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Scanning the Cell Surface Proteome of Cancer Cells and Identification of Metastasis-Associated Proteins Using a Subtractive Immunization Strategy

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Identification of the cell surface proteome and comparison of their expression between cells with different phenotypic characteristics is crucial to the discovery of novel cancer drug targets as well as elucidating the basic biologic processes of cancer. However, cell surface proteomics are complex and technologically challenging, and no ideal method is currently available. Here, we describe a strategy that allows scanning of the entire cell surface and identification of molecules that exhibit altered expression between two cell types. Concurrently, this method gives rise to valuable reagents for further characterization of the identified proteins. The strategy is based on subtractive immunization of mice, and we used the two isogenic cell lines, NM-2C5 and M-4A4, derived from the MDA-MB-435 cancer cell line, as a model system. Although the two cell lines are equally tumorigenic, only M-4A4 has metastatic capabilities. Our results yielded a large panel of monoclonal antibodies (mAbs) that recognized cell surface markers preferentially or exclusively expressed on metastatic vs nonmetastatic cancer cells. Four mAbs and their corresponding antigens were further characterized. Importantly, analysis on an extended panel of breast cancer cell lines demonstrated that the four mAbs bound preferentially to cell lines known to be metastatic in vivo, suggesting that these markers have general applications. Immunohistochemical analysis showed that mAb 11E6 reacted preferentially with neuroendocrine tumors while exhibiting no or very weak reactivity with normal tissues. mAb 15C7 stained a variety of cancers as well as some normal lymphoid organs and was subsequently identified to react with HLA-DR-β. A third mAb, 31D7, that also specifically recognized HLA-DR-β was capable of inhibiting the growth of MZ2 melanoma cells in vitro. Further, we found that the reduced expression of HLA-DR antigens in nonmetastatic cells of this model was not regulated by class II transactivator, but by posttranscriptional mechanisms. The study demonstrates the advantage of using the exquisitely discriminating recognition system of the immune system itself to scan the cell surface proteome for differentially expressed proteins. The subtractive immunization strategy should be broadly applicable as a quantitative and comparative proteomic approach for screening the cell surface and also allow generation of mAbs to study these cell surface antigens in more detail.

Keywords: Subtractive immunization • HLA-DR • breast cancer cell lines • metastases • M-4A4 • NM-2C5 • CIITA

Introduction

Cancer is one of the most common diseases in the western world, and despite significant therapeutic progress, mortality remains high in cases where the primary tumor has metastasized. The spread of cancer cells from a primary tumor to form metastases at distal sites is a complex multistep process requiring genetic and epigenetic changes.[1−3] During this process, the cell-surface protein composition changes due to alterations in expression levels,[4] reappearance of fetal antigens,[5] or changes in the post-translational modifications of cell-surface proteins.[6] Several specific alterations have been correlated to tumor progression, metastatic potential, and patient survival.[7,8] A large number of studies have investigated the use of these altered proteins as targets for cancer therapy, resulting in several useful clinical reagents.[7,9,10] Advancing this therapeutic utility requires further study of the metastatic process to identify novel surface markers that are preferentially or exclusively expressed on metastatic breast cancer cells as potential targets for cancer treatments. Novel proteomic approaches in combination with subcellular fractionation procedures has recently made it possible to study the plasma

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membrane proteome in more detail. We and others have optimized methods for plasma membrane fractionation of SILAC-labeled cells and subsequent LC-MS/MS analysis, which has led to promising results.[11−13] Alternative strategies involving biotinylation of the cell surface (either or not in combination with SILAC labeling) followed by affinity-purification of plasma membranes and LC-MS/MS have been developed with interesting results.[14,15] However, even these improved methods have limitations in the number of identified plasma membrane proteins with altered expression, and not all of these are exposed on the cell surface. Another problem with MS-based proteome identification of cell surface proteins may be the lack of useful reagents to further characterize the potentially interesting cell surface proteins in case no commercial antibodies are available.

Subtractive immunization of mice has previously been shown to be an effective technique for the generation of monoclonal antibodies against weakly immunogenic or rare antigens.[16] In addition, subtractive immunization may allow for generation of monoclonal antibodies capable of distinguishing between two highly similar cell types, as demonstrated by using a metastatic human epidermoid carcinoma cell line system and a human melanoma cell line system.[17,18]

The use of cell lines with similar genetic backgrounds, but different phenotypes with respect to metastatic potential, may provide a model system for identification of key cell surface proteins involved in the metastatic process. The two isogenic cell lines, NM-2C5 and M-4A4,[19] derived from the MDA-MB-435 cell line, could serve this purpose in that they are equally tumorigenic but have diametrically opposite metastatic capabilities. In athymic mice inoculated with M-4A4 cells, lung metastases were detected in 74%, while no metastases developed in mice inoculated with NM-2C5 cells.[19] Identifying differentially expressed surface proteins on these cell lines could potentially provide novel clinically relevant prognostic markers and/or targets for cancer immunotherapy.

The expression of HLA class II molecules on cancer cells has been reported in several cancers[20,21] and cancer cell lines[22] with various degrees of prognostic significance. In breast tissue, HLA class II molecules are expressed more often on benign lesions than invasive breast cancer tumors, and only rarely on metastatic breast cancer carcinomas,[23] although no significant correlation between HLA-DR expression and prognosis has been identified. Transcription of HLA-DR is regulated by the class II transactivator (CIITA), a non-DNA binding protein that also regulates transcription of CD74.[24] CIITA, in turn, is transcriptionally regulated by a number of mechanisms, including promoter methylation[22] and post-translational modifications.[25] Several studies have shown that the loss of HLA expression often observed in metastatic cell lines and metastatic lesions is caused by loss of CIITA.[22,26]

This study was designed to scan the cell surface proteome and identify proteins expressed at a higher level in metastatic vs nonmetastatic cancer cells. Mice were therefore immunized with the nonmetastatic cancer cell line NM-2C5 and treated with cyclophosphamide to render them immunotolerant to NM-2C5 expressed antigens. Following tolerization, mice were immunized with the metastatic cancer cell line M-4A4, and hybridomas produced by standard protocols, followed by screening on whole cells. Several of the generated mAbs reacted exclusively or preferentially with the metastatic M-4A4 cell line, and among this group of mAbs, some recognized unknown cancer-associated markers while others recognized HLA class II antigens that we subsequently demonstrated to be exclusively expressed on the metastatic cell line because HLA-DR was down-regulated on the nonmetastatic NM-2C5 cells. We confirmed that this was not caused by loss of CIITA, and that transcripts for HLA-DR-α, HLA-DR-β, and CD74 were present in both cell lines, indicating that, in the NM-2C5 cell line, HLA-DR is regulated post-transcriptionally.

Experimental Procedures

Cell Lines. The cell lines M-4A4 and NM-2C5 were a kind gift of Dr. D. Tarin, UCSD Medical Center, La Jolla, CA. The colon cancer cell line Colon 137 was obtained from Dr. P. Ebbesen, Danish Cancer Society, Aarhus, Denmark, and the medullary breast cancer cell line BrCa-MZ-01 was a gift from Dr. V.J. Möbus, University of Ulm, Ulm, Germany. All other cell lines were obtained from American Type Culture Collection and were maintained in DMEM (Gibco, Invitrogen, Taastrup, Denmark) supplemented with 10% FBS (Gibco), penicillin/streptomycin (Gibco), and nonessential amino acids (Gibco). Cultures were grown in a humidified atmosphere of 5% CO_2 at 37 C.

