Research paper

Antigens for the selection of pan-variable number of tandem repeats motif-specific human antibodies against Mucin-1

Jonas Persson, Mats Ohlin *

Department of Immunotechnology, Lund University, BMC D13, S-221 84 Lund, Sweden

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Abstract

Epitopes found on Mucin-1 are differentially expressed on tumour versus normal tissue. Such epitopes have also been shown to have a potential in immunotherapy and tumour detection. The major epitope explored in this context is located within the variable number of tandem repeats. It has however recently been demonstrated that this epitope exists in several sequence variants. The standard sequence is highly antigenic while the most common sequence variant is much less so. We have now explored routes employing defined synthetic antigens to ensure the development of human recombinant antibody specificities targeting both sequence variants of this epitope. These antibodies may serve as a platform for the development of human antibodies for efficient targeting of Mucin-1 in human disease.

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1. Introduction

Several of the mucins are interesting in the context of tumour biology as they are functionally important not only for normal but also for tumour cells. In particular Mucin-1 (MUC1) has received a lot of attention since it displays epitopes connected to different forms of cancer. When compared, the expression of MUC1 in these cancers is heterogeneous with various traits in common such as overexpression, and truncated or incomplete glycosylation. Importantly, a mouse monoclonal antibody (HMFG-1) specific for MUC1 has been used successfully in radioimmunotherapy for the treatment of ovarian carcinoma (Epenetos et al., 2000) reinforcing the viability of MUC1 as a target for tumour therapy. This mouse monoclonal antibody and a range of other antibodies, most of them also of murine origin, have been raised against a repeated immunodominant epitope in a sequence motif entitled the variable number of tandem repeat (VNTR) that is exposed on tumour cells (Price et al., 1998).

The extracellular domain of MUC1 is in the Northern European population made up of a VNTR carrying between 21 and 125 copies of a peptide sequence (Gendler et al., 1990), which in turn consists of 20 amino acids (GVTSAPDTRPAPGSTAPPAH). Although MUC1 has until recently been considered to contain this standard peptide sequence, more recent studies have shown that polymorphism exists with respect to the occurrence and...
location of sequence variants (Engelmann et al., 2001). In particular, the PDTR sequence associated with the most important epitope in VNTR is often modified to PESR and clusters of these sequence variants are interspersed between clusters of the major peptide sequence (Fowler et al., 2003). However, this major sequence variant has been reported not to react with many mouse monoclonal antibodies specific for the standard sequence (von Mensdorff-Pouilly et al., 2005). Furthermore, we have recently shown that the substitution of aspartic acid/threonine of the major MUC1 sequence variant for glutamic acid/serine of the major sequence variant of MUC1 results in a reduction of the antigenicity of the peptide sequence (Persson et al., 2005), suggesting that it will be difficult to develop antibodies against it in many systems. In agreement with this finding it was observed that although natural immunity has a component of antibody reactivity targeting this major sequence variant, patients affected by adenocarcinoma preferentially induced an antibody repertoire targeting the major sequence found within MUC1 VNTR (von Mensdorff-Pouilly et al., 2005). To allow for induction and selection of new antibodies targeting also the major sequence variant within MUC1 VNTR, we have now explored different approaches to secure the development of appropriate human antibodies that recognise also this poorly antigenic target.

