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Selection strategies for anti-cancer antibody discovery: *searching off the beaten path*

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Abstract

Antibody based drugs represent one of the most successful and promising therapeutic approaches in oncology. Large combinatorial phage antibody libraries are available for identification of therapeutic antibodies, and a variety of technologies exist for their further conversion into multivalent and multispecific formats optimized for the desired pharmacokinetics and the pathological context. However, there is no technology for antigen profiling of intact tumors to identify tumor markers targetable with antibodies. Such constraints have led to a relative paucity of tumor-associated antigens for antibody targeting in oncology. Here we review novel approaches aimed at the identification of antibody-targetable, accessible antigens in intact tumors. We hope that such advanced selection approaches will be useful in the development of next-generation antibody therapeutics for cancer.

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ER has a patent for *in vivo* screening of random libraries of compounds that Sanford-Burnham Medical Research Institute has licensed to various companies. The other authors declare no competing financial interests.

Keywords

Therapeutic antibodies; tumor-associated antigens; phage display; advanced phage selections

Monoclonal antibodies in cancer therapy

One of the most promising strategies for the development of cancer therapies relies on monoclonal antibodies (mAbs). Tumor-specific mAbs can be therapeutically active as such, they can be used in targeted delivery of therapeutics to tumors, or they can serve in both capacities. The number of tumor-specific molecules available for antibody targeting is limited, and identification of new targets can expand the utility of the approach [1, 2].

According to the available information [3–5], there are currently 19 therapeutic mAbs for cancer indications on the market and two more were undergoing their first regulatory review as of January 2015. Seven of the 18 marketed mAbs are used against hematological malignancies, and 12 are for the treatment of solid tumors (Table 1). In the first group, only 4 different CD antigens are targeted (CD19, CD20, CD30 and CCR4), with 4 mAbs directed against anti-CD20. This paucity of targets is also found in mAbs aimed to the treatment of solid tumors. Of the 12 mAbs on the list, 6 are directed against cancer cell surface molecules. Two growth factor receptors (EGFR and HER2) are the targets of 5 mAbs. Other antibody-based therapeutics in cancer do not focus on the tumor itself, but instead aim at inhibiting tumor angiogenesis (with anti-angiogenic mAbs targeting VEGF or VEGFR-2) or promoting immune responses (immunostimulatory mAbs blocking CTLA-4 or PD1) [3–5]. In summary, all the therapeutic mAbs either approved or in advanced clinical trials are directed against a limited number of targets. Moreover, most of the target antigens are also expressed and accessible in normal tissues.

This paucity of target antigens may be a result of current target identification methods, such as searching for differences between tumor and non-tumor cells at the DNA, RNA and protein levels [6]. Availability of biological source material such as cell lines and clinical samples is a prerequisite to target identification. Cell lines are widely used because they provide readily accessible starting material for any common target search strategy. As shown in Table 1, cancer cell membrane proteins are popular targets for antibody selection; however, the extent to which their presence and abundance in established cell lines correspond to those of primary tumors is not clear. In a recent article, a significant portion of the “membranome” (genes encoding plasma membrane proteins) lost their up-regulated state upon transition from *in vivo* to *in vitro* conditions [7]. This information has important implications for antibody selection, as one can miss potential targets. On the other hand, fresh tumor biopsy material may be difficult to obtain in sufficient amounts for conventional selection strategies, and although pieces of primary human tumors can be propagated in immunodeficient mice, changes in cell composition of the tumor stroma can impact tumor cell expression pattern [8].

Moreover, genomic and proteomic studies involve a complex sample preparation process that destroys tissue architecture. The loss of “topographical” information is a major handicap in the identification of cancer targets. In fact, tumors are complex organ-like structures

containing multiple and highly interactive cell types, which include the cancer cells and a variety of non-cancer cell types (endothelial cells, pericytes, fibroblasts, immune cells, etc.), soluble factors, and extracellular matrix components. The non-cancer cell components can promote neoplastic transformation, support tumor growth and invasion, and protect the tumor from host immunity [9]. Furthermore, current protocols for target identification often ignore the tumor vasculature; which is an important site of accessible and stably targetable tumor-associated markers.

Tumor blood vessels are abnormal both structurally and functionally [9]. This creates a microenvironment characterized by elevated fluid pressure, transient hypoxia and repeated hypoxia-reperfusion injury, all factors thought to contribute to the limited diffusion of therapeutic antibodies into the tumors [10].

For many years, scientists focused their research on strategies that directly target tumor cells, and spent little time studying other components of the tumor microenvironment. However, this view is changing, and recent data show the value of targeting the tumor microenvironment in combination with therapies aimed at the tumor cells [11]. Nevertheless, the information available on antigens associated with tumor stroma that might potentially amenable to antibody targeting is limited. Here we discuss some emergent strategies that are based on antibody display technology and its adaptation to complex selection scenarios. This technology can expand the repertoire of targetable tumor molecules, while simultaneously generating new antibodies for the targeting. It can also be useful in the optimization of the structure of an antibody, and when applicable, its therapeutic payload.