Subtractive Immunization and Hybridoma Production. C56 BL6 male mice, 6−8 weeks old and weighing 16−18 g, were purchased from Taconic Europe (Ejby, Denmark). Subtractive immunization was performed as previously described.[17] In brief, on day 1, subconfluent NM-2C5 cells were washed 3× with sterile PBS and harvested by scraping. Single cell suspensions were washed and resuspended in sterile PBS. Two × 10^6 NM-2C5 cells in 500 μL PBS were injected ip into the C56 BL6 mice. At 24 and 48 h, mice were injected ip with 200 mg/kg cychlophosphamide (Sigma-Aldrich, Brøndby, Denmark) in PBS. On day 18, M-4A4 cells were harvested as described above and 2 × 10^6 cells were injected ip into the mice. On day 39, the mice received an ip boost of 3 × 10^6 M-4A4 cells, and on day 43, the mice were sacrificed and spleens recovered. Hybridomas were produced as previously described.[27] Briefly, spleens were removed, washed in ice-cold RPMI (Gibco), homogenized, and spleen cells fused 4:1 with myeloma (SP2 IL6) cells. The cells were plated in 96-well tissue plates (CM lab, Vordingborg, Denmark) (2.5 × 10^4 cells/0.1 mL HAT medium (Gibco)), and culture supernatants were screened in whole cell ELISA. Positive clones were subcloned by serial dilution three times.

Whole Cell ELISA. Five × 10^3 cells/well were seeded in a 96-well tissue plate (CM lab) one day prior to antibody screening. Culture medium was removed and cells fixed for 10 min with 4% formaldehyde (Sigma-Aldrich) in PBS. The plates were washed 2× with 0.05% Tween-20 in PBS and blocked for 1 h with 2% BSA in PBS. Plates were washed once, and hybridoma supernatants were added to the wells and incubated for 1 h at room temperature. Subsequently, plates were washed 3× with 0.05% Tween-20 in PBS and bound antibody detected with HRP-labeled goat antimouse mAb 1:5000 (Jackson Immunotech, Suffolk, UK). The plates were washed, and 100 μL of OPD substrate (Kem-En-Tec, Taastrup, Denmark) added and incubated for 10 min at room temperature. The reaction was stopped by adding 1 M H_2SO_4, and the plate was read at 492 nm on a Sunrise plate reader (Tecan Group Ltd., Männedorf, Switzerland).

Monoclonal Antibody Purification. Hybridoma conditioned media was centrifuged at 3000*g* for 30 min and precipitated overnight at 4 C by adding 4.1 M ammonium sulfate (Sigma), pH 7.0 (500 mL media to 500 mL ammonium sulfate). The

suspension was centrifuged at 4000*g* for 30 min and the supernatant carefully removed. The pellet was resuspended in 25 mL of PBS and 2.7 mL 1 M NaAcetate (Sigma), pH 5.0, was added prior to purification. The resuspended pellet was sterile filtrated and applied to a HiTrap Protein G HP column (GE Healthcare, Hillerød, Denmark) connected to a fast protein liquid chromatography (FPLC) system (GE Healthcare). The antibody was eluted with 0.1 M glycine (Sigma), pH 2.7. Purified mAbs were immediately neutralized with 1 M Tris (Sigma), pH 8.6, dialyzed against PBS, and stored at 4 C.

Flow Cytometry. Cells were harvested by scraping, washed once in PBS, and counted. 2.5×10^5 cells were incubated for 1 h with antibody in 500 μL of PBS containing 5 μg/mL mAb. Cells were washed twice with PBS and incubated with FITC-conjugated goat antimouse IgG (Dako, Glostrup, Denmark) 1:500 in 500 μL of PBS for 1 h. After washing the cells twice in PBS, the stained cells were resuspended in 500 μL of PBS and analyzed on a FACS Calibre (Becton Dickinson labware). The collected data was analyzed using FlowJo software (TreeStar Inc., Ashland, OR).

Western Blotting. SDS-electrophoresis was run in a 4−20% Precise gradient gel (Pierce, VWR, Herlev, Denmark) and transferred to a Hybond-P membrane (Amersham Biosciences, GE Healthcare, Hillerød, Denmark). The membrane was blocked in 5% nonfat dry milk/0.05% PBS-Tween overnight at 4 C. The membrane was rinsed once in PBS-Tween and incubated with primary antibody (1−10 μg/mL) for 1 h. The following commercial antibodies were used: anti-HLA-DR-α (TAL 1B5) (Abcam, Cambridge, UK), anti-HLA-DR-β (TAL 14.1) (Abcam), and anti-p53 (sc-126) (Santa Cruz Biotechnology, Santa Cruz, CA). Subsequently, the membrane was washed and incubated with HRP-antimouse (Dako) 1:5000 for 1 h. Following another wash, visualization was performed with ECL-kit (Pierce).

Immunoprecipitation. mAb 15C7 (1 μg) was incubated with 100 μL of total MZ2 cell lysate, 50 μL of protein G Sepharose slurry (GE healthcare), and 400 μL of PBS for 4 h at 4 C, rocking. The protein G Sepharose was washed three times with 400 μL of PBS on Sigmaprep (Sigma) columns and bound proteins eluted by adding 50 μL of SDS-PAGE sample buffer and boiling for 5 min. These samples were separated by SDS-PAGE, and Western blotting was performed as described above.

Cell Growth Inhibition Assay. Cells (5000 per well) in 100 μL of DMEM (Gibco) supplemented with 10% FBS (Gibco), penicillin/streptomycin, and nonessential amino acids were seeded in a 96-well culture plate. Antibody was added to a final concentration of 0.5, 5, 50, and 100 μg/mL and incubated for 4 days. The number of viable cells was determined using the MTT assay (Invitrogen). All concentrations were done in pentaplicate and repeated $\times$4. Standard deviations were calculated using Excel.

Immunocytochemistry. Plasma/thrombin cell blocks were generated from the different cell lines by adding 50 μL of plasma to 5×10^5 cells followed by 35 μL of bovine thrombin (Biofac A/S, Ejby, Denmark), resulting in formation of a clot surrounding the cells. The plasma/thrombin cell clots and patient tissues biopsies were fixed in 4% formaldehyde, pH 7.4, for 24 h. Sections were cut, deparaffinized, and processed as for immunohistochemistry, as described below. For immunocytochemistry on live cells, cells were harvested by scraping, washed once in PBS, and counted. 2.5×10^5 cells were incubated for 1 h with antibody 1:50 in 100 μL PBS. Cells were washed twice with PBS and incubated with FITC-conjugated goat antimouse (Dako) 1:200 for 1 h. After washing the cells twice with PBS, the stained cells were resuspended in 10 μL of ProLong Gold antifade reagent (Invitrogen), mounted on a microscope slide, and analyzed on a Leica fluorescence microscope.

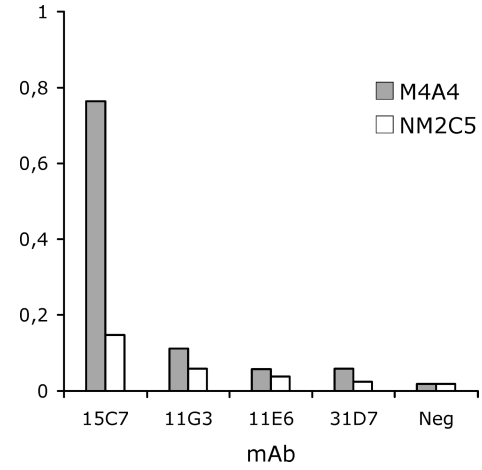

Figure 1. Binding of mAbs 31D7, 15C7, 11G3, and 11E6 to NM-2C5 and M4A4 in whole cell ELISA. An isotype-matched mAb was included as a negative control. The ELISA was repeated three times. Results from a representative experiment are shown. The four isolated mAbs exhibited higher reactivity with the metastatic cell line M-4A4 than with NM-2C5.

