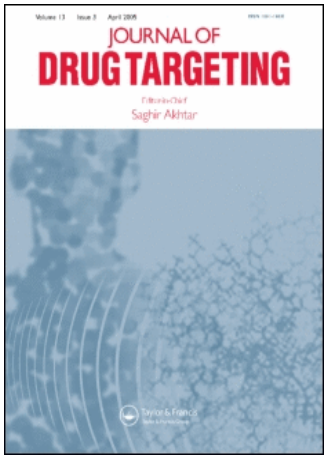

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Bacteriophage biopanning in human tumour biopsies to identify cancer-specific targeting ligands

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Abstract

Intravenous targeting of anticancer agents should improve both efficacy and therapeutic index. However, rational design of targeting constructs requires detailed definition of receptor targets and must take account of polarised tissue architecture that may restrict access to chosen receptors from the bloodstream. Bacteriophage biopanning provides a solution to this problem, identifying targeting sequences by functional selection rather than design, although reiterative panning in polarized human tumours has not previously been attempted. Here, we report an *ex vivo*, intra-arterial method for biopanning in freshly-resected human tumours, enabling reiterative selection of oligopeptide sequences capable of intravascular targeting to human colorectal tumours. Significant consensus was observed after two rounds of panning in tumours from different patients, and lead sequences demonstrated tumour targeting in samples from unrelated patients. This novel approach may be applicable to a wide range of settings, thus enabling iteration of consensus targeting sequences for tumour imaging and selective delivery of anticancer agents.

Keywords: *Tumour targeting, colorectal cancer, phage display, gene therapy, receptor, endothelium*

Introduction

Tumour-selective targeting is a major pharmacological goal that promises increased anticancer activity coupled with lower toxicity against normal tissues. The identification of tumour-selective ligands capable of intravenous targeting could enable improved delivery of a variety of therapeutic agents, such as cytotoxic drugs or therapeutic genes, to disseminated tumour metastases or to facilitate imaging for improved disease management.

Candidate targeting agents, such as antibodies (including fragments and single chain Fv molecules (Coll et al. 1997; Kashentseva et al. 2002)) and growth factors (notably basic fibroblast growth factor (FGF2)(Fisher et al. 2001) and vascular endothelial growth factor (VEGF) (Backer and Backer 2001) have been rationally selected and evaluated with varying success. This empirical approach to the identification of targeting ligands has been largely superceded by the use of library-based screening systems, designed to allow iterative selection of high affinity ligands by repeated screening and enrichment of living libraries (Lorimer et al. 1996; Rajotte and Ruoslahti 1999). Phage-based peptide display libraries have shown particular promise for identifying receptor binding peptides, and have

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recently been used in cell culture-based studies to identify receptor binding mimetics of FGF2 (Maruta et al. 2002) and VEGF (Binetruy-Tournaire et al. 2000) as well as tumour cell-binding sequences with unknown targets (Zitzmann et al. 2005). Unfortunately, these *in vitro* studies take no account of tumour anatomy and polarisation, and accordingly they cannot predict whether the sequence iterated will have targeting activity in a clinical disease setting. Similarly, they do not consider the presence of non-cancerous tissue within solid tumours (fibroblasts, stromal connective tissue and endothelial cells) that may provide the cancer-associated targets that are most accessible from the bloodstream. However, a major strength of reiterative bacteriophage panning is its suitability for application in complex biological systems (notably *in vivo*) to enable selection of sequences capable of functional targeting to the specific cells and tissues in a disease context (Kolonin et al. 2001; Pasqualini and Ruoslahti 1996). Performing the selection procedure *in vivo* avoids artifacts of *in vitro* culture and ensures that ligands are selected only for receptors that are accessible in the polarised *in vivo* cellular anatomy from the appropriate route of administration. Ruoslahti and Pasqualini have pioneered this approach, and have identified several ligands capable of mediating organ-selective and tumour-selective targeting in rodents *in vivo* (Arap et al. 2002a; Pasqualini and Ruoslahti 1996) and several other studies have also been reported (Landon and Deutscher 2003; Ho et al. 2004; Bockmann et al. 2005). The oligopeptides iterated have been used successfully for targeting cytotoxic drugs to tumours growing as xenografts in mice, and have demonstrated encouraging therapeutic activity (Arap et al. 1998).

There have been attempts to utilise phage display technology to identify targeting ligands of clinical relevance, but the usefulness of this has been limited by the animal model systems available since even human tumour xenograft systems are supported by host-derived vasculature and fibroblasts. In 2002, Arap et al. (2002b) reported the first case of biopanning in a human patient, where the library was administered to a terminal patient shortly before death and phage were recovered *post mortem*. This study yielded interesting insights into oligopeptide sequences with selectivity for specific human tissues, but reiterative panning was not performed and no high-affinity sequences were deduced. Reiterative biopanning in living human subjects is generally considered difficult because of the ethical considerations, potential risks to the patient (arising from possible transfer of pathogens from one patient to the next) and the practical requirement for tissue biopsies, hence the development of methodologies to enable reiterative biopanning in human clinical samples *ex vivo* could yield high-affinity peptide ligands for organ or tumour selective vasculature *in vivo*.

