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Identification of a Novel Peptide Ligand of Human Vascular Endothelia Growth Factor Receptor 3 for Targeted Tumour Diagnosis and Therapy

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Human vascular endothelia growth factor receptor 3 (VEGFR-3) is up-regulated in a variety of human cancers. It is a potentially rational target for drug delivery. To identify novel ligands with specific binding capabilities to VEGFR-3, we screened a phage display peptide library and found a consensus motif of the peptides which is displayed by the positive phages CSDxxHxWC (x is any amino acid). The phage displaying peptide CSDSWHYWC (designated as P1) exhibited the highest affinity to VEGFR-3 in phage ELISA and the chemically synthesized P1 could bind to VEGFR-3 specifically in a dose-dependent manner. In addition, the flow cytometry assay and immunoflourescence showed that the FITC labelled P1 could bind to VEGFR-3 positive carcinoma cells with specificity. Our study suggests that P1 may be a homing peptide for treatment of tumours.

Key words: phage display, peptide, VEGFR-3.

Abbreviations: FITC, fluorescein isothiocyanate; HRP, horseradish peroxidase; OPD, o-Phenylenediamine; PBS, phosphate-buffered saline; VEGFR-3, vascular endothelia growth factor receptor 3.

During the past 20 years, the experimental proof that some receptors can be used successfully as molecular targets for cancer diagnosis and therapy has been provided. It is on the basis of the discovery that these receptors are overexpressed in cancer in comparison to normal tissues (*1*). Once some molecules which could bind to these receptors with high affinity are found, they can be radiolabelled or coupled with anti-tumour drugs, such as proteins, peptides and chemicals. These complexes will have better applications in clinical diagnosis or treatment for tumours. The molecules that have been found include monoclonal antibodies and peptides. The latter are more suitable for tumour treatment because of their less molecular weight and because they can be easily synthesized. Targeting the receptors which overexpressed in tumour cells by small peptides will be one of the most promising fields for the next decades (*2*).

Human vascular endothelia growth factor receptor 3 (VEGFR-3) is a new promising target which has been identified recently. VEGFR-3 belongs to III receptor tyrosine kinase family and the mature VEGFR-3 consists of extracellular region, transmembrane region and intercellular (*3*). VEGFR-3 is initially expressed in all embryonic endothelia, but its expression in the blood vessel endothelium decreases during development, and it becomes largely restricted to the lymphatic endothelium in adult tissues (*4*). Recently, more and more data showed that VEGFR-3 expression was increased in a variety of human tumours (*5–11*). In addition, some animal assays showed that inhibiting the signal pathway of VEGFR-3 could prevent the growth and metastasis of tumour (*12–14*). All these features of VEGFR-3 make it a new promising target for tumour diagnosis and therapy.

In order to find a new peptide that can bind VEGFR-3, we screened the phage display peptide library with human VEGFR-3. The result revealed that we obtained a novel peptide (P1) which had a high affinity with VEGFR-3, suggesting that P1 may be a new targeting peptide for tumour diagnosis and treatment.

MATERIALS AND METHODS

Cell Lines and Proteins—The human colon cancer cell line (HT29), human umbilical vein endothelial cell line (EVC304), the human retinoblastoma cell line (Y79), the human amino cell line (WISH), human hepatic cancer cell line (HepG2) and human alveolar epithelial cell line (A549) were routinely cultivated as monolayer at 37°C and in 5% CO_2 atmosphere in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum containing 0.1 mg/ml ampicillin and 0.1 mg/ml streptomycin. All the cell lines were purchased from the National Rodent Laboratory Animal Resource (Shanghai, China). Human VEGFR-3 (hVEGFR-3), human VEGFR-2(hVEGFR-2), human VEGFR-1(hVEGFR-1) ectodomain Fc fusion

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proteins and human IgG1 Fc were purchased from R&D Systems, Minneapolis, MN, USA.

Phage Library and Biopanning—A total of three successive rounds of panning with hVEGFR-3 ectodomain Fc fusion protein were performed with a random phage display constrained 7-mer peptide library (New England Biolabs, Beverly, MA). The procedure for screening the phage display library was modified according to instruction of the manufacturer of the kit (New England Biolabs, Beverly, MA). Briefly, 96 well-plate was incubated with human IgG1 Fc or human VEGFR-3 ectodomain Fc fusion protein (15 μg/well) at 4°C. Wells were blocked for 1 h with 1% bovine serum albumin (BSA) in TBS, and rinsed with binding buffer (TBS, 0.1% Tween-20 or 0.5% Tween-20). For pre-absorption, 2×10^{11} plaque forming units of the phage library in 100 μl binding buffer were added to the human IgG1 Fc coated wells. After 1 h incubation at room temperature, the pre-absorbed phages were applied to the hVEGFR-3 ectodomain Fc fusion proteins coated wells for specific screening. Unbound phages were removed by extensive washing with TBS/0.5% Tween 20. Bound phages were eluted with 0.2 mol/l Glycine–HCl/0.1% BSA, pH 2.2 and immediately neutralized with 15 μl of 1 mol/l Tris–HCl, pH 9.0. Phages were amplified in *Escherichia coli* ER2738, precipitated from the bacterial culture supernatant with polyethylene glycol, then titred before the next round of biopanning. Titre determination utilized aliquots of the elution or the amplification that were plated in serial dilutions on Luria broth agar plates (20 μg/ml tetracycline).

