A Peptide Selected by Biopanning Identifies the Integrin $\alpha_v\beta_6$ as a Prognostic Biomarker for Nonsmall Cell Lung Cancer

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Abstract

The development of new modes of diagnosis and targeted therapy for lung cancer is dependent on the identification of unique cell surface features on cancer cells and isolation of reagents that bind with high affinity and specificity to these biomarkers. We recently isolated a 20-mer peptide which binds to the lung adenocarcinoma cell line, H2009, from a phage-displayed peptide library. We show here that the cellular receptor for this peptide, TP H2009.1, is the uniquely expressed integrin, $\alpha_v\beta_6$, and the peptide binding to lung cancer cell lines correlates to integrin expression. The peptide is able to mediate cell-specific uptake of a fluorescent nanoparticle via this receptor. Expression of $\alpha_v\beta_6$ was assessed on 311 human lung cancer samples. The expression of this integrin is widespread in early-stage nonsmall cell lung carcinoma (NSCLC). Log-rank test and Cox regression analyses show that expression of this integrin is significantly associated with poor patient outcome. Preferential expression is observed in the tumors compared with the surrounding normal lung tissue. Our data indicate that $\alpha_v\beta_6$ is a prognostic biomarker for NSCLC and may serve as a receptor for targeted therapies. Thus, cell-specific peptides isolated from phage biopanning can be used for the discovery of cell surface biomarkers, emphasizing the utility of peptide libraries to probe the surface of a cell. [Cancer Res 2007;67(12):5889–95]

Introduction

During carcinogenesis, genetic, epigenetic, and proteomic changes occur, resulting in an alteration of cell surface features (1). The difference in the surface profile between cancerous cells and their nonmalignant counterparts can serve as a molecular address for targeting reagents to deliver a molecule of choice to the desired cell type. Identifying biomarkers for cancerous cells is critical in terms of developing new targeting reagents for cell-specific delivery of chemotherapeutics or imaging reagents.

Genomic approaches have been the primary method for target identification in cancer (2). However, gene expression and protein levels do not always correlate. Also, gene expression does not provide information, such as receptor turnover rate, posttranslational modifications, or the ability to function as an internalizing receptor. Mass spectrometric-based proteomic approaches have been used to identify biomarkers that are of value as diagnostic, prognostic, and/or drug targets (2, 3). However, it is difficult to detect low abundance proteins, and certain classes of proteins are underrepresented, especially membrane proteins. This is particularly problematic if the goal is to identify cell surface proteins for targeted drug delivery. Next, once the targets are identified, researchers are still faced with the task of isolating ligands that are specific for that cellular feature.

Phage display biopanning on viable cells has proved to be a powerful approach for identifying cell-specific peptides that mediate binding to a target tumor type even in the absence of knowledge of the target cellular receptor (4–7). The isolated peptides are able to discriminate between their target cell type and closely related cell types. Thus, the peptides are recognizing distinct cell surface receptors that may be of clinical value. Hence, biopanning can identify targeting reagents and the peptides can be used for the discovery of unique cell surface biomarkers.

Within the United States, 170,000 new cases of lung cancer are diagnosed per year and 60% of these patients will die within 1 year (8). We have isolated several cell-targeting peptides for different lung cancer cell lines (9). These peptides are able to discriminate between cancerous and normal cells and display high cell specificities even among tumors with the same pathologic classifications. One such peptide was isolated from panning our peptide library on the lung adenocarcinoma cell line H2009. This 20-mer peptide, named TP H2009.1, has the sequence RGDLATLRQLAQEDGVVGVR. The peptide is able to deliver a chemotherapeutic agent, resulting in the death of the target cell (10). Our results suggest that this peptide may have value as a diagnostic and cell-targeting reagent. However, the cellular receptor for this peptide remained unidentified. Here, we have identified the cellular receptor for the TP H2009.1 peptide to be a restrictively expressed integrin, $\alpha_v\beta_6$. Importantly, we show that this integrin is up-regulated in many human nonsmall cell lung carcinoma (NSCLC) compared with normal lung tissue and expression of this integrin is an independent prognostic factor for a poor patient outcome.