New antibody phage selection strategies in oncology

A number of human antibody libraries with different formats and diversities have been generated [12]. The *in vitro* selection of antibodies against purified antigens is a relatively straightforward process, generally accomplished within a few rounds of affinity selection [12]. The power of this *in vitro* antibody selection can be further enhanced by precise control of the selection conditions. In contrast to immunization of animals, where there is little control over the nature of antibodies generated, the selection conditions can be readily manipulated *in vitro*. This can be accomplished for example, by presentation of specific conformations of the target antigen or by including competitors for unwanted antigens, so as to direct the selection towards targets or epitopes of interest. *In vitro* selection methods also overcome immunological tolerance, allowing the selection of antibodies against highly conserved targets and self-antigens [12].

However, there are more complex scenarios for antibody selection, which require design of specific selection strategies. Examples include isolation of antibodies against whole cells, where the desired target is only a fraction of the available epitopes present on the cell, and performing selection on heterogeneous cell populations or molecular preparations. Unfortunately, antibody discovery by phage display on whole cancer cells is complicated for a number of reasons, including high background of non-specifically bound phage particles, selection bias toward highly expressed epitopes, and emergence of dominant phage clones, particularly when multiple rounds of selection are used.

According to the information summarized in Table 2, only in a little more than half (29 out of 52 articles) of the selections performed on cancer cells or tissue the antigens recognized by the retrieved antibodies were identified. Most of the identified antigens were highly expressed markers, e.g. EGFR [13–15], HER2 [13, 14, 16–19], CD44 [13, 14, 20], and transferrin receptor [13, 14, 19, 21], which at that time were already known as cancer markers. In some cases, the phage selection was specifically designed to favor antibodies against a given antigen e.g. by using a cell line expressing high levels of a particular antigen, or by secondary screening of the clones obtained by ELISA against a purified form of the antigen.

A few publications have identified novel tumor-associated antigens (Table 2) suggesting that new antibody selection methods should be developed to provide the means for discovering all potentially useful antigenic differences between tumors and normal tissues. Such methods should be suitable for discovery of antibodies for the targeting of low abundance antigens, sub-populations of rare cells, and antigens present in the tumor microenvironment. Phenotypic selections are being developed as alternative methods to identify novel targets. This phenotypic approach relies on the selection of antibodies based on their effect on the target, rather than previous knowledge of the target [22–25]. In order to enable efficient selection of antibodies in an unmodified or minimally modified tumor environment that preserves antigenic diversity and topographic location, researchers need to develop new methods that deviate from the trodden track of target-directed selection. Such selections can be performed *ex vivo* on freshly isolated cancer tissue or patient derived biological fluids, or *in vivo* in tumor-bearing mice or in cancer patients.

Ex vivo phage selection

Tissue slide selection

It has been shown that the removal of any cell, including tumor cells, from their native environment will impact gene expression [26, 27]; thus caution has to be exercised when applying tumor cell lines to biomarker identification. Ideally, conditions as close to the natural environment as possible should be used. Another important issue affecting antigen discovery is tissue heterogeneity. To deal with both issues, novel antibody phage display selection strategies have been devised. Firstly, laser capture microdissection (LCM) has been utilized to obtain tumor specific phage antibodies because this technique allows one to isolate small clusters of target cells from tumor sections previously incubated with a phage library [28, 29]. Similarly to the commonly used subtraction methods, performing the selection before LCM creates competition for library variants recognizing antigens shared by tumor cells and surrounding tissue (pan-cell binding phage), which may increase the proportion of specific phage in the LCM-isolated cell cluster. An inherent problem with LCM is that the slide preparation conditions required for successful dissection, especially the dehydration step, drastically reduce phage infectivity [28, 30–33]. Therefore, alternative procedures to retrieve the sequences of the peptides or antibodies encoded by the inserts in the phage DNA have been used, including subcloning of PCR-amplified inserts [30, 31].

Alternatively, investigators have searched for conditions that are compatible with both LCM and phage viability. For example, freeze drying the slides after selection preserves phage

infectivity after LCM [33]. Most recently, Sun *et al.* [29] protected the phage from drying while performing LCM by using an overlying membrane that creates a moist environment capable of preserving phage viability for up to 6 hours. It is also possible to isolate clusters of cells by LCM before incubating them with the phage library [28, 34, 35]. Although LCM allows isolation of single cells from tissue sections, the general approach has been to dissect clusters of 20–50 cells. Ruan *et al.* [31] observed that antibody phage was recovered from single cell isolations only occasionally, and then as a single clone. Thus, the potential of antibody selection using LCM has hitherto been restricted to the use of clusters of multiple cells as the target.