2. Materials and methods

2.1. Antigens

The synthetic biotinylated peptides used during the selection procedures presented herein were obtained from either Thermo Electron (Ulm, Germany) or Innovagen (Lund, Sweden). The selection peptides were designed so as to mimic the dominant sequence variant and in several cases also modified so as to maximise the peptides ability to expose the key residues ES and hide the C-terminus of the peptide. The biotinylated peptides were a linear 12-mer peptide (12ES, biotin-GVTSAPDTRPAP), a linear 15-mer peptide (15ES, biotin-GVTSAPESRPAGST), a cyclic 17-mer peptide (12ESC, biotin-AAACGVTSAPERSPAPC), a cyclic 18-mer peptide (13ESC, biotin-AAACGVTSA PESRPAPGC), and a cyclic 19-mer peptide (14ESC, biotin-AAACGVTSA PESRPAPGC). The three peptides containing cysteines had all been made circular through the formation of disulfide bonds. The peptides were made so as to efficiently expose the target epitope, to reduce peptide flexibility (Ladner, 1995) and to hide the C-terminus which otherwise may have an undesirably large role in the selection procedure as has been observed in the past. Additional peptides included biotin-alat-MUC-13 (biotin-alat-[GVTSAPDTRPAP STAPPAH]3) that was obtained from Elias Krambovitis (Institute of Molecular Biology and Biotechnology, Heraklion, Crete, Greece) (Krambovitis et al., 1998). A linear biotinylated 12-mer peptide (12DT, biotin-GVTSAPDTRPAP) was synthesized by Thermo Electron. A biotinylated and glycosylated peptide G15DT (biotin-GVTSAPDT(Tn)RPAPGST; Tn=α-D-GalNAc) was obtained from Biosyntan GmbH (Berlin, Germany) in order to allow for an evaluation of the ability of antibody fragments to recognise the major VNTR even when it was partially shielded by carbohydrates. The peptide AD-2 (biotin-ANETIYNTTLKYGDV) (Genosys Biotechnologies Inc. (Cambridge, GB)) that mimics an epitope found on cytomegalovirus glycoprotein B was used as a negative control in several experiments (Lantto et al., 2002).

2.2. Anti-MUC1 antibodies

A number of murine anti-MUC1 antibodies were used for comparison during this work (BC2, SM3, HMFG-1 (kindly provided by Antisoma, London, UK) and HMFG-2). The epitopes recognised by these antibodies are associated with the PDTR motif of the VNTR peptide sequence motif as described in the report on the ISOBM TD-4 workshop (Price et al., 1998) and by Franke et al. (2001).

2.3. Naïve antibody library

A stock of phages from a large naive library of single-chain antibody fragments (scFv), a member of the n-CoDeR family of antibody libraries (Söderlind et al., 2000), with approximately 2·10^10 independent members was obtained from BioInvent International AB (Lund, Sweden). The n-CoDeR library family has been created on a scaffold based on the use of the IGHV3-23 and IGLV1-47 heavy and light chain framework regions. Complementary determining regions, CDR, that contribute the diversity to the library originate from the immunoglobulin-encoding genes of human B cells. They have been introduced into the framework regions through CDR shuffling technology (Jirholt et al., 1998).

2.4. Selection of specific binders

The selections were performed after immobilisation of the biotinylated synthetic peptides on magnetic beads coated with streptavidin (Dynal A/S, Oslo, Norway)
essentially as previously described (Persson et al., 2005). Briefly, 50 μl streptavidin-coated magnetic beads were washed with 1 ml TPBSB (phosphate buffered saline (PBS) containing 0.05% Tween-20, 3% bovine serum albumin (BSA)) and the phage solution diluted in TPBSB was subsequently added. The mixture was incubated by slow rotation for 30 min at room temperature as a pre-selection to remove phages carrying scFv specific for streptavidin or those with otherwise undesirable binding properties. After this incubation the beads were discarded and the remaining phages were added to magnetic beads that had been coated in 1 ml TPBSB for 15 min at room temperature with a peptide at a concentration of 10^{-7} M. As flexible, linear peptides previously had been shown to be ineffective in selecting appropriate scFv specific for the major sequence variant harbouring within MUC1 VNTR (Persson et al., 2005) we either used constrained peptides or alternated between the biotinylated 12-mer and 15-mer linear peptides during the selection procedure with an aim to limit the selection of scFv dependent on an interaction with the C-terminus of the peptide. The phages and peptide-coated beads were rotated for 2 h at room temperature. The beads were washed five times in TPBSB and subsequently three times in PBS to remove unbound phages. Bound phages were eluted through cleavage of a protease-sensitive site found in-between the displayed scFv and the phage particle by addition of 250 μl trypsin (0.5 mg/ml in PBS) solution (GIBCO/Life Technologies, Paisley, UK) for 30 min with slow rotation at room temperature. Trypsin was inactivated by the subsequent addition of 250 μl aprotinin (0.1 mg/ml) (Boehringer Mannheim, GmbH, Werk Penzberg, Germany). The eluted phages were used to infect Escherichia coli (HB101F'; BioInvent International AB, Lund, Sweden), grown in 2 x YT (10 g/l Difco bactotryptone, 10 g/l Difco yeast extract and 5 g/l NaCl) with 10 μg/ml tetracycline. After incubation for 30 min at 37 °C, the bacteria were pelleted by centrifugation and the bacteria were spread out on two plates per culture (2 × YT-agar, 0.1 mg/ml ampicillin, 10 μg/ml tetracycline, 1% glucose). The plates were incubated at 37 °C until colonies became visible. Bacteria were retrieved from plates and used for the production of new phage stocks using VCSM13 helper phage (Stratagene, La Jolla, CA). Selections were repeated 3 times.