Colony Screening Assay—After three rounds of biopanning, colony screening for selection of specific phage clones was performed. The 96-well plate was incubated with hVEGFR-3 ectodomain Fc fusion protein (100 ng/well) or human IgG1 Fc (100 ng/well) overnight at 4°C. After blocking, 60 phage clones (2×10^9/well) were added to the wells and incubated for 1 h at room temperature. Wells were washed 10 times with TBS/0.5% Tween 20 and bound phage was detected using a horseradish peroxidase (HRP)-conjugated anti-M13 monoclonal antibody (Amersham Biosciences, Freiburg, Germany). The reaction was developed with o-Phenylenediamine (OPD, Sigma, St Louis, MO, USA). After ending the development reaction with 2 mol/l sulphuric acid, the absorption value was read at 490 nm with a Bio-Rad ELISA reader. Positive clones were amplified and identified by DNA sequencing.

DNA Sequencing—Single strand phage DNA was prepared with 20% polyethylene glycol-8000 (PEG)/NaCl according to the random phage display constrained 7-mer petide library kit (New England Biolabs, Beverly, MA). Prepared DNA amount was verified by EtBr2/0.8% agarose gel under UV illumination. DNA sequencing was done by Sangon (Shanghai, China).

Phage 1 Specifically Binding to hVEGFR-3—The phage which exhibit the best affinity to hVEGFR-3 was designated as phage 1. To study whether it could specifically bind to hVEGFR-3, the hVEGFR-3, hVEGFR-2 and hVEGFR-1 ectodomain Fc fusion proteins were coated on the 96-well plate (100 ng/well) overnight at 4°C. After blocking, the phage 1 (2×10^9/well) was added to the wells and incubated for 1 h at room temperature. Wells were washed 10 times with TBS/0.5% Tween 20 and bound phage was detected using an HRP-conjugated anti-M13 monoclonal antibody. The reaction was developed with OPD. After ending the development reaction with 2 mol/l sulphuric acid, the absorption value was read at 490 nm with a Bio-Rad ELISA reader.

Peptide Synthesizing—Peptide C**SD**SW**H**Y**W**C (P1) and control peptide CSSSPSKYC (P5) were synthesized using Fmoc [*N*- (9-fluorenly) methoxycarbonyl] chemistry and purified by high-pressure liquid chromatography. For easy observation, fluorescein isothiocyanate (FITC) labelled peptides were also synthesized. Matrix-assisted laser desorption ionization-time of flight mass spectrometry was used to verify proper peptide synthesis and purity. Stock solution of the peptide was dissolved in phosphate-buffered saline (PBS, Irvine Scientific, Santa Anna, CA). Peptide concentration was calculated based on OD280.

P1 Specifically Binding to hVEGFR-3—The 96-well plate was incubated with different diluted solution of the synthesized peptide P1 and P5 (control) overnight at 4°C. After blocking with BSA, hVEGFR-3, hVEGFR-2 or hVEGFR-1 ectodomain Fc fusion protein, respectively were added to the well, and incubated at room temperature for 1 h. The wells were washed with PBST (PBS, 0.5% Tween 20) for six times and then the mouse anti human IgG1 Fc monoclonal antibody (R&D Systems, Minneapolis, MN) (1 : 2000) was added to the wells, incubating at room temperature for 1 h. After washing, the HRP-conjugated goat anti mouse IgG (ZhongShang Golden Bridge Biotechnology, Beijing, China) (1 : 1000) was added to the well and incubated for 1 h at room temperature. The reaction was developed with OPD(Sigma, St Louis, MO, USA). After ending the development reaction with 2 mol/l sulphuric acid, the absorption value was read at 490 nm with a Bio-Rad ELISA reader. In order to find whether P1 binding to VEGFR-3 in a dose-dependant manner, the different diluted solution P1 were coated on the 96-well plate overnight at 4°C, and after blocking, the VEGFR-3 and the human IgG1 Fc as a control were added. The remaining process was same as the above.

Identification of hVEGFR-3 Positive Cell Lines With Flow Cytometry—HT29 cells, Y79 cells, EVC304 cells, HepG2 cells, WISH cells and A549 cells were blocked with heat-inactivated normal goat serum in PBS pH 7.4 (1 : 20), washed and incubated at 4°C for 30 min with mouse anti hVEGFR-3 monoclonal antibody (1:10) or the isotype control mouse IgG1 (1 : 10). Samples were washed twice with PBS and then incubated with FITC labelled goat anti mouse IgG for 30 min at 4°C. After washing, the samples were fixed in 1% paraformaldehyde/PBS. All samples were analysed (Becton Dickinson FACS caliber) using Cellquest software (Becton Dickinson, San Jose, CA).

