepG2 and HL-7702 were inoculated in the 96 well plate in a density of 1×10^4/well and incubated at 37°C in an incubator with 5% CO_2 for 24 h and the cells were subjected to serum-free treatment for 1 h; the cells were fixed with 4% paraformaldehyde for 20 min and rinsed with PBS for three

times; after the cells were blocked with 2% PBS-BSA for 1 h, phages in a titer of about 10^{10} were added and the cells were incubated at 37°C for 2 h, the cells were rinsed with 0.05% PBST for three times; HRP-antiM13 antibody (diluted with 2% PBS-BSA in 1:5000) and the cells were incubated at 37°C for 1 h then they were rinsed with 0.05% PBST for three times; TMB was used for coloration for 10 min and 2 M H_2SO_4 was used to stop the reaction. The plates were subjected to measurements on a micro-plate reader at 450 nm. The blue colonies in the original phage library were randomly selected as the random control, the wild-type M13 phage was used as the negative control and PBS was used as the blank control. The experiments were repeated for three times, the mean OD value and the binding specificity were calculated. OD490 nm phage clone/OD490 nm control >2 was considered as positive result.

Sequencing and peptide synthesis: The single-strand DNA was extracted from the positive clones with reference to the method for small-scale preparation of single-strand DNA from M13 phage in molecular cloning the fifth edition and it was sequenced by Beijing Huada Biotechnological Co., Ltd. the primer for sequencing was -96 gIII, 5'-CCC TCA TAG TTA GCG TAA CG-3'. The amino acid sequence of the exogenous septa-peptides fused to pIII protein were deduced according to the open reading frame of *pIII* gene in the coding chain; sequence homology analysis was carried out in SWISS-PROT, PepBank protein and polypeptide databases; Clustal X 1.83 program was used for multiple comparison and analysis on sequences; ANTHEPROT 5.0 program and ExPASy (http://www.expasy.org/tools) Software were used to analyze the biochemical characteristics of the polypeptides. The septa-peptide AS1-2 and the non-specific random control peptides were synthesized by Shanghai Ji'er Company and FITC was labeled.

Peptide-phage competitive inhibition test: HepG2 were inoculated in the 96 well plate in a density of 1×10^4/well and cultured overnight and the cells were subjected to serum-free treatment for 1 h and blocked with 2% BSA for 1 h. The polypeptides AS1-2 were diluted in series with PBS (0, 0.1, 1, 10, 100 and 1000 nM) and then were incubated with the cells at 4°C for 1 h, P13 and P15 (the titer was about 10^{11} pfu) were incubated with the cells at 4°C for 1 h. The phages were eluted by using the same method as mentioned above and the titer was determined.

The non-specific septa-peptides not related to phages in the peptide library were used as the negative control. The inhibition rate/Percentage = (The titer in the blank control well−The titers in the wells of different concentrations)/ The titer in the blank control well ×100%.

Immunocytochemical and immunohistological identification for the polypeptide: Cell climbing-slices were prepared according to conventional methods and HL-7702 cells were used as the control cells and the difference in the binding activities of the polypeptide FITC-AS1-2 on HepG2 cells and control cells was identified by using immuno-cytochemical staining (SP Method) after the blocking according to conventional immuno-cytochemical staining method. The slices were firstly incubated with FITC-AS1-2 (4 μg mL^{-1}) at 4°C overnight. Then, the slices were successively incubated with rat anti-FITC monoclonal antibody, anti-rat IgG secondary antibody and SP compound and DAB coloration was performed. After Mayor's hematoxylin counterstain, the staining of the cells was observed under a microscope and evaluations were carried out by combining the positive intensity of brown-color response and the percentage of positive cells with reference to the scoring method by Shimizu *et al.* (1990). In the mean time, blank control and negative control of non-specific phage clones were also set up.

The samples from human hepatoma tissues and the samples from corresponding paracancerous tissues were collected from the samples of surgical resection in the Second Affiliated Hospital of Jilin University from 2009 to 2010 and they were subjected to conventional frozen sectioning after definite diagnosis by pathological physicians. The immunohistochemical tests were all the same to those in the immuno-cytochemical tests.

Immmunofluorescence identification for FITC-tagged polypeptide: HepG2 and HL-7702 cells were inoculated in the 24 well plate in a density of 1×10^5/well and treated according to conventional methods, after blocking with 5% goat serum for 1 h, 200 μL FITC-AS2 (4 μg mL^{-1}, dissolved in PBS) was added and the cells were incubated at 4°C overnight and the cells were observed under an inverted fluorescence microscope after rinsing with PBS for six times.