2.5. Immunological analysis

Initial analyses were performed on phages carrying scFv with respect to their binding to biotinylated synthetic peptides captured on streptavidin by enzyme-linked immunosorbent assay (ELISA). Streptavidin was adsorbed onto polystyrene microtiter plates (Nunc A/S, Roskilde, Denmark) at a concentration of 1 μg/ml in PBS over night at room temperature. The peptides were added at 1 μg/ml in blocking buffer (PBS, 1% BSA, 0.05% Tween-20) and they were allowed to bind to immobilised streptavidin for two h at 37 °C. Culture supernatants containing phage particles displaying scFv were diluted in 2× blocking buffer. After washing with 0.9% NaCl, 0.05% Tween-20, bound phages were detected by adding horse-radish peroxidase-labelled mouse monoclonal anti-M13 pVIII (Amersham Bioscience, Uppsala, Sweden). Orthophenylenediamine was used as the detection substrate.

2.6. Production of VNTR-specific IgG

To allow for the production of VNTR-specific, intact antibodies (human IgG1λ), two different plasmids were used, one encoding the light chain (λ-dhmK7) and another encoding the heavy chain (G1ncmd5) (BioInvent International AB). The genes encoding heavy and light chain variable domains were separately amplified by PCR with Platinum Pfx DNA polymerase (Invitrogen, Carlsbad, CA, USA). PCR products and vectors were cut with BstWI and Bsml (Roche Diagnostics GmbH, Mannheim, Germany) for 3 h at 55 °C to create sticky ends. The digested plasmids were treated with alkaline phosphatase (Roche Diagnostics GmbH) to avoid self-ligation. The cut vectors and PCR products were ligated, after purification (JetQuick PCR Purification Spin Kit (Genomed GmbH, Löhne, Germany)), with T4 DNA ligase (New England Biolabs Inc., Beverly, MA, USA) for 1 h at room temperature. The ligated plasmids were transformed into chemically competent E. coli (Top10F', Invitrogen). To minimise the levels of endotoxin in plasmid preparations that were to be used for transfection they were prepared with EndoFree Plasmid Maxi Kit (Qiagen GmbH, Hilden, Germany).

CHO-K1 cells, kindly provided by Prof. G.C. Hansson (Department of Medical Biochemistry, Göteborg University, Gothenburg, Sweden) were used for production of intact antibodies in IgG-format. The cells were grown in Iscove’s Modified Dulbecco’s Medium (Invitrogen) containing 10% fetal calf serum (Invitrogen) at 37 °C in a humidified atmosphere containing 5% CO₂. The transfection of the two plasmids, one encoding the light chain and the other the heavy chain, was done with Lipofectamine™ 2000 (Invitrogen). On the day prior to transfection 5 × 10^5 cells were added to each well in a 6-well Nuncolon culture plate (Nunc). The cells were grown overnight reaching a
confluency of approximately 90%. At that time 2 μg of each plasmid per transfection was diluted in 250 μl Opti-MEM (Invitrogen) and incubated for 5 min at room temperature. Lipofectamine™ 2000 was also diluted in Opti-MEM (10 μl in 250 μl medium per transfection) and incubated at room temperature for 5 min. The diluted DNA and the diluted Lipofectamine™ 2000 was mixed and incubated for 20 min at room temperature after which the DNA/Lipofectamine™ 2000/Opti-MEM mixture was added to the CHO-K1 cells. After transfection the cells were cultures as above for 2–4 days and the media containing the produced antibodies was retrieved. Cell debris was removed by centrifugation and the supernatant was sterile filtered (0.2 μm).