Immunohistochemistry. All tissues were fixed in 4% formaldehyde, pH 7.4, for 24 h and embedded in paraffin. Sections were deparaffinized and treated with 1.5% H2O2 in Tris-buffered saline (TBS), pH 7.5, for 10 min to block endogenous peroxidase activity. Antigen retrieval was performed by microwave boiling in T-EG buffer for 15 min, followed by a wash in TNT buffer (0.1 M Tris, 0.15 M NaCl, 0.05% Tween-20, pH 7.5). Sections were then incubated with primary antibody in antibody diluent (S2022, Dako) for 1 h at room temperature, washed with TNT buffer, and incubated with horseradish peroxidase (HRP)-conjugated "Ready-to-Use" EnVisionTM polymer K4001 (mouse mAb) or polymer K4003 (rabbit pAb)(Dako) for 30 min, followed by another wash with TNT buffer. The final reaction product was visualized by incubating with 3,3′-diaminobenzidine (DAB)+substrate-chromogen (Dako) or 3-amino-9-ethyl-carbazole (AEC)+ for 10 min, followed by washing with H_2O and counterstaining of sections with Mayers hematoxylin before mounting in AquaTex (Merck Inc., Whitehouse Station, NJ). For each experiment, samples of either an isotype-matched antibody or no primary antibody were included as controls.

RNA Purification, cDNA Synthesis, and Real-Time PCR. RNA was isolated from MDA-MB-435, M-4A4, and NM-2C5 cells using TRIzol reagent (Invitrogen) and reverse transcribed using SuperScript III reverse transcriptase (Invitrogen). Real-time PCR was performed on a Applied Biosystems 7500 real-time PCR system using Power Sybr Green master mix (Bio-Rad, Copenhagen, Denmark) according to manufacturer's instructions. The following primers were used: CIITApIV (for: gaa cag cgg cag ctc aca; rev: tct cca gcc agg tcc atc tg), HLA-DR-α (for: gcc aac ctg gaa atc atg aca; rev: agg gct gtt cgt gag cac a), HLA-DR-β (for: ggc ctg atc cag aat gga gat; rev: atg agg cgc tgt cat caa tgc), CD74 (for: tga cca gcg cg acct tat ct; rev: gag cag gtg cat cac atg gt), GAPDH (for: ccg tct aga aaa acc tgt c; rev: gcc aaa ttc gtt gtc ata cc). Thermocycler parameters were 3 min at 95 C and 40 cycles of denaturation at 95 C for 15 s, annealing/extension at 60 C for 1 min.

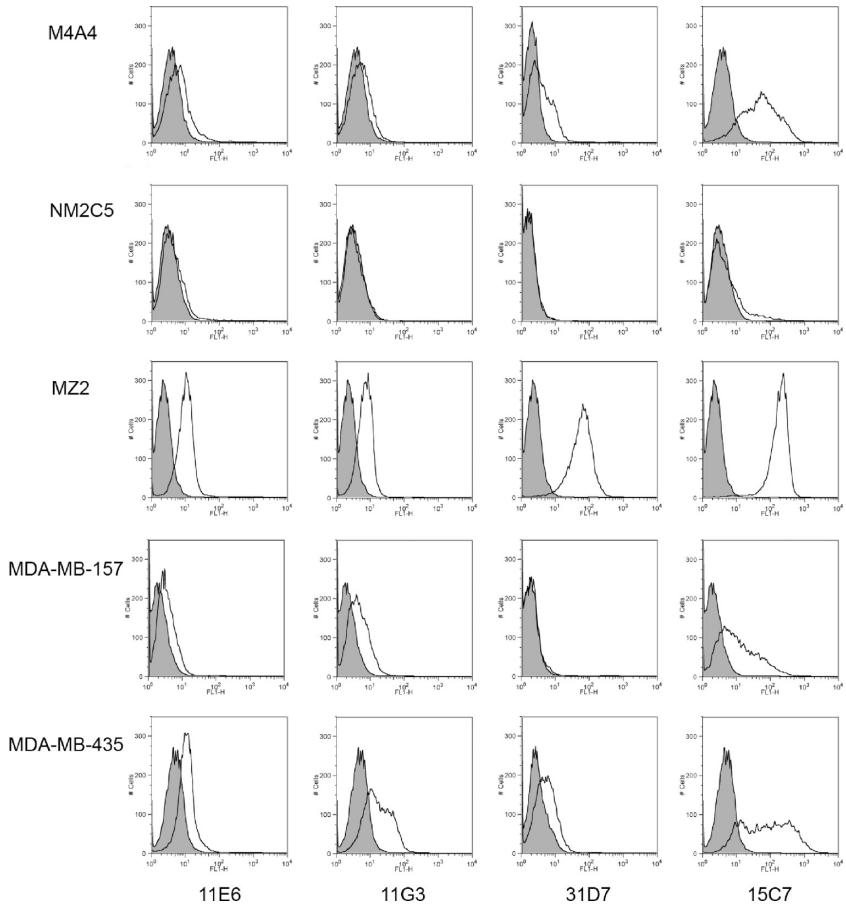

Figure 2. Binding of mAbs 31D7, 15C7, 11G3, and 11E6 to the four breast cancer cell lines, M4A4, NM2C5, MDA-MB-157, and MDA-MB-435, and the melanoma cell line, MZ2, as determined by flow cytometry. MZ2 exhibited high expression, while the breast cancer cell lines exhibited only moderate expression of the antigen recognized by mAbs 31D7, 11G3, and 11E6. mAb 15C7 exhibited strong reactivity to most metastatic breast cancer cell lines and MZ2. The shaded peak represents the background staining measured without the primary antibody, while the clear peak represents the binding with the mAb followed by FITC-conjugated secondary antibody.

Results

Comparative Proteomic Using Subtractive Immunization and Generation of mAbs. Immune-tolerance to the cell surface antigens on live NM-2C5 cells was induced in three mice that were subsequently immunized with live M-4A4 cells. The spleens of all three mice were used for fusion, and the resulting hybridomas were screened for binding to NM-2C5 and M-4A4 cells in a whole cell ELISA. Four of approximately 500 hybridoma supernatants examined showed exclusive or a marked preferential binding to M-4A4 cells compared to NM-2C5 cells (Figure 1). These four clones (15C7, 11G3, 31D7, and 11E6) were subcloned and mAb purified from culture supernatant.

