Aim of the study: Tumour endothelial cells have been proven to have molecular markers distinct from normal endothelial cells. These specific molecular markers allow for targeting of the tumour vasculature with specific pharmacological vehicles to direct diagnostic or therapeutic modalities at the endothelial cells. By performing a phage display-based screening, this study aimed to identify a certain short peptide that could specifically bind to osteosarcoma vasculature.

Material and methods: We performed *in vivo* screening in the murine models of osteosarcoma with annular Ph.D.-C7C library in the present study. To explore the *in vivo* binding specificity of the retrieved peptide, we conjugated the peptide with fluorescein isothiocyanate (FITC) and injected it intravenously into osteosarcoma-bearing BALB-c mice.

Results: CTKPDKGYC was the dominant sequence isolated from *in vivo* screening and was named as NF-1. Fluorescence staining found that FITC-NF-1 peptide could be specifically homed to osteosarcoma vasculature while being almost undetectable in the heart, brain, lung and liver. Simultaneously, a small amount of fluorescence could also be detected in the renal glomerulus and renal tubule but not in renal vascular endothelium, indicating that FITC-NF-1 peptide might be excreted mainly through the renal-urinary route.

Conclusions: Our data suggest that, with high binding specificity to osteosarcoma vasculature, peptide NF-1 may have potential value in early diagnosis or targeted therapy for osteosarcoma.

Key words: osteosarcoma (OS), phage display, peptide, angiogenesis.

Targeting osteosarcoma vasculature with peptide obtained by phage display

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Introduction

Osteosarcoma is the most common primary malignant bone tumour in children and adolescents [1]. The standard treatment strategy of osteosarcoma at present is the complete removal of the tumour by wide excision and aggressive chemotherapy [2]. Although this strategy has significantly improved the survival rate from 11% to 70% [3], it seems that improvements in survival have reached a plateau in recent years [4]. Metastases, chemoresistance and serious side effects remain the major reasons for the failure of osteosarcoma treatment [5]. Thus, novel agents that can enhance the local drug concentration in the tumour while reducing undesirable side effects are needed in order to achieve further improvement in the survival rate. Tumour angiogenesis is known to be an indispensable process during tumour growth and metastasis [6]. Accumulative evidence indicates that tumour endothelial cells possess molecular markers distinct from normal endothelial cells [7–9]. Specific molecular markers would allow targeting of the tumour vasculature [10–12]. This creates the possibility of developing specific pharmacological tools to direct diagnostic or therapeutic modalities at the endothelial cells [13, 14]. Because of several clinical advantages, small peptides obtained by phage display technology seem to be promising pharmacological tools. For instance, small peptides display good tissue penetrating ability due to their small molecular weight (average less than 50 amino acids), low immunogenicity, high affinity to targets, acceptable stability and integrity *in vivo* and easy manipulation for synthesis and conjugation with other agents [15–17]. To date, several functionally relevant homing peptides have been identified in tumour vasculature. They may be used as promising vehicles to transport more selectively diagnostic or therapeutic cargo to tumour endothelial cells [18–21]. In a recent study [22], the authors successfully identified a new peptide by *in vitro* phage screening technology using human osteosarcoma cells but not osteosarcoma vasculature as the selected target. When the peptide was labeled with fluorescein isothiocyanate, successful noninvasive PET imaging of osteosarcoma tumours was possible in nude mice, indicating peptide imaging as a promising strategy for early detection of osteosarcoma.

In the present study, we firstly established osteosarcoma xenograft with osteosarcoma cell line Saos-2, which was obtained from the American Type Culture Collection (ATCC), primarily cultured from an 11-year-old female Caucasian. We then performed *in vivo* phage screening process using tumour vasculature as the selected target. The *in vivo* binding specificity of the peptide obtained from the phage display was then confirmed under laser scanning microscope (LSCM) when it was conjugated FITC.

Material and methods

Materials

The disulphide-constrained (seven amino acids with a flanking cysteine residue at both ends of the peptide) cyclic M13 phage display library (Ph.D.C7C system; New England Biolabs, Hitchin, UK) was used. Dulbecco's modified Eagle's medium (DMEM) and foetal calf serum (FCS) were purchased from Hyclone (Thermo Scientific, USA).