2.7. DNA sequencing

Genes encoding scFv and antibody variable domains were sequenced from plasmids prepared with QIAprep Spin Miniprep Kit (Qiagen GmbH). The sequencing procedure itself was carried out at a large-scale sequencing centre (MWG-Biotech, Martinsried, Germany).

2.8. Flow cytometry

The T47D breast carcinoma cell line was used for the determination of the ability of specific scFv and antibodies to bind not only peptides carrying VNTR sequences but also native MUC1. The MOLT4 T cell leukaemia cell line was used as a negative control cell line in these studies. The cell lines were cultured in RPMI-1640 (Invitrogen) containing 10% fetal calf serum and 2% L-glutamine (Invitrogen) at 37 °C in a humidified atmosphere with 5% CO₂. In the primary analysis, 50 μl phages were added to cells in 50 μl PBS containing 1% BSA. The mixture was incubated on ice for 30 min. After a threefold wash with PBS containing 1% BSA, the cells were incubated with a mouse monoclonal anti-M13 pVIII antibody (Amersham Bioscience). After a second wash, R-phycoerythrin-labelled rabbit anti-mouse immunoglobulin (DakoCytomation A/S, Glostrup, Denmark) diluted in PBS containing 1% BSA was added and incubated as above. Flow cytometric analysis was carried out on a FACScan cytometer (Becton Dickinson, San Jose, CA, USA). Flow cytometric analysis of the binding of intact recombinant human IgG was performed in a similar manner using R-phycoerythrin-labelled goat anti-human IgG (Caltag Laboratories, Burlingame, CA, USA) as the detection reagent.

The mouse monoclonal anti-MUC1 antibodies BC2 and HMFG-1 were used in an attempt to block binding of 12ESC-6 to MUC1 on the surface of the T47D cell line. The analysis was performed as previously described with the adding of an incubation step with a blocking antibody (BC2 or HMFG-1) for 30 min under standard conditions after which 50 μl undiluted 12ESC-6 was added directly to the mix without a washing step. The mixture was incubated for another 30 min under standard conditions after which the original protocol was resumed. The R-phycoerythrin-labelled goat anti-human IgG used for detection was diluted in 10% mouse sera to avoid cross reactivity with mouse IgG. A monoclonal antibody specific for CD71 (Ber-T9; DakoCytomation) was used to ensure the specificity of the blocking procedure. The activity of each antibody was determined by the detection of their binding to T47D by R-phycoerythrin-labelled rabbit-anti-mouse IgG (DakoCytomation).

2.9. Epitope mapping

To determine the epitope specificity of antibodies and fragments thereof, inhibition studies were performed using a set of alanine-scanned and otherwise mutated peptides based on two 9-mer peptides mimicking the conserved sequence (SAPDTRPAP) and the major sequence variant 1 (SAPESRPAP) of the extracellular domain of MUC1. Detection of remaining binding activity in the presence of various peptides was performed on a Biacore Upgrade (Biacore International AB, Uppsala, Sweden). The target peptide antigen coupled to streptavidin immobilised on a CM5 sensor chip (Biacore AB) was either biotinylated ala3-MUC-13 (for 12ESC-6) or biotin-12DT.

3. Results

The rarity of antibodies, in particular fully human antibodies specific not only for the major sequence found within VNTR but also for the major sequence variants of MUC1 prompted us to initiate a programme to identify