Materials and Methods

Reagents and cell lines. Human lung cancer cell lines were obtained from the Hamon Center for Therapeutic Oncology Research (UTSW) and maintained according to standard protocols (11). Small airway epithelial cells (SAEC) were obtained from Clonetics. The SW480 human colon carcinoma cell line transfected with the full-length gene for the human β_6

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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integrin subunit (B611.6) and the corresponding mock-transfected cells were a gift from Dean Sheppard (University of California San Francisco; ref. 12). Peptides were synthesized in-house. The mass of the peptides was confirmed by the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, and all peptides are >90% pure as determined by reverse-phase HPLC. Peptide stock solutions were prepared in PBS (pH 7.4). Function-blocking antibodies for $\alpha_v\beta_5$ (MAB1961Z, clone P1F6), $\alpha_v\beta_3$ (MAB1976Z, clone LM609), $\alpha_v\beta_6$ (MAB2077Z, clone 10D5), and $\alpha_5\beta_1$ (AB1950, polyclonal) were purchased from Chemicon International. The $\alpha_v\beta_6$ antibody MAB2074Z, clone E7P6 (Chemicon) was used for flow cytometry. Qdot Innovator's tool kit Carboxyl Qdot605 was obtained from Quantum Dot, Inc. EDC-coupling reagent and streptavidin were purchased from Pierce Chemical. Spin columns with 100K NMWL membrane were obtained from Millipore.

Determination of phage uptake. Phage uptake was determined as previously described (9). Antibodies were added at the indicated concentrations with no incubation before the addition of phage. Percentage blocking was calculated as the ratio of phage uptake in the presence of antibody compared with the phage uptake in the absence of antibody. All experiments were repeated in duplicate, a minimum of three times.

Expression of $\alpha_v\beta_6$ as determined by flow cytometry analysis. The indicated cells were trypsinized and counted, and 100,000 cells were placed into a polypropylene tube containing 1 mL media. Cells were incubated overnight at 37°C in a 5% CO_2 incubator. The following day, cells were collected by centrifugation at 4°C. All of the following steps occurred at 4°C. The supernatant was removed, and cells were blocked for 30 min in 2% normal goat serum in staining medium consisting of HBSS containing 2% FCS, 10 mmol/L HEPES (pH 7.4), and 0.4% EDTA. Following blocking, cells were incubated for 30 min with a 1:50 dilution of mouse anti-$\alpha_v\beta_6$ antibody. After washing, cells were incubated for 30 min with a 1:24 dilution of FITC-conjugated goat anti-mouse IgG. Samples were washed with staining medium, and the cells resuspended in 400 μL of staining medium. Cells were filtered through nylon mesh before fluorescence-activated cell sorting analysis on a Becton Dickinson FACSCalibur. Analysis was done using Cell Quest (v3.1f), measuring 5000 events per sample.

Peptide-mediated delivery of a fluorescent nanoparticle. SAQDot605 were purchased from Quantum Dot or prepared in-house. Streptavidin derivatization was done by following manufacturer's recommended procedure. Presence of streptavidin on Qdot was confirmed using a standard biotin assay. Formation of SAQdot605-peptide conjugate was done by mixing the biotinylated tetrameric H2009.1 peptide {sequence, [RGDLATLRQLAQEDGVVGVRK(biotin)PEG_6]$_4$$Lys_2$-Lys-β-Ala-COOH} with SAQDot605. A 3:1 peptide to Qdot ratio was used for the commercial Qdots, whereas a 5:1 ratio was used for the in-house derivatized Qdots. The peptide-SAQdot605 solution was diluted with RPMI to a final concentration of 20 nmol/L based on the concentration of the Qdot. Cells were grown to 80–90% confluency in an eight-well chamber slide. After incubation in serum-free RPMI for 2 h, the cells were incubated with 20 nmol/L H2009.1 tetrameric peptide-SAQdot605 (based on Qdot concentration) at 37°C for 10 min. The cells were washed and fixed, and the nuclei were stained with Hoechst 33342.

Case selection and tissue microarrays construction. Archival, formalin-fixed and paraffin-embedded material from surgically resected lung cancer specimens (lobectomies and pneumonectomies) containing tumor tissues was obtained from the Lung Cancer Specialized Programs of Research Excellence Tissue Bank at M.D. Anderson Cancer Center (Houston, TX) from 1997 to 2001. Tumor tissue specimens obtained from 311 lung cancers were histologically examined, classified using the 2004 WHO classification (13), and selected for tissue microarrays (TMA) construction. They consisted of 18 SCLC and 293 NSCLC, including 180 adenocarcinomas, 6 adenosquamous carcinomas, and 107 squamous carcinomas. All adenocarcinomas were mixed histology subtype, except for three bronchioloalveolar carcinomas. After histologic examination, tumor TMAs were prepared using triplicate 1-mm diameter cores per tumor, obtaining tissue from central, intermediate, and peripheral tumor areas.

