original research report

Serine and proline-rich ligands enriched via phage-display technology show preferential binding to BCR/ABL expressing cells

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Hematol Oncol Stem Cell Ther 2014; 7(1): 32–40

DOI: http://dx.doi.org/10.1016/j.hemonc.2014.01.001

BACKGROUND AND OBJECTIVES: Despite the use of targeted therapy, chronic myelogenous leukemia (CML) currently remains incurable with drug therapy, with patients requiring life-long treatment. Developing either a vaccine to prevent the disease or another novel drug to specifically target and eradicate the CML cell will require the identification of CML-associated cell-surface markers and molecules that can bind specifically to the cell surface. In an attempt to discover peptides that bind specifically to cells in the early chronic phase of the disease, we used phage-display technology to identify heptapeptides that bind specifically to the surface of BCR/ABL-expressing fibroblasts.

METHODS: An *in vitro* system using NIH3T3 stably transfected with pGD210 (BCR/ABL) was used as a model for the chronic phase of the disease. The cells were panned using a linear heptapeptide phage library (Ph.D 7.0) in a negative/positive panning strategy with NIH3T3 containing only the plasmid vector as the wild type control.

RESULTS: We identified four novel peptides that were enriched through this technique. These peptides contained either multiple proline residues or serine/threonine–proline pairs and showed a confirmed binding preference for BCR/ABL+ fibroblasts. The peptide Y-R-A-P-W-P-P also showed a binding affinity for granulocytes from untreated CML patients.

CONCLUSION: We have identified several novel peptides that can be used in future studies to identify specific CML cell-surface antigens or provide a novel drug-delivery mechanism.

Although CML is one of the best described leukemic diseases, the only definitive cure continues to be bone marrow transplants. Treatment with specific tyrosine kinase inhibitors (TKI) offers a targeted therapy approach and is currently the standard of care for chronic phase CML, yet in most cases life-long therapy is required to prevent a relapse. In addition, despite remission being rapidly achieved with TKI therapy in many patients, some do not respond at all, suffer severe side-effects, or acquire resistance mutations and relapse while on therapy.[1,2] Alternative therapies, including the development of a CML vaccine[3–5] and targeted drug-delivery mechanisms,[6] both of which are based on the concept of a unique CML or leukemic cell-surface antigen, are thus currently being investigated. Although BCR/ABL, the novel fusion protein responsible for chronic phase CML development, can be processed and presented on the surface of clonal cells, it is not highly immunogenic and has shown limited success in vaccine trials with patients in the chronic phase.[7–9] Despite the use of global microarray techniques[10] and cancer/testis antigen profiling,[11] CML cell-surface antigens remain poorly defined. Research is thus still ongoing to identify a CML-specific antigen which holds potential for the development of a vaccine and targeted drug-delivery.

Phage-display technology, using either peptide or antibody libraries to identify ligands and receptors, is a technique that is proving useful in the search for cancer-specific markers.[12–14] Despite the technical difficulties associated with whole-cell panning (i.e.,

low level targets, suitable negative control cell types), in the arena of haematological malignancies, this technique has already generated useful targets. This includes potentially new drug-delivery mechanisms in acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL),[6] identification of growth and proliferation modifying ligands in AML[15] and potential diagnostic markers for IFN-α sensitive CML cells.[16]

In the search for a CML-specific antigen, we used phage-display technology to identify peptides that bind to the surface of BCR/ABL expressing cells, as a means of identifying ligands that are associated specifically with chronic phase CML disease. Due to the lack of suitable human cell-lines that represent chronic phase disease and normal physiology, and the difficulty associated with stable transfection of human haematopoietic stem cells,[17] we chose to use a BCR/ABL-expressing NIH3T3 mouse fibroblast cell-line as a model. Mouse and rat fibroblast models have been used extensively in defining the pathogenic role of BCR/ABL[18–21] and allow for the development of a well-controlled, genetically defined system for the whole-cell panning phage-display approach. We generated stably transfected clones using the *pGD210* BCR/ABL expression vector,[22] as well as a modified vector-only control (*pCVT*). These cells were assessed for cell-surface associated changes previously attributed to BCR/ABL expression and then panned using a linear heptapeptide phage library in a negative/positive panning approach to identify peptides that bind preferentially to BCR/ABL expressing cells.