Shadow-stick selection

Selecting antibodies against rare cells is often impeded by insufficient access of the phage to the target cells. To circumvent this problem, Sørensen and Kristensen [36] have presented a selection method targeting only a single cell within a heterogeneous population. The method has been dubbed ‘shadow-stick selection’ due to the use a minute disk attached to a glass pipette (resembling a hockey-stick), which is used to selectively protect the target cell, and the phage bound to it, from UV irradiation. By reducing the requirement to a single identified cell it was possible to select antibodies against an individual cultured K562 cell spiked into peripheral blood mononuclear cells (PBMC) [32] and against cells identified as fetal microchimeric cells in maternal blood [23]. This method is especially useful if the target cell is present in a very low frequency. For example, for some cancer types, circulating tumor cells (CTC) have been estimated to occur with a frequency of only one in a million of PBMCs [37]. In the shadow-stick protocol a heterogeneous cell suspension containing a few or only one target cell is applied to a glass slide (Figure 1). The cells are treated with a fixative to preserve cellular architecture and to keep the cells attached during the selection procedure and subsequent washing steps. Thereafter, the single target cell is identified and protected by a UV-impermeable disc attached to a glass capillary. By means of micromanipulation the disc is aligned above the cell of interest and the slide is exposed to UV-C light. The irradiation crosslinks the DNA of the unprotected phages bound to the surrounding non-target cells and thereby prevents their replication in *E. coli*. Thus, only protected phages give rise to colonies after infection, which will typically be in the range of 1–8 unique clones per single cell selection [36].

The single cell selection method targets one cell in a large population of cells. For the method to be successful, the target cell should be present at a low frequency (preferably between one out of 10^{-4} – 10^{-6} cells). This will increase the probability of selecting antibodies that target antigens specific for the target cell. There are only 10 to 1000 copies of any given antibody present in the first round of selection, and the abundant non-target cells should capture the antibodies that bind to epitopes common to all cells [36]. This combined with gentle and controlled phage elution provides a method making it possible to select antibodies recognizing the target cell. Sufficient selectivity is achieved by using a protease sensitive helper phage [38], which can reduce the background of bound non-displaying phages. In phage antibody libraries where the antibody fusion is introduced by a phagemid only 1–10 % of the resulting phage particles will present an antibody on the surface. Thus 90–99 % of the phages binding to the single target cell are not captured through a specific

antibody-antigen interaction. This background is removed by applying the protease sensitive helper phage, resulting in a low output of only 1–8 unique clones per target cell [36]. Performing multiple rounds of selection generally favors enrichment of antibodies that have high affinities or target highly expressed antigens [39]. However, iterative selections, normally required when using conventional helper phages, may compromise the identification of specifically expressed antigens, as the most interesting biomarkers might not be expressed in high abundance or recognized by antibodies with high affinity; therefore only a single round of selection is preferred.

The strength of this selection method lies in the targeting of a single cell. Single cell analyses at the mRNA level have shown that phenotypically identical cells display transcriptional differences [40]. In contrast to selections on a pool of target cells, which are likely to be heterogeneous in their protein expression patterns, the single cell method can identify biomarkers uniquely expressed by individual cells.

***In vivo* phage selection**

***In vivo* selection of phage peptides**

Repertoires of peptides on the surface of the bacteriophage T7 have been successfully used in mouse models *in vivo*. Traditionally, phage display has been used in screens that are performed *in vitro* against a pre-defined target molecule or cell in order to identify probes recognizing the target molecule or cell. However, this strategy relies on pre-existing knowledge of the target. In the *in vivo* approach the phage library is screened in a live animal [41]; reviewed in ref [42] or human patient [43, 44]. The main advantage of *in vivo* screening is that the target is probed as it exists *in situ*, which eliminates the possibility that changes introduced by the removal of a tissue or cells from their natural environment. A prime example of a protein that is expressed differently *in vivo* and *in vitro* is the $\alpha v \beta 3$ integrin; it is only expressed in highly activated cells, including tumor endothelial cells *in vivo*, whereas essentially all cultured cells express this integrin. Thus, screens and analyses performed with cultured cells would miss the fact that $\alpha v \beta 3$ is an excellent marker for tumors and wounded/inflamed tissues, and is much used in targeting drugs, imaging agents, and nanoparticles to tumors [45]. In an *in-vivo* screen, the phage library is allowed to circulate through the organism and to interact with the molecules accessible from the blood, particularly, in the luminal surface of the vessels. Those phages harboring peptides that do not interact (or do so weakly) with any anchored molecule are washed away during the selection process. Further, the *in vivo* selection includes a built-in negative selection step because the whole vascular bed acts as a sink for those phages that harbor peptides capable of interacting with commonly expressed molecules. Finally, those phages displaying peptides that interact with molecules that are specific for a certain organ accumulate in that organ or tissue and can be retrieved, amplified and analyzed [41]. This *in vivo* selection has been very effective to map a variety of markers that distinguish the luminal surface of vessels in different organs, and that can discriminate between normal vessels and tumor vessels (vascular “zip codes”) [42].