Here, we describe a novel technique that is simple, safe and non-invasive, and enables identification and assessment of oliopeptide sequences capable of binding to human tumours following intravascular delivery. In this strategy, phage library is directly injected into freshly-resected human colon cancer specimens *via* the feeding artery, allowing phage to reach the vascular aspect of solid tumours and to bind to accessible targets. Unassociated phage are removed by extensive arterial flushing and phage associated with tumour are subsequently reclaimed from isolated samples for expansion and reiterative panning in samples from subsequent patients. Analysis of the peptide sequences from phage isolated allows identification of consensus sequences that should be capable of binding selectively to tumour tissue, ultimately suitable for incorporation into pharmacological agents for systemic imaging and therapeutic targeting.

Materials and methods

Patients contributing to this study

Twelve patients undergoing colectomy for colorectal cancer at Selly Oak Hospital, Birmingham, UK were recruited into this study. Patients with lower rectal cancer were excluded because the middle rectal artery, the main feeding artery for cancers of the lower rectum, is normally too small to be canulated for injection of phage solution. Some individuals were also excluded because of the impossibility of canulating the appropriate tumour-feeding artery, mainly due to arterial involvement within the tumour mass or arteriosclerosis. The informed consent of patients, and approval of the South Birmingham Local Research Ethics Committee, were obtained prior to experimentation.

Arterial delivery to freshly-resected colorectal tumours

Immediately following surgical resection of the tumour, the canula (Venflon, 22 G, Becton Dickinson, Helsingborg, Sweden) was inserted into the main tumour-feeding artery from the stump of the artery, and tied firmly to the artery with surgical thread to avoid backflow (Figure 1a). The artery was then perfused with 50–100 ml of Dulbecco's phosphate-buffered saline (PBS, Invitrogen, Paisley, UK) *via* the inserted canula to wash the vasculature of the specimen. Fluid emerging from the corresponding vein was initially bloody, but typically became clear after injection of 30–40 ml PBS. A second canula was then inserted into the vein to collect the perfusate.

Bacteriophage library and biopanning procedure

Biopanning was performed using the Ph.D-12 M13 peptide phage display library (New England BioLabs Inc., Beverly, MA, USA), which displays a library