Cell culture

The human osteosarcoma cell line Saos-2 was obtained from the American Type Culture Collection (ATCC) and maintained in DMEM supplemented with 10% FCS (v/v) in a 5% CO_2 atmosphere.

Osteosarcoma xenograft

The osteosarcoma xenograft was established by subcutaneous injection of Saos-2 cells (1×10^7 cells in 200 μl DMEM) into the front flank of BALB-c mice (Southern Medical University, China) at 4 weeks of age. All animal experiments were approved by the ethics committee of Southern Medical University.

In vivo panning

We continued with the *in vivo* experiment when the tumour volume reached at least 80 mm^3 (about 9 weeks after cell injection).The phage library [1×10^{11} plaque-forming units (PFU) in 200 μl PBS] was injected into the tail vein of anaesthetised animals. After 10 minutes, the mice were perfused via the left ventricle with 20 ml of DMEM to ensure phage clearance from the blood. The inferior vena cava was cut for the outlet. Tumours and control organs (heart, brain, lung, liver and kidney) were extracted, weighed and quick-frozen in liquid nitrogen for 5 minutes and were then ground in DMEM with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 20 mg/ml aprotinin, and 1 mg/ml leupeptin) and 0.5% bovine serum albumin (BSA) and washed five times by centrifugation at 6000 rpm. Phages were eluted from the tissues with 1.6 ml of 0.1 M glycine, pH 2.0, and after 10 minutes of incubation, were neutralised with 36 μl of 2 M Tris base. To determine and compare the number of phages in the eluate from tumours and control organs, in each round of selection, 100 μl of the eluate was added with the Escherichia coli host ER2738 into melted Luria-Bertani (LB) agar tops, which were then plated onto isopropyl-β-D-thiogalactopyranoside (IPTG)/X-Gal LB agar plates. After overnight incubation at 37°C, the peptide phages, appearing as blue plaques, were counted and the yield of phage localising to each individual tissue was determined. The residual eluate from the tumour was amplified and used as the input phage for the next round of *in vivo* selection. Three further cycles of *in vivo* selection were performed to enrich for specificity to osteosarcoma vasculature. The eluate of the last cycle from the tumour was re-injected into the tail vein of normal non-tumour-bearing BALB-c mice for 10 minutes to clear phages that bound to the normal vasculature and other non-tumour antigens.

Elimination of the phages without inserted sequences

After subtractive screening with normal non-tumour-bearing BALB-c mice, 72 individual clones were picked at random and their DNA was amplified by PCR. Then the clones without inserted sequences were identified and eliminated by agarose gel electrophoresis with the PCR product, since the DNA with inserted sequences was about 300 bp with lower speed in agarose gel electrophoresis, while the one without inserted sequences was only 258 bp with higher speed.

Sequence analysis

In total 61 phage clones with inserted sequences were sequenced (Beijing Huada Gene Corp, China). The primer used for sequencing was 5′-HOCCC TCA TAG TTA GCG TAA CG-3′ (−96 gIII sequencing primer, provided in a C7C kit, New England Biolabs). The amino acid sequences of displayed peptides were then deduced from the DNA sequence. The three peptide sequences with highest frequency (CTKPDKGYC, CEGHSHRHC and CYGPNNHIC) were identified and sequentially named as NF-1, NF-2 and NF-3 for further experiments.

Enriching comparison of selected phage clones in tumour and control organs

Phages NF-1, NF-2 and NF-3 (1×10^{11} PFU in 200 μl PBS) were respectively injected into the tail veins of anaesthetised mice ($n = 6$ per group). The number of phages bounding to osteosarcoma vasculature and control organs (heart, brain, lung, liver and kidney) were determined and compared.