MATERIALS AND METHODS

Stable clone generation

The BCR/ABL expression vector p*GD210*,[22] (kindly supplied by Prof G. Daley) was used to generate the p*CVT* control plasmid, by excising the 7.2 kb *bcr/abl* cDNA fragment with *XhoI* and re-ligating the vector. Endotoxin-free plasmid DNA (4 μg p*GD210* and 2 μg p*CVT*) (Nucleobond Xtra EF, Macherey–Nagel) was used to transfect NIH3T3 mouse fibroblasts (2×10^6) using Nucleofector technology (LONZA, program U-023). Transfected NIH3T3 were maintained in RPMI/10% FBS media (Gibco) and 400 μg/ml G418 (Promega) antibiotic selection was started 48 h post-transfection for a 14-day period to select transfected clones. Surviving clones were expanded in the presence of G418, and cryopreserved for later use.

BCR/ABL mRNA expression

Ribonucleic acid (RNA) was extracted using Total RNA isolation reagent (TRIR) (ABgene) and the levels of BCR/ABL mRNA expression were determined using standardised methodology (1 μg RNA) for minimal residual disease monitoring of this transcript,[23–25] by the NHLS diagnostic service (Groote Schuur Hospital, Cape Town).

Adherence independent growth

Agar clonogenic assay

Fibroblasts (3×10^5) were mixed with 3 ml 0.3% agar/RPMI/10% FBS and plated on top of a solidified layer of 0.6% agar/RPMI/10% FBS in six well plates (Corning). Cells were incubated for 21 days and colonies visualised using phase-contrast microscopy.

Methylcellulose assay

Fibroblasts (5×10^3) were mixed with 100ul 1.5% methylcellulose (Sigma–Aldrich) RPMI/10% FBS media and plated into polyHEMA (Sigma–Aldrich) coated 96-well plates. Following incubation for the specified time periods, the colony expansion was measured by the addition of 20 μl CellTiter 96 AQ_{ueos} One reagent (Promega), incubation for 2 h at 37 °C/5% CO_2 and spectrophometry at 490 nM (Anthos2001 plate reader). Triplicate blank readings were subtracted.

In vitro cell motility assay

Confluent cell layers were established in six well plates in RPM1/10% FBS and a linear wound was created by scratching through the monolayer using a sterile 200 μl pipette tip. To remove cell debris, the growth medium was replaced and mitomycin C (10 μg/ml) (Sigma–Aldrich) was added to prevent further cell proliferation. Cells were incubated at 37 °C/5% CO_2 and the wound width was measured at three reference points at 0 and 6 h post treatment using light microscopy and the distance migrated recorded (μm).

Phage-display analysis

The phage-display linear heptapeptide library Ph.D 7.0 (New England Biosciences) with a complexity of 10^9 sequences, was used to pan NIH3T3 clones, using a combination of negative/positive panning in each round.[26] Each panning round was performed using cells at P3 post-thaw (to ensure similar membrane composition) and both 3T3B.p*GD210* and 3T3C.p*CVT* cells were of the same passage following transfection. Cells (3×10^6) were plated in 10 cm dishes, left to adhere O/N and then prewashed with

PBS/3% BSA at room temperature (RT) (20–22 °C) prior to panning. All planning stages were performed at RT in PBS/3% BSA. Phages (2×10^{11}) were pre-incubated in 10 volumes PBS/3% for 1 h at 4 °C prior to each round of panning and then used to first pan the control cells (3T3C.p*CVT*) for 30 min at RT. The supernatant was removed and used to negatively pan a second set of 3T3C.p*CVT* cells for 30 min at RT. This phage-depleted supernatant was used to positively pan against the BCR/ABL$^+$ cells (3T3B.p*GD210*) for 1 h at RT. Unbound/weakly bound phage was removed with 10 PBS washes at RT and cell-surface bound phage was eluted with 1 ml 0.2 M Glycine pH2.2/1 mg/ml BSA (15 min), followed by neutralisation with 150 µl 1 M Tris pH 9.1. This eluted phage was amplified in ER2738 cells and purified according to manufacturers' recommendations (NEB), with the extension of phage precipitation to O/N at 4 °C and phage collection of 45 min at 14,000 rpm at 4 °C. This process was repeated for four complete panning cycles. Post-panning, the eluted phage-extract was also titred by plating on LB^{tet} XGal/ITPG agar plates and 10–30 separate phage plaques were selected for sequence analysis using the 96gII primer (NEB) (rounds 3 and 4).