The FITC Labelled P1 Binding to hVEGFR-3 Positive Cells—HT29 cells, Y79 cells, EVC304 cells, HepG2 cells, WISH cells and A549 cells were blocked with heat-inactivated normal goat serum in PBS pH 7.4 (1 : 20), washed and incubated at 4°C for 30 min with FITC

labelled P1 or P5 (0.8 μg/ml). Samples were washed twice and then fixed as mentioned earlier. All samples were analysed (Becton Dickinson FACS caliber) using Cellquest software (Becton Dickinson, San Jose, CA).

Immunoflourescence Analysis of P1 Binding to HT29—HT29 cells and EVC304 cells were grown on glass coverslips to 50–85% confluence. The slides were washed with PBS for three times and fixed in cold acetone for 10 min, and then the cells were blocked with normal goat serum for 2 h at room temperature. After washing, FTIC labelled P1 and P5 (0.5 μg/ml) were applied to the cells and incubated for 1 h. Cells were washed and the propidium iodide (PI, Sigma, USA) (20 μg/ml) were applied to the cells for 5 min. After washing, the cells were mounted with PBS-glycerine (1 : 1). Results were analysed using a confocal microscope (Bio-Rad, CA, USA).

RESULTS

Biopanning and Colony Screening—A library of random constrained 7-mer peptides was screened with hVEGFR-3. As the first indicator of successful biopanning, an increase of phage titres during rounds of panning was observed. The phage titre increased from 9×10^6 plaque forming units(pfu)/ml (first round) to 1.19×10^8 pfu/ml (second round) and finally to 1.11×10^{10} pfu/ml (third round). A total of 60 phage clones were randomly chosen for colony screening after three successive rounds of biopanning. Among them, 29 clones with the highest affinity with hVEGFR-3 but not with the hIgG1 Fc and BSA were found, amplified and sequenced. Nine amino acid sequences were deduced from the sequencing result (Table 1).

Binding of hVEGFR-3 to the Peptides Displayed On Phage—The result of colony screening was confirmed by ELISA in a separate test system. All positive phages candidates were specifically recognized by hVEGFR-3, but not by human IgG1 Fc or the blocking protein (BSA) (Fig. 1). Among them, phage 1 has the best binding ability with hVEGFR-3. Moreover, hVEGFR-3 did not react with control phages, which were washed off in the first round biopanning. Our results also observed that the phage 1 only binds to hVEGFR-3 but not hVEGFR-2 or hVEGFR-1 (Fig. 2).

To determine whether the peptide still has the ability to bind to hVEGFR-3 after it departs from the phage, P1 which exhibited the highest affinity to hVEGFR-3 in phage ELISA was chemically synthesized. The ELISA assay showed that P1 could specifically bind to hVEGFR-3 but not control protein (Fig. 3A). In addition, a dose-dependent increase of the binding of P1 to hVEGFR-3 was observed in the ELISA (Fig. 3B).

VEGFR-3 Expressions on HT29 Cells and Y79 Cells Are Positive—To identify VEGFR-3 positive cell line, six human cell lines were analysed with the mAb against VEGFR-3 by flow cytometry. The results show that VEGFR-3 was highly expressed on HT29 cells (42.3%) and Y79 cells (53.1%) and weakly expressed on A549 cells (8.8%), while EVC304 cells, WISH cells and HepG2 cells were VEGFR-3 negative (2.3%, 3.4% and 3.9%, respectively) (Fig. 4).

Binding of the FITC Labelled P1 to HT29 Cells and Y79 Cells—To determine whether P1 could bind to the natural VEGFR-3 on cells, the FITC labelled P1 was also chemically synthesized and the association ability of P1 with the six cell lines above were analysed by flow cytometry assay. The result indicated that the FITC labelled P1 could specifically bind to VEGFR-3 positive cells, including HT29 cells (41.3%) and Y79 cells (47.7%), but not bind to the VEGFR-3 negative cells, such as EVC304 cells (1.2%), WISH cells (1.2%) and HepG2 cells (7.2%) (Fig. 5).

P1 Binding to HT29 Cells—The result of immunoflourescence assay was according with the result of FCM. P1 could specifically bind to HT29 cells but not to EVC304 cells (Fig. 6).

Table 1. **Analysis of the positive phage clones in colony screening assay by DNA sequencing**

No. of peptide	Amino acid sequences	Frequency[a]
1	C**SD**SW**H**Y**W**C	**10/29**
2	C**SD**WQ**H**P**W**C	**8/29**
3	C**SD**YN**H**H**W**C	**4/29**
4	C**SD**GQ**H**Y**W**C	2/29
5	CY**D**SW**H**Y**W**C	1/29
6	CF**D**GNHI**W**C	**1/29**
7	CTDFPRSFC	**1/29**
8	CTQDRQHPC	**1/29**
9	CLSRYLDQC	**1/29**

[a]Frequency of peptide appearance/sequenced 29 positive clones.

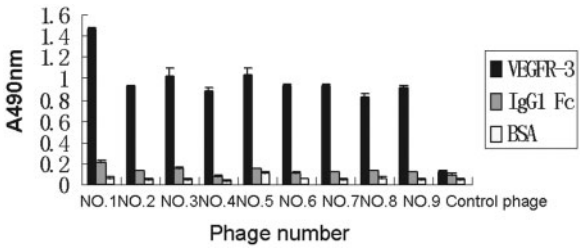

Fig. 1. **VEGFR-3 specifically recognized positive phages screened by biopanning (ELISA)**. VEGFR-3 recognized phages displaying the peptide inserts, sequences were shown in Table 1.