Several peptides discovered by *in vivo* phage library selections have been used as targeting elements in delivering payloads to a chosen tissue, such as a tumor [46]. While peptides

have their uses (for example, they are particularly well suited for the targeting of nanoparticles [45]), as we will discuss in the next subsection, antibodies have substantial advantages as targeting agents and therapeutics [2].

***In vivo* selection of phage antibodies**

Some of the first attempts to apply the *in vivo* phage screening technology to antibodies have been in human patients. Krag *et al.* [44] combined peptide and recombinant antibody screening to run a Phase I clinical trial on 9 patients with melanoma, pancreas or breast cancer using recombinant antibody (and peptide) phage display. Two of the patients received the antibody library, and the tumor nodules were excised after 30 min of circulation to recover the bound antibodies for analysis. The study concluded that the administration of the phage was safe, and that some of the recovered antibodies were able to bind *in vitro* to tumor cells, but not to blood cells from the patients. However, no further characterization of the target, biodistribution, or the ability to localize the tumor *in vivo* was done. More recently, Shukla *et al.* [47] took this approach a step further by infusing a scFv library into three stage IV-patients and then evaluating 998 different phages for binding to tumor sections. They obtained 45 soluble scFv that were able to bind to tumor sections, of which only 2 did not bind to normal tissue sections. However, the binding to normal sections does not necessarily mean that the target antigen would be accessible in normal tissues *in vivo*, so it is possible that additional scFv would have been tumor-specific if *in vivo* assays had been available [47]. Interestingly, these authors identified a possible tumor-specific IL-17α mimic among the isolated phages on the basis of sequence homology.

In another *in vivo* phage antibody screen, Deramchia *et al.* [48] performed selection of human recombinant antibodies in an atherosclerotic mouse model. After three rounds of selection, with a very short circulation time, several clones were isolated from the atherosclerotic aorta. Some of the recombinant antibodies showed positive immunohistochemical staining in sections of atherosclerotic rabbit aorta and in sections of atherosclerotic lesions of human carotid and coronary arteries; however, neither the ability of the recombinant antibody to localize in the model *in vivo* nor the localization of the proposed target were studied.

Despite the fact that tumor blood vessels are abnormal, fragile, and hyperpermeable, and that phage can have opportunities to interact directly with cancer cells and stromal components, *in vivo* antibody screening in tumor-bearing mice has not been accomplished. Some of the technical limitations that may account for the lack of success include the high background with common phage selection procedures, as some organs non-specifically capture too many phages to be used as target organs for selection [41].

Sequential *ex vivo* and *in vivo* phage antibody selection

Recently, Sánchez-Martín *et al.* [25] reported having overcome some of the limitations of *in vivo* phage antibody screening and described the isolation of a tumor-homing antibody from a human single-domain antibody library. Using a prostate cancer mouse model, they combined *ex vivo* and *in vivo* rounds of selection of human recombinant antibodies from phage libraries. Initial *ex vivo* selections with freshly disaggregated tumors provided an

enriched, but highly diversified repertoire of binders to tumor components, while minimizing the number of animals needed for the selection (Figure 2). The subsequent *in vivo* selection pruned the binders whose target is not available or otherwise not reachable *in vivo*, as well as the phages that bind to blood or tissue components, but are not tumor-specific. At that time, extensive conventional DNA sequencing was needed to minimize the rounds of selection needed and to preserve a high diversity that unveiled consensus sequences. High-throughput sequencing, which has since become available, makes these goals much easier to achieve. While helpful in all types of selection, high throughput sequencing particularly useful in the analysis *in vivo* results because of the substantial amount of work that goes into every round of selection. The ability of the selected antibodies to home to tumors and to deliver a payload to the tumors was examined by studying individual phage clones from the screen. Some of the phage clones stood out in that the distribution of the phage was not restricted to the endothelium but, after a 24 h circulation time, phage particles were widely distributed in perivascular areas. The proteasome activator complex PA28 was identified as the target of one of the antibodies by mass spectrometry, and verified by ELISA [25]. Further, the relevance of PA28 as a tumor marker was confirmed in studies on human primary and metastatic prostate cancer tissue [25]. These studies showed that it is possible to perform *in vivo* selection of human recombinant antibodies with the ability to selectively home to tumors from systemically administered injection. The target identification made possible by the availability of a tumor-homing probe can provide new tumor markers and unveil new functions for the target molecules.