Phage binding assay

Single phages were re-amplified, purified and titred, and 1×10^{10} phages simultaneously panned against 1×10^{6} 3T3B.p*GD210* and 3T3C.p*CVT* (plated in 6-well plates), K562 vs. HL60 cultured cells (2 ml suspension cultures) or density-gradient prepared granulocyte preparations from two healthy volunteers and two untreated CML patients (2 ml suspension cultures) (ethics approval REC REF:360/2006). All cells were pre-washed in PBS/3%BSA and binding was performed for 1 h at RT in PBS/3% BSA. The cells were washed in PBS and the phages eluted with Glycine/Tris treatment as described above. Phage extractions were titred on LB^{tet} XGal/ITPG agar plates.

RESULTS

Generation and characterisation of stable NIH3T3 BCR/ABL expressing clones

To control cell-surface changes associated with the transfection methodology and the vector backbone (i.e., gentamycin resistance), we modified the original p*GD210* BCR/ABL vector[22] to create p*CVT*. This vector lacks only the *bcr/abl* cDNA insert and was a preferred control to untransfected cells in the phage-display analysis. Three separate clones were isolated and expanded following transfection with either the BCR/ABL expression vector or the control vector. The p*GD210* clones expressed variable levels of *bcr/abl* messenger RNA (mRNA) transcripts (2.2×10^{3}–2.6×10^{4}) (Fig. 1A), while none of the p*CVT* clones expressed this transcript. Although the ratios of *bcr/abl*:*abl* transcripts (0.091–0.555) did not approach 1, which is observed in BCR/ABL positive CML cells (K562), a lower ratio (0.196) was also shown in 3T3-*MIG210*, a BCR/ABL expressing NIH3T3 cell-line which has been used successfully in several BCR/ABL studies.[27–29] Clone 3T3B.p*DG210*, which expressed the highest intracellular *bcr/abl*:*abl* ratio of 1:2 (0.555) and shown to express the BCR/ABL protein via western hybridisation analysis (results not shown), was chosen for characterisation and phage-display analysis. Clone 3T3C.*pCVT* was used as the BCR/ABL negative control in all further experiments.

To validate the use of the 3T3B.p*DG210* cells as a suitable model for phage-display analysis, these cells were characterised for key features of BCR/ABL expressing CML stem cells. One such feature is reduced cellular adherence to the stroma substrate, which results in increased mobility and migration of the cells. Additionally, these stem cells are capable of anchorage-independent growth-signalling, which leads to uncontrolled proliferation.[19,30–34] These consequences of BCR/ABL expression were of particular interest as they involve both intracellular and cell-surface protein alterations, with the latter being the main target in phage-display analysis. The NIH3T3 *pGD210* cells demonstrated a significantly altered adhesion phenotype compared to the control cells. Agar clonogenic assays provided a qualitative assessment of this phenotype, with 3T3B.p*GD210* forming large, round, suspended colonies when grown in the dual-layer agar (21-day growth), compared to small cell clusters of <10 cells in the controls (Fig. 1Bi). Methylcellulose/polyHEMA suspension cultures allowed for a quantitative analysis, and although colony size was restricted compared to agar growth, the majority of the 3T3B.*pGD210* cells formed colonies and clearly showed a growth advantage over the control in this adhesion-restricted environment (Fig. 1Bii). A BCR/ABL-associated increase in mobility was demonstrated using the wound closure assay, with 3T3B.*pGD210* showing a 3.5-fold increased rate of wound closure compared to the 3T3C.*pCVT* control (Fig. 1C). Combined, these results indicated that the stable BCR/ABL$^+$ mouse fibroblasts showed anchorage-independent growth and increased migration/mobility, key characteristics of BCR/ABL