Immunohistochemical staining of the phage NF-1

The phage NF-1 (1×10^{11} PFU in 200 μl PBS) was injected into the tail veins of anaesthetised mice. After 10 minutes, the mice were perfused via the left ventricle with 20 ml of DMEM. The inferior vena cava was cut for the outlet. Tumours and control organs (heart, brain, lung, liver and kidney) were harvested and fixed with formalin and embedded in paraffin. The paraffin-embedded specimens were cut into 4 mm sections and baked for 1 h at 65°C. All sections were deparaffinised with xylenes and rehydrated through a graded ethanol series into distilled water. Then, the sections were submerged into EDTA antigenic retrieval buffer (pH 8.0) and microwaved for antigenic retrieval. The sections were blocked with 0.3% H_2O_2 (3% in PBS) at room temperature for 510 minutes. After being washed by PBS for 5 min at 37°C, the sections were incubated with normal sheep serum for 20 minutes at 37°C. Subsequently, sections were incubated overnight at 4°C with mouse anti-M13 phage antibody with a work dilution of 1 : 1000. The next day, the sections were rinsed three times (10 minutes for each rinse) in PBS and incubated with secondary antibody for 1 hour at room temperature. Subsequntly, the sections were rinsed three times (5 minutes for each rinse) in PBS. The bound antibody was visualised using DAB. Sections were rinsed three times (5 minutes for each rinse) in running tap water before staining by haematoxylin and eosin. Finally, sections were rinsed for 10 minutes in running tap water before dehydration and mounting. The samples were

examined with a BX50 microscope (Olympus, Tokyo, Japan) equipped with an AxioVision system (Carl-Zeiss, Germany).

Synthesis and conjugation of peptide NF-1

The NF-1 peptide, containing nine amino acids with a disulphide bridge connecting the two terminal cysteines was synthesised by Bai Ao Tai Biotech Corp (Guangzhou, China) using standard FMOC chemistry in a solid-phase synthesiser. Fluorescein (FITC)-conjugated peptide (FITC-NF-1) contained a spacer of two glycines at the amino-terminus of the cysteine.

In vivo distribution analysis of FITC-NF-1

FITC-NF-1 was injected intravenously into BALB-c mice bearing osteosarcoma. After 4 hours of circulation, mice were perfused with PBS and then with 4% paraformaldehyde before their tumours and control organs were obtained, fixed and prepared for scanning by LSCM (Leica, Germany).

Statistical analysis

Statistical analyses were performed with SPSS 16.0 for Windows. Means were compared using one-way analysis of variance (ANOVA); p values < 0.05 were considered statistically significant.

Results

Enrichment of phages homing to osteosarcoma vasculature

After the four rounds of *in vivo* selection, the number of phages recovered from each gram of tumour was increased for 1.76 folds (from 4.5×10^7 PFU/g to 7.9×10^7 PFU/g), while there was a decrease in the number of phages recovered from each gram of control organs (heart, brain, lung, liver and kidney) as shown in Fig. 1. In order to further clear the phages that bound to the normal vasculature and other non-tumour antigens, we re-injected 1.0×10^{11} PFU of the eluate from the tumour of the last cycle into the tail vein of normal non-tumour-bearing BALB-c mice and recovered 3.3×10^{10} PFU from serum after 10 minutes of circulation.
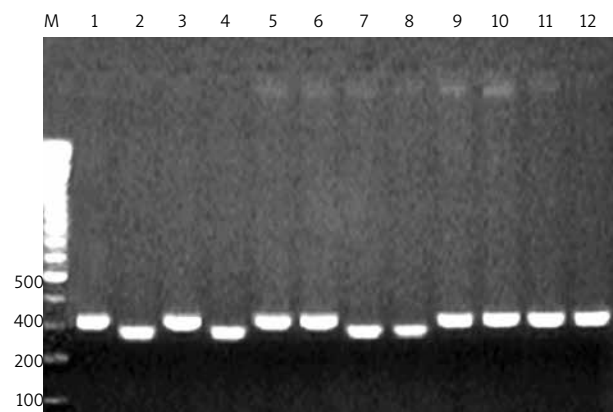

Fig. 2. Elimination of the phages without inserted sequences. DNA clones without inserted sequences with higher speed could be seen in lane numbers 2, 4, 7 and 8

