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Original Article

Use of Phage Display to Isolate Specific Human Monoclonal Antibody Fragments Against a Potential Target for Multiple Myeloma

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Abstract

Introduction: Multiple myeloma (MM), a malignancy of plasma cells, accounts for 10% of all haematological malignancies and is currently incurable. Although it can be treated, the disease tends to relapse after several years and becomes increasingly resistant to conventional therapy. Investigations into using humoral therapy for MM are now underway with a view that novel therapeutic agents may provide a more targeted therapy for MM. Materials and Methods: Here, phage display, a faster and more efficient method compared to classical hybridoma fusion technology, was used as a proof-of-concept to isolate several single-chain Fragment variables (scFv) against Ku86. Results: Anti-Ku86 polyclonal scFvs biopanning was successful where third round scFvs (A_{450}~1.1) showed a 1/3 increase in binding as compared to the first round scFvs (A_{450}~0.4) with 100ug/mL of antigen (purified human Ku86). Subsequent selection and verification of monoclonal antibodies using third round biopanning revealed 4 good affinity binding clones ranging from A_{450}~0.1 to A_{450}~0.15 on 12.5ug/mL of antigen as compared to low binders (A_{450}~0.07) and these antibodies bind to Ku86 in a specific and dose-dependent manner. Comparative studies were also performed with commercially available murine antibodies and results suggest that 2 of the clones may bind close to the following epitopes aa506-541 and aa1-374. Conclusions: These studies using phage display provide an alternative and viable method to screen for antibodies quickly and results show that good affinity antibodies against Ku86 have been successfully isolated and they can be used for further studies on MM and form the basis for further development as anti-cancer therapeutic agents.

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Key words: Antibody isolation, Ku86, Phage display, ScFv

Introduction

Multiple myeloma (MM) is a malignant plasma-cell neoplasm that is characterised by an excess of mature B-cells in the bone marrow (plasmacytosis), in association with secretion of monoclonal immunoglobulins (Ig) in serum and/or urine, decreased normal Ig and lytic bone disease.[1,2] MM accounts for 10% to 20% of all malignant haematological cancers and myeloma patients often have extensive skeletal destruction, which are a main cause of morbidity and mortality.[2-4] Although MM can be treated, the disease ultimately relapses in most cases and becomes increasingly resistant to therapy until conventional options are exhausted, prompting an urgent need for novel treatment.[5] Major advances have been made in the understanding of plasma cell biology and the importance of the microenvironment in MM development[6-9] in the past decade, leading to the development of newer agents such as thalidomide, its analog lenalidomide[10] and the proteasome inhibitor bortezomib.[11] These agents have been used in combination with existing agents against MM, but prospects of a cure of MM with these agents is still uncertain and major limitations exist, such as patient resistance and toxicities relating to the use of these drugs.

Investigators are currently looking to immunotherapy as a means of treatment for MM.[12,13] Tumour-directed monoclonal antibodies (mAb) or humoral therapy, have

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caused much excitement since the 1970s and with the development of mAbs such as Rituximab (anti-CD20) and Campath-1H (anti-CD52) for the treatment of lymphoma and leukaemia.[14] However, in the context of MM, the major milestone to be crossed in this area is the selection of a suitable surface antigen (Ag) to allow targeting by antibodies against MM cells.

The Ku heterodimer is made up of 2 sub-units of approximately 86 and 70kDa in higher eukaryotes which binds to DNA. Identified in 1981 from Japanese patients with scleroderma-polymyositis overlap syndrome, Ku has now been found to play major roles in many cellular processes.[15] Foremost, Ku86 in association with Ku70 and DNA protein kinase C plays a critical role in DNA repair especially in non-homologous end joining (NHEJ) where the lack of functional Ku proteins in the cell typically results in genomic instability and hypersensitivity to DNA damage as well as an increased likelihood of tumour development and immunodeficiency.[16-18] A comprehensive review of Ku and its potential role in cancer, especially myeloma, has been written by Gullo et al.[19] Other cellular processes which Ku has been found to partake in include antigen-receptor-gene arrangements (Variable [Diversity] Joining {V(D)J} recombination), gene transcription, apoptosis, telomere maintenance, heat shock-induced responses and cell cycle regulation[20] which are important processes affecting tumour metastasis. Therefore, it is clear that the Ku protein has an integral and intrinsic role in cellular and organismal tumour biology. Further, Ku86 has been discovered to translocate to the cell surface of MM cells upon CD40L treatment[21] and also mediate the binding of MM cells to fibronectin and bone marrow stromal cells.[22] Most importantly, the mAb 5E2 directed against Ku86 were seen to induced apoptosis of MM cells.[22] Therefore, with Ku's role in tumour biology and its subsequent surface translocation, surface bound Ku in MM provides an attractive target for therapeutic antibodies.

In this present study, we have raised several monoclonal single-chain Fragment variable (scFv) against Ku86. Isolation of scFv was performed using phage display of a human monoclonal antibody library instead of conventional hybridoma technology, allowing us to bypass steps of humanisation of the scFv to avoid issues of human anti-mouse antibody (HAMA) reactions commonly associated with antibodies produced using hybridoma. Finally, the isolated scFvs' specificities and affinities were compared to other known mAbs using competitive ELISAs.