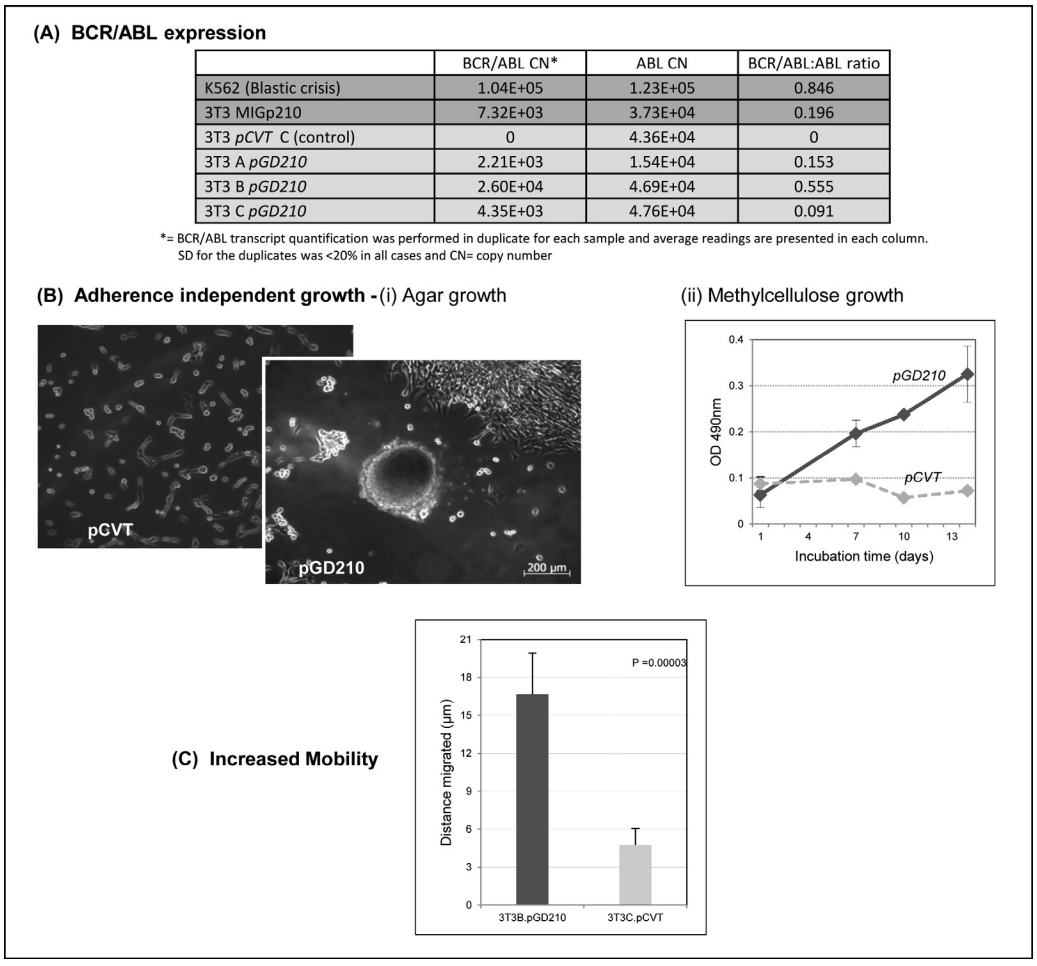

Fig. 1. Characteristics of BCR/ABL$^+$ NIH3T3 fibroblast clones. (A) *BCR/ABL* mRNA expression levels in 3T3.*pGD210* clones and controls. B(i) Colony formation in the double-layer agar clonogenic assay (21-day incubation). B(ii) Colony growth in methylcellulose media assayed by MTS conversion (triplicate analysis). (C) *In vitro* motility assay indicating distance migrated over a 6-h period (4–6 scratches analysed).

expression that are associated with CML-diseased stem cells. These cells thus represented an appropriate model to study the cell-surface effects of BCR/ABL expression by phage-display.

Phage-display analysis of NIH3T3 BCR/ABL clones

A phage-display library expressing linear heptapeptides was used to screen the fibroblast cell-surface for BCR/ABL-associated molecules in a whole-cell panning approach. A combined negative/positive panning strategy was adopted, where phages were first hybridised against control fibroblasts (3T3C.*pCVT*) to remove phages that bind generally to fibroblast cell-surface molecules. This depleted phage pool was then panned against the BCR/ABL$^+$ cells (3T3B.*pGD210*) to positively select bound phages, which were harvested and re-amplified for the following round.