The mAbs Bind Preferentially to Cell Lines Known to Be Metastatic. To further characterize the binding of the isolated mAbs, they were tested for binding to M-4A4 and NM-2C5 by flow cytometry (Figure 2). The results correlated with those from the whole cell ELISA and revealed that the clones reacted almost exclusively with M-4A4 except for 15C7, which showed very weak binding to NM-2C5. To further explore the expression pattern of the isolated mAbs, we tested their binding pattern to an additional 10 breast cancer cell lines and correlated the reactivity to these cell lines with their ability to form metastases in mice (Table 1). The mAbs 15C7, 11G3, and 11E6 showed a marked trend toward stronger reactivity with cell lines known to be metastatic in mice. The correlation was weaker for mAb 31D7, exhibiting some binding to the cell line BT-20, which has been shown to be nonmetastatic in mice. In general, only moderate binding was observed to most breast cancer cell lines tested with the exception of mAb 15C7, which exhibited strong binding to M-4A4 and MDA-MB-435. The expression level of the antigen recognized by mAb 15C7 appeared to vary between cells within each of the different breast cancer cell lines, with the exception of MDA-MB-435, in which homogeneous staining was observed. Because most of the isolated antibodies reacted only moderately with breast cancer cell lines, we also tested their binding to cancer cell lines of other origins, including the glioblastoma cell line U87MG, the colon cancer cell line Colon 137, and the melanoma cell line MZ2 (Table 1) Interestingly, all of the antibodies exhibited strong reactivity with the melanoma cell line MZ2, which adds to the ongoing controversy concerning the origin of the MDA-MB-435 cell line because several studies have shown that this cell line, despite generally being reported as a breast cancer cell line, also shows a high resemblance with melanoma cell lines.[28−30] Antibody 11G3 exhibited strong binding to U87MG and to a subpopulation of Colon 137 cells, while 31D7 only showed weak reactivity to U87MG and did not bind Colon 137 cells. mAb 15C7 also exhibited strong binding to U87MG and

Table 1. Binding of mAb 31D7, 15C7, 11G3, and 11E6 to a Panel of Breast Cancer Cell Lines (A), and Other Cancers (B) as Determined by Flow Cytometry

					reactivity			
cell line	mouse strain	tumor formation	metastatic growth	ref	15C7	11G3	11E6	31D7
A								
M-4A4	nude	+	+	Urquidi[19]	++++	+	+	++
NM-2C5	nude	+	−	Urguidi[19]	+	−	−	−
MDA-MB-435	SCID	+	+	Prince[32]	+++++	+++	++	++
MDA-MB-231	SCID	+	+	Muller[4]	++	+	+	−
MDA-MB-157					+++	++	++	−
MDA-MB-468	nude	+	infrequent	Mukhopadhyay[33]	−	−	−	−
T47D	nude	+	+	Harrell[39]	++	+	+	−
		+	−					
MCF7	nude	+	+	Harrell[39]	+	+	−	−
	nude	+	−	Mukhopadhyay[33]				
BT-474	SCID	+	+	Clinchy[37]	−	−	−	++
	nude	+	−	Mukhopadhyay[33]				
	Bg-nu-xid	+	+	van Slooten[36]				
BT20	nude	+	−	Mukhopadhyay[33]	−	−	−	++
HTB-126	nude	+	−	Sheridan[35]	−	−	−	−
BrCa	nude	+	NE[a]	Möbus[41]	−	−	−	−
B								
U87MG					++++	+++	−	+
MZ2					++++	+++	+++	++++
Colon137					++	+	−	−

[a] NE: Not evaluated.

Colon 137. In contrast to the heterogeneous expression of the antigen recognized by mAb 15C7 observed between cells in each of the breast cancer cell lines, homogeneous expression was found on the cell lines U87MG and MZ2.

Immunocytochemistry. To further validate the cell surface expression of the antigens recognized by the mAbs and to examine whether the antigens were also expressed in the cytoplasm or nucleus, sections of an array containing 20 different formalin-fixed and paraffin-embedded cancer cell lines were stained with the different mAbs. Only mAbs 15C7 and 11E6 exhibited cell staining (Figure 3A); antibody 15C7 exhibited distinct plasma membrane staining of the cell lines MDA-MB-435 and M-4A4, while weaker cell surface staining was observed on MDA-MB-157 (data not shown), in agreement with the results from the flow cytometry study. The staining of MDA-MB-157 was not solely restricted to the plasma membrane because weak cytoplasmic staining was also observed in some cells. The mAb 11E6 exhibited cytoplasmic staining with a slight intensification at the cell surface, and some extracellular granules were intensely stained. These granules were present only in the preparations of the cell lines MDA-MB-435, M-4A4, and NM-2C5, suggesting that the antigen recognized by mAb 11E6 might be secreted. To study the cellular staining pattern of mAb 31D7 and mAb 11G3, we stained freshly frozen cancer cell lines and found similar staining patterns in the three cell lines tested with distinct plasma membrane staining of M-4A4 and MZ2 cells, while NM-2C5 cells were not stained (Figure 3B).

To investigate the cell surface staining in more detail, live MZ2 cells were stained with the four mAbs and examined by immunofluorescence microscopy (Figure 4). mAbs 15C7 and 31D7 showed intense staining that appeared to be confined to distinct patches on the cell surface membrane, with significantly more patches being stained by 15C7 than 31D7. The staining of mAbs 11E6 and 11G3 was less intense but also exhibited staining of very distinct spots on the cell surface

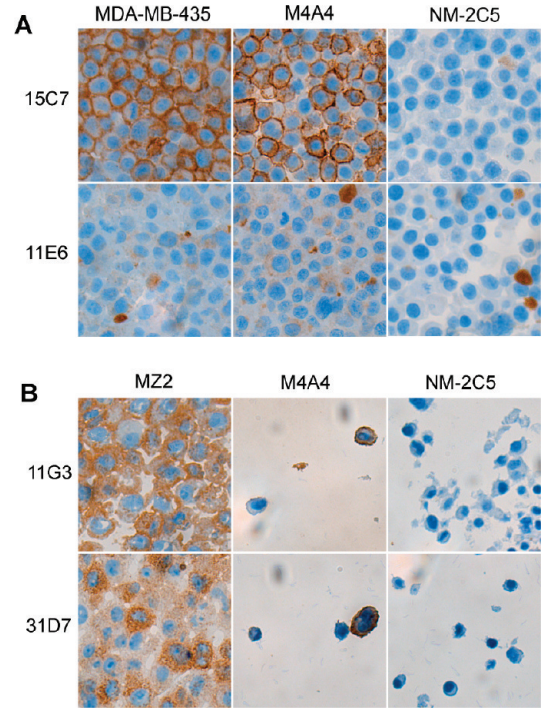

Figure 3. Comparison of the expression pattern of the antigens recognized by mAbs 31D7, 15C7, 11G3, and 11E6 in different cancer cell lines as determined by immunocytochemical. (A) mAbs 15C7 and 11E6 were tested on formalin-fixed, paraffin-embedded cell lines, while (B) mAbs 31D7 and 11G3, which did not recognize their epitope when they were formalin-fixed and paraffin-embedded, was tested on freshly frozen cell lines. All antibodies exhibited plasma membrane staining of M4A4 and no staining of NM2C5. In addition to plasma membrane staining, mAbs 11G3 and 31D7 stained the cytoplasm of MZ2 cells. Isotype-matched control mAbs were included in all experiments.

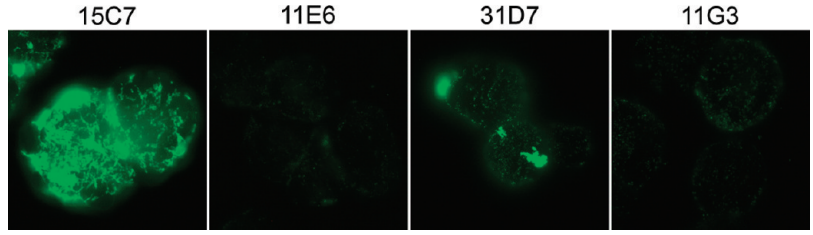

Figure 4. Immunofluorescence staining of live MZ2 melanoma cells with mAbs 15C7, 11E6, 31D7, and 11G3. Intense membrane staining localized to distinct patches on the cell surface was observed for mAbs 15C7 and 31D7, while weaker patchy staining was observed for mAbs 11E6 and 11G3. Isotype-matched control mAbs were included in all experiments.

