Isolation of Human Tumor-specific Antibodies by Selection of an Antibody Phage Library on Melanoma Cells

Jörg-Michael Kupsch, Nick Harold Tidman, Norbert V. Kang, Hazel Truman, Stephen Hamilton, Nimesha Patel, Julia A. Newton Bishop, Irene May Leigh, and James Scott Crowe

Department of Immunopathology, Glaxo Wellcome Medicines Research Centre, Hertfordshire SG1 2NY [J-M. K., J. S. C.], Centre for Cutaneous Research [J-M. K., N. H. T., I. M. L.] and Imperial Cancer Research Fund Skin Tumor Laboratory [J. A. N. B.], Queen Mary and Westfield Medical School, London E1 2AT; and RAFT Institute of Plastic Surgery, Mount Vernon Hospital, Northwood, Middlesex HA6 2RN [J-M. K., N. V. K., H. T., S. H., N. P.], United Kingdom

ABSTRACT

A human single-chain Fv (scFv) library as fusion to phage was constructed from donors with a high titer of autoantibodies. The library was subjected to three rounds of positive selection on human melanoma cells and negative selection on human peripheral blood mononuclear cells. Two scFv clones, B3 and B4, were isolated that bound melanoma cells in cell ELISA and fluorescence-activated cell sorting. The scFvs were characterized further by immunohistochemistry on a large number of normal human tissues. No cross-reactivity with normal tissues was observed. On the other hand, the target antigens were expressed in sections from several different melanoma patients and in some breast cancer and basal cell carcinoma sections. The unusually high tumor specificity of the B3 and B4 antigens makes them attractive targets for the specific therapy of melanoma. The selection strategy used should be generally applicable to the identification of novel cell surface antigens by antibody phage display.

INTRODUCTION

MM is among the few types of tumors that are still on the increase, affecting a rising number of patients who can be as young as in their early 30s. The principle treatment for MM is excision surgery. Surgery can be curative if it is performed at an early stage. However, MM is resistant to standard radiotherapy and chemotherapy, and there is no effective treatment for metastatic disease (1). Consequently, experimental forms of treatment such as immunotherapy and gene therapy have been studied extensively in MM. A promising approach is targeted tumor therapy that is directed at tumor-specific antigens or genes.

The concept of targeted tumor therapy relies on the assumption that tumor-specific markers exist. However, despite extensive efforts over the past two decades to identify such markers by Mabs, few have been described (see Refs. 2 and 3 for a review). More recently, T-cell lines have been used with more success to identify tumor-specific antigens (4). These markers are antigens expressed on a variety of tumors including MM but not on most normal adult tissues. Melanocytic lineage-specific antigens are not tumor markers in the strict sense but nevertheless are quite melanoma specific. However, the intracellular expression of these antigens makes them unsuitable as targets for antibody-mediated therapy and prone to immune escape by modulation of HLA (allele) expression (5). In addition, the expression of these antigens does not seem to be essential for tumor biology and makes immune escape by antigenic loss possible (6). A number of tumor-associated (as opposed to tumor-specific) markers, the expression of which is up-regulated on tumor cells as compared to normal cells, have also been described for MM and other tumors. When these were used as therapeutic targets, clinical responses were observed, as were side effects due to damage of normal tissues expressing the marker (7).

The most melanoma-specific tumor antigen described is HMW. HMW is a surface marker expressed on MM only and not on a large panel of normal tissues including normal melanocytes and some fetal tissues or on the non-MM tumors tested (8, 9). Therefore, HMW is not melanocytic lineage-specific and is probably not an oncofetal antigen. HMW has been used extensively as a target for the diagnosis (10) and therapy of MM, and a vaccine-induced patient anti-idiotype immune response correlates with prolonged survival (11). However, HMW is shed (12), and the formation of immune complexes may interfere with some forms of antibody-mediated tumor targeting.

The abbreviations used are: MM, malignant melanoma; FACS, fluorescence-activated cell-sorting; HMW, high molecular weight melanoma-associated proteoglycan; Mab, monoclonal antibody; PBMC, peripheral blood mononuclear cell; scFv, single-chain Fv.