Materials and Methods

Cell and Cell Culture

COS-7 cell line, a derivation of CV-1 which was established from the kidney of an African green monkey, RPMI 8226 human MM (CCL-155) and CHO-K1 (CCL-61) was purchased from American Type Culture Collection (ATCC, Rockville, MD). EBV-negative SGH-MM5 human MM cell line (CD10+, CD19-, CD20-, CD38+, CD40+, CD45+, CD56+, CD138+) was developed previously in our laboratory[23] from a patient with MM. COS-7 and CHO were cultured in complete media consisting of 89% Dulbecco's modified Eagle medium (DMEM), 10% fetal bovine serum (FBS) and 1% MEM non-essential amino acids while RPMI 8226 and SGH-MM5 were cultured in 90% RPMI 1640 with L-glutamine media, 10% FBS, 25IU/mL penicillin, 25ug/mL streptomycin and additional 5mM L-glutamine. All cell lines were maintained at 37^{o}C with 5% CO2 in a humidified atmosphere.

Construction and Cloning of Ku86 gene into Mammalian Expression Vector

Full-length 2.4kb Ku86 cDNA was obtained by RT-PCR using QIAGEN RT-PCR kit (QIAGEN, Hilden, Germany) according to manufacturers' protocol. RT-PCR was performed on total RNA extracted from SGH-MM5 and RPMI cell lines using QIAGEN RNAeasy (QIAGEN) kit and the following oligonucleotides, Ku1 (forward: 5'-TGTATGGACGTGGGCTTTA CCAT-3') and Ku2 (reverse: 5'-TCCACAGAGAATTAGATGATCCGCC-3'). PCR products were then separated by 1.5% agarose gel electrophoresis and cloned into Topo II TA vectors (Invitrogen, Carlsbad, USA) for amplification in *Escherichia coli* (*E. coli*) with 100ug/mL ampicillin pressure and blue/white colony selection. Sequencing was performed on the cloned Ku86 gene to ensure no mutations. Next, 2 restriction enzyme sites, *Hind*III at 5' end and *Xba*I at 3' end were engineered at the ends of the cloned Ku86 using high fidelity PWO SuperYield DNA polymerase (Roche Diagnostics, Penzberg, Germany) with the following oligonucleotides (forward: 5'-ATTAAAGCTTCCGGCAACATGG TGCGGTCGGGGAATAAGGCAGCTGTTGTGC TGTGTATGGACGTGGGC-3') and reverse: 5'-ATTATCTAGACTTATC ATGTCCAATAAATC-3'). The engineered Ku86 gene was then sub-cloned into the transient mammalian expression vector pcDNA3.1/myc-His B (Invitrogen) via digestion of the vector and cloned gene at the 2 restriction sites with *Hind*III/*Xba*I before ligating with DNA ligation kit (Stratagene, San Diego, USA) at 4^{o}C overnight. The resultant ligated product was analysed with 1% agarose before transforming into *E.coli* with 100ug/mL ampicillin selection for amplification. Following overnight incubation, the recombinant plasmid was extracted from transformed *E.Coli* via QIAprep Spin Miniprep kit (QIAGEN) and sequenced.

Expression and Purification of Ku86 Recombinant Protein

The purified mammalian expression vector was transfected into COS-7 cells for transient expression of Ku86 recombinant protein. Transfection was performed by incubating the plasmid and Lipofectamine2000 (Invitrogen) with COS-7 cells grown to 90% confluence in 175cm^2 large flasks as per manufacturer's protocol. CHO cell lines were transfected similarly. After 24 hours, the cells were harvested for assays and purification. For gene expression assays, RNA of transfected COS-7/CHO cells were extracted using QIAGEN RNAeasy (QIAGEN) as above and RT-PCR with primers Ku1 and Ku2 was performed and analysed on 1% agarose stained with ethidium bromide under UV light. Transfected COS-7/CHO cell lysates, lysed with EBC1 lysis buffer which contains 50mM tris pH 8.0, 150mM NaCl, 0.1% NP-40, 0.5ug/mL phenylmethylsulfonyl fluoride (PMSF), 50mM NaF, 1mM $NaVO_4$ and one Complete® protease inhibitor tablet (Roche Diagnostics GmbH, Mannheim, Germany) in every 50mL of lysis buffer, was used for western immunoblotting as described. Untransfected COS-7 was used as negative control. Ku86 recombinant protein was affinity purified using ProBond Nickel-Chelating Resin column (Invitrogen) as per manufacturer's protocol and purified Ku86 was confirmed by western immunoblotting as described. SYPRO Ruby Gel staining was performed according to manufacturer's protocol.