Detailed clinical and pathologic information, including demographic, smoking history (never-smokers and ever-smokers), and status (never, former, and current smokers), clinical and pathologic tumor-node-metastasis (TNM) staging, overall survival, and time of recurrence, were available in most cases. Follow-ups on the patients were initially done at 6 months and then yearly. Only disease-related deaths were included in the analysis. Patients who had smoked at least 100 cigarettes in their lifetime were defined as smokers, and smokers who quit smoking at least 12 months before lung cancer diagnosis were defined as former smokers. Tumors were pathologic TNM stages I-IV according to the revised International System for Staging Lung Cancer (14).

Immunohistochemical staining and evaluation. Five-micron-thick, formalin-fixed and paraffin-embedded tissue histology sections were deparaffinized, hydrated, heated in a steamer for 10 min with 10 mmol/L sodium citrate (pH 6.0) for antigen retrieval, and washed in Tris buffer. Peroxide blocking was done with 3% H_2O_2 in methanol at room temperature for 15 min, followed by 10% bovine serum albumin in TBS containing Tween 20 for 30 min at room temperature. The slides were incubated with mouse anti-β_6 antibody (EMD Calbiochem) at 1:300 dilution for 65 min at room temperature. After washing with PBS, incubation with biotin-labeled secondary antibody was done for 30 min followed by incubation with a 1:40 solution of streptavidin-peroxidase for 30 min. The staining was then developed with 0.05% 3′,3-diaminobenzidine tetrahydrochloride, freshly prepared in 0.05 mol/L Tris buffer (pH 7.6) containing 0.024% H_2O_2 and then counterstained with hematoxylin, dehydrated, and mounted. A panel of 10 lung cancer formalin-fixed and paraffin-embedded tissue specimens was used as positive control. As negative control, the same specimens were used as positive controls replacing the primary antibody with PBS.

Cell membrane staining was quantified using a computerized image analysis system (Ariol SL-50, Applied Imaging). Staining in each sample was quantified from tumor TMA cores using a four-value membrane score (0, 1+, 2+, and 3+) based on algorithms developed and standardized for Her-2/Neu immunohistochemical expression in breast cancer, which takes into account cell membrane staining intensity, extension (percentage of cells with a given membrane intensity), and completeness of membrane staining. An average from the three cores was considered for each case.

Statistical analysis. Statistical analysis was done using SAS 9.1.3 Service Pack 3 XP_PRO platform. Association between integrin expression and categorical covariates, such as cancer stages and smoking status, was examined using χ^2 test or Fisher's exact test when appropriate. General survival times in β_6-positive and β_6-negative patients were compared using log-rank test. Cox regression model was used to adjust for five other covariates (age at surgery, smoking status, recurrence, gender, and stage). A P value of 0.05 or less is considered as statistically significant.

Results

Antibodies against the functional domain of the $\alpha_v\beta_6$ integrin block uptake of the TP H2009.1 phage on H2009 cells. The TP H2009.1 peptide contains the known tripeptide integrin–binding motif RGD (15). Integrins are a family of heterodimeric cell surface proteins. The different combinations of α and β subunits result in more than 20 different integrin receptors, 12 of which recognize the RGD motif (16). A peptide BLAST search of the TP H2009.1 peptide against the SwissProt database was done. The first eight amino acids of TP H2009.1 are an exact match to a region in the GH loop of the viral coat protein VP1 of SAT-1 type foot-and-mouth disease virus (FMDV; accession no. 15420033; ref. 17). This RGD domain is responsible for binding and viral invasion into the epithelial host cell. Integrins $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_6$, and $\alpha_5\beta_1$ have been postulated as the cellular receptors that FMDV uses to mediate internalization (18–22).

To address if one of these integrins might be the cellular target for the peptide, uptake of the TP H2009.1 phage by H2009 cells was determined in the presence of antibodies against these integrins that block binding of their respective RGD ligands.