<table>
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<th>Table 1</th>
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<tr>
<td>Pattern of selection of different clones depending on the selection antigen used</td>
</tr>
<tr>
<td>scFv</td>
</tr>
<tr>
<td>LV-11</td>
</tr>
<tr>
<td>12ESC-6</td>
</tr>
<tr>
<td>12/14ESC-7</td>
</tr>
<tr>
<td>12/13ESC-10</td>
</tr>
<tr>
<td>12ESC-17</td>
</tr>
<tr>
<td>13ESC-2</td>
</tr>
<tr>
<td>13ESC-17</td>
</tr>
<tr>
<td>14ESC-8</td>
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such specific binders and antigens suitable for their isolation. To allow for identification of such specific antibody fragments, we used a scFv display library (n-CoDeR) (Söderlind et al., 2000). As this epitope had previously proven to be very poorly antigenic in vitro (Persson et al., 2005) we chose two different approaches. In one we made use of two peptides of different length that were used alternately during the selection procedure. In the other approach we made use of constrained (Ladner, 1995), circular peptides as selection antigen. Selected scFv were following selection displayed on phages and their specificity was evaluated by ELISA. In these analyses approximately 25% of the clones show specificity for their selecting antigen (data not shown). Several of these clones were found to be genetically identical as expected from a selection process that proceeded under substantial selection pressure. Nevertheless, a diverse array of scFv was identified based on sequence differences including sequence motifs in and length of heavy chain CDR3 (data not shown), the key determinant of antibody specificity (Xu and Davis, 2000). Some clones were even identified after selection on multiple antigen variants suggesting that constrained peptides differing in length at least to some extent displayed similar epitopes to the antibody repertoire (Table 1). Eight clones, seven of which were selected on constrained peptides, with a different VNTR peptide recognition pattern (Fig. 1) were further evaluated. All eight scFv could recognise other peptides than the one they had been selected on, and four of them (LV-11, 12ESC-6, 12/13ESC-10 and 12ESC-17) were found to have a broad MUC1 specificity including a recognition...
of not only the major peptide sequence variant of VNTR but also of the classical sequence (Fig. 1). In addition, two of them also have the ability to bind a peptide carrying a carbohydrate modification within the part of the sequence (PDTR) that is considered to be most antigenic in MUC1 VNTR (Fig. 1). Thus, these selection strategies, in particular those involving constrained peptides were successful for the isolation of pan-VNTR peptide motif-specific scFv.

Identification and selection of antibodies and their fragments based on their binding to peptides often selects for binders that do not recognise the protein from which the peptide was derived. This was the case for antibody fragments selected previously against the major sequence variant of MUC1 VNTR (Persson et al., 2005). We therefore assessed the binding specificities of the eight diverse clones identified above for their ability not only to bind peptides, but also for their ability to bind to MUC1 on the surface of a cell line, T47D. The cell line T47D has previously been shown to upregulate MUC1 (Apostolopoulou and McKenzie, 1994) and to have a high frequency of the variant sequence in its extracellular domain (Müller et al., 1999). The cell line MOLT4, which does not overexpress MUC1, was used as a negative control. Importantly, it was found that two scFv, 12ESC-6 and 12ESC-17, selected on peptide antigens bound intact MUC1 as found on this tumour cell line (Fig. 2). Thus, the peptide these clones were selected on mimicked native MUC1 sufficiently to allow for selection of binders recognising MUC1 as found on tumour cells. The genes encoding these scFv were transferred into an expression vector system allowing for the production of intact human IgG1. Although one clone lost its specific recognition of MUC1 in this format, 12ESC-6 retained its specificity for MUC1 as found on a tumour cell line (Fig. 3). Importantly it was possible to block the binding of 12ESC-6 to the cell line T47D by pre-blocking the antigen on the surface of the cells by either one of the two mouse monoclonal anti-MUC1 antibodies BC2 and HMFG-1 (Table 2), but not by a control antibody. Thus, the binding of 12ESC-6 to T47D is specific for MUC1.

Many antibodies raised by immunisation with or selected on MUC1 and in particular on peptides derived from the major sequence found in VNTR, i.e. the one with the PDTR sequence, do not recognise sequences carrying the PESR motif (Fig. 4A; see also von Mensdorff-Pouilly et al., 2005). In contrast, 12ESC-6 that had been selected on the major sequence variant found in VNTR bound to peptides carrying the PDTR sequence (Fig. 1). Although shorter peptides poorly recognised this antibody, alanine scanning mutagenesis employing such epitope-mimicking peptides nevertheless demonstrated that the epitope still resided close to the PDTR/PESR sequence, although it was slightly shifted in the direction of the C-terminus as compared to the epitope recognised by several murine antibodies or other human MUC1 VNTR-specific recombinant antibodies (Fig. 4). We thus conclude that the constrained peptide 12ESC, resembling the major sequence variant

Table 2

<table>
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<tr>
<th>Detected antibody</th>
<th>Background (MFI)</th>
<th>Signal without blocking antibody (MFI)</th>
<th>Signal with blocking antibody (MFI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12ESC-6</td>
<td>5</td>
<td>21–24</td>
<td>11</td>
</tr>
<tr>
<td>BC2</td>
<td>476</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HMFG-1</td>
<td>8</td>
<td>105</td>
<td></td>
</tr>
<tr>
<td>anti-CD71</td>
<td>704</td>
<td></td>
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The ability of the blocking antibodies to bind the cell line is also shown.

a Mean fluorescence intensity.