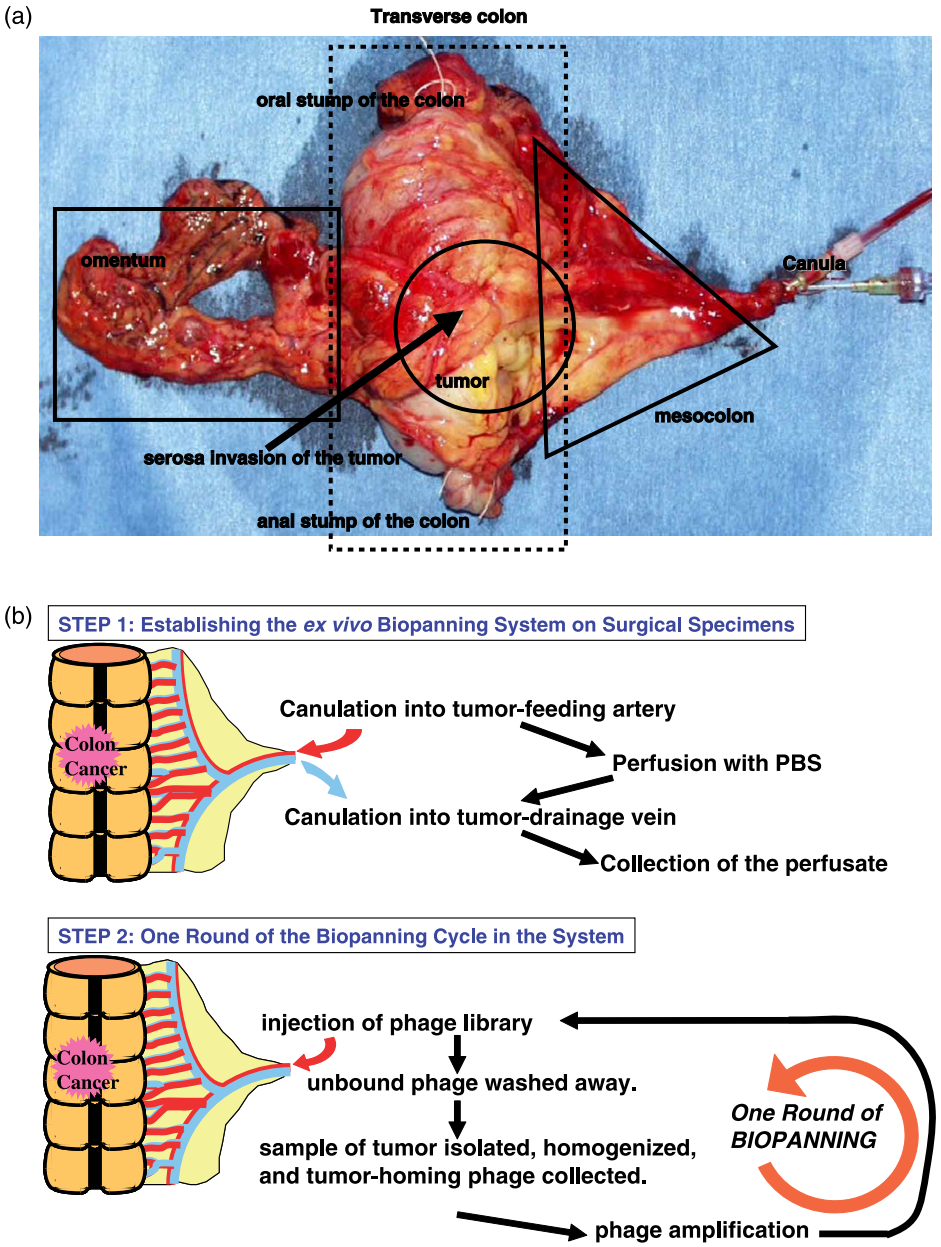

Figure 1. Arterial delivery to freshly-resected colorectal tumours. Immediately following surgical resection of the tumour, the canula (Venflon, 22 G, Becton Dickinson) was inserted into the main tumour-feeding artery from the stump of the artery, and tied firmly to the artery with surgical thread to avoid backflow (A). Schematic representation of the biopanning procedure (B).

of 12-mer linear oligopeptides. Phage library (2×10^{11} plaque forming units (pfu) diluted in 5 ml PBS) was injected into the feeding artery through the canula and allowed to bind for 10 min at room temperature. In order to maximise use of viable tissues, phage library was injected into all specimens used in this study within 30 min after ligation of the main tumour-feeding artery. Unbound phage were removed after 10 min incubation by perfusion with PBS (100 ml) *via* the canula into the feeding artery (Figure 1b). The colon was then opened, and samples of tumour and adjacent normal colorectal wall (3 cm oral from tumour edge) were collected.

Titration of phage clones recovered from tumour specimens

Resected tumour and normal tissue samples were weighed, washed with PBS and homogenized in PBS (5 ml) containing a cocktail of protease inhibitors (Sigma, St Louis, MO, USA; 0.5%) using a motor-driven teflon-on-glass homogenizer. The homogenate was centrifuged at 1500 rpm for 3 min (CS-6R Centrifuge, Beckman Coulter, Buckinghamshire, UK) and washed twice with PBS containing protease inhibitors. After incubation of the homogenate with glycine–HCl (5 ml, 0.2 M, pH 2.2, 3 min) the acid-eluted fraction, containing phage that bound weakly

to the cell surface, was removed by centrifugation. The pellet (containing tightly bound phage) was neutralized by adding Tris–HCl (750 μl, 1 M, pH 9.1). The pellet was then washed with PBS containing protease inhibitors, lysed with Tween 20 (polyoxyethylene-sorbitan monolaurate, Sigma; 3 ml, 0.5%) and phage titre was determined. Numbers of eluted phage were established by titering a small proportion on agar plates containing *Escherichia coli* strain ER2537 supplemented with 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-gal, Bioline Ltd., London, UK) and isopropyl-β thiogalactopyranoside (IPTG, Bioline Ltd.). The remaining phage were amplified by addition to an early log phase culture of ER2537 (20 ml, 5 h, 37°C with vigorous shaking (200 rpm)). Amplified phage were isolated from the resulting culture according to the manufacturer's recommended protocol, concentrated, titered and used in the subsequent round of biopanning. In total, three consecutive rounds of biopanning were performed and three phage populations were recovered after each—those tightly associated with tumour, those weakly associated with tumour and those isolated from normal colon.

Sequencing of bacteriophage oligopeptide inserts

Several clones were picked from each phage population and their total DNA was isolated according to the recommended protocol of the sequencing kit manufacturer (Biosystems, Perkin Elmer, Foster, CA, USA). The resulting DNA was used for sequencing across the insert region of *pIII* using the −96 primer together with the BigDye Terminator Cycle Sequencing Kit (Biosystems, Perkin Elmer). DNA sequences were determined using an automated ABI PRISM 3700 sequence detector (Perkin Elmer).

Evaluation of tumour binding activities of selected phage clones

To assess the selectivity of binding of phage clones displaying consensus oligopeptides to human colorectal cancer, 1×10^{10} pfu of selected phage clones displaying the consensus motifs VPNTNSLPAAVN (a, patients 4–6), LIAKTALPQTNK (b, patients 7–9) or no peptide insert (c, patients 10–12) were injected into the tumour feeding artery immediately following resection of colon from patients and incubated for 10 min to allow binding. The colon was then opened and sections of tumour and normal colon wall were collected for assessment of phage binding. The results are presented as plaque forming units per milligram tissue (a) for individual patients, with solid bars representing tumour and hatched bars representing normal gut tissue, and (b) as means ± SD, where NS is not significant, and ** $p < 0.05$.