1 To whom requests for reprints should be addressed, at the RAFT Institute of Plastic Surgery, Mount Vernon Hospital, Northwood, Middlesex HA6 2RN, United Kingdom. Phone: 44-1923-844-212; Fax: 44-1923-844-031; E-mail: kupschj@raft.ac.uk.
2 Present address: Imperial Cancer Research Fund Cancer Medicine Research Unit, St. James University Hospital, Becket Street, Leeds LS9 7TF, United Kingdom.
3 Present address: Imperial Cancer Research Fund Cancer Medicine Research Unit, St. James University Hospital, Becket Street, Leeds LS9 7TF, United Kingdom.

4 The abbreviations used are: MM, malignant melanoma; FACS, fluorescence-activated cell-sorting; HMW, high molecular weight melanoma-associated proteoglycan; Mab, monoclonal antibody; PBMC, peripheral blood mononuclear cell; scFv, single-chain Fv.
Traditionally, tumor-associated antigens have been identified by hybridomas derived from mice immunized with tumor cells. More recently, antibody phage display has been used for the production of antibodies (13, 14). The main advantage of this technique is that human antibodies can be produced much easier than is possible with human hybridomas. These can then be developed for therapeutic use without the need to humanize mouse Mabs. Furthermore, by changing the selection conditions, the antigen binding properties of antibody phages can be influenced, e.g., by selection for high-affinity antibodies or specificity for a predetermined epitope (15, 16). The random pairing of V domains has the potential to generate artificial antibody specificities and could overcome the problem of the immune dominance of some antigens and epitopes in mice that limit the spectrum of specificities obtained with hybridomas (17). However, a major weakness of the phage display technique is the requirement of pure antigen for phage selection. Very few tumor antigens are available in this form. Therefore, only a few novel antigens have been identified by phage display.

In this communication, we describe a simple and generally applicable positive/negative selection strategy for antibody phage that obviates the need for pure antigen. Using this selection strategy, we have isolated two antibody phage clones that identify two novel melanoma antigens with high specificity for MM.

**MATERIALS AND METHODS**

**Materials.** The control scFv's used in this study were LHM2, RAFT3, and anti-CD18. The LHM2 scFv is derived from mouse Mab LHM2 and binds the melanoma-specific HMW antigen (9). RAFT3 scFv is derived from LHM2 by chain shuffling and binds the same epitope on HMW but with higher affinity. Anti-CD18 scFv is a human scFv that binds human CD18 that is not expressed on MM and was used as a negative control (9).

The cell lines used were human melanoma cell lines DX3 (18) and HMB2 (19), the human fibroblast cell line J5, the human hepatoma cell line Hep2 (American Type Culture Collection), and the human keratinocyte cell line SVK14 (20).

**Cell ELISA.** Donor sera were screened for increased autoantibody titer by ELISA on the human melanoma cell lines DX3 and HMB2. Serial dilutions of sera (in RPMI 1640 + 10% FCS + 0.05% sodium azide) were incubated with cells grown in 96-well microtiter plates for 1 h at room temperature followed by rabbit antihuman immunoglobulin horseradish peroxidase conjugate (DAKO). p-Phenylenediamine dihydrochloride was used as a substrate.

Cell ELISA is difficult to quantify due to background variability from plate to plate. Therefore, we defined sera as positive when >5 × background staining was seen at a dilution of 1:20. Initial testing of sera was on human melanoma cell lines DX3 and HMB2. Candidate positive sera were rescreened on DX3 and HMB2 together with several negative sera. Sera that were positive on melanoma cell lines were further screened on human nonmelanoma cell lines SVK14, Hep2, and J5. Sera that were not reproducibly positive on all five cell lines were scored as negative and excluded from further analysis. The serum immunoglobulin titer of positive sera and a representative number of negative sera chosen at random was measured by direct ELISA using the same secondary reagents and a standard of human immunoglobulin of known quantity.

Preliminary screening of scFv clones by cell ELISA was as described previously (9). Briefly, serial dilutions of periplasmic extracts of *Escherichia coli* HB2151 (21) containing scFv were incubated with DX3 or J5 cells in 96-well microtiter plates followed by anti-c-myc hybridoma supernantant 9E10 (American Type Culture Collection) detecting the COOH-terminal tag for scFv encoded by the vector pCantab-5 (Pharmacia) and rabbit antimouse immunoglobulin horseradish peroxidase conjugate (DAKO). For a more detailed analysis, pure scFv was used with the human melanoma cell line DX3.