RESULTS AND DISCUSSION

Enriching of phages specifically binding to hepatoma carcinoma cells: HepG2 cells were used as the target cells and HL-7702 cells were used as negative subtractive cells and the phage clones specifically binding to HepG2 cells were obtained after four rounds of subtractive screening

on the Ph.D-C7C™ Phage Display Peptide Library. After four rounds of screening, the phage clones binding to HepG2 were enriched for about 10^2 times and the results were shown in Table 1.

ELISA identification for phage clones specifically binding to hepatoma carcinoma cells: After four rounds of subtractive screening on the Ph.D-C7C™ Phage Display Peptide Library, 60 phage clones were randomly selected and the affinity of the phage clones to HepG2 was preliminarily identified by using ELISA and they were successively named as P1-60. The results showed that 18 clones from the 60 selected phage clones from the fourth round of screening showed relatively high affinity to HepG2 cells, in other words, $OD450\ nm_{phage\ clones}$/ $OD450\ nm_{control}$ >2 while their affinity to HL-7702 cells was relatively low and the results had positive significance indicating that the screening had satisfactory efficacy in enriching. The results were shown in Fig. 1.

DNA sequencing and sequence analysis for phages specifically binding to hepatoma carcinoma cells: The positive clones were amplified and the single-strand DNA was extracted and sequencing was carried out by Beijing Huada Company using -96 g primer and the amino acid sequence was deduced (Table 2). The 18 positive phage clones were deduced to 14 polypeptide sequences among them sequence loss was detected in two clones and the sequences were both positively charged; the homology analysis on amino acid sequence by using NCBI Blast

Table 1: Enrichment of phages for each round of selection from phage displayed peptide library

Round	Selected phage (input, cpu)	Eluted phage (output, cpu)	Ration (output/input)
1	4×10^{10}	4.3×10^{3}	1.075×10^{-7}
2	1×10^{11}	2.0×10^{4}	2.00×10^{-7}
3	6×10^{10}	9.0×10^{4}	1.5×10^{-6}
4	7×10^{10}	8.1×10^{5}	1.157×10^{-5}

Table 2: Screening results of the chosen phage clones for HepG2

Phage clones	Peptide No.	Peptide sequence (N-C)	Frequency
P13/P46	AS1	KNHRISL	2
P15/P29	AS2	FIHRIQL	2
P1	AS3	RILKISR	1
P7	AS4	KSTKSIR	1
P10	AS5	RLMMLRT	1
P11	AS6	RIQRMNI	1
P21	AS7	KLTRTIP	1
P24	AS8	NQPRMNI	1
P28	AS9	KPSRTIR	1
P34	AS10	KINRISL	1
P41	AS11	LTQRIIN	1
P47	AS12	RIMKMLI	1
P48	AS13	PIRPSLN	1
P53	AS14	PTIKSTK	1
P4/P57		Sequence loss	2