Results

Freshly isolated human tumours were used for intra-arterial biopanning using a phage display library in order to identify consensus oligopeptide sequences capable of targeted delivery to human tumours following intravascular delivery. This novel methodology enables reiterative biopanning in consecutive tumours from different patients and provides a powerful means to identify sequences capable of binding tumour-associated targets that are accessible from the luminal surface of polarised human tumour vasculature.

Enrichment of the phage library by biopanning in consecutive colorectal tumours

Three consecutive rounds of biopanning were performed on surgically resected colorectal cancer specimens from patients 1–3 (Table I) using an M13 phage protein III 12-mer linear oligopeptide phage display library, in order to identify oligopeptide sequences capable of binding to colorectal tumours. Phage library was injected directly into the tumour feeding artery in PBS (Figure 1a), allowed to bind for 10 min at room temperature and then unbound phage were removed by washing (Figure 1b). Injection of phage library directly into the tumour feeding artery should allows for negative selection (depletion) of the library for peptides binding to ubiquitously expressed receptors on non-transformed endothelium, *en route* to the tumour. Tissue samples were then isolated and phage association with normal tissue and malignant material was quantified by titering on Xgal IPTG containing agar plates after each round, and phage tightly associated with tumour tissue (i.e. which could not be removed by an acid wash) was amplified in *E. coli* to produce the library for panning in the subsequent specimen. Phage association with the tumour (both weakly and tightly bound) increased more than 100-fold between patients 1 and 2, suggesting that phage expressing receptor-binding oligopeptides were being selected. Tightly bound phage rose from 30 to 3200 pfu/mg tissue, while weakly bound phage rose from 55 to 5100 pfu/mg tissue. Interestingly, between patients 2 and 3 the number of tightly bound phage increased further (to 5300 pfu/mg tissue), while the recovery of weakly bound phage decreased (to 180 pfu/mg tissue; Figure 2). Since only the tightly bound phage population is amplified after each step for use in the next biopanning procedure, these data fit with the selective amplification of phage expressing oligopeptide sequences mediating high affinity binding to tumour specific receptors.

Analysis of peptide inserts to identify consensus tumour-targeting sequences

After each round of panning, several individual phage clones were isolated from the population of tightly

Table I. Details of patients in this study.

Case	Age	Sex	Tumor			Stage	Procedure	Injected artery	Injected phage
			Location	Size	Histology				
1	79	M	S	45	mod	B	S	S	Library
2	78	F	uR	60	mod	B	AR	SR	Library
3	79	F	uR	25	well	A	AR	SR	Library
4	81	F	uR	30	mod	C	AR	SR	VPNTNSLPAAVN
5	67	M	A	70	mod	C	RH	RC	VPNTNSLPAAVN
6	67	M	S	60	mod	C	S	S	VPNTNSLPAAVN
7	58	M	uR	60	mod	B	AR	SR	LIAKTALPQTN
8	55	M	D	30	mod	B	LH	LC	LIAKTALPQTN
9	82	F	S	30	mod	A	S	S	LIAKTALPQTN
10	78	M	C	40	muc	A	RH	IC	Control
11	66	F	uR	40	mod	B	AR	SR	Control
12	77	F	S	45	mod	B	S	S	Control

Tumour location: S, sigmoid colon; uR, upper rectum above the peritoneal reflection; A, ascending colon; D, descending colon; C, caecum.
Histology: mod, moderately differentiated; well, well differentiated; muc, mucinous adenocarcinoma.
Stage: dukes stage.
Procedure: S, sigmoidectomy; AR, anterior resection of rectum; RH, right hemicolectomy; LH, left hemicolectomy.
Injected artery: S, sigmoidal artery; SR, superior rectal artery; RC, right colic artery, LC, left colic artery; IC, ileocolic artery.
Injected phage: library, unselected library; VPNTNSLPAAVN, phage clone containing TNSLP consensus targeting sequence; LIAKTALPQTNK, phage clone containing AxPxTN consensus targeting sequence; control, insertless phage.

tumour-bound phage, their DNA isolated, sequenced and the corresponding amino acid sequences of the inserts deduced. After biopanning in the sample from the first patient, the oligopeptide sequences displayed no discernible homology (Figure 3a). After panning in the tumour sample from the second patient the consensus sequence NxxP (where x represents any amino acid) appeared in 9 out of 20 clones sequences (45%) (Figure 3b). As two phage contained identical inserts, and may have arisen from the same selection event, the frequency of NxxP iteration was conservatively regarded as 8/19. The probability that any such dipeptide motif will arise at this frequency by chance is low ($p = 0.0013$), hence this represents a significant observation. The sequence NSLP was the most well represented, appearing as a complete tetrapeptide three times in the eight independent NxxP sequences, suggesting a positive selection for this sequence ($p = 0.016$).