**Antibody Phage Library Construction.** PBMCs (5 × 10^9 each) of 10 human donors with increased autoantibody titer were purified, and total RNA (RNA Isolation Kit; Stratagene), mRNA (Dynabeads mRNA Purification Kit; Dynal), and first-strand cDNA (SuperScript cDNA Kit; Life Technologies, Inc.) were prepared. Equal amounts of cDNAs were pooled for V gene segment-specific PCRs. Primers for the first round of PCR were as described previously (22), with the following modifications: the linker-encoding sequences were redesigned to minimize slippage mispairing and to encode a NarI site that results in a Gly<sub>4</sub>AlaGly<sub>4</sub>Ser<sub>2</sub> linker. A primary V<sub>H</sub> library of 1.7 × 10<sup>9</sup> clones was constructed as NarI/NorI inserts in pUC18Not (pUC18 with a 740-bp BamHI/PstI insert of pCantab-5; Pharmacia). Similarly, a V<sub>λ</sub> library of 1.6 × 10<sup>9</sup> clones as SfiI/NarI inserts in pCantab-5 was made. DNA of the V<sub>H</sub> and V<sub>λ</sub> libraries was extensively digested with NarI, ligated, and digested with SfiI and NorI, and the ~750-bp fragment was purified. The scFv-encoding fragment was reamplified using the SfiI and NorI primers and ligated with pCantab-5. Aliquots of the ligation were electroporated into *E. coli* TG1 cells and plated at 30°C in the presence of 2% D-glucose. Colonies (9.3 × 10<sup>9</sup>) were obtained and rescued by superinfection with the M13KO7 helper phage as described previously (9).

**Library Selection.** Library selection was essentially as described previously (9), except for an additional preclearing step. Briefly, 5 × 10<sup>12</sup> tdu of polyethylene glycol-precipitated phages were incubated with 5 × 10<sup>9</sup> human PBMCs in 1 ml of PBS + 3% skimmed milk + 0.05% sodium azide (1 h at 4°C or room temperature). The precleared phage supernatant was then incubated for 1 h with 5 × 10<sup>6</sup> DX3 melanoma cells. Cells were washed six times with 12 ml of PBS, and bound phages were eluted at pH 2.8. The eluate was neutralized and used to transduce *E. coli* TG1 cells. The phage eluate titer after selection rounds 1, 2, and 3 was 4.4 × 10<sup>9</sup>, 2.4 × 10<sup>9</sup>, and 5.6 × 10<sup>8</sup>, respectively. Detailed analysis of clones was performed after transduction of eluate 3 into *E. coli* HB2151.

For further characterization, scFvs were subcloned into pUC119 His<sub>6</sub>Xba (a kind gift of G. Winter, Cambridge Centre for Protein Engineering, MRC, Cambridge, United Kingdom), and scFv protein was purified by immobilized metal ion affinity chromatography.

**Immunohistology.** ScFv-containing bacterial periplasmic extracts were incubated with 10-μm cryostat sections obtained from human biopsies or cadavers followed by anti-c-myc hybridoma 9E10, rabbit antimouse immunoglobulin biotin (DAKO), and avidin fluorescein (Amersham). Sections were counterstained with propidium iodide. For the analysis of
non-MM sections, MM sections were stained in parallel using anti-HMW scFv RAFT3 as a positive control.

**FACS Analysis.** DX3 melanoma cells (5 $\times$ 10$^5$) were incubated with 5 $\mu$g of pure scFv in PBS + 0.1% BSA for 1 h on ice. Cells were washed three times with PBS and incubated with 9E10 hybridoma supernatant followed by goat antimouse IgG FITC for 1 h each. Samples were analyzed on a Becton Dickinson FACScan.