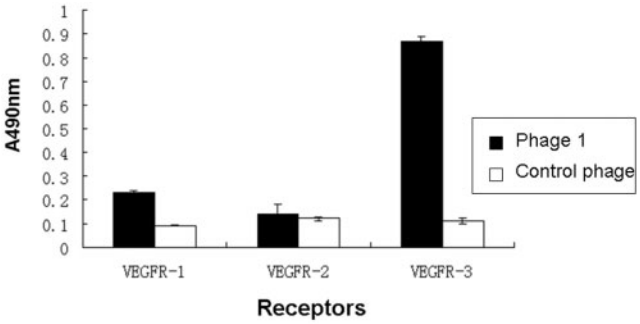

Fig. 2. **Phage 1 binding to hVEGFR-3 specifically**. Binding of phage 1 with hVEGFR-1, hVEGFR-2 and hVEGFR-3 was detected. Phage 1 could only bind to hVEGFR-3 but not to hVEGFR-1 and hVEGFR-2.

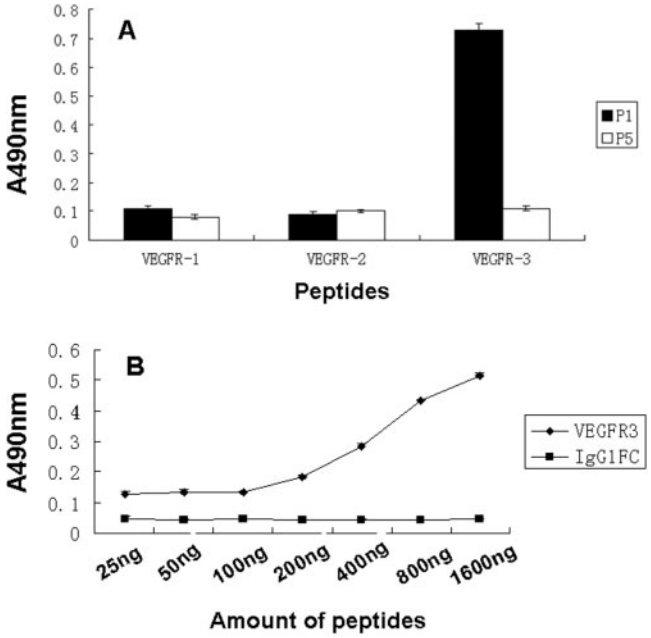

Fig. 3. **Synthesized peptide (P1) binding to hVEGFR-3 specifically**. (A) P1 could associate with the hVEGFR-3 specifically but not with the hVEGFR-1 or hVEGFR-2. P5 was as a peptide control. A representative result of three perform was shown. (B) P1 binding to hVEGFR-3 is dose-dependent. The 96-well plate was coated with P1. The binding ability of hVEGFR-3(filled diamond) or IgG1FC(filled square) with P1 was analysed. A representative result of three perform is shown.

DISCUSSION

The rapid development in current pharmaceutical drug discovery has resulted in the emergence of increasing numbers of novel therapeutic drugs for the treatment of a variety of diseases. However, at present the main problem associated with systemic drug administration is likely to include even biodistribution of pharmaceuticals throughout the body, the lack of drug-specific affinity toward a pathological site, non-specific toxicity and other side effects resulting from high doses (*15*). An attractive strategy to enhance the therapeutic index of drugs is to specifically deliver them to the defined target cells, not to healthy cells which are sensitive to the toxic effects of the drugs. In order to make this strategy into application, two problems have to be solved. The one is to identify the specific molecules on target cells. The other is to find ligands which can recognize and associate those molecules. Phage display is a powerful tool for screening novel ligands for various target proteins (*16*). Especially it has been used extensively *in vitro* and in animal models to generate ligands and to identify cancer-relevant targets. Many tumour-homing peptides have been found with this method, such as NGR (Asn-Gly-Arg), RGD (Arg-Gly-Asp) and GSL (Gly-Ser-Leu) (*17*). Coupling anticancer drugs or peptides to the RGD or NGR peptides yields compounds with increased efficacy against tumours and lowered toxicity to normal tissues in mice (*18*).