Elimination of the phages without inserted sequences and sequence analysis

Using the method described in Materials and methods herein, 11 clones without inserted sequences were identified and eliminated from the 72 clones (Fig. 2). The other 61 individual clones with inserted sequences were sequenced last. The amino acid sequences of the displayed peptides were then deduced from the DNA sequences of the phages. The three peptide sequences with the highest frequency of occurrence were identified: NF-1 (23 times, 37.7%), NF-2 (12 times, 19.7%) and NF-3 (4 times, 6.6%).
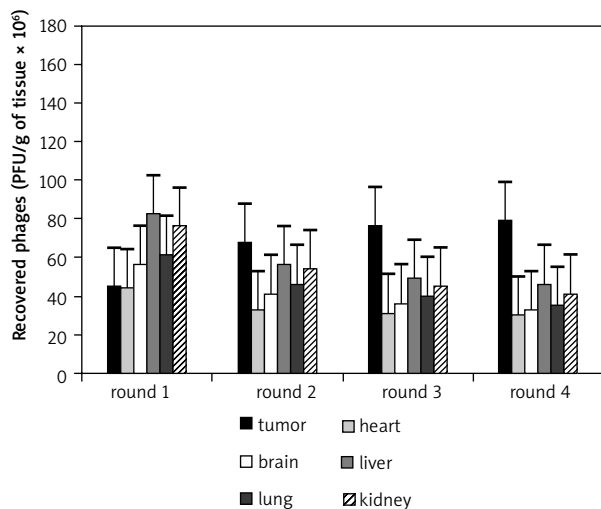

Fig. 1. Recovery of phages from the tumour and control organs during the four *in vivo* screening rounds. The library (1×10^{11} PFU) was injected into the tail vein of the tumour-bearing mice. After four rounds of screening, the phages recovered from each gram of tumour increased from 4.5×10^7 PFU/g to 7.9×10^7 PFU/g, while there was a decrease in the number of phages recovered from each gram of control organs
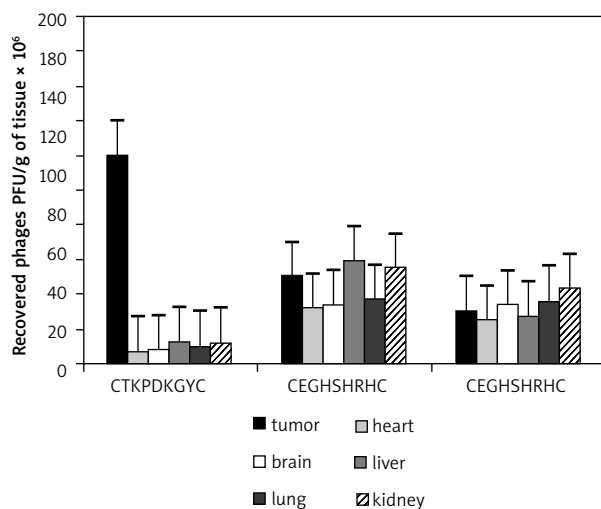