This panning strategy was performed in two independent experiments, with 10 phages initially sequenced following three rounds of panning in each experiment. Although no dominant motif was observed, the combined analysis showed a predominance of ligands containing proline, serine and threonine residues, with 71% containing at least one proline residue and 57% containing serine or threonine residues (results not shown). Round 4 panning was performed and 20/27 phages were sequenced respectively. No significant difference was observed between the two experiments in terms of sequence dominance and thus the 47 sequences were analysed collectively to look for predominant motifs. Again, a similar enrichment of phage peptides containing serine, threonine and proline residues was observed (Fig. 2). The majority (76%) of the peptides encoded at least one proline residue, while as many as 36% encoded two or three. One of the serine-rich ligands sequenced in round 3

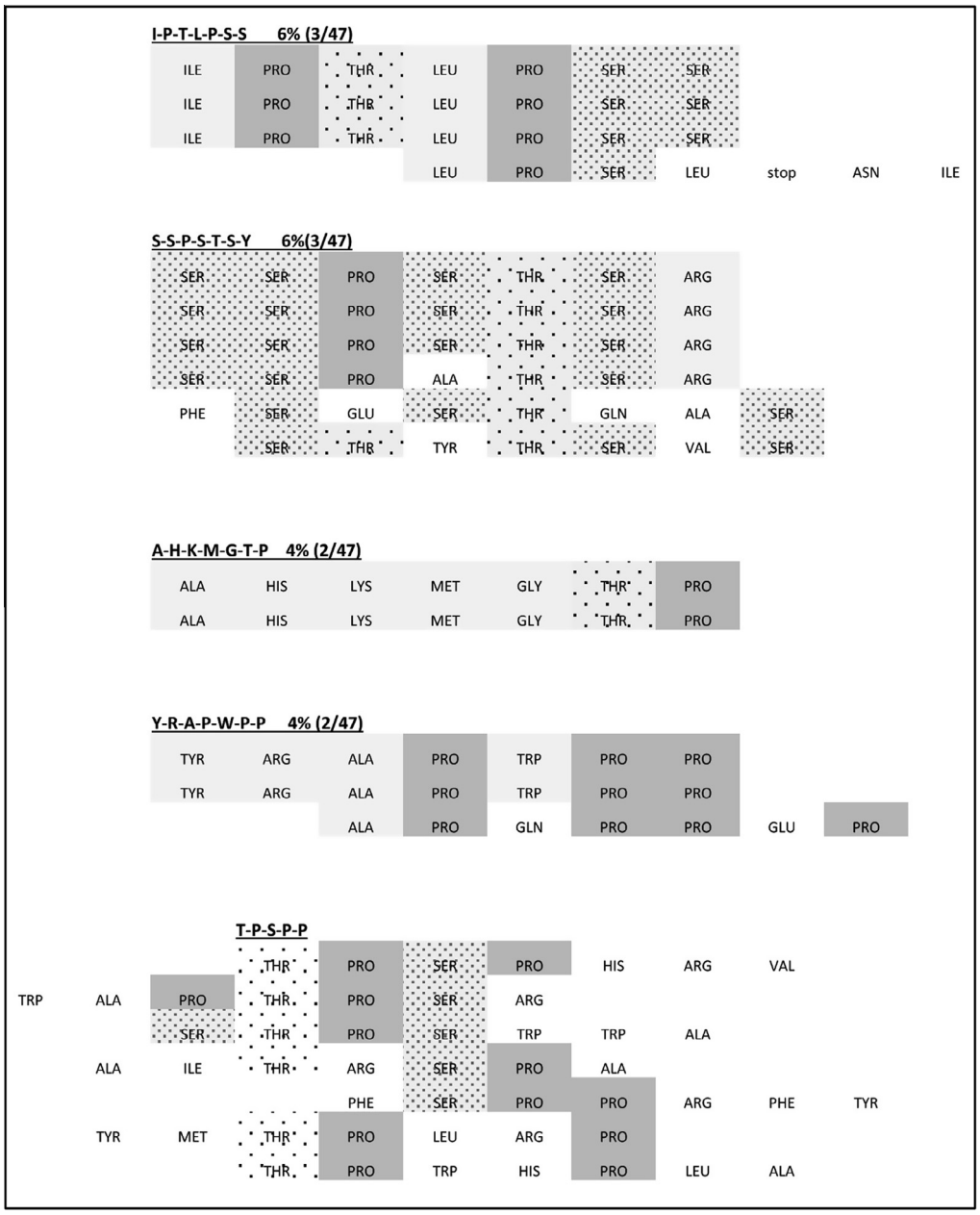

Fig. 2. Alignment of phage-peptide sequences from 4 rounds of panning. Phage-peptide sequences showing an alignment of at least 3 residues to one of the 5 enriched motifs are represented. PRO/SER/THR residues are highlighted to show their predominance and light grey blocks indicate additional amino acid identity.

was encoded by several phages in round 4 (S-S-P-S-T-S-Y), demonstrating a definite enrichment via this methodology (Fig. 2). In total, four separate 7-mer motifs were present in multiple copies following four rounds of panning: I-P-T-L-P-S-S, S-S-P-S-T-S-Y, A-H-K-M-G-T-P, Y-R-A-P-W-P-P. A 5-mer motif, T-P-S-P-P, also showed some level of enrichment. While 15/47 phages contained at least two amino acids that agreed with this