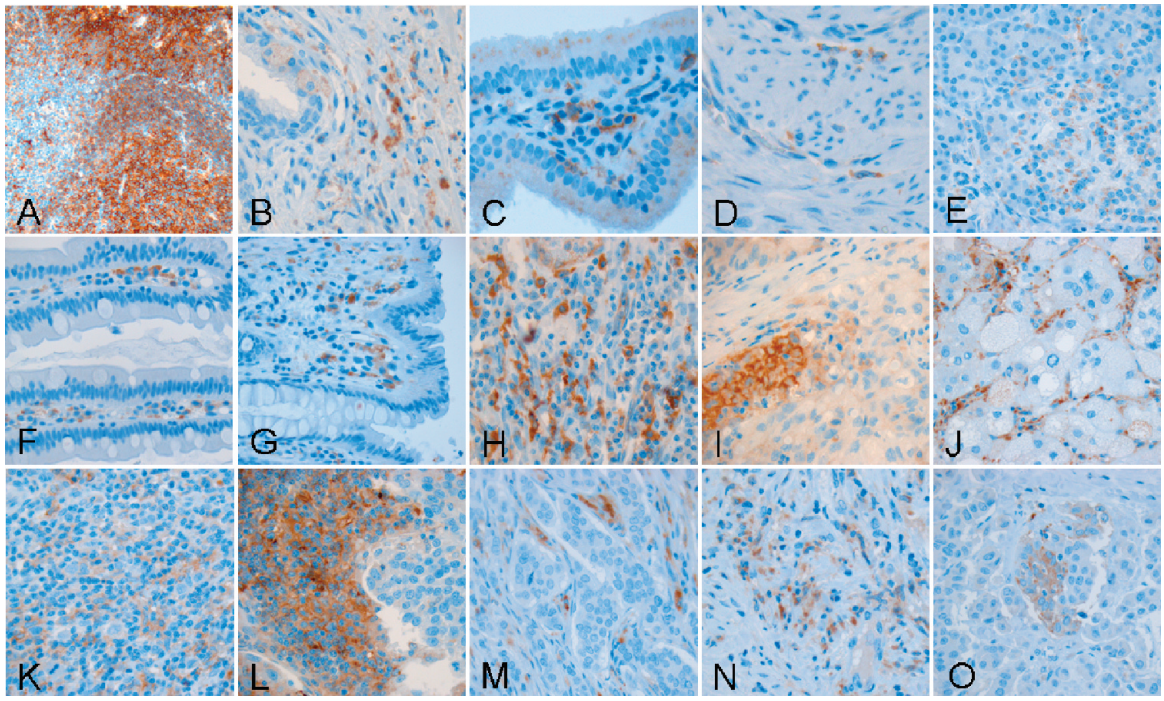

Figure 5. Immunohistochemical analysis of mAb 15C7 on normal and malignant tissues: (A) tonsil, (B) prostate, (C) gallbladder, (D) corpus ut., (E) pancreas, (F) duodenum, (G) colon, (H) colon carcinoma, (I) lung adenocarcinoma, (J) adrenal adenoma, (K) Hodgkin's lymphoma, (L) malignant melanoma, (M) breast carcinoma, (N) ovary carcinoma, (O) medullary thyroid carcinoma.

membrane. The staining of very large extracellular aggregates with mAb 31D7, as observed in the fresh-frozen MZ2 cells preparations, was also observed in the live cell preparations, making it unlikely that it was caused by antigen-independent antibody cross-linking.

Normal and Malignant Tissue Distribution of Antigens Recognized by mAbs as Determined by Immunohistochemical Analysis. The distribution of the antigens recognized by mAb 15C7 and mAb 11E6 was initially studied by immunohistochemistry using an array of 25 normal formalin-fixed, paraffin-embedded tissues. Similar to observations from the immunocytochemistry experiments, neither mAb 31D7 nor mAb 11G3 stained formalin-fixed, paraffin-embedded tissues. As shown in Figure 5 and Table 2, the mAb 15C7 stained lymphoid cells in several lymphoid tissues and also stained single cells in several other tissues, such as the submucosa of the gallbladder and duodenum. In contrast, mAb 11E6 only exhibited weak staining of a subgroup of pancreatic cells and single cells in esophagus, cerebellum, portio uteri, gall bladder, and skin (Figure 6, Table 2). These single cells were generally observed in the connective tissues and could potentially be disseminated neuroendocrine cells known to be present at these locations.[31] Immunohistochemical analysis of a large panel of formalin-fixed and paraffin-embedded malignant tissues showed that mAb 15C7 intensely stained the cytoplasm of several tumors, including malignant melanoma, medullary thyroid carcinoma, lung adenocarcinoma, and colon carcinoma. Weaker staining was observed in adrenal adenoma, Hodkin's lymphoma, seminoma, and breast carcinoma. Interestingly, mAb 11E6 stained primary neuroendocrine cancers, including 75% of medullary thyroid carcinomas (3/4), 50% of lung carcinoids (2/4), and 50% of malignant melanomas (7/14). One renal cell carcinoma also exhibited weak, primarily cytoplasmic, staining (Figure 5 and 6 and Table 2). Because the staining pattern exhibited by mAb 15C7 localized to various tumors as well as to lymphoid cells in several lymphoid organs, we wanted to determine whether the recognized antigen could be an HLA or an HLA-associated molecule and thus tested the staining pattern of commercially available HLA-DR-α- and HLA-DR-β-specific antibodies on tissue sections adjacent to those stained with mAb 15C7. The HLA-DR specific antibodies exhibited a staining pattern identical to mAb 15C7 on the panel of normal and malignant tissues (data not shown).

Western Blotting. To determine the approximate molecular mass of the antigens recognized by the four mAbs, lysates of M4A4 and NM2C5 were separated by SDS-PAGE, blotted onto

Table 2. Immunohistochemical Analysis of the Reactivity of mAbs 15C7 and 11E6 with Formalin-Fixed, Paraffin-Embedded Normal (A) and Cancerous (B) Tissues ($n_{positive}/n_{total}$)[a]

tissue	15C7	11E6
A		
bladder	−	sc
cerebellum	−	−
gallbladder	+	sc
kidney	−	−
liver	−	−
lung	−	−
portio uteri	−	sc
cervix uteri	−	−
corpus uteri	+	−
muscle	−	−
esophagus	−	sc
thyroid gland	−	−
colon	+	sc
duodenum	+	sc
placenta	−	−
prostate	+	−
pancreas	+	+
skin	−	sc
testis	−	sc
thymus	+	sc
tonsil	+	−
stomach	−	−
glandula parotis	−	−
spleen	−	−
B		
breast carcinoma	1/2	0/25
lymphoma	1/1	0/1
lung carcinomas, total	1/1	2/25
nonsmall cell carcinoma	1/1	0/19
small cell carcinoma	0/0	0/2
lung carcinoid	0/0	2/4
malignant melanoma	1/1	7/14
mesothelioma	0/1	0/1
ovary carcinoma	0/1	0/1
renal cell carcinoma	0/1	1/1
seminoma	1/1	0/1
medullary thyroid carcinoma	1/1	3/4
colon carcinoma	1/1	0/1
pheochromocytoma	0/1	0/1

[a] Single cells, sc.

nitrocellulose membranes, and probed with the mAbs. Antibody 15C7 stained a distinct band of approximately 34 kDa (Figure 7), while no specific bands were stained by the three remaining mAbs, suggesting that they recognize conformational-dependent epitopes (data not shown). Because the immunohistochemistry staining pattern suggested that mAb 15C7 recognized an HLA-DR antigen, parallel strips of the Western blot were stained with mAb 15C7, an HLA-DR-α-specific antibody, and an HLA-DR-β-specific antibody. Both mAb 15C7 and the HLA-DR-β-specific antibody stained a distinct band of approximately 34 kDa of lysates of M4A4 cells, while the HLA-DR-α-specific antibody stained a broader band at a slightly higher molecular mass (Figure 7). None of the three antibodies stained a distinct band of lysates of NM2C5 cells, while a control antibody did.