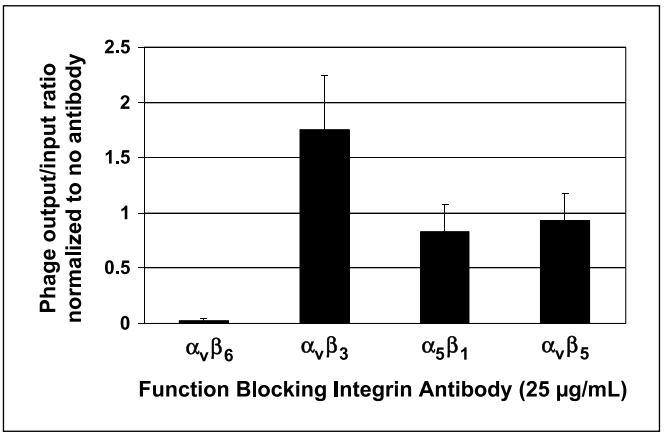

Figure 1. The TP H2009.1 peptide binds $\alpha_v\beta_6$ through an integrin-binding domain. Blocking of phage uptake is observed when the binding assay is done in the presence of anti-$\alpha_v\beta_6$ antibody. All values are normalized to phage uptake in the absence of any antibody.

At the concentration of 25 μg/mL, the anti-$\alpha_v\beta_6$ antibody obstructs almost all phage uptake (Fig. 1). Antibodies against $\alpha_v\beta_3$, $\alpha_5\beta_1$, and $\alpha_v\beta_5$ are unable to block phage uptake. Surprisingly, anti-$\alpha_v\beta_3$ antibodies increase phage uptake. The reason for this phenomenon is unknown but one possibility is that the antibodies block a nonproductive or weaker binding event of the TP H2009.1 phage. The blocking of phage binding by the anti-$\alpha_v\beta_6$ antibody is concentration dependent (Supplementary material). Phage uptake is unaffected when this antibody is added to another phage clone that is specific for H1299 cells, a large cell lung carcinoma cell line that does not bind TP H2009.1 (data not shown). The almost complete inhibition of phage uptake by the function blocking anti-$\alpha_v\beta_6$ antibody suggests that the $\alpha_v\beta_6$ integrin on H2009 cells is the functional receptor for the TP H2009.1 peptide.

Binding of the TP H2009.1 peptide is correlated to expression of the $\alpha_v\beta_6$ integrin. To determine if peptide binding correlates to $\alpha_v\beta_6$ expression, expression levels of the integrin on the cell surface of several cell lines were determined by flow cytometry analysis (Fig. 2). We previously assessed the binding of the TP H2009.1 phage clone on a panel of lung cancer cell lines (9, 10). The TP H2009.1 peptide has high affinity for the cell line on which it was selected (H2009), another adenocarcinoma cell line (H1648), and a human SAEC. Moderate affinity is observed for the adenocarcinoma line H441. The peptide has no significant affinity for the large cell lung carcinoma lines H1299 and H460. All cell lines that display $\alpha_v\beta_6$ on the cell surface bind TP H2009.1, whereas low-expressing cell lines show dramatically reduced peptide binding. Furthermore, the higher the expression levels of $\alpha_v\beta_6$, the greater the observed binding of the TP H2009.1 peptide. A linear correlation of the mean fluorescence intensity and the selectivity value for peptide binding is observed with an R^2 correlation coefficient of 0.96. Cell surface expression of $\alpha_v\beta_6$ on H2009 cells was also confirmed by immunofluorescence. H1299 cells have substantially reduced $\alpha_v\beta_6$ expression, and it is not located at the cell surface (Supplementary material). Thus, peptide binding is directly correlated to the cell surface expression of the $\alpha_v\beta_6$ integrin.

Additionally, the expression levels of the other putative integrin targets, $\alpha_v\beta_3$, $\alpha_5\beta_1$, and $\alpha_v\beta_5$, for the peptide were determined on both H2009 and H1299 cells by flow analysis. All three of these integrins are expressed on both cell lines with similar expression levels (Supplementary material). As TP H2009.1 peptide binds to the H2009 cells and not to the H1299 cells; this indicated that the peptide is specific for $\alpha_v\beta_6$ and does not bind the other RGD-binding integrins.

Expression of $\alpha_v\beta_6$ converts a nonbinding cell line into a TP H2009.1–binding cell line. The binding of TP H2009.1 to a nonbinding cell line and that same cell line transfected with the β_6 gene was determined to confirm $\alpha_v\beta_6$ as the receptor. The human colon carcinoma cell line SW480 expresses the α_v subunit but not the β_6 subunit. Transfection of this cell line with the full-length human β_6 gene results in expression of functional $\alpha_v\beta_6$ at the cell surface (12). As predicted, the parental line that does not express $\alpha_v\beta_6$ is resistant to binding of the TP H2009.1 phage. However, the β_6-transfected cells bind TP H2009.1 phage 260 times better than the parental cell line (Supplementary material). The increased phage uptake is due to the expression of $\alpha_v\beta_6$, as uptake can be reduced by >90% by anti-$\alpha_v\beta_6$ antibody. Thus, expression of $\alpha_v\beta_6$ can convert a resistant cell to a TP H2009.1 peptide–binding cell.