Fig. 3. Flow cytometric analysis of background signal (A) and of the binding of 12ESC-6 IgG (B) to T47D breast carcinoma cell line. The binding of murine monoclonal antibody BC2 is shown for comparison (C). 12ESC-6 IgG did not bind the negative control MOLT4 cell line (not shown).
found in VNTR, was able to overcome the problems of antigenicity associated with the PDTR→PESR modification (von Mensdorff-Pouilly et al., 2005; Persson et al., 2005) and successfully selected for human antibody fragments with the ability to recognise both the major sequence and its major sequence variant of MUC1 VNTR.

4. Discussion

MUC1 has been widely recognised as a potential target for cancer diagnosis/therapy due to the differences in levels of protein expression and glycosylation between MUC1 on healthy cells and that on malignant cells. A variety of murine antibodies have been raised against this target (Price et al., 1998) and tested for their ability to target MUC1 as found on tumour cells. One antibody, HMFG-1, has even shown promising effects in a radiolabelled form for the treatment of ovarian carcinoma (Epenetos et al., 2000). The recent realisation that several sequence variants of the repeated unit in VNTR exists and the observation that many antibodies poorly recognise the most important variant (von
Mensdorff-Pouilly et al., 2005) (Fig. 4A) suggests that much remains to be explored with respect to antibodies targeting this antigen. Indeed, natural humoral immunity targeting this epitope develops in normal subjects but the response shifts towards the major sequence motif in VNTR in patients suffering from adenocarcinoma (Mensdorff-Pouilly et al., 2005) suggesting that this particular sequence is a better immunogen than the major sequence variant. Previous studies also identified the major alternative sequence to be a poor antigen in vitro (Persson et al., 2005). Furthermore, as antibodies that recognise the alternative sequence are of murine origin, their usefulness with respect to tumour treatment and in vivo diagnosis is very limited. Indeed, it has proven difficult to raise high affinity antibodies of human origin against MUC1 in general (Andersson et al., 1999; Jirholt et al., 2002) and when the matter has been investigated, selected specificities do not recognise the major sequence variant of VNTR. By employing structurally constrained peptides we have now, in contrast to approaches employing simple linear peptides (Persson et al., 2005), been able to by-pass the low antigenicity of this sequence, possibly caused by an increased side chain entropy and/or reduced levels of a defined stable structure in the sequence variant in comparison to the major sequence found in VNTR (von Mensdorff-Pouilly et al., 2005; Persson et al., 2005), and we have efficiently develop antibody specificities targeting the major sequence variant of VNTR. Importantly these peptides selected for specificities that were not dependent on a free carboxy terminus, a characteristic that would prevent binding to intact MUC1. Indeed, several selected scFv variants bound not only the peptide on which it had been selected but also other peptides with other lengths and some of them even bound peptides displaying the most common VNTR sequence carrying the PDT sequence motif. Thus, in contrast to many established murine antibodies developed against synthetic peptides originating from MUC1 VNTR (von Mensdorff-Pouilly et al., 2005) these specificities recognised both the major sequence of VNTR and its major sequence variant, i.e. they displayed a pan-VNTR motif specificity. Importantly, these structurally constrained peptides had selected for scFv that were able to recognise the intact protein as displayed on a tumour cell line. Altogether this set of data suggests that such structurally constrained peptides may also allow for the development of MUC1 specific antibody repertoires in vivo, including those that recognise both the major sequence and the major sequence variant of MUC1 VNTR, following immunization.

In conclusion, we have defined strategies to circumvent the poor antigenicity of the major sequence variant of MUC1 VNTR and raised human antibody specificities that target this structure. The herein identified binders may serve as starting points to develop human antibodies with tumour-targeting potential in vivo.

Acknowledgements

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References