Recently, several studies reported the successful identification of the peptide ligands, such as vascular

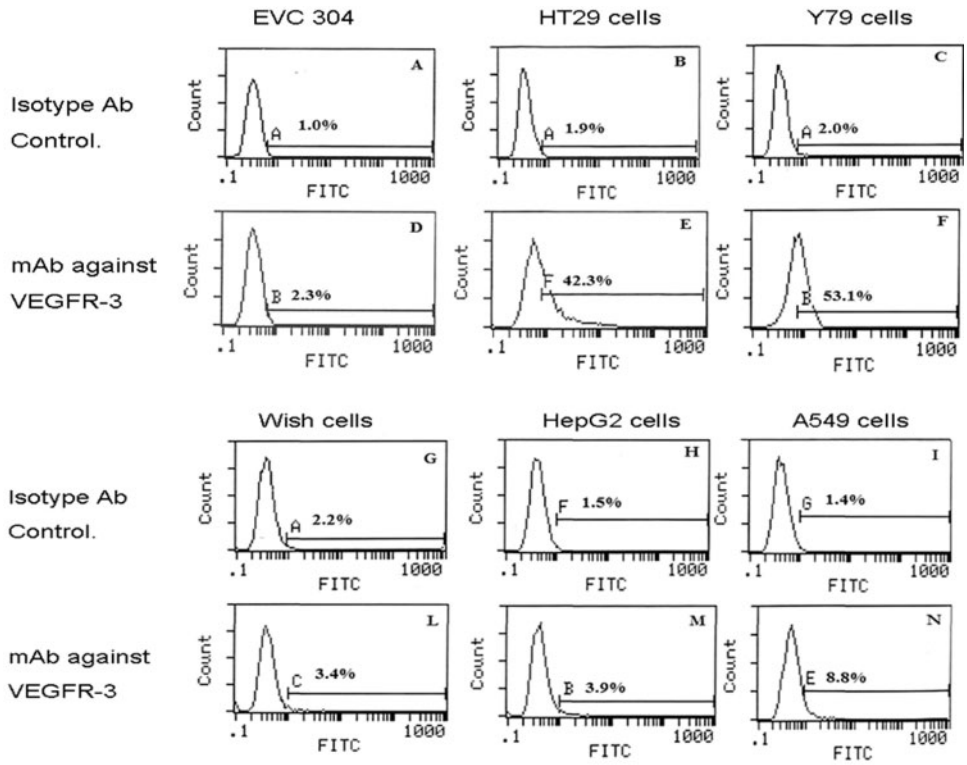

Fig. 4. **HT29 cells and Y79 cells are VEGFR-3 positive**. (A, B, C, G, H, I) The isotype Ab reacted with the six cells for gating. (D, E, F, L, M, N) The mAb against VEGFR-3 did not react with EVC304 cells, WISH cells or HepG2 cells but reacted weakly with A549 cells and reacted strongly with HT29 cells and Y79 cells. A representative result of three perform was shown.

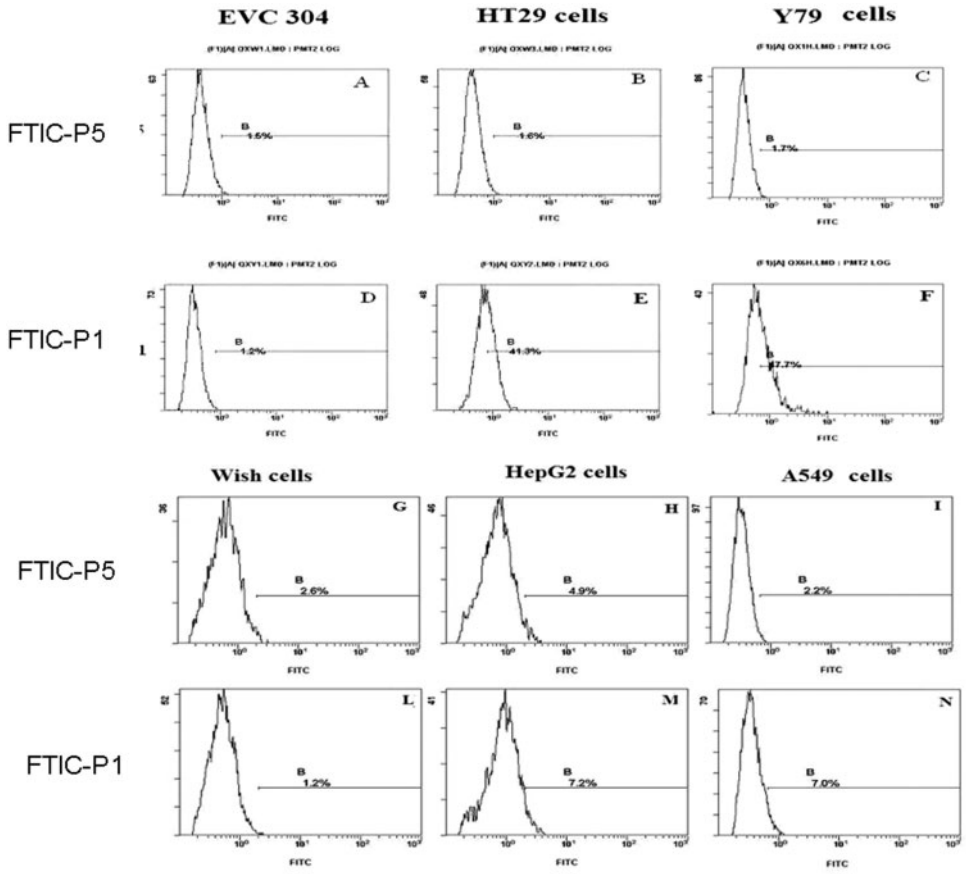

Fig. 5. **P1 specifically binding to HT29 cells and Y79 cells**. (A, B, C, G, H, I) FTIC-P5 (control peptide) did not bind to the six cells for gating.(D, E, F, L, M, N) FTIC-P1 did not react with EVC304 cells, WISH cells or HepG2 cells but reacted weakly with A549 cells and reacted strongly with HT29 cells and Y79 cells. A representative result of three perform is shown.