The H2009.1 peptide can deliver a fluorescent nanoparticle via the $\alpha_v\beta_6$ receptor. We have previously synthesized the TP H2009.1 peptide on a trilysine core (10). To confirm that the free peptide mediates cellular uptake via $\alpha_v\beta_6$, the biotinylated peptide [RGDLATLRQLAQEDGVVGVRK(biotin)PEG_6]$_4$Lys$_2$-Lys-β-Ala-COOH was conjugated to a streptavidin coated quantum dot

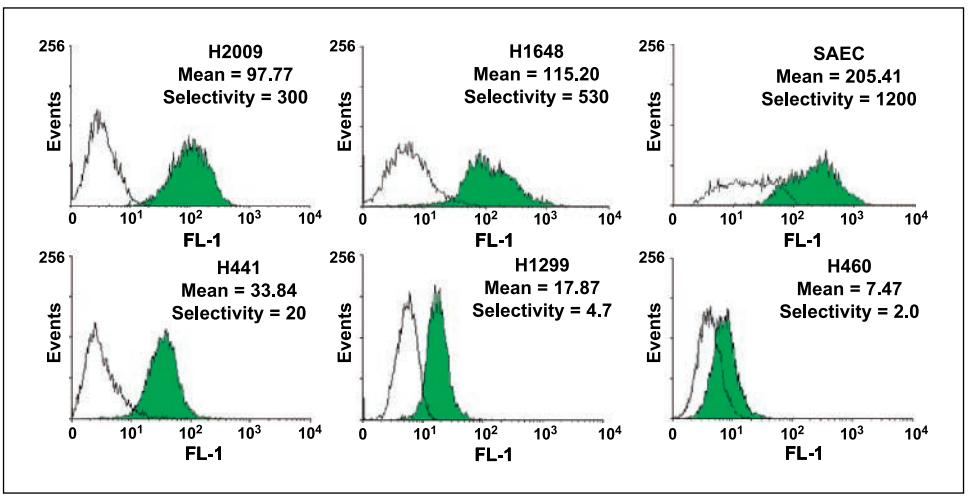

Figure 2. Binding of the TP H2009.1 peptide is correlated to the expression of β_6. Flow cytometric analysis was done to determine surface expression of $\alpha_v\beta_6$. High expression of $\alpha_v\beta_6$ is observed on cell lines that also have affinity for the TP H2009.1 peptide. The selectivity values are shown in the inset for each cell line.