motif, no single phage-peptide sequenced encoded all five amino acids (Fig. 2), which suggested a fairly weak consensus motif. Overall, 47% had at least 3/7 residues that matched the five motifs described here, while 81% possessed two residues that aligned. This indicated a progressive enrichment of possible consensus ligand sequences. Unfortunately, further panning rounds did not improve the motif enrichment.

To confirm the binding specificity of the peptide motifs for BCR/ABL expressing cells, single phage preparations, representing the five motifs, as well as

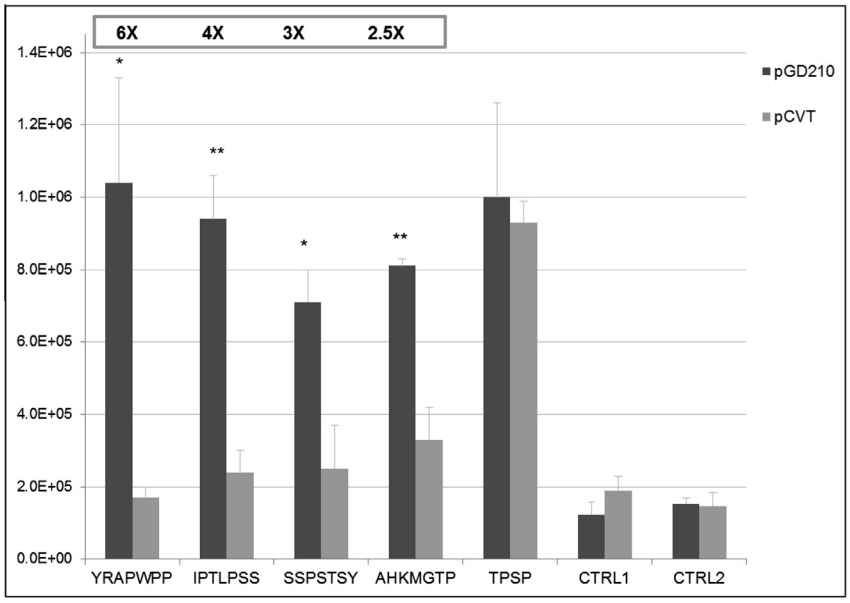

Fig. 3. Analysis of phage-binding preference to NIH3T3 transfected clones. Graphical representation of the number of specific phages bound to either 3T3B.*pGD210* or 3T3C.*pCVT* cells (triplicate analysis). The binding preference for BCR/ABL$^+$ cells is indicated. Ctrl 1:H-W-G-M-W-S-Y; Ctrl 2: N-D-I-N-N-Q-S. $P = <0.01^*, <0.001^{**}$.

two unrelated control peptides (H-W-G-M-W-S-Y/N-D-I-N-N-Q-S), were panned against 3T3B.*pGD210* and control 3T3C.*pCVT* cells separately. Fig. 3 indicates the number of phages remaining bound to the two cell types following hybridisation and washing. All four of the 7-mer motifs showed an increased binding preference for the BCR/ABL + fibroblasts, with Y-R-A-P-W-P-P motif showing the highest affinity with a sixfold increase in phage binding. The T-P-S-P-X-X-X phage representing the T-P-S-P-P motif showed no binding preference, but a much higher level of binding to both cell types. This result may indicate a ubiquitous, highly expressed epitope on mouse fibroblasts; an increase in the number of negative panning rounds would be needed to prevent the preferential amplification of this motif.