Concluding remarks and future perspectives

The generation of antibodies against cell surface markers selectively expressed by rare tumor subpopulations, but still with a crucial role in cancer progression, and the identification of antibody-targetable tumor-associated antigens in intact tumors are important bottlenecks in the development of novel immunotherapies for cancer. Hence, there is an urgent need to implement technological platforms and selection strategies for anti-cancer antibodies. Recently, considerable efforts have been devoted to optimizing phage display selection methods using antibody libraries *ex vivo* and *in vivo*. Advanced selection conditions make feasible the use of phage antibody libraries for selecting tumor-homing antibodies and the subsequent identification of their antigens for validation in primary and metastatic human cancer samples.

Among emerging techniques with high potential for applications to phage display selection against cells are the microfluidic-/lab-on-a-chip devices used for rare cell sorting. A few attempts to apply this technology for phage display selections have been made [49], but not on whole cells. This technique will be especially useful for selection against rare cells in suspension, such as circulating tumor cells.

The use of viral particles (e.g. bacteriophages) *in vivo* has several disadvantages. Systemically administered virus must avoid destruction by immune effector mechanism, must avoid nonspecific adhesion and sequestration, and must extravasate selectively at tumor sites. However, phages are inefficient at crossing endothelial barriers. Therefore, it would be of significant value to use other repertoire display particles that traffic to tumors more

efficiently, such as macrophages, T cells, and stem cells. Although several mammalian cell surface display platforms have been developed [50], the display of antibodies on the surface of primary T lymphocytes, as a part of a chimeric antigen receptor (CAR) [51, 52], could simplify the selection process, since effective triggering would promote CAR-T cell survival and expansion. One of the biggest hurdles to overcome would be the production of large diversity repertoires. However, mammalian platforms could be used as a second round selection method using *ex vivo* pre-enriched phage pools.

A possible way of circumventing the limitation on the size of antibody repertoire would be to create an antibody library by inserting into the antibody framework a pool of peptide sequences obtained by screening on a desired target cell or tissue. The resulting library could then be screened on the same target to obtain antibodies for that target. The feasibility of this approach is suggested by antibody engineering work performed 20 years ago; grafting the integrin-binding RGD sequence into an antibody CDR resulted in an integrin-binding antibody [53]. Moreover, this antibody could be further evolved by introducing mutations to the CDR and selecting for additional binding properties [54]. It may be possible to adapt this strategy to screening against unknown, complex targets, including tissues targeted *in vivo*. All these approaches could also be easily adapted to *in vivo* selection processes in tumor-bearing mice, and in animal models for other diseases. Clearly, *in vivo* screening of large antibody libraries has great potential that has not been fully tapped.

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Figure 1. Schematic illustration of the shadow-stick selection method. Phage affinity selection is performed on cells placed on a glass slide, including a target cell of interest (green cell). The shadow-stick is placed above the target cell before UV irradiating the slide, selectively protecting the phage bound to the target cell while “killing” all phage bound to non-target cells. Illustration by Simon Lykkemark.

Figure 2.

Schematic representation of the sequential selection process to obtain tumor-homing antibodies. The initial phage repertoire is pre-enriched on cell suspensions prepared from freshly excised tumors (*ex vivo* strategy) and the resulting enriched phage pool then systemically injected into tumor-bearing mice (*in vivo* strategy). Retained phage particles are recovered from the tumor. Illustration by Simon Lykkemark.

Table 1

Therapeutic monoclonal antibodies for cancer indications marketed worldwide

International non-proprietary name	Tradename	Target/Antibody type	Indication First approved	First US (EU) [Japan] approval
Hematological malignancies				
Rituximab	MabThera, Rituxan	CD20, Ch IgG ₁	NHL, CLL	1997 (1998)
Ibritumomab tiuxetan	Zevalin	CD20, Mu IgG ₁ , ⁹⁰ Y-radiolabeled	NHL	2002 (2004)
Ofatumumab	Arzerra	CD20, Hu IgG ₁	CLL	2009 (2010)
Obinutuzumab	Gazyva	CD20, Hz IgG ₁ , glycoengineered	CLL	2013 (2014)
Brentuximab vedotin	Adcetris	CD30, Ch IgG ₁ , ADC	HL, <i>systemic ALCL</i>	2011 (2012)
Mogamulizumab	Poteligeo	CCR4, Hz IgG ₁ , glycoengineered	T cell leukemia-lymphoma	NA (NA) [2012]
Blinatumomab	Blincyto	CD19 × CD3 (bispecific), BiTe	ALL	2014 (NA)
Solid tumors				
Nimotuzumab	CIMAher, BIOMAb-EGFR	EGFR, Hz IgG ₁	SCCHN, glioma, nasopharyngeal cancer	NA (NA) [NA] [#]
Cetuximab	Erbix	EGFR, Ch IgG ₁	CRC	2004 (2004)
Panitumumab	Vectibix	EGFR, Hu IgG ₂	CRC	2006 (2007)
Trastuzumab	Herceptin	HER2, Hz IgG ₁	Breast cancer	1998 (2000)
Trastuzumab emtansine	Kadcyla	HER2, Hz IgG ₁ , ADC	Breast cancer	2013 (2013)
Pertuzumab	Perjeta	HER2, Hz IgG ₁	Breast cancer	2012 (2013)
Catumaxomab	Removab	EPCAM × CD3 (bispecific) Rat/Mouse IgG	Malignant ascites	NA (2009)
Denosumab	Xgeva	RANKL, Hu IgG ₂	Bone metastasis, bone giant cell tumors	2010 (2011)
Ipilimumab	Yervoy	CTLA-4, Hu IgG ₁	Melanoma	2011 (2011)
Pembrolizumab	Keytruda	PD1, Hz IgG ₄	Melanoma	2014 (In review)
Nivolumab	Opdivo	PD1, Hu IgG ₄	NSCLC, renal cell carcinoma, melanoma	2014 (In review) [2014]
Bevacizumab	Avastin	VEGF, Hz IgG ₁	CRC	2004 (2005)
Ramucirumab	Cyramza	VEGFR2, Hu IgG ₁	Gastric cancer	2014 (In review)