**RESULTS AND DISCUSSION**

It has been shown that the antibody phage of a given antigen specificity can be isolated more readily from libraries derived from immunized donor mice than from those derived from naive donor mice (23). In an attempt to construct a human antibody phage library with an increased abundance of antibody phages with anti-self specificities, we screened the sera of 183 melanoma patients and relatives of patients with hereditary melanoma for autoantibodies by cell ELISA on the human melanoma cell lines DX3 and HMB2. The LHM2 scFv directed at HMW and, in some experiments, its derivative, RAFT3 (9), were used as a positive control. Anti-CD18 scFv directed against human CD18 that was not expressed on MM was used as a negative control in these and all other experiments. Sixteen sera were reproducibly positive on melanoma cells (see “Materials and Methods”). An example is shown in Fig. 1. The same sera also had an increased anti-self antibody titer when tested on the nonmelanoma cell lines SVK14, Hep2, and J5 (data not shown). The level of serum immunoglobulin varied in the same range for MM-positive and -negative sera; therefore, it does not correlate with increased serum reactivity in cell ELISA (Fig. 2). There was no correlation between autoantibody titer and clinical status or prognosis within the melanoma patient group (data not shown). PBMCs of 10 such individuals were used as a source for cDNA preparation.

V gene segments were amplified by PCR, and a scFv fusion phage library of 9.3 $\times$ 10$^7$ clones in vector pCantab-5 (Pharmacia) was obtained. The vector contains a COOH-terminal c-myc tag that allows detection of scFv with the anti-c-myc Mab 9E10. To avoid the isolation of antibody phages binding common rather than tumor-specific antigens, we included a negative selection step on normal human cells. Each round of phage selection consisted of preclearing on human PBMCs and positive selection on DX3 human melanoma cells followed by antibody phage amplification.
in bacteria. We observed a significant increase in the phage titer in the eluate of the third round of selection.

ScFv clones from the third round of selection were initially screened by cell ELISA on DX3 human melanoma cells versus the human fibroblast cell line J5. Of 44 clones tested, 36 were strongly positive on DX3 cells and weakly positive on J5 cells (Fig. 3). When these clones were tested by BstO I digestion, two different restriction patterns were observed. Clone B3 was isolated 34 times, and clone B4 was isolated twice. Specific melanoma cell binding of the B3 and B4 scFvs was confirmed by FACS staining (Fig. 4). ScFv RAFT3, a derivative of LHM2 scFv specific for HMW (9), was used as a positive control, and antihuman CD18 scFv (9) was used as a negative control.

An attempt was made to isolate MM binding scFvs other than B3 and B4 by using MM cells for phage selection that had been saturated with B3 and B4 scFv to block these antigens. Twelve scFv clones each were tested after selection rounds 3, 4, and 5. No BstO I restriction patterns similar to those of B3 and B4 were observed, indicating that the block with scFv was complete. However, when the clones were tested for MM reactivity by cell ELISA, no binding to MM was observed (data not shown). This shows that B3 and B4 are the only anti-MM scFv fusion phage specificities in our library that can be selected by our method (see below).

The scFvs B3 and B4 were tested on a panel of sections of MM and 17 normal human tissues. Neither B3 nor B4 cross-reacted with normal tissues, except for the weak reactivity of B3 with normal liver (Table 1). Both scFvs were positive on all melanoma sections obtained from several different patients (Fig. 5; Table 2). Staining by B3 and B4 scFvs was granular, whereas staining by the RAFT3 scFv used as a control was diffuse. The scFvs were further tested on a small panel of non-MM tumor sections available to us (Table 2). Both scFv clones stained basal cell carcinoma sections. B3 also stained breast carcinoma sections.

Several groups have successfully selected antibody phage libraries on whole cells. Portolano et al. (24) used pairs of untransfected and transfected COS cells for library preclearing/selection. Similarly, combinations of erythrocytes of different blood groups have been used to select blood group-specific antibody phages (25). This approach is impractical for MM because normal melanocytes are difficult to grow in numbers sufficient for preclearing.

Although effective in principle, these approaches require the availability of cloned genes or mutant cell lines, which is difficult to achieve for novel antigens. Cai and Garren (26) have therefore selected antibody phages on melanoma cells without preclearing. Of a very large number of melanoma cell-binding scFv
clones tested in cell ELISA, 2% did not cross-react with fibroblasts and endothelial cells used as a negative control. However, antigen expression in cell lines is not representative of antigen expression in situ (27). For example, the reactivity of B3 and B4 scFvs in cell ELISA is different from antigen binding in situ when tested by immunohistology (compare the weakly positive staining of J5 fibroblasts in Fig. 3 with no staining of fibroblasts present in normal skin; Table 1). Therefore, the MM specificity of scFv clones tested only on cell lines has yet to be demonstrated.