After biopanning in the sample from the third patient, however, although the overall number of tightly bound phage increased (Figure 2) the frequency of NxxP sequences decreased (2 of 35 samples). The most obvious motif was AxP, which appeared 9 times

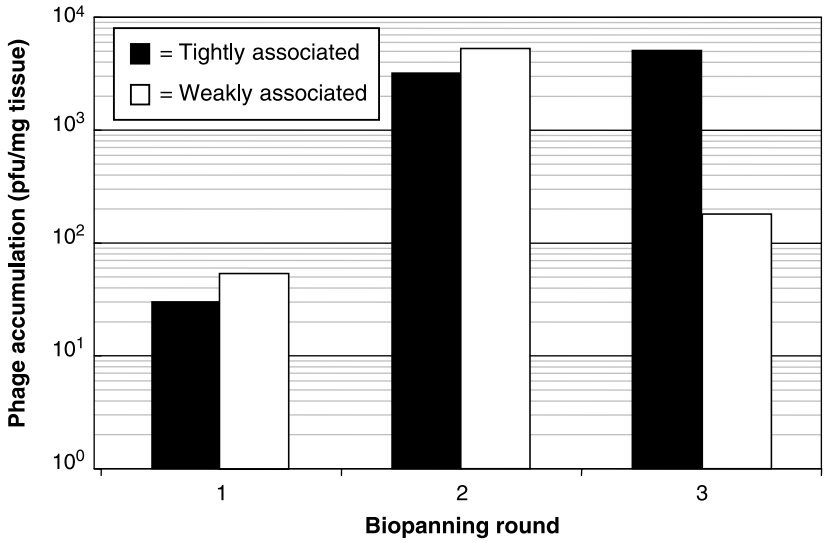

Figure 2. Comparison of the numbers of phage clones recovered after three consecutive rounds of panning in different tumours. Three sequential rounds of biopanning were performed to identify peptides capable of binding to human tumour tissue *ex vivo*. Three phage populations were recovered after each—those tightly associated with tumour, those weakly associated with tumour and those isolated from normal colon.