Abbreviations: ADC, antibody-drug conjugate; ALCL, anaplastic large cell lymphoma; ALL, acute lymphoblastic leukemia; BiTe, bispecific T cell engager; CD, cluster of differentiation; Ch, chimeric; CLL, chronic lymphocytic leukemia; CML, chronic myelogenous leukaemia; CRC, colorectal cancer; CTLA-4, cytotoxic T lymphocyte antigen 4; EGFR, epidermal growth factor receptor; EPCAM, epithelial cell adhesion molecule; HER2, human epidermal growth factor receptor 2; HL, Hodgkin lymphoma; Hu, human; Hz, humanized; Mu, murine; NA, not approved; NHL, non-Hodgkin lymphoma; NSCLC, non-small cell lung cancer; PD1, programmed death 1; RANK-L, receptor activator of NFκB ligand; SCCHN, squamous cell carcinoma in head and neck; VEGF, vascular endothelial growth factor; VEGFR2, vascular endothelial growth factor receptor 2.

[#] Argentina, China, Cuba and India, among over 20 countries.

Table 2

Antibody selections performed by phage display on cancer cells or cancer-derived tissues.

Cancer type	Cells targeted	Selection system	Library Type (Diversity)	Identified Ag	Ref.
Brain and Neural system					
Glioblastoma	PDT and X spheres	<i>Ex vivo</i> + <i>in vitro</i> , S/I/PA	NIL-h scFv (Sheets, 6.7×10^9)	No	[55]
	JF, SK-N-SH, IMR-32	<i>In vitro</i>	IL-h, PD scFv	No	[56]
Breast					
ACa	MFC-7	<i>In vitro</i>	NIL-h scFv (Sheets, 6.7×10^9)	Mucin 1	[57]
	8701BC	<i>In vitro</i> , PA	IL-h, PD scFv (5×10^8)	No	[58]
	PDT/C	<i>Ex vivo</i> fresh PDT, PA	IL-h, PD Fab (1.7×10^6)	Cytokeratin 10	[59]
		<i>Ex vivo</i> , S/I	NIL-h (Griffin 1, 1.2×10^9)	GRP78	[60]
	SK-BR-3	<i>In vitro</i> , I/PA	IL-h, PD scFv (5×10^9 and 8×10^8)	HER2	[16]
		<i>In vitro</i> , LCM	NIL-h scFv (Tom I, 1.5×10^8)	No	[28]
		<i>In vitro</i> , I/PA	NIL-h scFv (Sheets, 6.7×10^9)	HER2, TFR, +	[19]
		<i>In vitro</i> , spiked, S/MACS	NIL-h scFv (Sheets, 6.7×10^9)	HER2	[17]
	MDA-MB-231	<i>In vitro</i> , S/I	NIL-h scFv (1×10^{11})	CD44, EGFR, +	[13]
	PDT	<i>Ex vivo</i> , PA	IL-h, PD Fab (10^{10})	gC1q-Receptor	[61]
Ca	SUM159	<i>Ex vivo</i> X, S/PA/FACS	NIL-h scFv (TomI+J; 1.5 and 1.4×10^8)	No	[62]
	PM-1	<i>In vitro</i> , S	NIL-h scFv (NBL library, 6.4×10^9)	CD166	[63]
	PDT	<i>Ex vivo</i> , S/PA	NIL-h scFv (Tom I, 1.5×10^8)	HER2	[18]
		<i>Ex vivo</i> , LCM/PA	NIL-h scFv (Tom I, 1.5×10^8)	No	[29]
		<i>In vivo</i> PT-BR	NIL-h scFv (Tom I, 1.5×10^8)	No	[44]
Cervix					
ACa	HeLa	<i>In vitro</i>	ILCL-1 sDAB	$\alpha_3\beta_1$ integrin	[64]
Colorectal					
ACa	PDT	<i>Ex vivo</i> , LCM	NIL-h scFv (Tom I, 1.5×10^8)	No	[28]
	Caco2	<i>In vitro</i> , PA	NIL-h scFv (Nissim $> 10^8$)	No	[65]
	SW480	<i>In vitro</i> , PA/S/DGC	ILCL-m Fab	No	[66]