In addition to these phages showing a binding preference in our fibroblast model, a preliminary experiment examining the clinical significance of these peptides also showed a binding preference for BCR/ABL+ myeloid cells. Phages encoding the proline-rich ligand Y-R-A-P-W-P-P and control H-W-G-M-W-S-Y were hybridised against K562 (a blastic crisis CML cell-line), HL60 (a promyelocytic BCR/ABL negative cell-line), as well as granulocytes from two healthy individuals, a chronic phase CML patient, and a patient in accelerated phase CML (Fig. 4). While the control phage showed no binding preference to any cell type, the proline-rich ligand showed a fivefold increase in binding to cells representing untreated chronic phase CML, compared to control cells. Cells which represented a more advanced disease state, such as accelerated and blastic crisis phases, showed a slightly lower, but still significant increased binding. This was demonstrated with a threefold increased binding to BCR/ABL + K562 compared to the HL60 control and a twofold increased binding to the accelerated phase CML cells compared to healthy granulocytes.

DISCUSSION

In the search for alternative strategies for CML treatment, vaccines and target drug-delivery systems are actively being investigated. For these kinds of therapies to be effective, it is necessary to identify antigens where expression is specifically linked to diseased cells, with preference being for a protein associated with early chronic phase disease. To further the identification of such an antigen, we investigated the direct effects of BCR/ABL expression, the primary oncogene in CML development, on cell-surface changes in a fibroblast model of disease. Through the use of phage-display technology with a linear heptapeptide library, we aimed to identify ligands that bound specifically to BCR/ABL$^+$ cells, which could then be used in future studies to characterise the associated cell-surface antigen or be investigated as a novel drug-delivery mechanism.

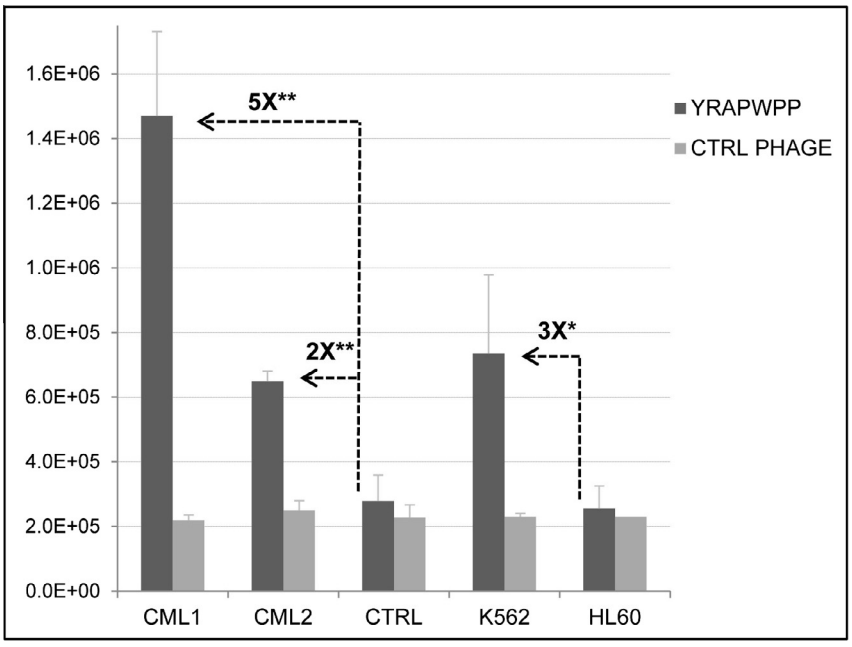

Fig. 4. Analysis of phage-binding preference to BCR/ABL$^+$ myeloid cells. Graphical representation of the number of specific phages bound to granulocyte and cell-line preparations (duplicate analysis). The binding preference for BCR/ABL$^+$ cells is indicated. Ctrl: H-W-G-M-W-S-Y; CML1: chronic-phase CML patient, CML2: accelerated-phase CML patient, K562: BCR/ABL$^+$ blastic crisis cell-line, HL60:BCR/ABL$^-$ myeloid cell line. $P = \leq 0.01^*$, $< 0.005^{**}$.