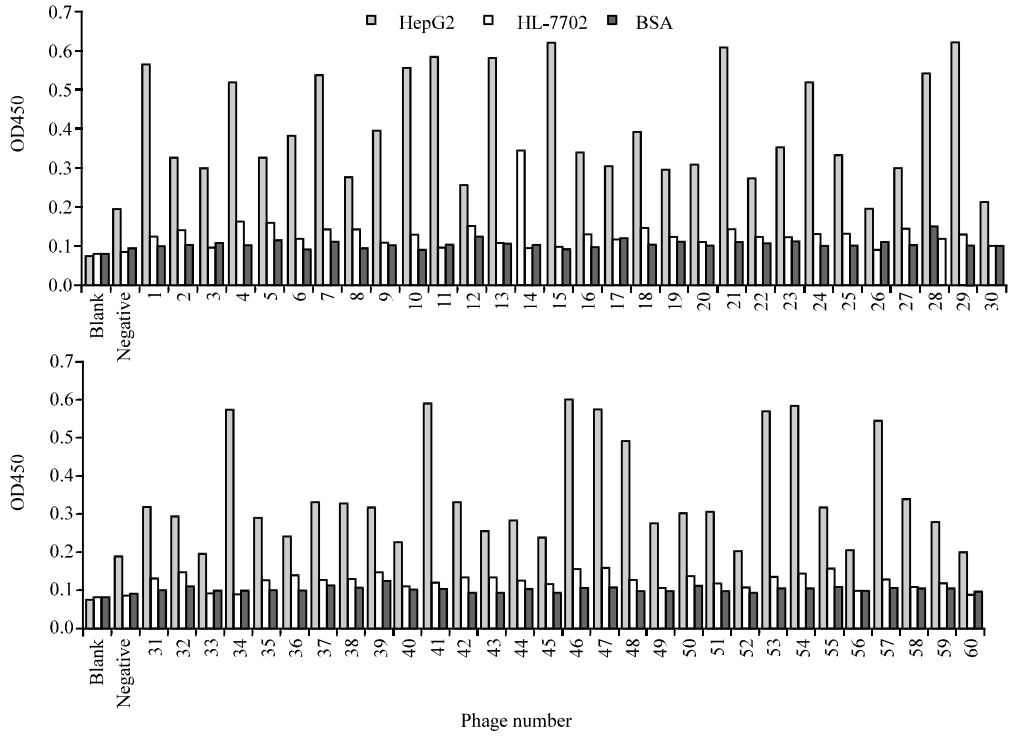

Fig. 1: ELISA identification for the affinity of 60 phage clones to HepG2 cells

showed that the similarity of the polypeptides to previously known sequences of proteins and polypeptides was not high and they were new polypeptides. Multiple comparison on the sequences was carried out by using clustalx1.83 and the result was shown in Fig. 2. The results showed that positive 1 and position 4 in the sequences were mostly Arg/Lys which were polar and positively charged amino acids, position 2 and 5 were mostly Ile/Leu which were nonpolar amino acids indicating that the sequences may be amphipathic.

Polypeptide-phage competitive inhibition test: Competitive inhibition test was carried out to confirm whether the synthesized polypeptide and corresponding phage monoclone compete for the same binding site. The result showed that the binding capacity of corresponding phage clones P13 and P15 to HepG2 cells showed a dose-dependent decrease to the polypeptides after pre-treatment with the synthetic polypeptide AS1-2 (Fig. 3) among them the inhibition rates of AS1 and AS2 on the binding of phages to HepG2 cells were 7.14 and 9% when the concentration of the polypeptide was 0.1 nmol L^{-1} and the inhibition rates on the binding of phages to HepG2 cells were 73.67 and 82.33% in average

Fig. 2: Results from the multiple comparisons on the sequence by using clustalx1.83. The results indicated that positive 1 and position 4 in the sequences were mostly Arg/Lys which were polar and positively charged amino acids, position 2 and 5 were mostly Ile/Leu which were nonpolar amino acids

when the concentration of the polypeptide was 1000 mmol L^{-1} and the inhibitory effects were obvious. The results were shown in Fig. 3.

Detection of the affinity of target peptide to HepG2 cells by using immuno-cytochemical and immunohistochemical methods: The affinity of the phage peptide to HepG2 was preliminarily identified in result 2.2, result 2.4 showed that the inhibition of the polypeptide AS1-2 on the binding of phages to HepG2 was obvious hence, the polypeptide AS1-2 was used for further immuno-cytochemical and immuno-histochemical identification in HepG2 and HL-7702 cells. After DAB staining and hematoxylin counterstain, the staining for the cells can be observed under the microscope (200x), the affinity of AS1-2 to HepG2 was relatively high, dark-brown staining can be detected in the cells and they showed positive result; the brown staining was seldom seen in the HL-7702 group indicating that the affinity of the polypeptide to HL-7702 cells was relatively weak and the result was negative almost no obviously dark-brown DAB staining was detected in the random peptide control, in other words, the binding of the random control polypeptide to HepG2 and HL-7702 was very weak and the result was negative indicating that AS1-2 had high affinity to hepatoma carcinoma cells while the affinity to normal HL-7702 cells was very weak (Fig. 4). The immunohistochemical results were consistent with those mentioned (Fig. 5).