Cancer type	Cells targeted	Selection system	Library Type (Diversity)	Identified Ag	Ref.
ACa/Ca		<i>In vitro</i> , S/PA/FACS	NIL-h scFv (de Kruif, 3.6×10^8)	EpCAM	[67]
	SW480, SW948, SW837	<i>In vitro</i> , S/DGC	ILCL-m Fab (7.1×10^6 and 2.1×10^6)	No	[68]
	Caco2, CW-2, SW480, +	<i>In vitro</i> , S	NIL-h scFv (AIMS-5)	@	[14]
Ca	PDT	<i>In vivo</i> PT-BR	NIL-h scFv (Tom I, 1.5×10^8)	No	[47]
Esophagus					
Squamous Ca	Eca109	<i>In vitro</i> , PA	NIL-h scFv (de Kruif, 3.6×10^8)	No	[69]
Gastric					
Ca	MKN45, NCI-N87, +	<i>In vitro</i> , S	NIL-h scFv (AIMS-5)	@	[14]
Hematological Malignancies					
AML	PDC	<i>Ex vivo</i> , S/PA	NIL-h scFv (de Kruif, 3.6×10^8)	NMNt, CD166, +	[70]
	PDC and HL-60	<i>In vitro</i> , S + <i>ex vivo</i> , S/FACS	NIL-h scFv (de Kruif, 3.6×10^8)	CLL-1	[71]
APML	HL-60	<i>In vitro</i> , PA/FACS	NIL-h scFv (Griffin 1, 1.2×10^9)	No	[72]
ATCL	Jurkat	<i>In vitro</i> , pathway+step-back	NIL-h scFv (Metha, 1.5×10^{10})	CD4, TIR	[21]
CLL	PDC	<i>Ex vivo</i> , S	IL-h, PD Fab	No	[73]
CML	K562	<i>In vitro</i> , single cell in PC	NIL-h scFv (TomI+J, 1.5 and 1.4×10^8)	No	[32]
Granulocytic Ly	PDC	<i>In vivo</i> PT-BR	NIL-h scFv (Tom I, 1.5×10^8)	No	[47]
Multiple myeloma	RPMI-8226	<i>In vitro</i> , S, Biotinylated PA	NIL-h scFv (Griffin 1, 1.2×10^9)	CD138	[74]
NHL	Raji and Daudi	<i>In vitro</i> , S	ILCL-m Fab (1.9×10^7)	No	[75]
	PDC	<i>Ex vivo</i> , S/PA/FPPE	NIL-h scFv (HuCAL, 1.6×10^{10})	Vimentin	[76]
Kidney					
Renal Ca	Caki-1, CFF-RC1, ACHN	<i>In vitro</i> , S	NIL-h scFv (AIMS-5)	@	[14]
Liver					
Hepatocellular Ca	PDC and HepG2, HLF, +	<i>Ex vivo</i> + <i>In vitro</i> , S	NIL-h scFv (AIMS-5)	@	[14]
	HepG2	<i>In vitro</i> , PA	IL-h Fab, PBMCs, (1.7×10^7)	No	[77]
Hepatocellular Ca	HepG2	<i>In vitro</i> , PA	NIL-h scFv (Griffin 1, 1.2×10^9)	No	[78]
Respiratory System					
NSCLC	A549	<i>In vitro</i> , S/PA	NIL-h scFv (NBL library, 6.4×10^9)	No	[79]
		<i>In vitro</i> , PA	NIL-1 sDab (5.4×10^8)	CEA6	[80]