More recently, Noronha et al. (28) have selected a semisynthetic scFv phage library on human melanoma cells. The authors performed four rounds of selection on melanoma cells, followed by extensive postabsorption on human B lymphoid cell lines, but without amplification in bacteria. About a quarter of the clones analyzed reacted weakly with melanoma and were shown to bind HMW. It was concluded that this antigen is immunodominant in vitro. However, the use of a semisynthetic phage library with identical V<sub>H</sub> domains and diversity in the V<sub>K</sub> CDR3 only (29) may have biased the result of this study. Although convenient, a potential disadvantage of using cell lines for library clearing is that immortal cells can be expected to up-regulate the expression of antigens associated with proliferation that could also be tumor markers and thereby result in the loss of tumor-reactive scFv clones during selection.

We adopted a different approach to the preclearing problem and used PBMCs as a highly diverse and abundant source of normal human cells. We analyzed only a relatively small number of anti-MM scFv clones and found two predominant clones that react with MM but not with normal human tissues. The frequency of isolation of MM-reactive scFv by our method is higher than in previous studies, suggesting a better efficiency of selection. The B3 and B4 scFvs also react very strongly with MM in vitro and in situ. The most likely explanation for this discrepancy with previous work is that the preclearing step included in our library selection procedure for each round of selection is critical. Bacterial amplification of phage after each round of positive/negative selection can be expected to favor positively selected clones and disadvantage PBMC-reactive phage at the same time. Another possibility is that the selection of donors with high autoantibody titers for library construction increased the abundance of scFvs with anti-self specificity.

It is possible that part of the scFv specificity for MM cells is derived from donors, although it is very unlikely that a phage library of 10<sup>8</sup> clones contains the original V<sub>H</sub>V<sub>K</sub> pairings, which would require a phage library of at least 10<sup>14</sup> clones. The V<sub>H</sub> segments belong to subgroups V<sub>H</sub>-1, V<sub>H</sub>-1 (B3), and V<sub>H</sub>-2, V<sub>K</sub>-2 (B4; Ref. 30; data not shown).

Table 1  Reactivity of the B3 and B4 scFvs with normal tissues

<table>
<thead>
<tr>
<th>Tissue</th>
<th>B3</th>
<th>B4</th>
<th>LHM2 scFv</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melanocytes</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Normal skin</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Spinal cord</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Esophagus</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Stomach</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Ileum</td>
<td>−</td>
<td>−</td>
<td>±</td>
</tr>
<tr>
<td>Colon</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Lymph node</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Spleen</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Breast</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Liver</td>
<td>±</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Kidney</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Ureter</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Ovary</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Synovium</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

<sup>a</sup> −, no staining; ±, very weak staining; +, intermediate staining.

The isolation of only two MM-reactive scFv clones by selection on whole cells was surprising and was not observed when another phage library was selected on MM cells by the same method. Interestingly, the frequency with which B3 and B4 were isolated correlated with the level of expression of the antigens (Fig. 4). We speculated that the predominant clones B3 and B4 could have overgrown other antibody fusion phases during selection that are less abundant or bind antigens expressed at lower levels. An attempt was made to select scFvs other than B3 and B4 using MM cells saturated with purified B3 and B4 scFv for phage selection. Although binding of B3 and B4 phage could be blocked completely, no antibody phages with different MM specificities were isolated (data not shown). This does not necessarily mean that there are only two MM-specific antibody phages in our library. The assay used to screen scFv clones is relatively insensitive. Greater than 10<sup>6</sup> antigens/cell and an antibody affinity of K<sub>a</sub> above 10<sup>6</sup> m are required for detectability in cell ELISA. Of note, our selection protocol failed to isolate anti-MM scFvs from the same semisynthetic antibody phage library used by Noronha et al. (28) and Nissim et al. (29). In spite of an increase of more than 10<sup>4</sup>-fold in phage titer, all scFv clones tested in cell ELISA after selection rounds 3 and 4 were scored as negative (data not shown). Therefore, our selection and screening procedure appears to be more stringent. It has been shown that high affinity of scFvs and high levels of target antigen expression are favorable for tumor targeting in vivo (31, 32). Therefore, we did not characterize clones that were scored negative in cell ELISA any further.