Western Immunoblotting

Proteins were quantified using Bradford's method (BioRad, Hercules, CA) and resolved (10ug/sample) in a 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. Subsequently, proteins were transferred to a polyvinylidine difluoride (PVDF) membrane (Schleicher & Schuell, Keene, NH) and blocked for 2 hours with 5% skimmed milk in tris-buffered saline – 20mM tris pH 7.6, 150mM NaCl and 1% Tween-20 (Sigma-Aldirch, St Louis, MO) with an additional 0.2% Tween-20 (TBST). Membranes were probed using mouse monoclonal antibody (mAb) S10B1 for primary hybridisation and goat anti-mouse-HRP as secondary mAB 1:1000 dilution for 2 hours, then washed thrice with TBST before carrying out chemiluminescene development with ChemiGlow reagents and detection with filmless imaging on the FluoChem Imager (both from Alpha Innotech, San Leandro, CA). For re-probing of membranes, the experiments were repeated as above but with the use of mouse anti-myc-HRP mAb.

Biopanning of Phage-scFvs against Ku86

The Tomlinson Library (MRC, Cambridge, UK) was used for phage-scFv biopanning. Four hundred micrograms of Ku86 in PBS was coated on Maxisorp immunotubes (Nalgene, Nunc, Roskilde, Denmark) for 4°C overnight and blocked with 2% skimmed milk in PBS (MPBS) before addition of total 10^{12} phage/mL phage-scFv library in 2% MPBS and hybridising for 2 hours at room temperature. Immunotubes were washed with PBS before and after blocking. Immunotubes were washed with PBS containing 0.1% Tween-20 ten times (20 times for rounds 2 and 3) before eluting phage-scFv. Panning was repeated twice with increased selection stringency; for rounds 2 and 3, 40 micrograms of Ku86 in PBS was used for coating. Thereafter, phage-scFv was eluted with either 0.1N glycine, 1% BSA, pH 2.2 (phages rescued with M13K07 helper phage) or 1mg/mL trypsin-PBS (phages rescued with KM13 helper phage) for latter rounds and quantitated by colony forming units (CFU). All eluted phage were amplified by TG1 cells with 100ug/mL ampicillin and 50ug/mL kanamycin selection before superinfection with either M13K07 or KM13 and purified by Polyethylene glycol/NaCl precipitation. For polyclonal phage ELISA, 10^{10} of amplified phage after panning were added onto immunoplates as described. For monoclonal phage ELISA, TG1 cells were infected with eluted phage for 30 minutes at 37°C and spread onto TYE/amp plates. After overnight incubation, TG1 colonies were randomly selected and inoculated into 100uL of 2xTY containing 100ug/mL ampicillin overnight before individual clones superinfection with M13K07 or KM13. Phage ELISA was performed as described by using 100 microlitres of culture supernatant containing phage particles. Monoclonal phage verification was performed similarly by using the 7 best binders and 7 randomly selected binders for ELISA.

Enzyme-Linked ImmunoSorbent Assay (ELISA) Analysis

To determine antigen-binding reactivity and sensitivity of phage particles, serial dilutions (100ug/mL, 50ug/mL, 25ug/mL, 12.5ug/mL and 6.25ug/mL) of purified Ku86 in PBS were used to coat 96-well microtiter Maxisorp immunoplate (Nunc) at 4°C overnight. For negative and positive controls, 100ug/mL of BSA for the former and 100ug/mL of anti-myc antibody for the latter was used. For monoclonal screening, 12.5ug/mL of purified Ku86 was used. After blocking the plate with 2% (w/v) MPBS, 10^{10} phage-scFv packaged with either M13K07 or KM13 in 2% MPBS was applied to each well for 1 hour at room temperature. After washing 3 times with PBS containing 0.1% Tween-20 (PBS-Tween) and 3 times with PBS, the bound phage was detected with anti-M13 antiserum conjugated with horseradish peroxidase (Amersham Biosciences, GE Healthcare, Chalfont St. Giles, UK). The signal was visualised with TMD substrate OptEIA reagent (BD, San Diego, USA) and quantitated with ELISA microplate reader (BioRad Model 3550, Hercules, CA) at OD 450nm after stopping the reaction with 1M H_2SO_4.

Monoclonal Phage-scFv characterisation Binding Assay

Characterisation of the binding epitopes of 2 of the best binding scFv clones were performed by phage serial dilution and competitive ELISA. Maxisorp Immunoplates were coated with 50ug/mL of purified Ku86 in PBS overnight at 4°C and 100ug/mL of BSA was used for negative control. For phage dilution, phage-scFv clones were serially diluted 2 fold for ELISA. For competitive ELISA, 1 mL of antibody mixture, which consisted of phage particles and known murine monoclonal antibodies (either S10B1, 111, S5C11 or N9C1), in 2xTY was prepared while the plate was blocked with 2% MPBS for 2 hours at room temperature; murine monoclonal antibodies were added in increasing amounts (2.0ug/mL, 4.0ug/mL, 8.0ug/mL, 16.0ug/mL) for each mixture. 100uL of the above antibody mixtures was hybridised in each well for 2 hours at room temperature before commencing washing and secondary ELISA hybridisation which was performed as above.