Immunofluorescence identification for FITC tagged polypeptide AS1-2: The results from further identification on affinity and specificity of AS1-2 to HepG2 and human hepatoma tissues by using immunofluorescence analysis indicated that the green fluorescence of HepG2 was relatively strong but the fluorescence in normal HL-7702 cells was very weak indicating that AS1-2 had high affinity to hepatoma carcinoma cells while the affinity to

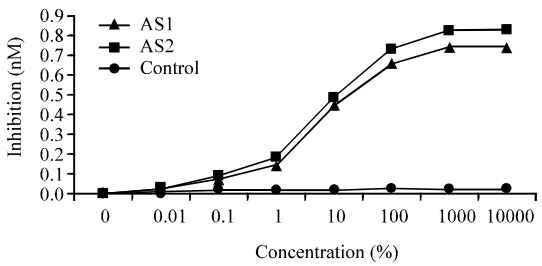

Fig. 3: Results from the competitive inhibition of the polypeptide AS-1-2

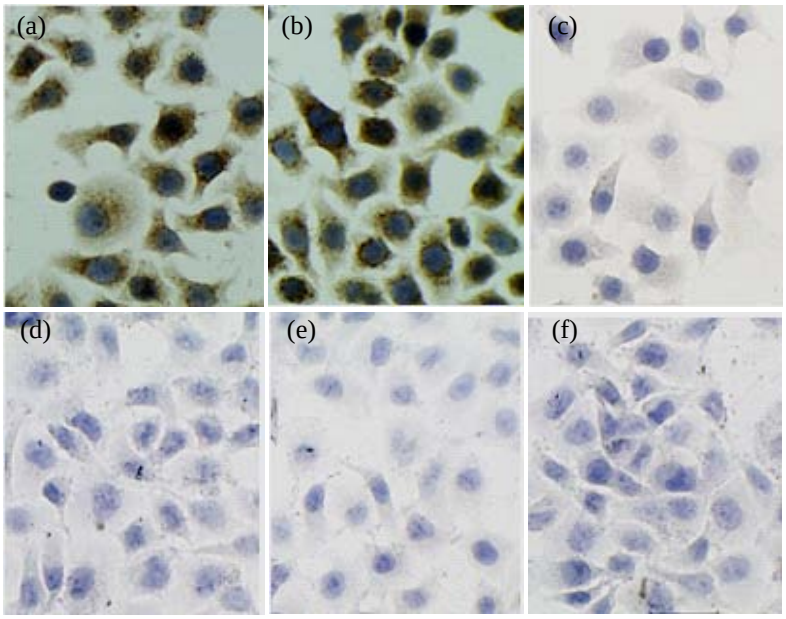

Fig. 4: Immuno-cytochemical detection for the affinity of the polypeptide to HepG2 and HL-7702 cells (400x). a-c) The coloration results after the reactions between the polypeptides AS-1, AS-2, the random control peptide and HepG2 cells; d-f) The coloration results after the reactions between the polypeptides AS-1, AS-2, the random control peptide and HL-7702 cells

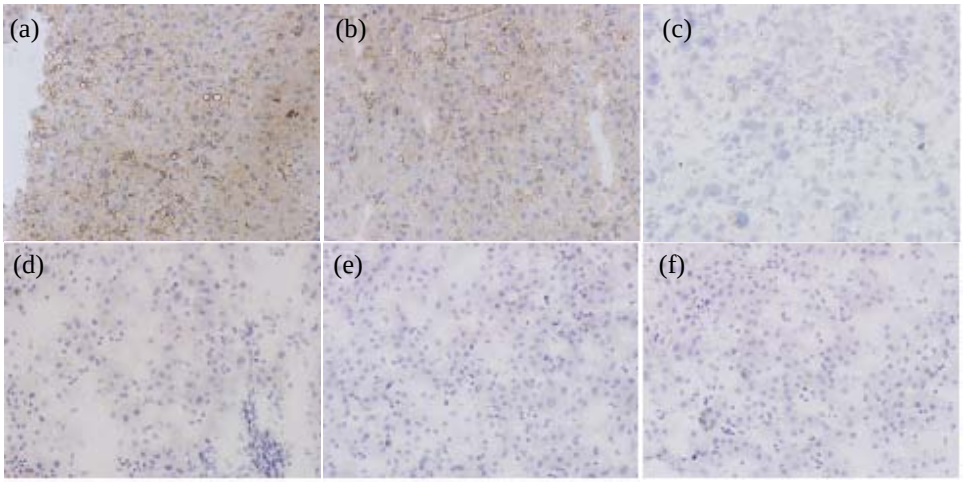

Fig. 5: Immunohistochemical detection for the affinity of the polypeptide to HepG2 cells (200x). a-c) The coloration results after the reactions between the polypeptides AS-1, AS-2, the random control peptide and human hepatoma tissues; d-f) The coloration results after the reactions between the polypeptides AS-1, AS-2, the random control peptide and normal hepatic tissues

normal HL-7702 cells was very weak (Fig. 6). Among them A was the negative control, B and C showed the fluorescence signals after the reactions of AS1 and AS2 with HepG2. The results from the fluorescence in different tissues were consistent with those mentioned above and the results were shown in Fig. 7. The sequences for the two polypeptides were KNHRISL and FIHRIQL.