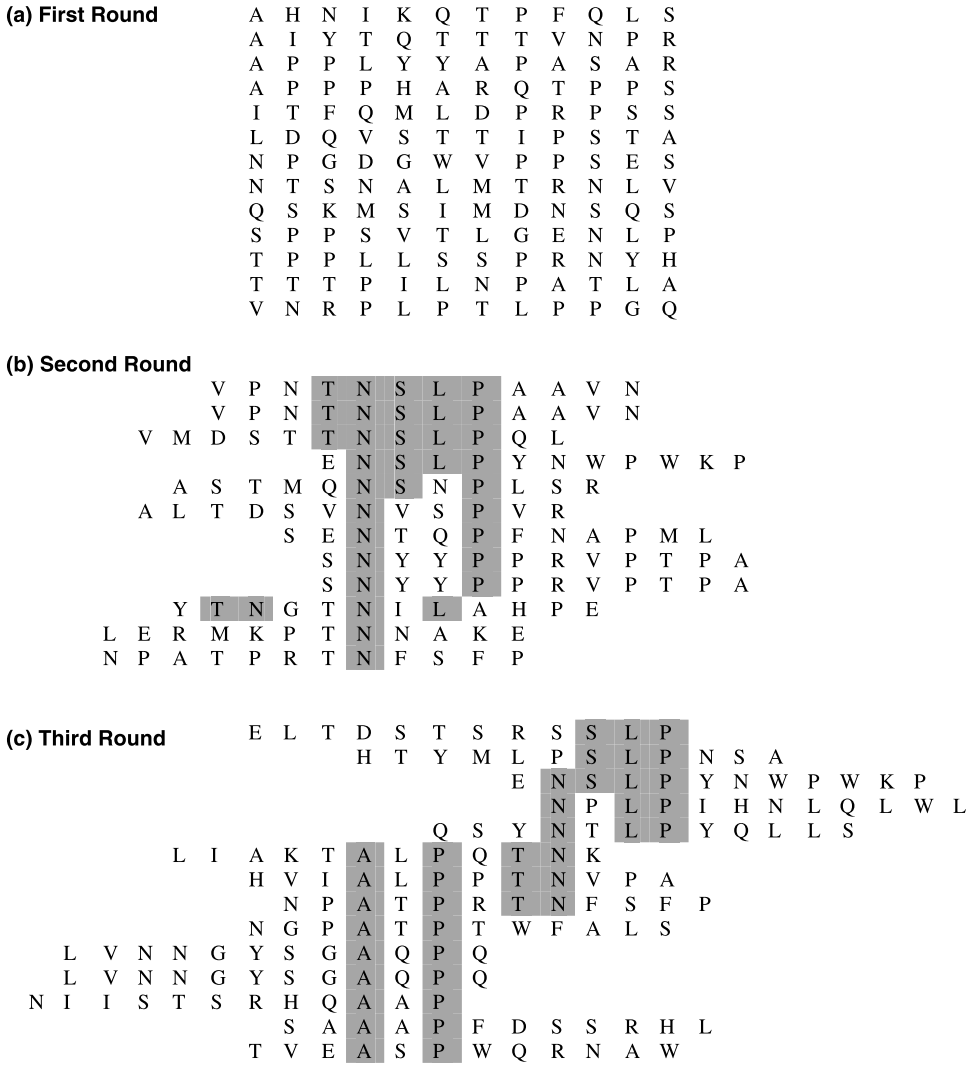

Figure 3. Iteration of consensus oligopeptides capable of binding to tumour tissue after panning rounds 1, 2 and 3. Sequences deduced from tightly bound phage isolated after the three rounds of biopanning. Only the consensus sequences containing NxxP, TN, SLP or AxP are shown (b) after the second round (from 20 samples) and (c) after the third round (from 35 samples).

in 35 sequences (Figure 3c), while the sequence AxPxTN appeared three times in the nine occurrences of AxP. It is hard to reconcile the increased frequency of tightly bound phage with the decrease in sequence homology, although this probably indicates variation in the profile of receptor expression between the tumours used for panning from patients 2 to 3. Indeed, whereas the tumours from patients 1 to 2 were both moderately differentiated Dukes B colorectal carcinomas, the tumour from patient 3 was a well differentiated Dukes A tumour.

No discernable consensus sequences were obtained at any stage from acid-eluted (weakly-bound) tumour-associated phage; neither did these phage show any significant homology with the tightly tumour-associated fraction (data not shown).

Assessment of tumour-targeting of consensus sequences in tumours from different patients

Based on the consensus sequences observed using tightly bound phage, phage clones expressing the oligopeptides VPNTNSLPAAVN (consensus sequence TNSLP) and LIAKTALPQTNK (consensus sequence AxPxTN) were selected for further investigation. To assess whether phage clones displaying consensus oligopeptides would be capable of selective targeting to human colorectal cancer, 10^{10} pfu of clonal phage solutions were prepared and injected *via* the tumour-feeding artery into freshly-resected colorectal cancer specimens from patients 4 to 9. Tumours resected from patients 10 to 12 were used to characterise binding of insertless phage as a control. In all cases, phage were allowed to bind for 10 min at room temperature, before