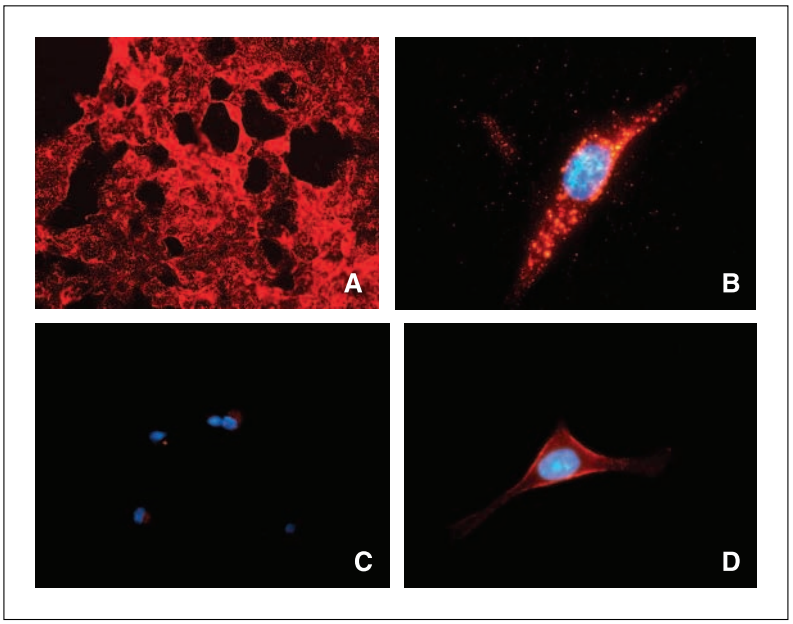

Figure 3. The tetrameric TP H2009.1 peptide mediates uptake of a fluorescent nanoparticle to H2009 cells via the $\alpha_v\beta_6$ receptor. H2009 cells were incubated with SAQdot605 conjugated to tetrameric TP H2009.1 peptide (20 nmol/L based on Qdot concentration). The fluorescence of the Qdot was visualized at magnification of ×100 (*A*). Higher magnification (1000×) shows punctate localization of the tetrameric TP H2009.1 peptide-SAQdot605 conjugate (*B*). In the presence of $\alpha_v\beta_6$ antibody, binding of the TP H2009.1 peptide–SAQdot605 conjugate is abrogated (*C*). Fixation of the H2009 before binding of the TP H2009.1 peptide–SAQdot605 conjugate results in only surface-bound Qdots (*D*).

(SAQdot605). The TP H2009.1 peptide is able to mediate selective binding of the SAQdot605 to H2009 cells (Fig. 3). Significantly reduced binding is observed when a tetrameric control peptide is conjugated to the particle, indicating that Qdot uptake is mediated by the TP H2009.1 peptide (Supplementary material). Furthermore, no uptake of the TP H2009.1-Qdot conjugate is observed on H460 cells (data not shown), consistent with the binding profile of H2009.1 phage clone (9). Higher magnification reveals significant amounts of punctate fluorescence within the cell with clear nuclear exclusion (Supplementary material; Fig. 3*B*). Importantly, peptide-Qdot uptake is inhibited when the cells are treated with function blocking anti-$\alpha_v\beta_6$ antibody (Fig. 4*C*), indicating that uptake of the free peptide is mediated by $\alpha_v\beta_6$. Fixation of H2009 cells with paraformaldehyde before TP H2009.1-Qdot binding results in cell surface–bound particles with no internalization (Fig. 4*F*), consistent with receptor-mediated endocytosis being blocked. Additionally, this result suggests that the peptide may have utility for probing fixed human tissues.

The β_6 subunit is expressed in human lung tumors and is associated with poor patient survival. A concern of using cell lines for identification of potential biomarkers is that the cell surface expression profile in culture may not reflect the state of the cancer cells in a tumor, although β_6 expression has been observed in a small set of lung tumors (23). To determine if $\alpha_v\beta_6$ is widely expressed in human lung carcinomas, we probed the expression of this integrin on a TMA of 311 patient samples in which clinical outcomes were known. The samples consisted of 18 SCLC and 293 NSCLC, including 180 adenocarcinomas, 6 adenosquamous carcinomas, and 107 squamous cell carcinomas (Supplementary material). Of these, 22 samples were excluded from the analysis as they contained mostly tumor stroma and normal lung tissue. Staining in each sample was quantified from tumor TMA cores using a four-value membrane score. Scores of 0 and 1 were

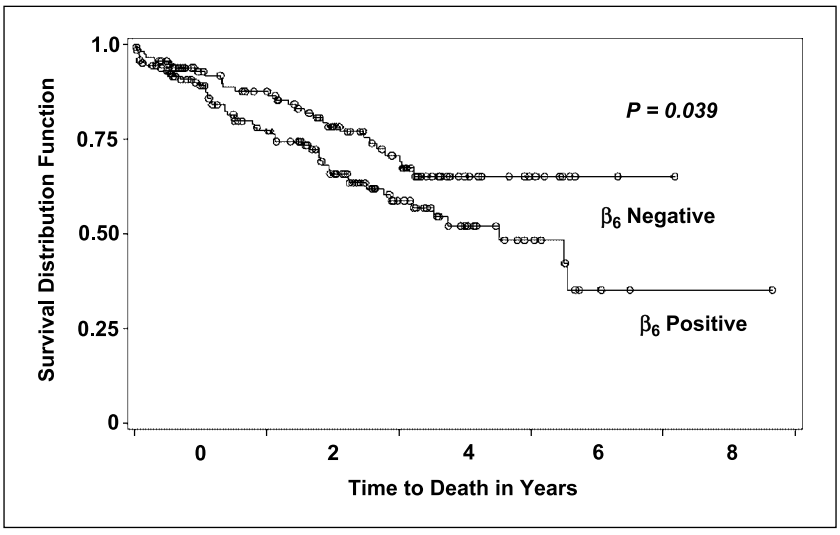

Figure 4. Kaplan-Meier survival analysis indicates that β_6 expression is correlated with reduced survival times. Patients were grouped according to β_6-positive or β_6-negative expression. Analysis was done using a log-ranked test. It should be noted that the survival time for this patient population is longer than that of the standard lung cancer patient population because of the high percentage of early-stage patients in the study.