Fig. 3. Phages NF-1, NF-2 and NF-3 were respectively re-injected into the tail veins of the tumour-bearing mice ($n = 6$ per group). The amount of phage NF-1 recovered from the tumour was significantly higher than that from control organs ($p < 0.01$). No statistical difference was detected in the amount of phage NF-2 and NF-3 recovered from the tumour and control organs
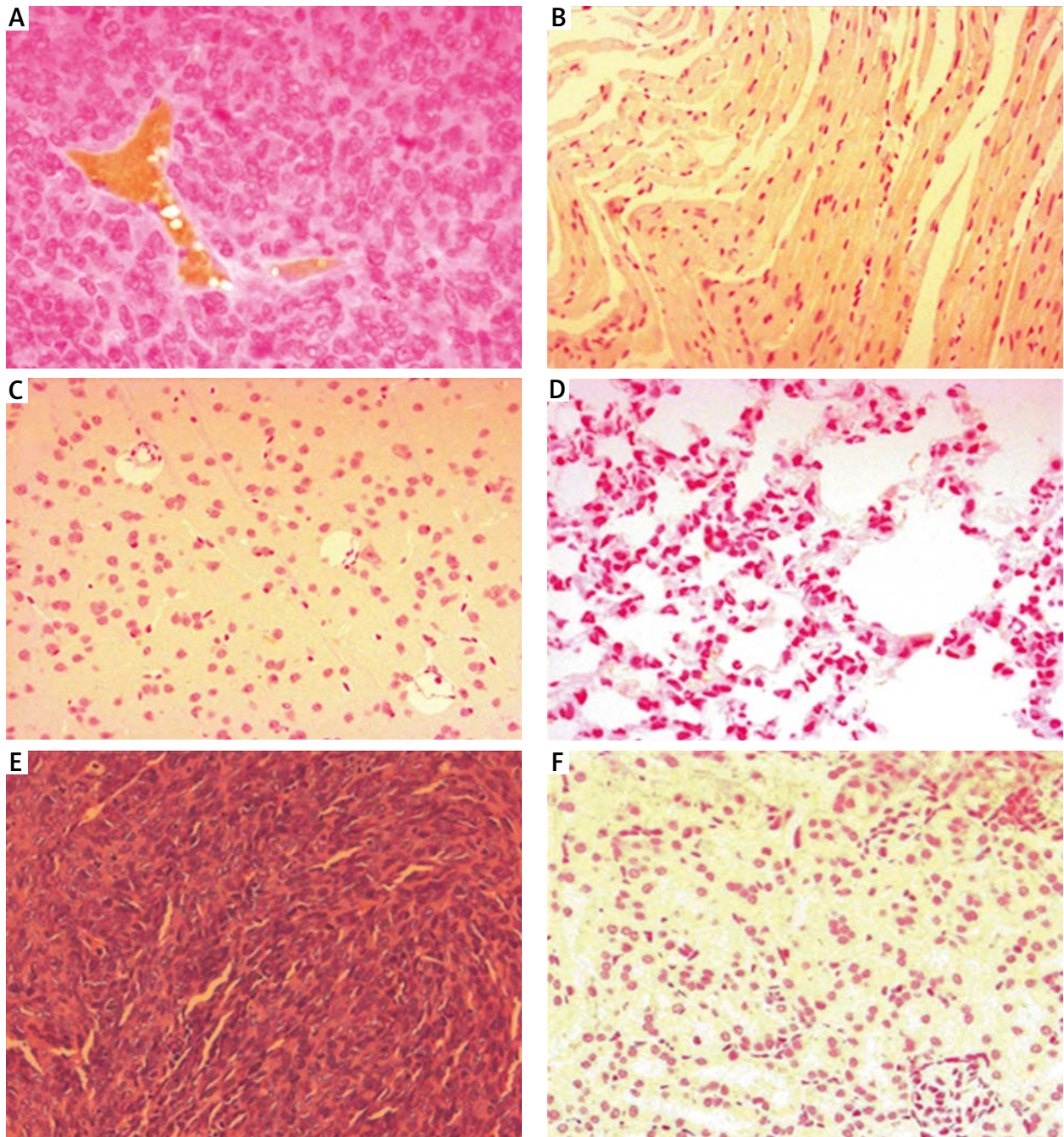
Fig. 4. Immunohistochemical staining of phage NF-1 after intravenous injection into tumour-bearing mice (magnification 200×). Obvious phage stains (yellow brown) could be seen in tumour vessels (**A**) but was almost undetectable in heart (**B**), brain (**C**), lung (**D**), liver (**E**) and kidney (**F**)

In vivo validation of phage clones NF-1, NF-2 and NF-3

We validated the three phages (NF-1, NF-2 and NF-3) with the highest frequency of occurrence by re-injecting them into the tail veins of the mice baring osteosarcoma ($n = 6$ per group). The phage NF-1 recovered from the tumours was 1.1×10^8 PFU/g, which was 15.7 times higher than the amount recovered from heart (7.0×10^6 PFU/g), 13.8 times higher than brain (8.0×10^6 PFU/g), 8.5 times higher than liver (1.3×10^7 PFU/g), 11.0 times higher than lung (1.0×10^6 PFU/g) and 9.2 times higher than kidney (1.2×10^7 PFU/g). No statistical differences were detected in the amount of phages NF-2 and NF-3 recovered from the tumour and control organs (Fig. 3). Additionally, the phage NF-1 showed abundant binding to the tumour vessel while being much less bonded to the control organs, as shown in immunohistochemical staining (Fig. 4).