Competition Flow Cytometry Assay. We also performed a competition flow cytometry assay to evaluate whether HLA-DR-α- and HLA-DR-β-specific antibodies could block the binding of mAbs 15C7, 31D7, 11E6, or 11G3 to THP-1 cells. The HLA-DR-β-specific antibody was capable of inhibiting the binding of mAb 31D7 to THP-1 cells in a dose-dependent manner (Figure 8), indicating that the antigen recognized by mAb 31D7 was HLA-DR-β. The HLA-DR-α-specific antibody exhibited no such inhibition (Figure 8). No competition was observed between the two commercial HLA-DR antibodies and mAb 15C7 or the other two isolated mAbs (data not shown). This suggests that although the antigen recognized by mAb 15C7 seem to be HLA-DR-β, it recognizes a different epitope than that of the commercial HLA-DR-β antibody.

Immunoprecipitation. To confirm that the antigen recognized by mAb 15C7 was HLA-DR-β, we performed an immunoprecipitation experiment using mAb 15C7 and MZ2 cell lysate. mAb 15C7 was capable of precipitating HLA-DR-β, while a control protein, p53, was not precipitated (Figure 9).

Inhibition of Cancer Cell Growth. We next tested the ability of the isolated mAbs to inhibit growth of several cancer cell lines. mAb 31D7 significantly inhibited the growth of the melanoma cell line MZ2 in a dose-dependent manner. These tests were performed in pentaplicate and repeated four times and revealed that some inhibition was observed at an antibody concentration of 0.5 μg/mL and reached maximal inhibition of approximately 20% at 100 μg/mL antibody (Figure 10). None of the three other mAbs influenced the growth of cancer cells.

Expression Level of CIITA, HLA-DR-α, HLA-DR-β, and CD74. Because the expression level of HLA-DR antigens is regulated by CIITA, we investigated whether the down-regulation of HLA-DR observed on NM-2C5 was caused by a down-regulation of CIITA. Surprisingly, we found that the expression of CIITA was 4-fold up-regulated in the NM-2C5 cell line compared to the M-4A4 cell line (Figure 11). Because CIITA regulates the expression of HLA-DR-α, HLA-DR-β, and CD74, we next examined the expression levels of these three genes at the transcriptional level in the three cell lines NM-2C5, M-4A4, and MDA-MB-435. All three cell lines expressed transcripts for all three genes, and although reduced expression of HLA-DR-β in the NM-2C5 cell line compared to M-4A4 and MDA-MB-435 was observed, the expression level in NM-2C5 was still relatively high and the observed reduction could not account for the significant reduction in surface HLA-DR expression on the NM-2C5 cell line. This suggests that the low expression of HLA-DR in the NM-2C5 cell line is likely regulated post-transcriptionally. Finally, to examine whether the loss of HLA-DR could be caused by a defect in CD74, as the assembly of a functional HLA-DR complex is dependent on CD74, we sequenced CD74 in all three cell lines but found no mutations or deletions.

Discussion

The development of metastases is a complex multistep process that may be elucidated by the use of different proteomic approaches that may also identify new targets for cancer therapy. In the present study, we used a comparative, semiquantitative proteomic approach that takes advantage of the exquisitely sensitive immune recognition system to scan for cell surface proteins preferentially or exclusively expressed on metastatic vs nonmetastatic cancer cell lines. An advantage of this proteomic strategy is that monoclonal antibodies against the proteins with altered expression are immediately generated, which allow for further analysis of these proteins. Using MS based proteomic, the availability of useful antibodies against the identified proteins is often a limiting factor, and frequently the available antibodies are only reactive with denatured

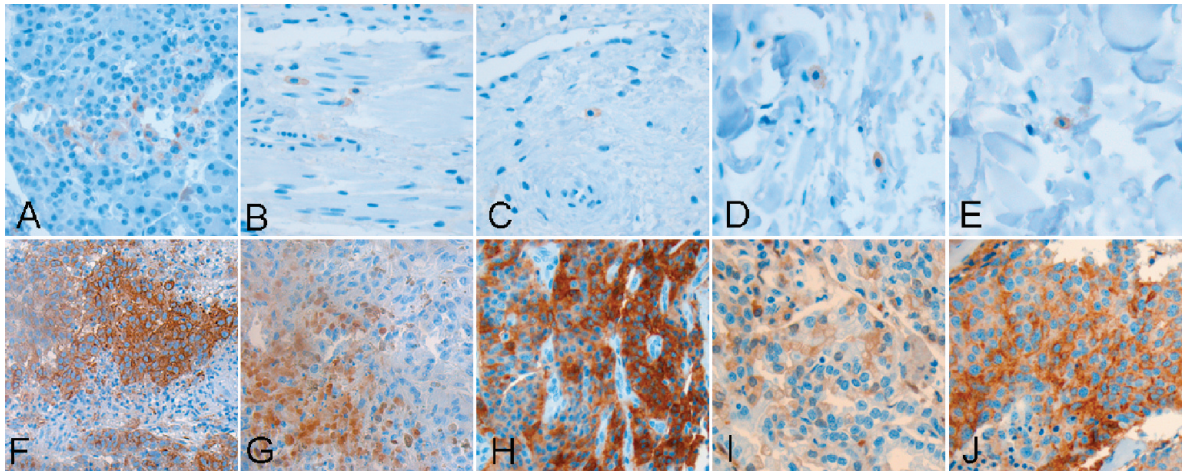

Figure 6. Immunohistochemical analysis of mAb 11E6 on normal and malignant tissues: (A) pancreas, (B) esophagus, (C) portio ut., (D) gallbladder, (E) skin, (F,G) malignant melanoma, (H) lung carcinoid, (I) renal cell carcinoma, (J) medullary thyroid carcinoma.

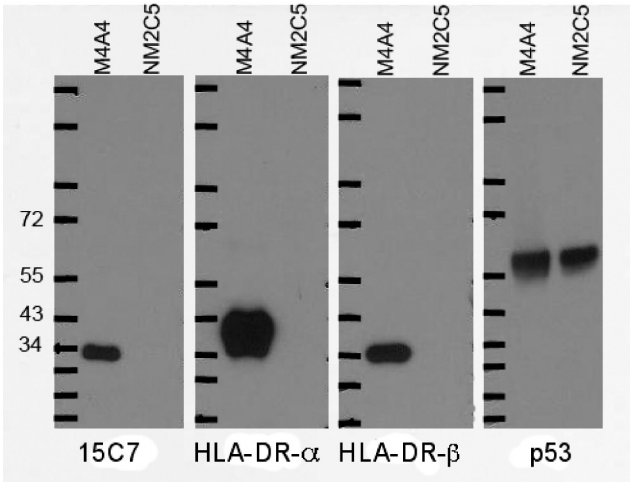

Figure 7. Western blot of M-4A4 and NM-2C5 lysates stained with mAb 15C7, HLA-DR-α-, and HLA-DR-β-specific antibodies. A p53-specific antibody was included as a positive control. mAb 15C7 stained a band of approximately the same molecular mass as the HLA-DR-β-specific antibody. No HLA-DR expression was observed in NM-2C5.