epithelia growth factor 1(VEGFR-1), VEGFR-2 and focal adhesion kinase (FAK) which is a tyrosine kinase interacting with VEGFR-3 (*19–21*). All these peptide ligands showed good affinity to the receptors *in vitro* and *in vivo* and therefore maybe potential molecules for tumour-targeted diagnosis and therapies. hVEGFR-3 is a new number of the VEGFR family. More and more studies indicated that VEGFR-3 is up-regulated in a wide range of cancers and it plays an important role in tumour growth and metastasis. These data showed that VEGFR-3 is a potential target for tumour diagnosis and therapy.

We screened the phage display constrained 7-mer peptide library with hVEGFR-3 ectodomain Fc fusion protein. After 3-round biopanning, 29 positive phages, which have higher affinity with VEGFR-3 but not with IgG1 Fc, were obtained and analysed by DNA sequencing. The primary DNA structure deduced from the DNA sequences revealed that there is a consensus motif among the peptides: C**SD**xx**H**x**W**C (x is any amino acid). In addition, BLAST online analysis indicated that no meaningful homogeneous sequence with the motif was found in proteins including VEGF-C and VEGF-D, suggesting that this motif could be a new one.

The phage displaying peptide C**SD**SW**H**Y**W**C (phage1) exhibited the best affinity to hVEGFR-3. Our study found that phage1 could only bind to hVEGFR-3 but not to hVEGFR-1 or hVEGFR-2. It demonstrated that the recognition of phage1 to hVEGFR-3 is specific. In addition, P1 is the peptide which has the highest frequency among all positive peptides, indicating that phage1 has more potential to bind to hVEGFR-3. In order to further identify the affinity of phage1 to hVEGFR-3 protein, we chemically synthesized peptide1 (P1) corresponding to phage1 and assessed its feature. The results showed that P1 could dose-dependently and specifically bind to VEGFR-3 protein in ELISA.

Although we proved that P1 could associate with VEGFR-3 protein, we still did not know whether P1 could bind to natural VEGFR-3. Consequently, we had to find a VEGFR-3-positive cell line for binding experiment. We detected the expression of VEGFR-3 on six cell lines with mAb against VEGFR-3 and found that HT29 and Y79 cell lines are VEGFR-3-positive cell lines, which is according to the references (*22*, *23*). Then we detected the binding of FTIC-P1 to the six cell lines and found that the binding of FTIC-P1 is similar to the binding of mAb against VEGFR-3, that is, FTIC-P1 could also bind to the VEGFR-3 positive cells (HT29 and Y79) but not bind to the VEGFR-3 negative cells (EVC304, WISH, HepG2). This suggests that FTIC-P1 could bind to the natural VEGFR-3 on cells. Therefore, HT29 cells were selected