In vivo verification of peptide FITC-NF-1

LSCM showed that FITC signals could be seen in single tumour vessels (Fig. 5A) and tumour vascular plexus (Fig. 5B) but were not detectable in heart (Fig. 5C), brain (Fig. 5D),
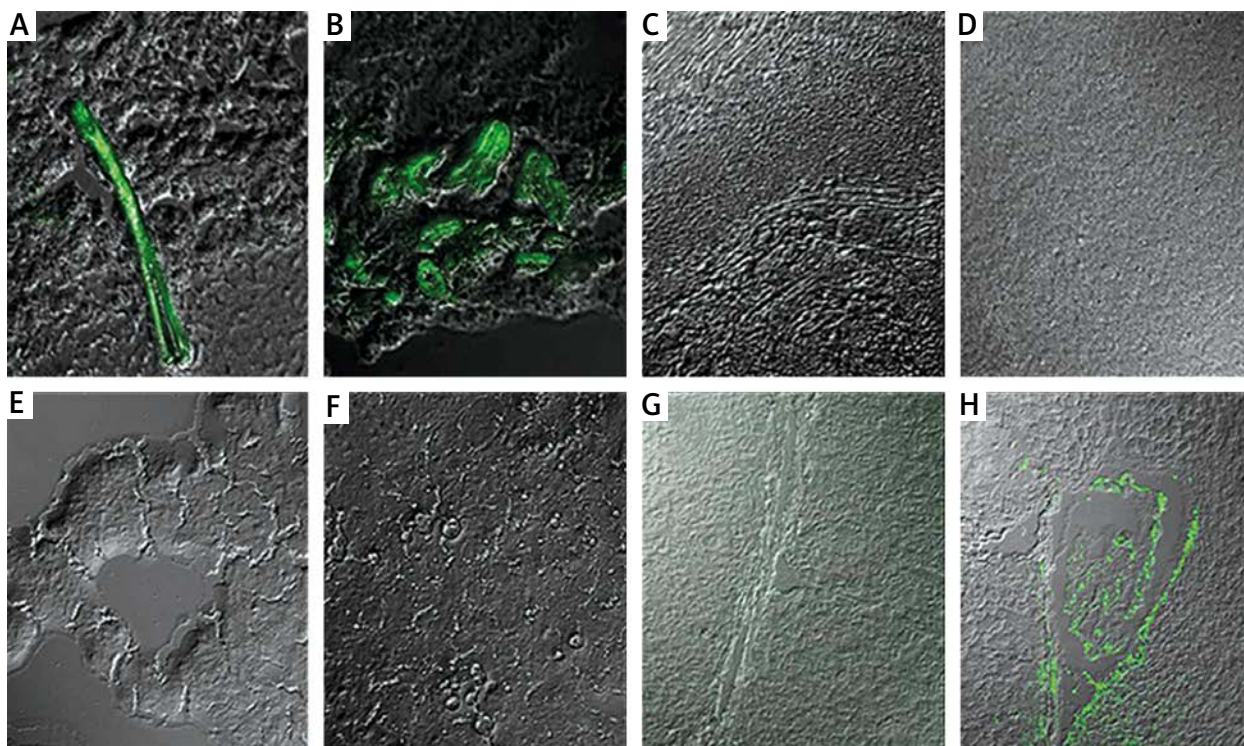
Fig. 5. *In vivo* banding specificity of FITC-NF-1 peptide to osteosarcoma vasculature confirmed under LSCM (magnification 600×). Obvious FITC signals (green) could be seen in single tumour vessel (**A**) and tumour vascular plexus (**B**) but was undetectable in heart (**C**), brain (**D**), liver (**E**) and lung (**F**). The signals could also be detected in renal glomerulus and renal tubule (**H**) but not in renal vascular endothelium (**G**)

liver (Fig. 5E) and lung (Fig. 5F). The signals could also be detected in renal glomerulus and renal tubule (Fig. 5H) but not in renal vascular endothelium (Fig. 5G), suggesting that FITC-NF-1 might be excreted mainly through the renal-urinary route.