We have shown that the expression of the B3 and B4 antigens is very tumor specific by immunohistology. The only known MM surface antigen of comparably high tumor specificity is HMW. This raises the question of whether the B3 and B4 scFvs could bind epitopes on HMW. We tried to characterize the B3 and B4 antigens further by Western blot and immunoprecipitation of MM cell extracts, but without success (data not shown). The identity of the B3 and B4 antigens is therefore unknown. The differences observed between B3, B4, and the anti-HMW scFvs LHM2 and RAFT3 in tissue section staining patterns, cellular distribution of antigen, and expression levels suggest that the antigens are not identical. Furthermore, we have selected our phage library on keratinocyte subpopulations that express HMW antigen without success, indicating that B3 and B4 are not directed against this antigen. However, we cannot exclude the possibility that these differences are caused by differ-

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7 Kang and J-M. Kupisch, unpublished observations.
8 Hall and J-M. Kupisch, unpublished observations.
ences in posttranscriptional modifications that could affect epitopes of the same antigen in different tumor cell types. The concept of specific tumor therapy is based on the assumption that tumor-specific antigens actually exist, and this has been proven only for private tumor antigens, which are of limited use in tumor therapy and diagnosis (4). We have shown that antibodies defining tumor-specific antigens can be selected from an antibody phage library by a relatively simple procedure that, unlike previously described selection methods, does not require the availability of purified antigen, cloned genes, or the analysis of large numbers of clones.

Hybridoma technology has been used with tremendous success for the identification of novel cell surface antigens by mouse Mabs. However, the production of human antibodies by hybridomas for therapeutic applications has consistently given poor results (33). Our scFv clones react with antigens that are also expressed on tumors other than MM. Therefore, our approach should be applicable to the isolation of human antibodies against tumor markers or novel cell surface markers in general.

The human antitumor scFvs B3 and B4 have a large number of potential clinical applications (34). Begent et al. (35) have recently shown that an anti-CEA scFv binds tumor cells with high specificity in patients. ScFvs are therefore of diagnostic value for the detection of metastatic disease by radioimaging of patients (36). Whole human tumor-specific antibodies or scFv fusions to effector molecules such as toxins could be engineered for use in passive antibody-mediated immunotherapy. Another application would be the use of recombinant antibodies in targeted drug or gene delivery (37).

The tumor specificity and limited variability of B3 and B4 antigen expression on tumor cells suggests that the antigens may play an important role in tumor biology that warrants further characterization. The B3 and B4 antigens may therefore have a potential use as targets for other approaches to specific tumor therapy such as tumor vaccine construction or gene therapy.

**Table 2** Reactivity of the B3 and B4 scFvs with tumor tissues

<table>
<thead>
<tr>
<th>Tissue</th>
<th>B3</th>
<th>B4</th>
<th>LHM2 scFv</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metastatic melanoma (5)</td>
<td>+/++ (5)</td>
<td>+/++ (5)</td>
<td>+/++ (5)</td>
</tr>
<tr>
<td>Superficial melanoma (1)</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Colon carcinoma (2)</td>
<td>-</td>
<td>-</td>
<td>+ (1)</td>
</tr>
<tr>
<td>Breast carcinoma (2)</td>
<td>+ (1)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Squamous cell carcinoma (2)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Basal cell carcinoma (2)</td>
<td>± (2)</td>
<td>+ (2)</td>
<td>++ (2)</td>
</tr>
</tbody>
</table>

*The number of sections from different patients is shown in parentheses. -, no staining; ±, very weak staining; +, intermediate staining; ++, strong staining; ++++, very strong staining.

Fig. 5 Staining of cryosections of human metastatic melanoma with scFv B3 (top left), B4 (top right), RAFT3 (bottom left), and anti-c-myc Mab 9E10 alone (bottom right).
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