It is the major development tendency to search for new specific markers for liver cancer and targeted therapy. The structures of small-molecule polypeptides are simple and the penetration to tumors is high, immunological responses are not liable to be induced in human body and thus they can be used as ideal targeting vector (Yang *et al*., 2008). It has been reported in previous

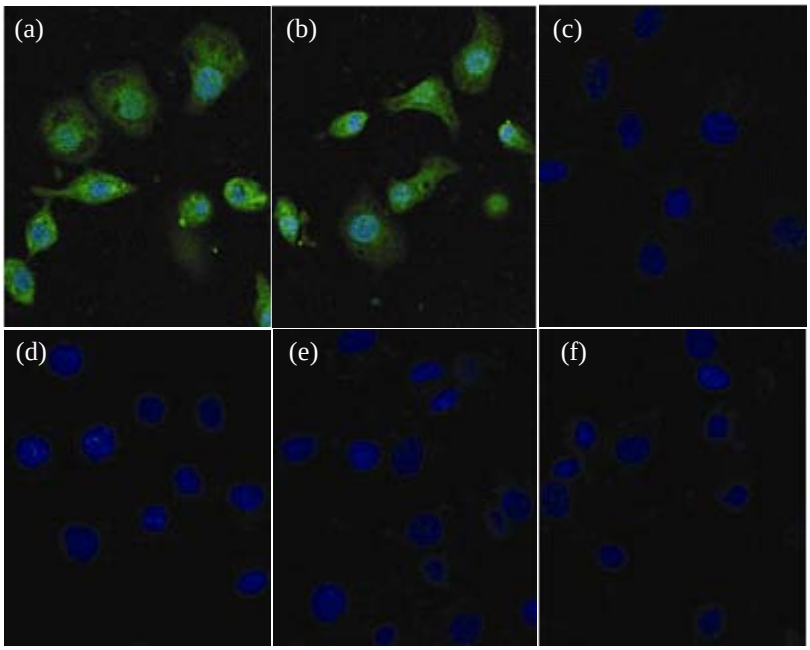

Fig. 6: Immunofluorescence cytochemistry identification for FITC tagged polypeptide AS1-2 (400x); a-c) showed the coloration results after the reactions between the FITC tagged polypeptide AS-2, the random control peptide and HepG2 cells; d-f) showed the coloration results after the reactions between the FITC tagged polypeptide AS-2, the random control peptide and HL-7702 cells; the nuclei were counterstained with Hochest33342 and the fluorescence was blue