Discussion

Novel peptides with high specificity to tumour tissue may be used as a promising tool to direct diagnostic or therapeutic agents at the tumour. Screening of phage libraries is one of the most commonly used ways to rapidly identify these tumour-selective peptides. Compared with *in vitro* phage screening, the *in vivo* phage screening process is more likely to maintain the native structure and functional conformation of the targeted cells or tissue. Accordingly, peptides obtained by *in vivo* phage screening may possess a higher potential value in clinical utilisation [23]. Conventional chemotherapy drugs at present are mainly designed to target tumour cells. However, it is often difficult for these drugs to be absorbed by tumour cells because they can seldom accumulate into the tumour mass due to poor blood perfusion, high interstitial pressure and abnormal vasculature inside the tumour mass [19, 23]. In addition, tumour cells are genetically unstable and often produce resistance to multiple chemotherapeutic drugs [24]. In contrast with tumour cells, tumour endothelial cells have good genetic stability, so they rarely produce drug resistance. Tumour blood vessels are also highly accessible to any intravenously administered agents [25]. Therefore, we performed *in vivo* phage screening using

osteosarcoma vasculature as the target in this study. We used Ph.D.-C7C library since it displays a random 7-residue peptide that is conformation restrained by the disulphide bond formed by the two cysteines on the two ends. This circular structure is thought to be more stable and natural than a linear structure [26]. For *in vivo* selection, circulation time of the phages is important. Phages need enough time to bind their target. However, if the circulation time is too long, they will be then taken up by the cells. A duration of 5–15 minutes is thought to be suitable for the phages to bind on the endothelial cells in the blood vessels while not being taken by the cells [27]. In this study, we kept the phages in circulation for 10 minutes. In order to clear phages that bound to the normal vasculature and other non-tumour antigens, we committed a subtractive screening with eluate of the last cycle from the tumour in normal non-tumour-bearing BALB-c mice. There is a certain percentage of phage clones without inserted sequences in the phage library. It would be wasteful and even misleading if this kind of clone were chosen to be sequenced. Therefore, we identified and eliminated them by agarose gel electrophoresis. Finally, a total of 61 clones with inserted sequences were sent to be sequenced, and phage NF-1, NF-2 and NF-3 were found to be most enriched of all sequenced clones. When the three phage clones were re-injected into the tail veins of the tumour-baring mice, respectively, phage NF-1 demonstrated high tumour vasculature-binding specificity confirmed by the much higher amount of phages recovered from tumours than from that recovered from control organs. The amount of phage NF-2 and NF-3 recovered

from the tumour was not obviously higher than that from the control organs. Binding specificity of phage NF-1 was further confirmed by immunohistochemical staining. We then synthesised and conjugated peptide NF-1 with FITC. The *in vivo* binding specificity to tumour vasculature of peptide FITC-NF-1 was confirmed under LSCM. The fluorescence could also be detected in renal glomerulus and renal tubule but not in renal vascular endothelium, indicating that peptide FITC-NF-1 may be mainly excreted through the renal-urinary route [22].

In future research, we would like to attempt to conjugate NF-1 with certain drugs and explore the efficacy of targeted therapy for osteosarcoma. In order to explore the diagnostic value for osteosarcoma, we would also like to try to radiolabel peptide NF-1 to examine its effect in tumour imaging [28]. In addition, the receptor of peptide NF-1 on tumour vasculature could also be identified and analysed [29].

In conclusion, the novel 7-residue peptide NF-1 demonstrated obvious binding specificity to osteosarcoma vasculature and may have the potential value to be used in the diagnosis and treatment of osteosarcoma.

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