Results

Generation of Ku86 Antigen for Phage Display

In order to produce ScFv antibodies against the MM surface antigen Ku86, the protein was cloned from MM cell lines. Briefly, full length gene encoding Ku86 was reverse engineered from MM cell lines and sub-cloned into mammalian expression vector pcDNA3.1/myc-His B. Restriction digest of the vector showed that the Ku86 gene was of the correct size (data not shown). In addition, the cloned gene was fully sequenced to ensure that the gene was in the correct orientation and contiguous reading frame without any mutation. The purified pcDNA3.1 vector was used to transfect COS-7 for expression of Ku86 and Ku86 antigen from COS-7 cells was purified through a Nickel-Chelating Resin column, which exhibits a high affinity and selectivity for the six His-tagged recombinant fusion protein of Ku86. Western immunoblotting was performed after purification to confirm that recombinant Ku86 was obtained (Fig. 1a). To examine and ascertain whether transfected COS-7 cells expressed recombinant Ku86 correctly, total protein from both transfected and untransfected COS-7 were either stained with SYPRO Ruby Protein Gel Stain or probed with anti-Ku86 antibodies (S10B1) in Western immunoblotting (Fig. 1b). Purified Ku86 was used as positive control, untransfected COS-7 was used as negative control and transfected/untransfected CHO cells were used as a reference. The stains and probing revealed that recombinant Ku86 was present in large amounts in transfected COS-7 while untransfected COS-7 cells do not express detectable levels of recombinant Ku86. Transfected and untransfected CHO cell lines did not express recombinant Ku86 at all.

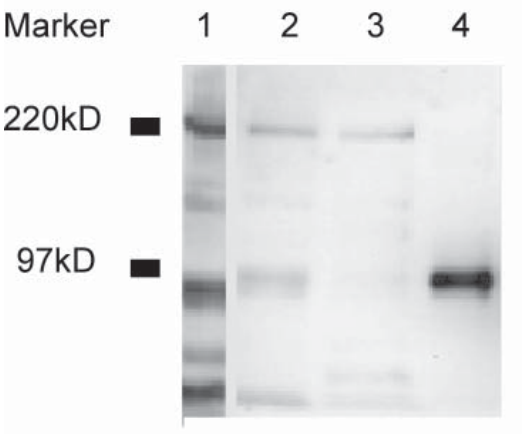

Fig. 1a. Western blot result for the purification of Ku86 protein. The Ku86 recombinant proteins were purified using ProBond Nickel-Chelating Resin column. ProBond Nickel-Chelating Resin exhibits high affinity and selectivity for 6xHis-tagged recombinant fusion proteins of Ku86. (Lane 1: Whole cell extract; Lane 2: Flow-through after binding; Lane 3: Flow-through after washing; Lane 4: Concentrated pure Ku protein (Eluted with 250 mM imidazole).

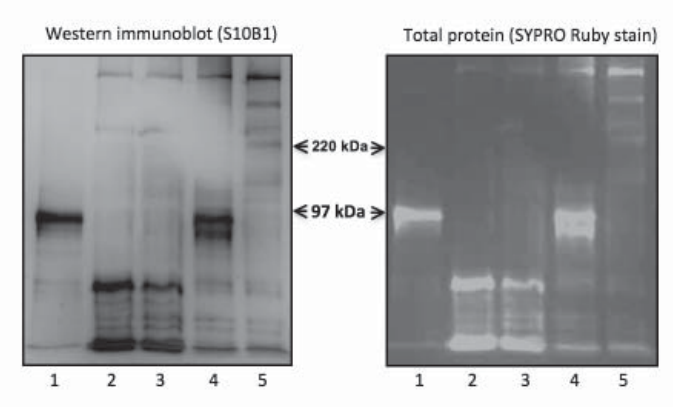

Fig. 1b. SYPRO Ruby Gel Stain and Western blot result of Ku86 protein expression. Total protein gel stain using SYPRO Ruby Stain (BioRad) and Western immunoblotting using anti-Ku86 antibodies (S10B1) revealed that Ku86 was present in large amounts in transfected Monkey cell line (COS-7) but not the Chinese hamster ovary (CHO) cell line. Untransfected COS cells do not express detectable levels of human Ku86. (Lane 1: Purified Ku protein; Lane 2: Transfected CHO cell line; Lane 3: Untransfected CHO cell line; Lane 4: Transfected COS-7 cell line; Lane 5: Untransfected COS-7 cell line.

Biopanning and Screening for Polyclonal Antibodies against Ku86 by Phage ELISA

Phage display biopanning allows for the simultaneous screening and enrichment of the highest affinity binders. The Tomlinson Library I was used to perform phage display biopanning against the full length Ku86 protein to obtain anti-Ku86 polyclonal antibodies. For the selection of good affinity polyclonal antibodies, panning stringency was increased from round 1 to 2 by decreasing the concentration of antigen 10-fold and increasing the number of washes to remove unspecific binders 2-fold. The phage library was rescued either with trypsin-sensitive helper phage KM13