Table 1. Survival analysis for lung cancer related deaths using Cox regression model for multivariate effects

Effects	Hazard ratio	95% Hazard ratio	*P*
Age at surgery	1.0	1.0–1.1	0.0007
Smoking status	1.1	0.62–2.0	0.73
Recurrence	2.6	1.5–4.6	0.0007
Gender	1.8	1.0–3.0	0.038
Stage II versus Stage I	1.7	0.92–3.3	0.087
Stage III versus Stage I	2.5	1.3–4.8	0.0082
Stage IV versus Stage I	3.2	0.42–24	0.26
Integrin membrane class	1.9	1.1–3.2	0.018

considered negative for $\alpha_v\beta_6$, whereas scores of 2 and 3 were considered positive. Using these variables, 54% of the samples stained positive for $\alpha_v\beta_6$. Thus, $\alpha_v\beta_6$ is expressed in certain human lung tumors, and β_6 expression in the lung tumor cell lines is not an artifact of the *in vitro* environment.

As $\alpha_v\beta_6$ is expressed in human tumors, we sought to determine statistically relevant correlations with its expression and clinical features of the disease. Significantly, more expression of $\alpha_v\beta_6$ was observed in the NSCLC samples than the SCLC samples ($P = 0.020$, χ^2 test), indicating that this integrin is a potential marker for NSCLC and may play a role in NSCLC but not in SCLC. For this reason, the remaining analysis was done only with NSCLC samples. Whereas there is a distinct difference in expression between SCLC and NSCLC, there is no significant difference in expression levels between the different histologic classes of NSCLC ($P = 0.90$, Fisher exact test). The sample set contained no large cell lung carcinoma samples, so expression levels in this subset of NSCLC cannot be addressed. Expression of $\alpha_v\beta_6$ is observed in a significant number of stage I tumors, and there is no significant relationship between expression and stage of diagnosis ($P = 0.63$, Fisher exact test). Thus, expression of the integrin occurs in early-stage cancers and is not confined to late-stage cancers. Additionally, there is no correlation between β_6 expression and node status ($P = 0.46$, χ^2 test). Expression level does not seem to be associated with patient gender ($P = 0.23$, χ^2 test). Surprisingly, there is a significant correlation between smoking history and β_6 expression. Nonsmokers diagnosed with lung cancer display a higher level of β_6 expression ($P = 0.033$, χ^2 test).

To determine if there is a relationship between $\alpha_v\beta_6$ expression and patient survival in NSCLC, a log-rank test was done (Fig. 4). There is significant survival difference between patients with β_6-positive tumors when compared with patients whose tumors were negative for expression of the integrin ($P = 0.039$, log-ranked test). Additionally, using a Cox's proportional hazard model, the hazard ratio for β_6 expression was determined adjusting for age, gender, stage, and reoccurrence (Table 1). Expectedly, the routine prognostic factors, such as age at surgery and cancer stage, were found to have significant effects on patient outcome. However, although the hazard ratio is high for stage IV cancers, the *P* value is high due to the small number of patients in this category. Expression of β_6 is predictive of poor patient survival, with a hazard ratio of 1.9 observed for patients with β_6-positive tumors. These data taken together suggest that patients with β_6-expressing tumors are at significant risk for poor clinical outcomes.

The $\alpha_v\beta_6$ integrin is expressed preferentially in lung tumors. For $\alpha_v\beta_6$ to be a molecular receptor for targeted cancer therapies, its expression must be higher in the tumor compared with normal tissues. Expression of $\alpha_v\beta_6$ was determined in 31 human tumor samples containing the surrounding normal bronchus or bronchiole