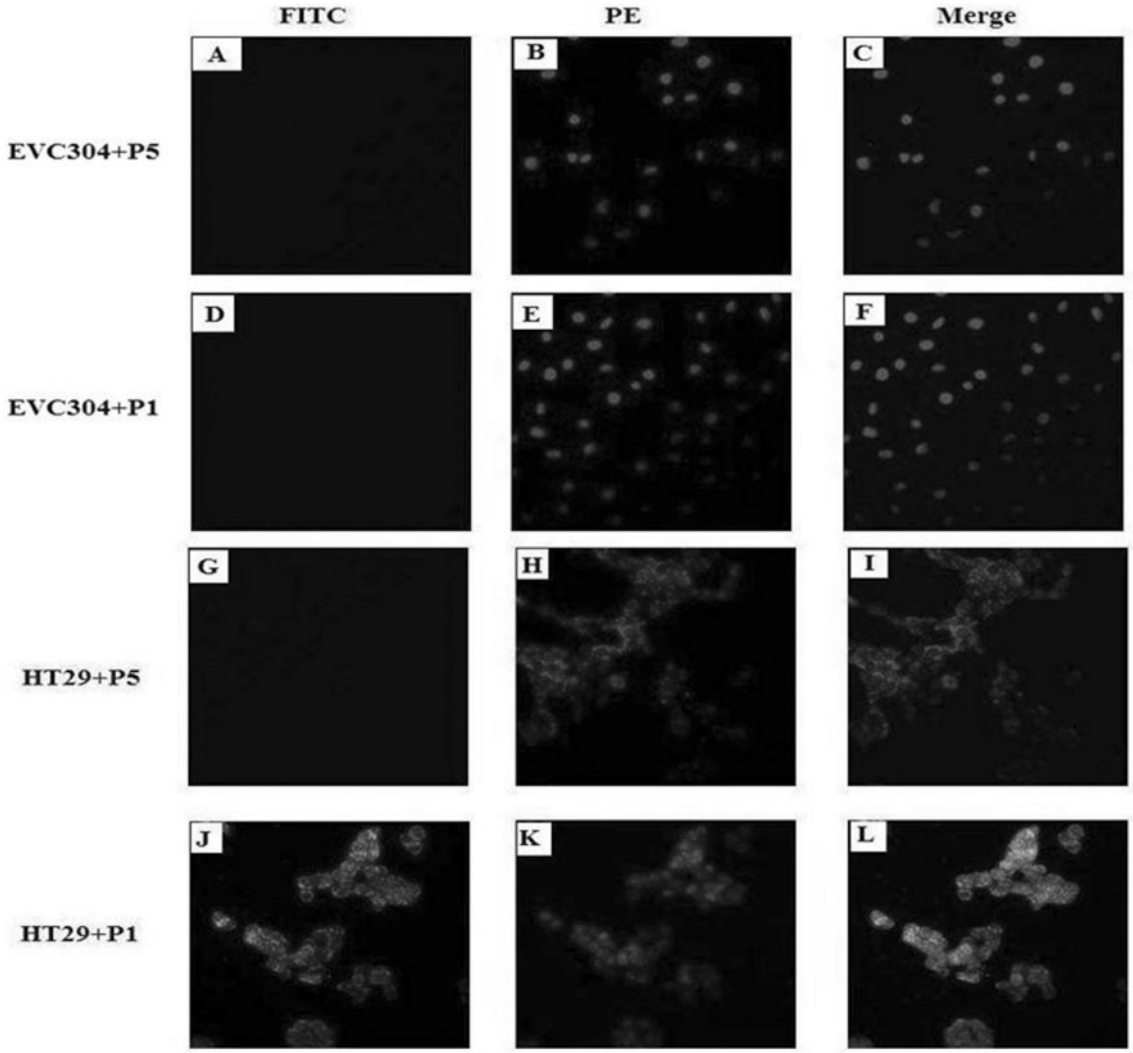

Fig. 6. **FTIC-P1 specifically binding on the membrane of HT29 cells**. (A–C) FTIC-P5 did not bind to EVC304 or HT29, no fluorescence was observed on the membrane of EVC304 cells or HT29 cells (G–I); (D-F) FTIC-P1 did not bind to EVC304 cells, no fluorescence was observed on the membrane of EVC304 cells; (J–L) FTIC-P1 specifically bind to HT29 cells and the fluorescence was observed on the membrane of the cells. A representative result of three perform was shown. All the cells were nuclear stained with PI.

to perform the immunoflourescence assay, and the result showed that FITC-P1 specifically bound to the membrane of HT29 cells, which is in agreement with the fact that VEGFR-3 expressed on the membrane of HT29 cells.

To our knowledge, this is the first study to identify peptide ligand with high affinity to hVEGFR-3. Our data indicated that P1 has potential to bind to hVEGFR-3 with specificity. Such a novel peptide ligand of hVEGFR-3 holds considerable promise for tumour diagnosis and therapy. Further studies to verify whether P1 could home anti-tumour drugs to the tumour tissue in animal tumour model are underway. This study provides a basis for further development of peptide ligand-based, human VEGFR-3-targeted cancer therapy.

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REFERENCES

1. Reubi, J.C. (2003) Peptide receptors as molecular targets for cancer diagnosis and therapy. *Endocr. Rev.* **24**, 389–427
2. Wagner, H.N. Jr. (1996) Nuclear medicine: 100 years in the making. *J. Nucl. Med.* **37**, 18N,24N, 37N
3. Valtola, R., Salven, P., Heikkila, P., Taipale, J., Joensuu, H., Rehn, M., Pihlajaniemi, T., Weich, H., deWaal, R., and Alitalo, K. (1999) VEGFR-3 and its ligand VEGF-C are associated with angiogenesis in breast cancer. *Am. J. Pathol.* **154**, 1381–1390
4. Stacker, S.A., Baldwin, M.E., and Achen, M.G. (2002) The role of tumor lymphangiogenesis in metastatic spread. *FASEB* **16**, 922–934
5. Williams, C.S., Leek, R.D., Robson, A.M., Banerji, S., Prevo, R., Harris, A.L., and Jackson, D.G. (2003) Absence of lymphangiogenesis and intratumoural lymph vessels in human metastatic breast cancer. *J. Pathol.* **200**, 195–206
6. Saaristo, A., Partanen, T.A., Arola, J., Jussila, L., Hytönen, M., Mäkitie, A., Vento, S., Kaipainen, A.,

Malmberg, H., and Alitalo, K. (2000) Vascular Endothelial Growth Factor-C and its receptor VEGFR-3 in the nasal mucosa and in nasopharyngeal tumors. *Am. J. Pathol.* **157**, 7–14