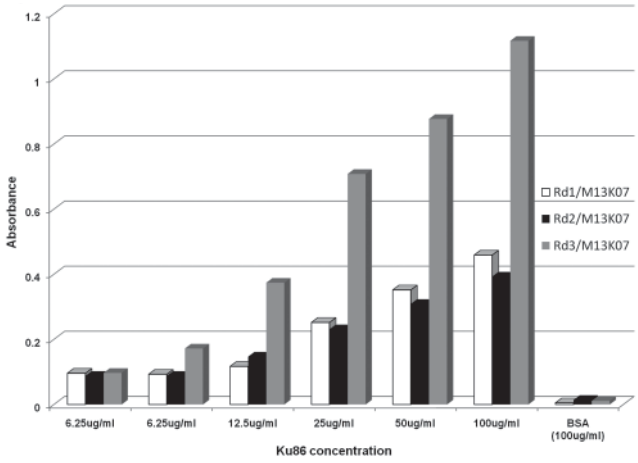

Fig. 2a. ELISA readings for 3 rounds of polyclonal antibodies biopanning rescued with M13K07 helper phage and eluted with non-specific acidic glycine buffer. Biopanning results were indistinct at low concentrations of antigen (6.25ug/mL). At antigen concentrations of 12.5ug/ml and above, Round 3, the expansion and amplification round of biopanning shows more than 1/3 increase in ELISA absorbance and binding as compared to Round 1 (⇔) and 2 (⇔). BSA (100ug/mL) was used as negative control.

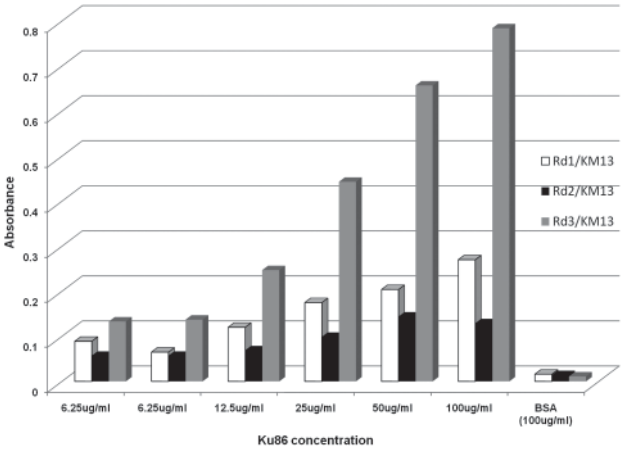

Fig. 2b. ELISA readings for 3 rounds of polyclonal antibodies biopanning rescued with KM13 helper phage and eluted with specific trypsin treatment. Biopanning results were inaccurate at low concentrations of antigen (6.25ug/mL). At antigen concentrations of 12.5ug/ml and above, Round 3 (⇔), the expansion and amplification round of biopanning shows more than 1/3 increase in ELISA absorbance and binding as compared to Round 1 (⇔) and 2 (⇔). BSA (100ug/mL) was used as negative control.

(eluted with tryptic treatment) or commercially available helper phage M13K07 (eluted with acidic glycine buffer). As KM13 contains a modified gene 3 protein (pIII) which is sensitive to tryptic digestion, rescued phages containing only KM13 pIII and lacking wild-type pIII-scFv fusion are rendered non-infective after tryptic treatment while rescued phages with fusions are not cleaved and remain infective for *E.Coli* amplification. In addition, to ensure that the ELISA signal reflects the phage-scFv binding activity of each round accurately, different concentrations of the antigen, Ku86, were used for ELISA plate coating. Figures 2a and 2b show the overall results of the biopanning and screening via ELISA. In general, the affinity of phage-scFv for Ku86 in both phage ELISAs were not substantial for the first 2 rounds of panning as these initial rounds of screening were used to weed out unspecific and low affinity binders; the decrease in binding seen in round 2 was probably due to the increased panning stringency. As initial rounds of biopanning are used to first select for strong binders before amplifying them in the third round, phage display biopanning was successful because polyclonal scFvs demonstrated an exceptional level of binding with more than one third increase in absorbance as compared to rounds 1 and 2. Interestingly, phage particles eluted non-specifically by acidic glycine buffer (Fig. 2a) showed a better binding to Ku86 as compared to phage particles eluted via trypsin treatment (Fig. 2b) by ~1.5 fold. It should be noted that comparison of phage ELISA data are made on a semi-quantitative basis.

Isolation of Individual Clones against Ku86 by Monoclonal Phage ELISA

Based on these results, the third round of selection with the library rescued with helper phage M13K07 was used for isolation of monoclonal antibodies against Ku86. The concentration of 12.5ug/mL of Ku86 was used as this concentration provided a good representation of the binding affinity of phage particles. To ensure that sufficient clones were screened, phage particles were infected into TG1, plated out such that individual colonies were well separated before inoculating each colony into 96-well plates (see methods). As each phage particle only encodes for 1 antibody, picking individual bacterium allows us to investigate the binding level of monoclonal antibodies where ELISA absorbance readings are directly correlated to strength and specificity of binding. Figure 3 illustrates the binding affinities of 80 clones that were screened; all clones bound to Ku86 in various degrees. The majority of the clones had a rather low binding as judged by their O.D. readings (0.03 to 0.06). There were approximately 13 modest affinity clones and 7 good binders (clones with absorbance readings below 0.06 were classified as weak binders, 0.06 to 0.08 as modest binders and readings above 0.08 as strong binders). As monoclonal phage particles bind to specific epitopes, it is expected that the majority of the clones do not bind well and that only a few clones would have a high specificity and avidity for Ku86 which was reflected in Figure 3. Furthermore, O.D. readings for monoclonal strong binders are expected to be lower as compared to polyclonal ELISA studies (O.D. ~ 0.22 at 12.5ug/mL) because the O.D. readings from polyclonal