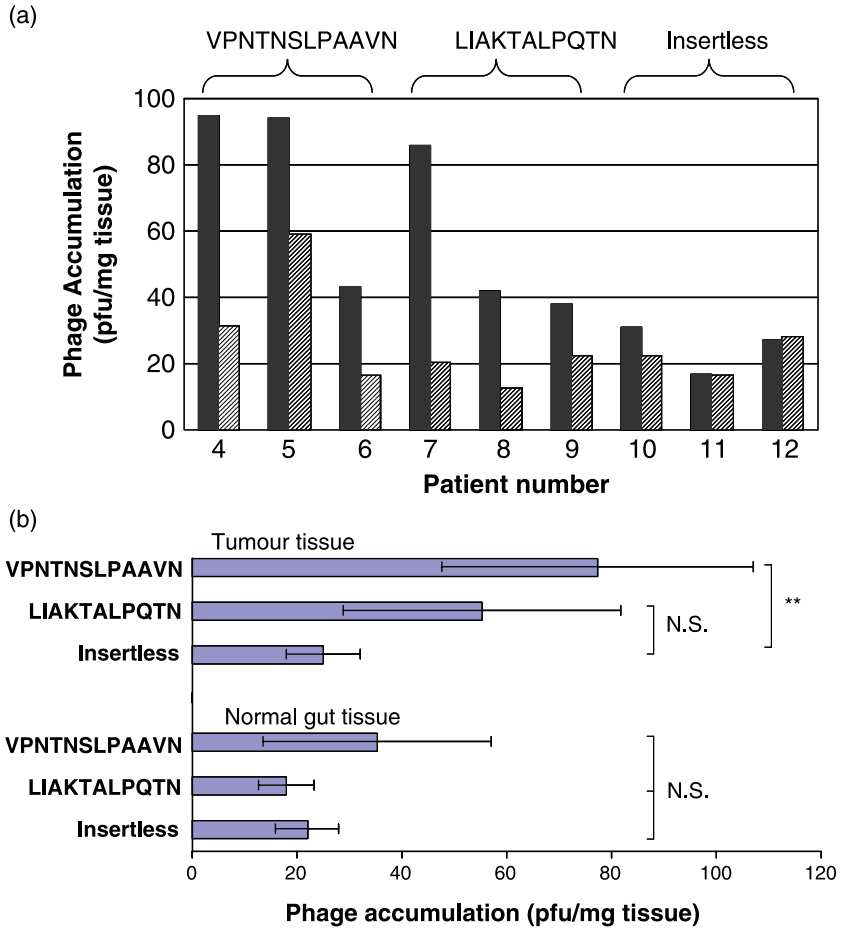

Figure 4. Evaluation of tumour binding activities of selected phage clones. To assess the selectivity of binding of phage clones displaying consensus oligopeptides to human colorectal cancer, 1×10^{10} pfu of selected phage clones displaying the consensus motifs VPNTNSLPAAVN (a, patients 4–6), LIAKTALPQTNK (b, patients 7–9) or no peptide insert (c, patients 10–12) were injected into the tumour feeding artery immediately following resection of colon from patients and incubated for 10 min to allow binding. The colon was then opened and sections of tumour and normal colon wall were collected for assessment of phage binding. The results are presented as plaque forming units per milligram tissue (a) for individual patients, with solid bars representing tumour and hatched bars representing normal gut tissue, and (b) as means ± SD, where NS, not significant, and ** $p < 0.05$.

unbound phage were washed away using PBS. Tissue samples were collected from both the tumour and the adjacent normal gut wall, tissue-associated phage were titred and data from individual patients are shown in Figure 4a,b. Control phage showed low levels of tissue association in samples from each patient 10 to 12 (two moderately differentiated Dukes B tumours and one Dukes A serous adenocarcinoma), reaching phage titres (mean ± SD) of just 25.0 ± 7.2 pfu/mg tumour tissue compared with 22.1 ± 6.0 pfu/mg in normal gut wall (Figure 4b). In contrast, the phage clone expressing VPNTNSLPAAVN showed average levels of 77.3 ± 29.7 pfu in tumour tissues isolated from patients 4 to 6, all of which were Dukes C moderately differentiated colorectal tumours. This level was significantly elevated compared with levels of control phage in tumours from patient 10 to 12 ($p = 0.04$). This phage accumulated only to the level of 35.3 ± 21.8 pfu/mg in normal tissue, not significantly different from the levels of accumulation of control phage.

The phage clone expressing LIAKTALPQTN achieved tumour levels of 55.3 ± 26.4 pfu/mg in tumours from patients 7 to 9 (moderately differentiated Dukes A and B tumors), not significantly elevated compared to binding of the control phage to tumours from patients 10 to 12 ($p = 0.13$). Similarly, levels of LIAKTALPQTN phage recovered from normal tissues (18.0 ± 5.3 pfu/mg) were not different from controls.

Selectivity of the phage clones for binding for tumour tissue may be estimated from the tumour/normal ratio of phage accumulation. For insertless phage this ratio was found to be 1.14 ± 0.24; for phage expressing VPNTNSLPAAVN the ratio was 2.44 ± 0.76 (significantly elevated compared to insertless control phage, $p < 0.05$) and for phage expressing LIAKTALPQTN the ratio was 3.18 ± 1.32 (not significantly elevated

compared to insertless controls, $p = 0.058$). Accordingly, phage expressing VPNTNSLPAAVN showed both increased levels of tumour uptake and increased tumour selectivity, while phage expressing LIAKTALPQTN did not show a significant effect.

Discussion

This relatively simple method is safe, non-invasive and allows selection of high affinity cell-binding oligopeptides by iterative intra-arterial biopanning in consecutive specimens isolated from different patients. The approach should be well suited to defining clinically useful oligopeptide targeting sequences, and the sequences identified are likely to bind to tumour components that are accessible from the bloodstream. This represents a distinct advantage over methodologies for panning in human tumour biopsies without vascular access (Shukla and Krag 2005a,b) which do not take account of polarised cellular anatomy.

Three rounds of biopanning using a phage library expressing random linear 12-mer oligopeptides was sufficient to mediate significant selectivity towards the diseased target cells (greater than 100-fold increases in recovery were observed compared to naïve library), with substantial sequence homology emerging in the deduced target cell-binding oligopeptides. Phage clones expressing consensus oligopeptides (TNSLP and AxPxTN) were isolated and phage expressing TNSLP associated preferentially with malignant tissue compared to normal tissue following intra-arterial injection into surgically resected clinical specimens, whilst the distribution of control phage (bearing no insert) was not found to be significantly different in malignant material compared to normal tissue.

The lead oligopeptides identified here have not been developed for therapeutic study since we consider that superior sequences may be iterated by more careful selection of patients for the reiterative panning procedure. In particular, we noted some oligopeptide epitopes that were strongly expressed in round 2 were less evident in round 3, most probably reflecting heterogeneity of receptor expression in the tumours used. This suggests that effective tumour-targeting epitopes may vary between tumour subtypes, probably as a function of individual tumour phenotype or genotype. Best results using this panning strategy may therefore be obtained by sequential panning in tumours of closely related genotype, leading to identification of a battery of targeting ligands that are differentially suited to different cancer subtypes. This represents a functional approach to cancer pharmacogenetics.