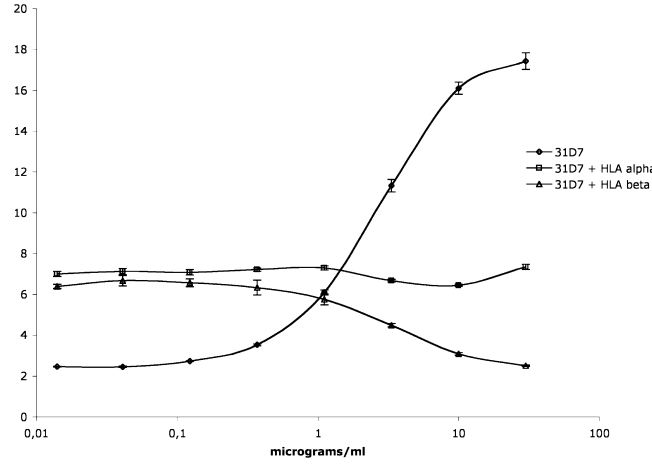

Figure 8. Inhibition of the binding of mAb 31D7 to THP-1 cells by an HLA-DR-β-specific antibody as measured by flow cytometry. Initially, mAb 31D7 vas titrated and an appropriate concentration (2 μg/mL) selected for the inhibition assay. At 30 μg/mL, the HLA-DR-β specific antibody completely blocked the binding of 31D7 to THP-1 (OD value at background level). No inhibition was observed with an HLA-DR-α-specific antibody.

protein and therefore only useful in some biochemical assays such as Western blotting. Because cell surface membrane proteins often lose their native confirmation when they are removed from the membrane microenvironment, it is often particularly difficult to generate antibodies that recognize these proteins in their native state.

The strategy, based on a whole cell subtractive immunization strategy in mice, resulted in generation of approximately 50 mAbs reacting with the surface of cancer cells. For further characterization, we focused on four mAbs that efficiently bound to M-4A4 cells but exhibited no, or only weak, binding to NM-2C5 cells. A considerable fraction of the mAbs that did not recognize cell surface molecules likely recognized cytoplasmic antigens. Although the immunizations were performed using freshly washed live cells, a small fraction might have disintegrated and expose intracellular antigens, which would stimulate the immune system and elicit antibodies against these intracellular antigens. This indicates that the strategy also can be used to identify cytoplasmic protein preferentially or exclusively expressed in one of the two cell lines.

Importantly, the strategy we used is not only comparative but also to some extent semiquantitative, as it gives rise to not only mAbs against proteins that are exclusively expressed on only one of the cell lines but also against proteins that were present on both cell types but expressed at significantly higher level on the metastatic cell line. The mechanism on how these mAbs are generated is not clear, but likely very low expression of a given cell surface protein does not give rise to tolerance, and thus immunization with the same cell surface protein at high concentration yields an immune response against this protein.

Flow cytometry analysis of the four mAbs on a panel of breast cancer cell lines showed a strong trend toward higher reactivity with breast cancer cell lines known to be metastatic in vivo. On the basis of the literature,[4,19,32−37] the breast cancer cell lines were grouped into three categories: (1) cell lines that most studies found were metastatic in vivo (M-4A4, MDA-MB-435, MDA-MB-231, and MDA-MB-157), (2) cell lines that some studies found to be nonmetastatic and others metastatic (T47D, MCF7, MDA-MB-468, and BT474), and (3) cell lines generally found to be nonmetastatic (NM-2C5, BT20, and HTB-126). The

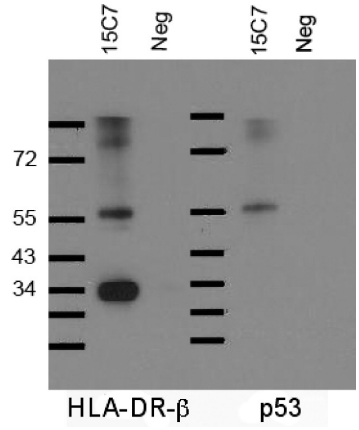

Figure 9. Immunoprecipitation of HLA-DR-β with mAb 15C7. mAb 15C7 was capable of precipitating HLA-DR-β from total cell lysate of MZ2 cells, giving rise to a band at 34 kDa, whereas a control protein (p53) was not precipitated. In both samples, unspecific bands at approximately 55 and 100 kDa were observed, corresponding to immunoglobulin fragments of mAb 15C7 detected by the secondary antibody. A sample including only protein G sepharose and cell lysate, but not mAb 15C7, was included as a negative control (Neg).

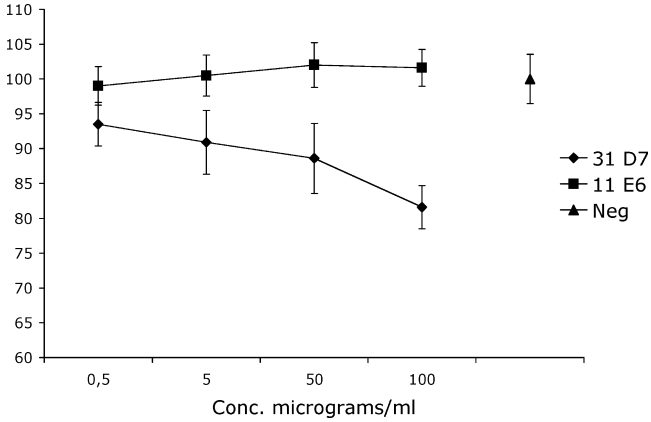

Figure 10. Inhibition of melanoma cell line MZ2 growth by mAb 31D7. mAb 31D7 was capable of inhibiting the growth of MZ2 in a dose-dependent manner, while mAb 11E6 and PBS alone (Neg) had no such effect. The experiment was performed in pentaplicate and repeated four times, with similar results.

reactive pattern of the isolated antibodies showed a clear trend toward preferential reactivity with breast cancer cell lines considered metastatic in vivo, suggesting that the mechanisms leading to the metastatic phenotype of the M-4A4/NM-2C5 model could also be relevant in other cell lines.

It should be noted that there is some controversy concerning the origin of the MDA-MB-435 cell line because several studies have shown that this cell line, despite generally being reported as a breast cancer cell line, shows a high resemblance with melanoma cell lines as well,[28,29] particularly the M14 melanoma cell line.[30] Although our study did not address this debate in that we simply used the M-4A4 and NM-2C5 cell lines as a general metastasis model, our results showed that all four of our generated mAbs bound most efficiently to the melanoma cell line MZ2 while binding to several breast cancer cell lines was relatively weak.

To analyze the cell-surface distribution of the antigens recognized by the four generated mAbs we performed immunofluorescence analysis on formalin-fixed and paraffin-embedded cells, freshly frozen cells, and live cells. We found the HLA-DR-β-reactive mAb 31D7 appeared to be localized in large patches on the cell surface on MZ2 cells. Similar observations have previously been reported for MHC class I, where it was further shown that the lateral movement of MHC class I in the plasma membrane was hindered.[38] Furthermore, we found that mAb 31D7 could inhibit the growth of the MZ2 melanoma cell lines, in agreement with previous findings using other anti-HLA-DR antibodies and cancer cell lines.[39]

In addition to being expressed on the cell surface, the antigen recognized by mAb 11E6 may also be a secreted protein given the intense staining of large extracellular complexes. Interestingly, analysis of this antibody on an array of normal and malignant tumors showed that it only stained tumors of neuroendocrine origin and malignant melanomas while exhibiting very low reactivity with normal tissues. Of the neuroendocrine tumors, the strongest reactivity was observed in lung carcinoid and medullary thyroid carcinomas, while renal cell carcinoma stained more weakly. Although strong surface staining was observed by mAb 11E6 on flow cytometry, the observed immunohistochemical staining also appeared to be cytoplasmic and granular. Neuroendocrine tumors are a very heterogeneous group and include carcinoid and noncarcinoid gastroenteropancreatic tumors, catecholamine-secreting tumors, medullary thyroid carcinoma, chromophobe pituitary tumors, small cell lung cancers, and Merkel cell tumors.[40,41] They express a variety of markers including chromogranin A and B,[42] neurone-specific enolase,[43] and carcinoembryonic antigen[44] and are also known to express receptors for small peptides such as somatostatin.[40] The observed staining of the malignant melanomas appeared focal and, as observed for the neuroendocrine tumors, cytoplasmic and granular. To our knowledge, this is the first marker described to exhibit the reactivity pattern in malignant melanomas and neuroendocrine tumors with mAb 11E6. Therefore, it is unlikely that the antigen recognized by mAb 11E6 is one of the classic neuroendocrine or melanoma markers, which suggests that mAb 11E6 recognizes a novel antigen. We are currently attempting to identify the specific antigen by affinity purification from MZ2 cell lysate using mAb 11E6 and mass spectrometry.