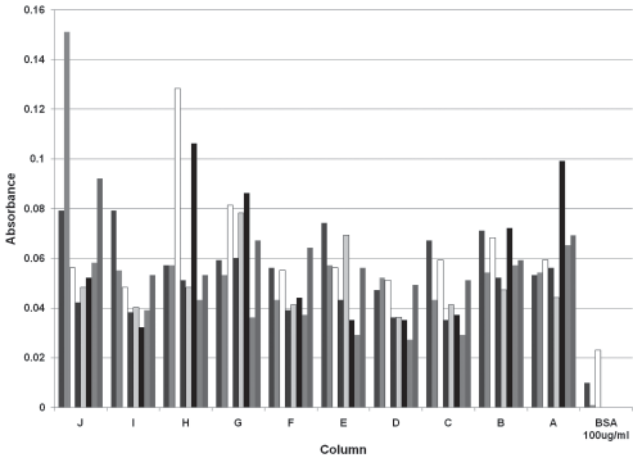

Fig. 3. ELISA readings for monoclonal antibody screening and selection. ELISA plate wells were coated with 12.5ug/ml.The abscissa (A-J) denotes the column which corresponds to the column of the 96-well plate and the colored bars denotes the row of the 96-well plate. The 'Strong binders' (above 0.08absorbance) were A6, G6, G3, H6, H3, J8 and J2 and used for ELISA phage verification

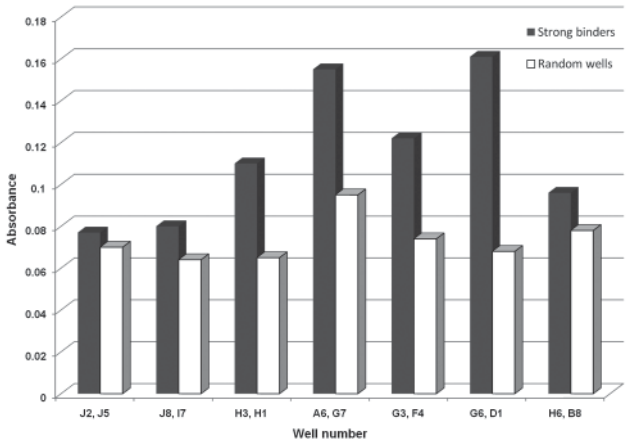

Fig. 4. ELISA results of monoclonal antibody phage verification. The ELISA plate was coated with 12.5ug/ml of antigen. All stronger binders (▭) had a better absorbance and affinity as compared to the random wells (▭) selected for comparison. However, 3 of the strong binders 'J2, J8 and H6' had lower than expected binding as compared to Figure 3. Wells H3, A6, G3 and G6 showed approximately 2-fold higher absorbance and increased binding compared to the random wells.

screening measures the overall combinatorial binding from all the monoclonal antibodies on different epitopes which allows for increased antibody-antigen interactions and higher readings.

Use of Phage ELISA to Verify Monoclonal Phage Particles

To ensure that the monoclonal phage-scFvs that were identified by ELISA screening were not false positives, strong binders were further examined with randomly chosen clones by phage ELISA at 12.5ug/mL Ku86 concentration. As depicted in Figure 4, all strong binders had better binding as compared to the 7 randomly selected binders in general. Interestingly, 3 of the strong binders which were chosen from monoclonal isolation (wells J2, J8 and H6) had absorbance readings of OD_{650nm} ~ 0.08-0.09 which were approximately similar to the randomly selected binders and were substantially lower as compared to previous findings. Thus, for further confirmation, PCR was performed on the 7 strong binders and analysed on agarose gel. The PCR analysis confirmed that wells H3, A6, G3 and G6 had full-length variable heavy (VH) and variable light chain (VL) while there were no bands seen for wells J2 and H6 and well J8 had only half the required length. In addition, the strong binders were sequenced and the Basic Local Alignment Search Tool (BLAST) was performed, confirming the above 4 wells had both VH and VL while the sequence of J8 showed that no VH was present and wells J2 and H6 revealed sequences from the cloning vector only.

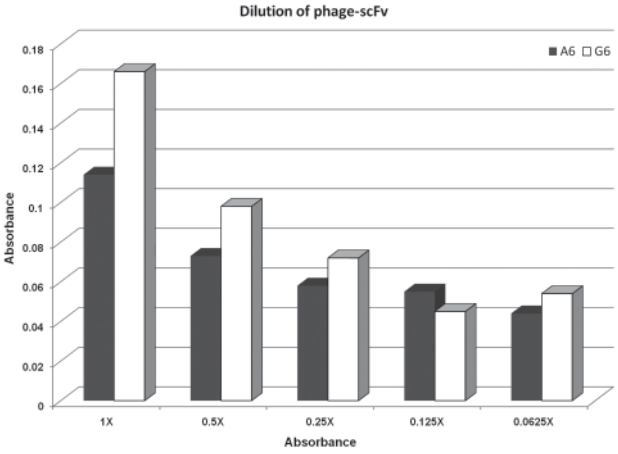

Fig. 5a. ELISA absorbance readings of phage ScFv-Ku86 dilution experiments. Both ScFvs, A6 (▭) and G6 (▭) showed antigen-dependent binding.