Cancer type	Cells targeted	Selection system	Library Type (Diversity)	Identified Ag	Ref.
SCLC		<i>In vitro</i> , PA	IL-h, PD scFv (6×10^8)	PrxI	[81]
	H889	<i>In vitro</i> , PA	ILCL-m scFv (3.5×10^4 and 7.0×10^4)	PDC-E2	[82]
Lung Ca	A549, PC-14, Calu-3, +	<i>In vitro</i> , S	NIL-h scFv (AIMS-5)	@	[14]
Mesothelium					
Mesothelioma	M28, VAMT-1	<i>In vitro</i> , I/PA	NIL-h scFv (O'Connell, 5×10^8)	No	[83]
Ovarian					
Ca	SK-OV-3, RMG-1/-2, +	<i>In vitro</i> , S	NIL-h scFv (AIMS-5)	@	[14]
	SK-OV-3	<i>In vitro</i> , S	NIL-h scFv (Nissim $>10^8$)	EEF1A1	[84]
Pancreas					
Ductal ACa/ACa/Ca	Panc-1, BxPC-3, +	<i>In vitro</i> , S	NIL-h scFv (AIMS-5)	@	[14]
Ca	PDT	<i>In vivo</i> PT-BR	NIL-h scFv (Tom I, 1.5×10^8)	No	[44]
ACa	SW1990, HPAF-II	<i>In vitro</i> , I/PA	NIL-h scFv (Gao)	$\alpha_3\beta_1$ integrin	[85]
Prostate					
ACa	PPC-1	1 st <i>Ex vivo</i> , 2 nd <i>in vivo</i> , X	NIL-h DAB (Garvan, 3×10^9)	PA28 $\alpha\beta$ complex	[25]
	C4-2B	<i>In vitro</i> , I/PA	NIL-h scFv (5×10^8)	ICAM-1	[86]
Ca	22Rv1	<i>In vitro</i> , I/PA	NIL-h scFv (Gao)	No	[85]
Sarcomas					
Chondrosarcoma	PDT	<i>In vitro</i> PT-BR	NIL-h scFv (Tom I, 1.5×10^8)	No	[47]
Fibrosarcoma	HT-1080	<i>In vitro</i>	ILCL-m scFv (10^7)	Hsp90	[87]
		<i>In vitro</i>	ILCL-m scFv (10^7)	CD44	[20]
Osteosarcoma	U2-OS	<i>In vivo</i> PT-BR	IL-h PD, (2.7×10^8)	No	[88]
Skin					
Melanoma	PDT	<i>In vivo</i> PT-BR	NIL-h scFv (Tom I, 1.5×10^8)	No	[47]
	MeCoP	<i>In vitro</i> , S	NIL-h scFv (ETH-2-Gold 3×10^9)	Syndecan-1	[89]
	DM341-I, DM341-II, +	<i>In vitro</i>	ILCL-h, PD scFv	No	[90]
Squamous Ca	A431	<i>In vitro</i> , S/I/PA	NIL-h scFv (Sheets, 6.7×10^9)	EGFR	[15]

Table divided by origin of the cancer cells on which the selection is targeted. Cell targeted is the cell type on which the selection was performed. Library type is the type of phage antibody repertoire used for the selection(s). Identified antigen is the antibody cognate antigen, if identified; "No" means no antigen identified in the reference (Ref.). Table does not include selections performed on transfected cells or cells co-cultured with cancer cells or serum.

Abbreviations: *ACx*, Adenocarcinoma; *Ag*, Antigen; *A(P)ML*, Acute (pro)myelocytic leukaemia; *ATCL*, Acute T-Cell leukaemia; *Ca*, Carcinoma; *CD*, Cluster of differentiation; *CL*, Cell line; *CLL*, chronic lymphocytic leukemia; *CCL-1*, Chemokine (C-C motif) ligand 1; *CEA6*, carcinoembryonic antigen 6; *CML*, Chronic Myelogenous Leukemia; *DABs*, single-domain antibody; *DGC*, density gradient centrifugation; *EEF1A1*, eukaryotic translation elongation factor 1 alpha 1; *EGFR*, epidermal growth factor receptor; *EPCAM*, epithelial cell adhesion molecule; *gC1q-R*, *GRP78*, 78 kDa glucose-regulated protein; *h*, Human; *HER2*, human epidermal growth factor receptor 2; *Hsp90*, heat shock protein 90; *I*, selection for internalization; *ICAM-1*, Intercellular Adhesion Molecule 1; *IL*, Immune library; *ILCL*, Immune library from immunization with cell line; *I Llama*; *LCM*, Laser Capture Microdissection; *Ly*, Lymphoma; *m*, Mouse; *NHL*, non-Hodgkin lymphoma; *NIL*, Non-immune library; *NMNt*, Nicotinamide nucleotide adenyltransferase 1; *NS*, Nervous system; *NSCLC*, Non-small cell lung cancer; *PA*, Library pre-absorbed on cells before selection; *PC*, primary cells; *PDC-E2*, Pyruvate Dehydrogenase Complex E2 component; *PDT/C*, Patient Derived Tissue/Cells; *PtxI*, Paired Related Homeobox 1; *PT-BR*, Patient Tissue, selection before removal; *S*, selection in suspension; *SCLC*, small cell lung cancer; *scFv*, single-chain fragment variable; *Tom*, Tomlinson; *TfR*, Transferrin Receptor; *X*, Xenograft; “+”, More cell lines used for cell target/more antigens identified; @, antigens identified, selection on multiple cancer cell types and cell lines, but not specific from which selections the antibody against the antigens were raised.