Although not commonly present on nonantigen presenting cells, HLA class II expression has been observed on tumor cells of a variety of cancers, including melanoma, gastric cancers, breast carcinomas, and lung carcinomas,[21] in agreement with our findings. Several studies have suggested a positive correlation between HLA-DR expression and prognosis[45,46] and proposed that HLA-DR expression allows the cancer cell to present peptides to the immune system and therefore be more readily eliminated. A recent study using a metastatic cell line model derived from the MDA-MB-435 breast cancer cell line showed the same correlation as observed with resected tumor samples,[22] with a down-regulation of HLA-DR on the highly metastatic cell line compared to the poorly metastatic variant. Surprisingly, we found that, in the M-4A4/NM-2C5 model, HLA-DR antigens were expressed preferentially on the metastatic breast cancer cell lines, in contrast to the expression pattern most often observed on resected tumors, and in the model described by Shi et al.[22] The selective expression of HLA-DR on M-4A4 cells and not on the NM-2C5 cell line was also recently demonstrated in a proteomic study using SILAC mass spectrometry.[11,12] The lack of correlation between the clinical findings and our model system could be explained by absent tumor shaping[46,47] because the cell model used was selected

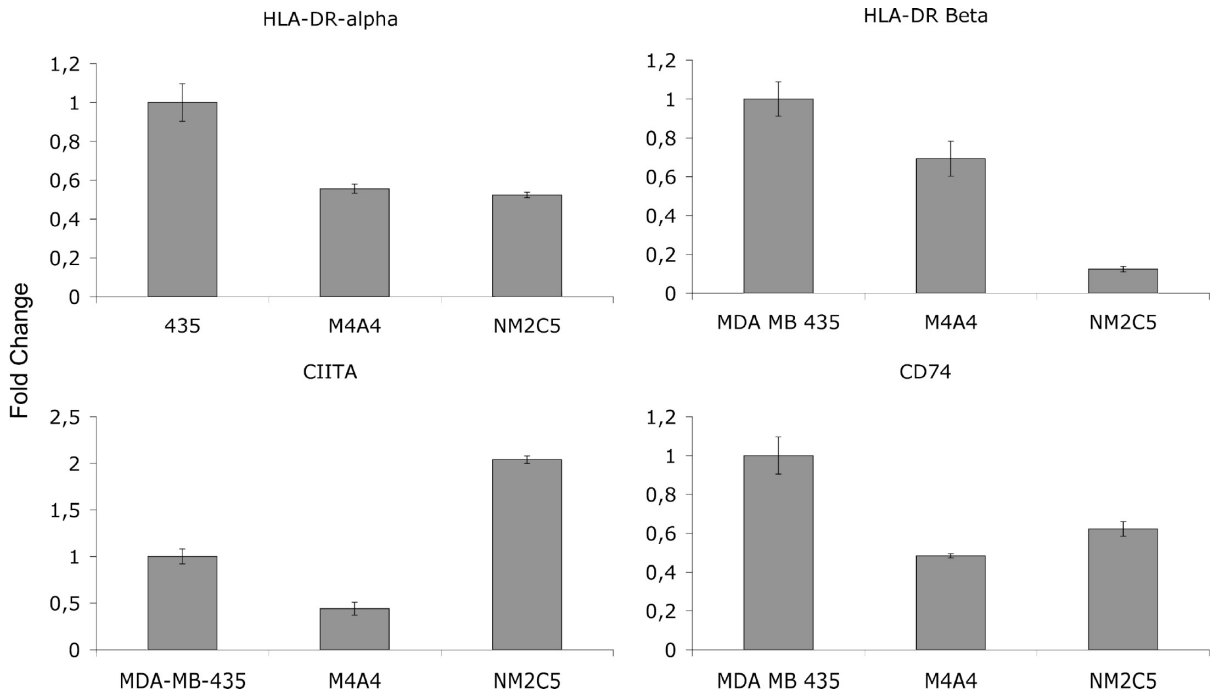

Figure 11. Real-time PCR quantification of the expression of CIITA, HLA-DR-β, HLA-DR-α, and CD74. HLA-DR-β expression was lower in NM-2C5 than M4A4, but the overall level was relatively high. The down-regulation of HLA-DR on the cell surface at the protein level could not be explained by reduced expression of CIITA.

on its in vivo metastatic capabilities in nude mice, meaning that no selective pressure would be present on an HLA-expressing tumor. It is, however, striking that HLA expression appears to be present in several breast cancer cell lines capable of forming metastatic lesions in nude mice and absent in breast cancer cell lines incapable of metastasis formation. This could indicate different functions of HLA and HLA-associated molecules in cell lines and primary tumors. Interestingly, it has been suggested that HLA molecules may play a role in adhesion of cancer cells.[48,49] Adding to this, we observed that the cellular distribution of HLA antigens differed significantly. While the cell lines exhibiting intense membrane staining and little cytoplasmic staining, the primary tumors primarily exhibited cytoplasmic staining. Further studies are needed to define the relevance of this observation.

The expression level of HLA-DR antigens is primarily regulated at the transcriptional level by CIITA,[25] and several studies have shown good correlation between CIITA and HLA-DR antigen expression.[22,26,50,51] Surprisingly, we found that CIITA was up-regulated in NM-2C5 cells compared to M-4A4 cells, showing that the down-regulation of HLA-DR in our model system was not due to reduced expression of CIITA. Because CIITA regulates the transcription of both HLA-DR-α, HLA-DR-β, and CD74, we investigated the expression level of these genes and found transcripts for all three proteins in both cell lines. Together, these data suggest that the down-regulation of HLA-DR is likely due to post-transcriptional regulation. To our knowledge, no post-transcriptional mechanism regulating HLA-DR expression has been previously described, although one study on the regulation of MHC class II expression in primary uveal melanocytes and ocular melanoma cell lines suggested that post-transcriptional regulation could account for the lack of surface MHC class II expression on ocular melanoma cell lines following IFN-γ treatment.[52] We hypothesized that the down-regulation of HLA-DR could be caused by a defect in CD74, but found no evidence of this as no mutations or deletions were found in the CD74 gene in the NM-2C5 cell line. Further studies are needed to identify the factors involved in the HLA-DR down-regulation.

In conclusion, this comparative, quantitative cell surface proteomic approach based on subtractive immunization lead to the isolation of a large panel of antibodies exhibiting preferential binding to the metastatic cell line of the M-4A4/NM-2C5 model. We identified the recognized protein of some of these antibodies to be HLA-DR antigens and found that HLA-DR antigens were down-regulated on NM-2C5 cells as well as on other nonmetastatic breast cancer cell lines due to post-translational regulation rather than loss of CIITA.

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