Phage ELISA and Inhibition Experiments for Characterisation of Binding Epitopes

Anti-Ku86 monoclonal antibody candidates, clones A6 and G6, which were identified by ELISA screenings, were further examined to determine their binding sites on Ku86 epitopes. They were thus tested as phage-scFvs in conjunction with other known antibodies for their potential to bind to Ku86 in competitive ELISAs. The first set of experiments, illustrated in Figure 5a, shows that both phage-scFv clones A6 and G6 bind to Ku86 in a specific

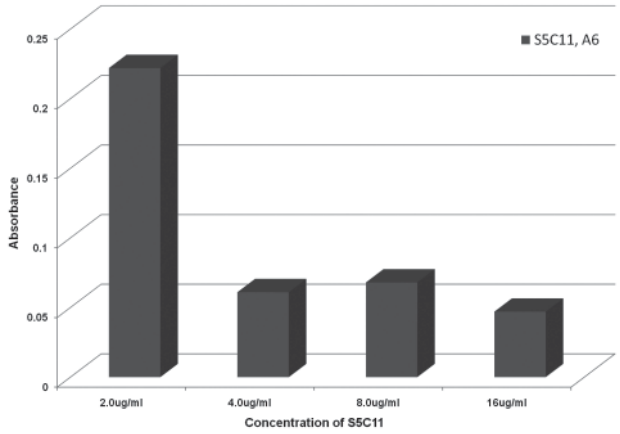

Fig. 5b. Competitive ELISA assay for phage-ScFv A6 with S5C11 antibody. Increasing amounts of S5C11 resulted in a 2/3 drop in absorbance reading from ~0.2 to ~0.05 where further increases had no further effect.

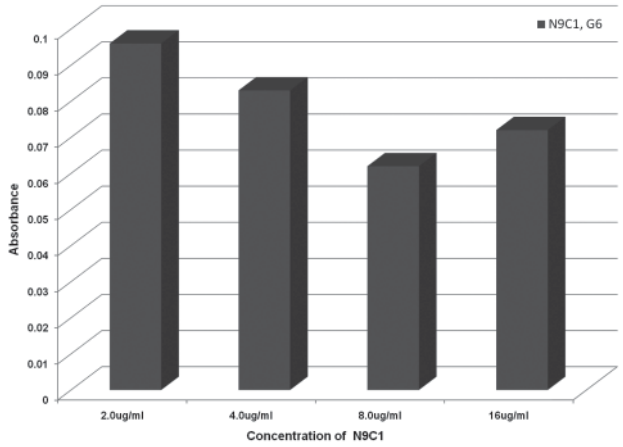

Fig. 5c. Competitive ELISA assay for phage-ScFv G6 with N9C1 antibody. Increasing amounts of N9C1 by half resulted in a ~1/5 drop in absorbance readings at each dilution point.

and dose-dependent manner. Subsequently, to determine the binding sites on Ku86, phage particles were incubated in competitive ELISAs with several murine monoclonal anti-Ku86 antibodies: S10B1, 111, S5C11 and N9C1, which are specific for human and monkey Ku proteins. For S10B1 and 111 competitive ELISA assays, no significant change in binding levels were seen with increasing amounts of antibodies (data not shown), indicating that clones A6 and G6 probably do not have recognition for epitopes targeted by S10B1 (C-terminal) or 111 (N-terminal). Conversely, competitive experiments of clone A6 with S5C11 revealed dramatically decreased binding of A6 when S5C11 concentrations were increased (Fig. 5b) while competitive experiments of clone G6 with N9C1 (Fig. 5c) showed a minor decrease. G6/S5C11 and A6/N9C1 competitive experiments did not reveal any significant change in binding levels (data not shown). These results suggest that clone A6 and G6 may be binding near the aa506-541 epitope (S5C11) and aa1-374 (N9C1) epitope, respectively.

Discussion

The development of therapeutic antibodies has been explored by many institutions and organisations because of their potency in finding, and binding to, typically any kind of molecule with high affinity and specificity.[24] Examples of antibodies which have progressed from the laboratory to the clinic include, but are not limited to, the following: Rituximab and Zevalin (Anti-CD20) for Non-Hodgkin lymphoma, Alemtuzumab (Anti-CD52) for B-cell lymphocytic leukemia and Mylotarg (Anti-CD33) for acute myeloid leukaemia. As studies to isolate antibodies against Ku using classical hybridoma technology were not very successful in our laboratory (unpublished data) and screening of recombinatorial phage display libraries for the isolation prototype antibody against cancer have become one of the main approach to therapeutic and diagnostic drug discovery and validation, phage display technology was used here to isolate several clones for further studies on MM in the hope of developing the clones for therapeutic use in the future. Antibody isolation was successful and enhancement of mAbs can be subsequently performed via molecular engineering or directed evolution if necessary. Synthesising of whole and humanised anti-Ku86 antibodies and subsequent scaling up antibody production in a GMP facility as well as performing animal proof-of-concept studies will be performed in future studies.