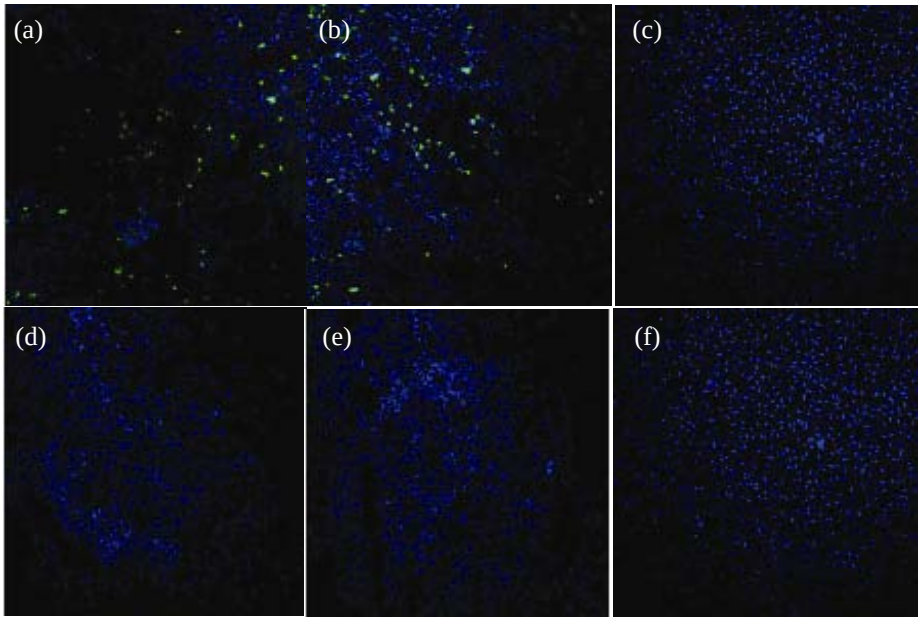

Fig. 7: Immunofluorescence histochemistry identification for FITC tagged polypeptide AS1-2 (200x); a-c) showed the coloration results after the reactions between the FITC tagged polypeptide AS-2, the random control peptide and human hepatoma tissues; d-f) showed the coloration results after the reactions between the FITC tagged polypeptide AS-2, the random control peptide and normal hepatic tissues; the nuclei were counterstained with Hochest33342 and the fluorescence was blue

studies that the proteins and polypeptides specifically recognizing or binding to cells can be separated by using phage peptide library technique (Shukla and Krag, 2005). Therefore, phage display peptide library has played important roles in the investigations on targeted therapy on various kinds of tumors. Subtractive screening on phage random peptide library by using living cells as targets has been widely used in the investigations on tumors (Zhang *et al*., 2009; Lou *et al*., 2001). When the structural information for a certain receptor molecule or epitope is not well understood, the receptors on the cell surface are unknown and integral membrane proteins are difficult to be isolated, living cells expressing the receptor or antigen are used as the targets for screening the phage peptide library which can completely simulate the interaction between protein molecules under natural conditions, obtain the target peptide and search for functional simulated peptides specifically binding to cells. Therefore, the screening is a unbiased process without selection pressure, the binding is dependent on the distribution density and the exposure degree of surface molecules as well as the affinity to polypeptides (Lou *et al*., 2001; De Kruif *et al*., 1995). It is very helpful for tumor cells with high surface complexity and variability (Itoh, 2007) and it provides an effective tool for the investigations on tumor-specific polypeptide vectors.

Phage peptide libraries can be divided into two categories according to whether the configuration of the exogenous fusion polypeptide is limited: some polypeptides are displayed on the surface of phage and their configurations are not limited in other words, disulfide bonds constrain the configuration of exogenous fusion polypeptide do not exist and the polypeptide is linear the other groups are composed of the peptide libraries with limited configuration which are characterized by Cys residues at both sides of the exogenous random polypeptide, a disulfide bond can be formed on the surface of phage all of the structure of the displayed polypeptide is confined in a dithio-loop and the binding configuration has higher stability and binding capacity in comparison to the polypeptides expressed in linear forms (Lulu and Chuan, 2002). During the screening of corresponding ligands for receptors if ideal ligand sequences are scarce or do not exist (not representative) or the polypeptide molecules can not be screened out since they have no sufficiently high affinity to bind to the target molecule, it is easier to make progress to use configuration-limiting peptide library in comparison to complete random peptide library. Therefore, the present study selected the Ph.D. C7C phage display library for the screening.

Efficacy of screening methods is a very important factor for obtaining targeting peptides and phage display peptide library has been successfully applied to the screening of peptides specifically binding to tumors (Chen *et al*., 2009; Wu *et al*., 2010; Staquicini *et al*., 2010; Pameijer *et al*., 2006). Since, several kinds of proteins, carbohydrates and lipids are expressed on the surface of tumor cells, they can bind to phages to certain extents and the tumor antigens for screening are all non-specific antigens therefore, subtractive screening or methods with previously known ligands are required to screen for targeting peptides (Pakkala *et al*., 2007). When the ligand is unknown, the present study carried out subtractive screening in which the phages in the peptide library were firstly kept to react with hepatoma carcinoma cells and normal hepatic cells were used for subtraction. During the screening, glycine buffer was firstly used to elute and collect the phages binding to the cell surface then the cells were digested with 0.1% TritonX-100 and lyzed, the permeability of cell membrane was increased and the possibly internalized phages were collected since, the characteristics for its internalization, it may be used as the penetrating peptide on the investigations for transmitting drugs or targeting vectors, both targeting binding peptides and internalized peptides have intensive usages in screening for anti-tumor peptides and drug-coupled targeted therapy on tumors.