7. Yu, X.M., Lo, C.Y., Chan, W.F., Lam, K.Y., Leung, P., and Luk, J.M. (2005) Increased expression of vascular endothelial growth factor C in papillary thyroid carcinoma correlates with cervical lymph node metastases. *Clin. Cancer Res.* **11**, 8063–8069
8. Branca, M., Giorgi, C., Santini, D., Bonito, L.D., Ciotti, M., Benedetto, A., Paba, P., Costa, S., Bonifacio, D., Bonito, P.Di., Accardi, L., and Favalli, C. (2006) Aberrant expression of VEGF-C is related to grade of cervical intraepithelial neoplasia (CIN) and high risk HPV, but does not predict virus clearance after treatment of CIN or prognosis of cervical cancer. *J. Clin. Pathol.* **59**, 40–47
9. Ueda, M., Hung, Y.C., Terai, Y., Kanda, K., Kanemura, M., Futakuchi, H., Yamaguchi, H., Akise, D., Yasuda, M., and Ueki, M. (2005) Vascular endothelial growth factor-C expression and invasive phenotype in ovarian carcinomas. *Clin. Cancer Res.* **11**, 3225–3232
10. Lu, Z.Q., Li, H.G., Xie, D.R., Zhang, H.Z., Sheng, X.M., Zeng, J.Y., and Zeng, H. (2005) Expression and clinical significance of vascular endothelial growth factor C and vascular endothelial growth factor receptor 3 in non-small cell lung carcinoma. *Chinese J. Cancer* **24**, 1132–1135
11. Zeng, Y.P., Opeskin, K., Baldwin, M.E., Horvath, L.G., Achen, M.G., Stacker, S.A., Sutherland, R.L., and Williams, E.D. (2004) Expression of vascular endothelial growth factor receptor-3 by lymphatic endothelial cells is associated with lymph node metastasis in prostate cancer. *Clin. Cancer Res.* **10**, 5137–5144
12. Kubo, H., Fujiwara, T., Jussila, L., Hashi, H., Ogawa, M., Shimizu, K., Awane, M., Sakai, Y., Takabayashi, A., Alitalo, K., Yamaoka, Y., and Nishikawa, S.I. (2000) Involvement of vascular endothelial growth factor receptor-3 in maintenance of integrity of endothelial cell lining during tumor angiogenesis. *Blood* **96**, 546–553
13. Pytowski, B. (2005) Complete and specific inhibition of adult lymphatic regeneration by a novel VEGFR-3 neutralizing antibody. *J. Natl Cancer Inst.* **97**, 14–21
14. He, Y., Rajantie, I., Pajusola, K., Jeltsch, M., and Alitalo, K. (2005) Vasular endothelial cell growth factor receptor 3 mediated activation of lymphatic endothelium is crucial for tumor cell entry and spead via lymphatic vessels. *Cancer Res.* **65**, 4739–4746
15. Qian, Z.M., Li, H., and Sun, H. (2002) Targted drug delivery via the transferrin receptor-mediated endocytosis pathway. *Pharmacol. Res.* **54**, 561–587
16. Wu, P., Leinonen, J., Koivunen, E., Lankinen, H., and Stenman, U.H. (2000) Identification of novel prostate-specific antigen-binding peptides modulating its enzyme activity. *Eur. J. Biochem.* **267**, 6212–6220
17. Meng, J.R, Ma, N., Yan, Z., Han, W., and Zhang, Y.Q. (2006) NGR enhanced the anti-angiogenic activity of tum-5. *J. Biochem.* **140**, 1–6
18. Sacchi, A., Gasparri, A., Curnis, F., Bellone, M., and Corti, A. (2004) Crucial role for interferon γ in the synergism between tumor vasculature-targeted tumor necrosis factor α (NGR-TNF) and doxorubicin. *Cancer Res.* **64**, 7150–7155
19. Mayada, E-M., Lioudmila, T., Ludmila, Y., Pietrzynski, G., Moreno, M., Stanimirovic, D., Ahmad, D., and Alakhov, V. (2003) A vascular endothelial growth factor high affinity receptor 1-specific peptide with antiangiogenic activity identified using a phage display peptide library. *J. Biol. Chem.* **278**, 46681–46691
20. Rubio, D.A., Marty, C., Console, S., Zeisberger, S.M., Ruch, C., Jaussi, R., Schwendener, R.A., and Ballmer-Hofer, K. (2005) Targeting human cancer cells with VEGF receptor-2-directed liposomes. *Oncol. Rep.* **13**, 319–324
21. Garces, C.A., Kurenova, E.V., Golubovskaya, V.M., and Cance, W.G. (2006) Vascular endothelial growth factor receptor-3 and focal adhesion kinase bind and suppress apoptosis in breast cancer cells. *Cancer Res.* **66**, 1446–1454
22. Troiani, T., Lockerbie, O., Morrow, M., Ciardiello, F., and Eckhardt, S.G. (2006) ZD6474, an inhibitor of VEGFR and EGFR tyrosine kinases, blocks VEGF-C-induced activation of VEGFR-3 and cell proliferation in human colon cancer cell lines. *J Clin Oncol*. 2006 ASCO Annual Meeting Proceedings Part I. Vol 24, No. 18S (June 20 Supplement), 13171
23. Pajusola, K., Aprelikova, O., Korhonen, J., Kaipainen, A., Pertovaara, L., Alitalo, R., and Alitalo, K. (1992) Flt4 receptor tyrosine kinase contains seven immunoglobulin-like loops and is expressed in multiple human tissues and cell lines. *Cancer Res.* **52**, 5738–5743