The Ku heterodimer plays a central role in several nuclear processes ranging from DNA repair, telomere maintenance to cell proliferation and apoptosis making it a highly important regulatory protein.[15,19,20,25] Indeed, recent reports have implicated that the Ku protein (or its variant) partakes in the development of several cancers, notably MM.[19] In MM, Ku has been shown to translocate to the cell membrane upon CD40-activation and are involved in homotypic and heterotypic cell-cell adhesion as well as adhesion to fibronectin which protects the MM cell from apoptosis triggered by irradiation or doxorubicin.[21,22] Further, reports on Ku86 variant protein (Ku86v) expression in MM have shown that increased expression of Ku86v is correlated with an increased sensitivity to DNA damage.[26] Finally, antibodies against surface Ku86 (5E2) were shown to induce apoptosis in MM cells.[22] Taken together, the Ku protein could be an important candidate for anti-MM drug development. Here, the generation of these human scFv clones against Ku86 provides a potentially important diagnostic tool, which can be used for further studies on

the role of Ku, its variant and understanding their link to the development of MM. Importantly, these clones form the basis upon which an anti-MM therapeutic agent could be engineered given that although there are several good affinity antibodies directed against the Ku protein available commercially at the moment, none of these are human in origin or suitable for use as therapy.

The functional display of antibodies on phage particles using large diverse combinatorial libraries opens possibilities to select specific antibodies against proteins or antigens of interest which subsequently allows for the basis with which the antibody could be studied, improved or altered.[27] Screening of these libraries typically allows for the selection of antibodies with high affinities, ranging from micromolar to nanomolar affinity constants, depending on the size and diversity of the library.[28] Following screening and binding selection, it is then possible to deduce the primary structure of the binding site of the antibody by sequencing the clone's DNA due to the linking of phenotype to the genotype encoding that molecule within the same virion. Here, in this study, several scFv clones have been isolated against the Ku86 protein using phage display as a preliminary effort to target the effect of Ku86 in MM. After selection, nucleotide sequence analysis have been performed on the clones to confirm that selected clones contained both human VH and VL sequences before subjecting them to comparative studies with commercially available Ku86 antibodies.

Since its advent approximately 30 years ago, the classical hybridoma method is still being used to generate mAbs.[29] Although this conventional technology has a successful history, it has several intrinsic limitations: namely, its relatively longer time span to isolate antigen-specific antibodies (a time measured in months as compared to weeks using phage display), its immunogeneticity where murine antibodies may result in patients developing HAMA reactions, presence of an affinity ceiling, the difficulty in controlling *in vivo* selection conditions and the subsequent antibody specificity and affinity. Antibody fragments or single chain Fvs', displayed on phage surfaces, bypass these problems and allow for an *in vitro* optimisation of antibodies against the specific antigen. Phage display has proven to be a faster, more flexible and more reliable alternative to generation and selection of antibodies.[30] Carried out in the test tube, with human V_H and V_L chain genes, the selection process is remarkably similar to the natural affinity maturation process of affinity selection and somatic hypermutation where it allows for tightly controlled conditions, comparatively shorter time selection without a need for superhumanising and isolation of human antibodies with higher affinities that are not easily achieved in hybridoma.

Despite the fact that phage display is relatively simple to use and its phage particles are robust and stable, this system has a few constraints such as library antibody diversity limitation and erroneous amplification of a low affinity clone[30-33] and difficulties in the reproducibility of the ELISA O.D. measurements. Antibody diversity and the subsequent possibility of isolating high affinity antibodies are directly linked to the size of the library. As the synthesis and assembly of phage particles occurs in bacteria, the DNA encoding the antibody needs to be imported artificially. Thus, the recombinatorial library is limited by bacterial transforming efficiency and this is presented as a disadvantage where the largest reported libraries are made of not more than $1x10^{10}$ to $1x10^{11}$ members currently. Amplification of antibodies during screening is directly linked to the phage with the highest growth advantage (with or without antibody fusion) and combined with the 'sticky' properties of phages, therein lies the possibility that only low affinity clones dominate a population after screening (erroneous amplification). A low affinity clone may arise due to several factors such as the failure of the antibody fragment to be expressed or folded properly, the susceptibility of the clone to proteolysis or the fact that the fragment is toxic to the host and forming aggregates which slows down or stops bacterial growth.[30]

In summary, the experiments described here have isolated good affinity scFvs against Ku86. These clones form the basis upon which further experiments can be performed to develop the antibodies into therapeutic anti-cancer agents. Further, here we show that an established method (phage display) other than the classical hybridoma technology could be used for antibody selection as a proof of principle that mAbs may be generated in a shorter period of time and, because a recombinatorial human antibody library was used, could offer a significant clinical advantage over murine antibodies by avoiding HAMA reactions.

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