The present study carried out four rounds of screening by using phage peptide library technique and 60 phage clones were randomly selected for preliminary identification and sequencing. It was found that the polypeptide sequences were positively charged which confirmed the assertion that the membrane surface of tumor cells was negatively charged in another viewpoint (Zachowski, 1993) the similarity analysis on the polypeptides showed that positive 1 and position 4 in the sequences were mostly Arg/Lys which were polar and positively charged amino acids, position 2 and 5 were mostly Ile/Leu which were nonpolar amino acids indicating that the sequences may be amphipathic. It still needs further studies to examine whether it is related to the motif in the receptor-binding peptide on the surface of hepatoma carcinoma cells. Due to the random error during exogenous nucleotide insertion in M13 filamentous phage, gene mutation and predominant amplification may occur during amplification and the antigens or receptors on the surface of tumor cells are complex and diverse (Midorikawa *et al*., 2010) it is very difficult to screen out homologous phage polypeptides but some or certain kinds of amino acids with relatively high frequency may exist in these polypeptides, they may be related to the motif in the receptor-binding peptides on the surface of

hepatoma carcinoma cells and it may play important roles in the interactions between receptors and ligands as well as those between proteins. Koivunen *et al.* (1993) screened out the α5β1 binding peptide with high affinity by using phage display peptide library in 1993 and obtained the binding motif RGD and it was confirmed in further experiments that it can be used as the penetrating peptide to reinforce the anti-tumor activity of anti-tumor drugs (Sugahara *et al*., 2010). Zahed *et al.* (2011) found during the screening for GRP94 carboxyl-terminal binding peptide by using Ph.D-C7C™ Phage Display Peptide Library that Proline (Pro) and Leucine (Leu) were frequently found in the polypeptide and it was supposed to be related to the binding of the polypeptide to GRP94 (Zahed *et al*., 2011). Therefore, it will be valuable for further investigations and applications when the obtained DNA sequence of the phage polypeptide was reliable and it can specifically bind to hepatoma carcinoma cells. The novel polypeptide obtained by us can specifically bind to hepatoma carcinoma cells and it has potential values in clinical applications as efficient targeting vectors for liver cancer.

CONCLUSION

Two polypeptides specifically binding to hepatoma carcinoma cells were obtained which established the experimental basis for further investigations on highly targeted drugs for liver cancer, early diagnosis of liver cancer and targeted therapy.

ACKNOWLEDGEMENTS

This research was supported by the Natural Science Foundation of Jilin Province, Major Research Plan (Grant No.: 201015165) and National Natural Science Foundation of China (Grant No.: 31101865) and Basic research and operating expenses of Jilin University (201103048). Guoxi Shao and Haiyang Yan contributed equally to this research.

REFERENCES

Chen, B., S. Cao, Y. Zhang, X. Wang and J. Liu *et al*., 2009. A novel peptide (GX1) homing to gastric cancer vasculature inhibits angiogenesis and cooperates with TNF α in anti-tumor therapy. BMC Cell. Biol., Vol. 10. 10.1186/1471-2121-10-63

De Kruif, J., L. Terstappen, E. Boel, and T. Logtenberg, 1995. Rapid selection of cell subpopulation-specific human monoclonal antibodies from a synthetic phage antibody library. Proc. Natl. Acad. Sci. USA., 92: 3938-3942.

Du, B., M. Qian, Z. Zhou, P. Wang and L. Wang *et al*., 2006. *In vitro* panning of a targeting peptide to hepatocarcinoma from a phage display peptide library. Biochem. Biophys. Res. Commun., 342: 956-962.

El-Serag, H.B. and K.L. Rudolph, 2007. Hepatocellular carcinoma: Epidemiology and molecular carcinogenesis. Gastroenterology, 132: 2557-2576.

Ferrer, M., B.J. Sullivan, K.L. Godbout, E. Burke and H.S. Stump *et al*., 1999. Structural and functional characterization of an epitope in the conserved C-terminal region of HIV-1 gp120. J. Pept. Res., 54: 32-42.

Hu, S., X. Guo, H. Xie, Y. Du and Y. Pan *et al*., 2006. Phage display selection of peptides that inhibit metastasis ability of gastric cancer cells with high liver-metastatic potential. Biochem. Biophys. Res. Commun., 341: 964-972.

Itoh, K., 2007. Development of diagnostically and therapeutically useful human antibody medicines by phage display system. Yakugaku Zasshi, 127: 43-53, (Article in Japanese).

Kelly, K.A., S.R. Setlur, R. Ross, R. Anbazhagan, P. Waterman, M.A. Rubin and R. Weissleder, 2008. Detection of early prostate cancer using a hepsin-targeted imaging agent. Cancer Res., 68: 2286-2291.