Cancer-selective ligands may be identified empirically in this system by evaluating individual sequences for differential selection in human and normal tissue, although a more powerful approach would to perform depletion panning by administering phage into the arterial supply of normal gut tissue and collecting those phage that are not retained, subsequently amplifying them for positive selection in tumour biopanning. The surgical procedure required to permit recovery of non-retained phage is straightforward and was performed here by inserting a canula into the corresponding vein to collect the perfusate.

The precise anatomical location of targeted receptors will vary between oligopeptides, and this will determine their relative usefulness for applications such as tumour imaging, delivery of cytotoxic drugs, radiosensitisation and targeting of oncolytic viruses. Careful development of this biopanning strategy could provide a powerful means to bridge the gap between the laboratory and clinical treatment strategies.

Acknowledgements

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References

Arap W, Pasqualini R, Ruoslahti E. 1998. Cancer treatment by targeted drug delivery to tumor vasculature in a mouse model. Science 279:377–380.

Arap W, Haedicke W, Bernasconi M, Kain R, Rajotte D, Krajewski S, Ellerby HM, Bredesen DE, Pasqualini R, Ruoslahti E. 2002a. Targeting the prostate for destruction through a vascular address. Proc Natl Acad Sci USA 99:1527–1531.

Arap W, Kolonin MG, Trepel M, Lahdenranta J, Cardo-Vila M, Giordano RJ, Mintz PJ, Ardelt PU, Yao VJ, Vidal CI, et al. 2002b. Steps toward mapping the human vasculature by phage display. Nat Med 8:121–127.

Backer MV, Backer JM. 2001. Targeting endothelial cells overexpressing VEGFR-2: Selective toxicity of Shiga-like toxin–VEGF fusion proteins. Bioconj Chem 12:1066–1073.

Binetruy-Tournaire R, Demangel C, Malavaud B, Vassy R, Rouyre S, Kraemer M, Plouet J, Derbin C, Perret G, Mazie JC. 2000. Identification of a peptide blocking vascular endothelial growth factor (VEGF)-mediated angiogenesis. EMBO J 19:1525–1533.

Bockmann M, Drosten M, Putzer BM. 2005. Discovery of targeting peptides for selective therapy of medullary thyroid carcinoma. J Gene Med 7:179–188.

Coll JL, Wagner E, Combaret V, Metchler K, Amstutz H, Iacono-Di-Cacito I, Simon N, Favrot MC. 1997. *In vitro* targeting and specific transfection of human neuroblastoma cells by chCE7 antibody-mediated gene transfer. Gene Ther 4:156–161.

Fisher KD, Stallwood Y, Green NK, Ulbrich K, Mautner V, Seymour LW. 2001. Polymer-coated adenovirus permits efficient retargeting and evades neutralising antibodies. Gene Ther 8: 341–348.

Ho IA, Lam PY, Hui KM. 2004. Identification and characterization of novel human glioma-specific peptides to potentiate tumor-specific gene delivery. Hum Gene Ther 15:719–732.

Kashentseva EA, Seki T, Curiel DT, Dmitriev IP. 2002. Adenovirus targeting to c-erbB-2 oncoprotein by single-chain antibody fused to trimeric form of adenovirus receptor ectodomain. Cancer Res 62:609–616.

Kolonin M, Pasqualini R, Arap W. 2001. Molecular addresses in blood vessels as targets for therapy. Curr Opin Chem Biol 5: 308–313.

Landon LA, Deutscher SL. 2003. Combinatorial discovery of tumor targeting peptides using phage display. J Cell Biochem 90: 509–517.

Lorimer IA, Keppler-Hafkemeyer A, Beers RA, Pegram CN, Bigner DD, Pastan I. 1996. Recombinant immunotoxins specific for a mutant epidermal growth factor receptor: Targeting with a single chain antibody variable domain isolated by phage display. Proc Natl Acad Sci USA 93:14815–14820.

Maruta F, Parker AL, Fisher KD, Hallissey MT, Ismail T, Rowlands DC, Chandler LA, Kerr DJ, Seymour LW. 2002. Identification of FGF receptor-binding peptides for cancer gene therapy. Cancer Gene Ther 9:543–552.

Pasqualini R, Ruoslahti E. 1996. Organ targeting *in vivo* using phage display peptide libraries. Nature 380:364–366.

Rajotte D, Ruoslahti E. 1999. Membrane dipeptidase is the receptor for a lung-targeting peptide identified by *in vivo* phage display. J Biol Chem 274:11593–11598.

Shukla GS, Krag DN. 2005a. Phage display selection for cell-specific ligands: Development of a screening procedure suitable for small tumor specimens. J Drug Target 13:7–18.

Shukla GS, Krag DN. 2005b. Selection of tumor-targeting agents on freshly excised human breast tumors using a phage display library. Oncol Rep 13:757–764.

Zitzmann S, Mier W, Schad A, Kinscherf R, Askoxylakis V, Kramer S, Altmann A, Eisenhut M, Haberkorn U. 2005. A new prostate carcinoma binding peptide (DUP-1) for tumor imaging and therapy. Clin Cancer Res 11:139–146.