Phage-display analysis of the BCR/ABL expressing fibroblasts generated a number of lead sequences which showed a preference for binding to BCR/ABL$^+$ cells: I-P-T-L-P-S-S, Y-R-A-P-W-P-P, S-S-P-S-T-S-Y and A-H-K-M-G-T-P. All four enriched ligands contained proline residues, but more specifically contained either multiple proline residues or SER/THR-PRO motifs. Proline-rich ligands are often associated with signalling proteins that have WW and SRC-homology domains (SH2 and SH3) and include many proteins involved in responses to extracellular signals and cytoskeletal rearrangements (i.e., ABL and actin-binding proteins).[35,36] Both the I-P-T-L-P-S-S and Y-R-A-P-W-P-P ligands, enriched in this study, specifically have the core P-x-x-P motif that is recognised by proteins with a SH3 domain.[35] Due to the formation of a hydrophobic binding surface, proline-rich ligands generally do not bind tightly to signalling protein domains, but this allows for a rapid on/off signalling mechanism.[36] The relatively low binding preference of these ligands (four and sixfold respectively) for BCR/ABL$^+$ fibroblasts observed in our study may thus represent a significant finding.

The SER/THR-PRO motif is common in ligands binding to proline-directed protein kinases such as cyclin-dependant kinases, kinases containing class IV WW or polo-box domains, and mitotic phosphoproteins. These proteins are crucial in cell-cycle regulation/proliferation, and signalling via these proteins is often aberrant in cancer.[37,38] Proline-directed kinases, such as Pin1, transduce a signal by cleaving the SER/THR-PRO bond present in a ligand, which results in several pathways, including mitotic division and microtubule formation, being activated.[37] Reversible phosphorylation of the serine/threonine residue in this motif is an important control mechanism, as it alters the binding efficiency of the ligand and can allow for rapid signalling changes. The S-S-P-S-T-S-Y peptide enriched in our study potentially possesses the key signalling motif S-pS/pT-P-x of the polo-box domain, while the pS/pT-P motif of the class IV WW domain may be found in the A-H-K-M-G-T-P peptide. Due to the phosphorylation of these ligands playing a role in ligand binding efficiency, the lower binding preference observed for these ligands may be due to the lack of phosphorylation of these residues in this *in vitro* system.

Phage-display analysis of BCR/ABL$^+$ mouse fibroblasts has generated several interesting and relevant lead ligands that may be of use in either defining the cell-surface antigens specific to chronic phase CML, or by providing tools for targeted drug-delivery. The peptides defined in this study contain motifs that

are often linked to ligands associated with proteins involved in cell-cycle regulation and cytoskeletal rearrangements, hallmark characteristics of BCR/ABL positive cells *in vivo*. Previous phage-display analysis on CML patients and cell-lines has concentrated on the blastic crisis stage of the disease, a complex disease stage characterised by many varied secondary genetic mutations.[6,15] In these studies, proline-rich ligands were not found to be enriched, indicating that the novel ligands identified in our study may represent markers that are specific to the chronic phase of the disease (BCR/ABL specific). Promising results from a preliminary evaluation of peptide-binding to myeloid cells from CML patients, cell-lines and controls, indicated that the proline-rich peptide tested showed a binding preference for BCR/ABL+ myeloid cells and a higher preference for chronic phase cells, which supports our hypothesis. Future studies would include the binding potential of purified peptides to various cell populations in a larger chronic phase CML patient cohort, isolation of the membrane-bound receptor and internalisation potential of the ligands for drug-delivery.

FUNDING SOURCE

National Health Laboratory Service Research Trust and CANSA provided funding for this research, with no influence on the research design.

CONFLICT OF INTEREST

None of the authors have a conflict of interest with the generation of this data or its submission.

Acknowledgements

Authors contributions: KS, NN and SM designed the study. KS, IS, SN and JJ provided the experimental data. KS wrote the manuscript, while SM and NN provided constructive comments. All authors have approved submission of this manuscript.

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