Koivunen, E., D.A. Gay and E. Ruoslahti, 1993. Selection of peptides binding to the α5β1 integrin from phage display library. Bournal Biol. Chem., 268: 20205-20210.

Kumar, S.R., T.P. Quinn and S.L. Deutscher, 2007. Evaluation of an 111In-radiolabeled peptide as a targeting and imaging agent for ErbB-2 receptor expressing breast carcinomas. Cancer Res., 13: 6070-6079.

Lou, J., R. Marzari, V. Verzillo, F. Ferrero and D. Pak *et al*., 2001. Antibodies in haystacks: how selection strategy influences the outcome of selection from molecular diversity libraries. J. Immunol. Methods, 253: 233-242.

Lulu, M. and L. Chuan, 2002. New advances in bacteriophage display peptide libraries screening against different targets. Bull. Acad. Military Med. Sci., 26: 135-138.

Midorikawa, Y., Y. Sugiyama and H. Aburatani, 2010. Molecular targets for liver cancer therapy: From screening of target genes to clinical trials. Hepatol. Res., 40: 49-60.

Pakkala, M., C. Hekim, P. Soininen, J. Leinonen and H. Koistinen *et al*., 2007. Activity and stability of human kallikrein-2-specific linear and cyclic peptide inhibitors. J. Pept. Sci., 13: 348-353.

Pameijer, C.R., A. Navanjo, B. Meechoovet, J.R. Wagner and B. Aguilar *et al*., 2006. Conversion of a tumor-binding peptide identified by phage display to a functional chimeric T cell antigen receptor. Cancer Gene Ther., 14: 91-97.

Scott, J.K. and G.P. Smith, 1990. Searching for peptide ligands with an epitope library. Science, 249: 386-390.

Shimizu, M., Y. Saitoh and H. Itoh, 1990. Immunohistochemical staining of Ha-ras oncogene product in normal, benign and malignant human pancreatic tissues. Hum. Pathol., 21: 607-612.

Shukla, G.S. and D.N. Krag, 2005. Phage display selection for cell-specific ligands: Development of a screening procedure suitable for small tumor specimens. J. Drug. Target., 13: 7-18.

Sidhu, S.S., W.J. Fairbrother and K. Deshayes, 2003. Exploring protein-protein interactions with phage display. Chembiochem, 4: 14-25.

Staquicini, F.I., R.L. Sidman, W. Arap and R. Pasqualini, 2010. Phage display technology for stem cell delivery and systemic therapy. Adv. Drug. Deliv. Rev., 62: 1213-1216.

Sugahara, K.N., T. Teesalu, P.P. Karmali, V.R. Kotamraju, L. Agemy, D.R. Greenwald and E. Ruoslahti, 2010. Coadministration of a tumor-penetrating peptide enhances the efficacy of cancer drugs. Science, 328: 1031-1035.

Thomas, M., 2009. Molecular targeted therapy for hepatocellular carcinoma. J. Gastroenterol., 44: 136-141.

Witt, H., K. Hajdin, K. Iljin, O. Greiner, F.K. Niggli, B.W. Schafer and M. Bernasconi, 2009. Identification of a rhabdomyosarcoma targeting peptide by phage display with sequence similarities to the tumour lymphatic-homing peptide LyP-1. Int. J. Cancer, 124: 2026-2032.

Wu, X., Q. Yan, Y. Huang, H. Huang and Z. Su, 2010. Isolation of a novel basic FGF-binding peptide with potent antiangiogenetic activity. J. Cell. Mol. Med., 14: 351-356.

Yang, W., D. Luo, S. Wang, R. Wang and R., Chen *et al*., 2008. TMTP1, a novel tumor-homing peptide specifically targeting metastasis. Clin. Cancer Res., 14: 5494-5502.

Zachowski, A., 1993. Phospholipids in animal eukaryotic membranes: Transverse asymmetry and movement. Biochem. J., 294: 1-14.

Zahed, M., T. Suzuki, K. Kita, S. Sugaya and N. Suzuki, 2011. Screening of novel peptide ligands bound to the carboxy-terminal domain of GRP94 by phage display. Chiba Med. J., 87: 61-68.

Zhang, B., Y. Zhang, J. Wang, Y. Zhang and J. Chen *et al*., 2009. Screening and identification of a targeting peptide to hepatocarcinoma from a phage display peptide library. Cancer Lett., 281: 246-254.