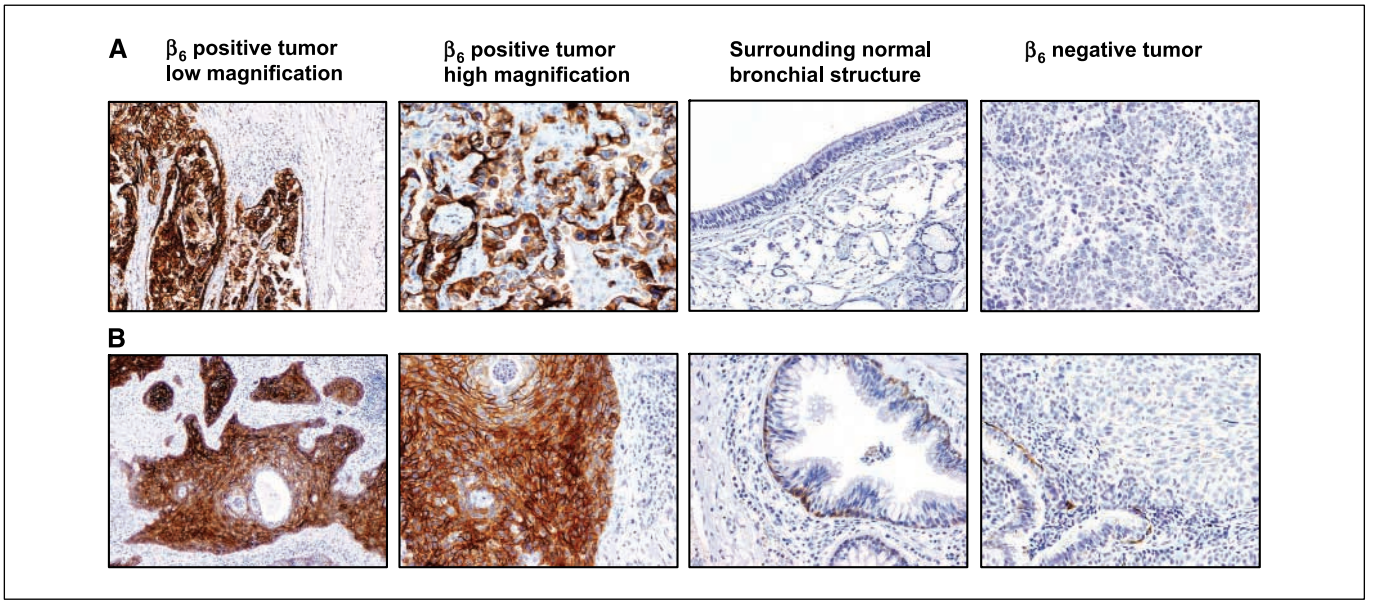

Figure 5. The integrin $\alpha_v\beta_6$ is expressed preferentially in lung tumors compared with surrounding nonneoplastic tissue. *A,* adenocarcinoma tumor samples were probed for the expression of $\alpha_v\beta_6$ in the tumor and surrounding normal tissue. Two magnifications (10× and 20×) of a β_6-positive adenocarcinoma tumor sample invading the surrounding stroma. Normal bronchial wall from the same sample, including a ciliated epithelium, displaying negative immunostaining. A representative sample of another adenocarcinoma case showing β_6-negative expression for comparison. *B,* squamous tumor samples were probed for the expression of $\alpha_v\beta_6$ in the tumor and surrounding normal tissue. Two magnifications (10× and 20×) of a β_6-positive squamous tumor sample invading the surrounding stroma. Normal small bronchial structure from the same case shows patches of β_6 expression in basal epithelial cells. A separate squamous cell carcinoma case with β_6-negative expression is shown for comparison.

tissue. Representative samples are shown in Fig. 5. A few key features are noted. First, the staining for β_6 in the tumors is stronger than that of surrounding normal tissue. Using the same ranking scheme as the TMA, the normal and tumor tissues were each assigned a rank score. Of the 17 adenocarcinoma tumor samples, 12 were positive for $\alpha_v\beta_6$ expression. Positive expression was only observed in the normal tissue for two of the samples. In both cases, expression was observed in the bronchiole and was less for the normal tissue than the tumor. Similarly, expression was higher in the squamous tumors compared with the normal tissue. Using the paired nonparametric signed rank test, the expression in the tumors is significantly higher than that of the surrounding normal tissue ($P < 0.0001$ for both groups combined, $P = 0.0009$ for adenocarcinomas, and $P = 0.011$ for squamous). Secondly, the staining intensity is fairly homogeneous in the tumor. However, the leading edge of the tumor as it invades the stroma was examined, and whole-tumor slices were not probed. Thus, the degree of expression throughout an entire tumor mass cannot be determined. Third, although the expression is significantly less in nontumor tissue, some expression is observed in the basal cells of the airway at the basement membrane and, to a lesser extent, the columnar cells. The expression level of $\alpha_v\beta_6$ is variable and is not seen in all samples or in all basal cells, including samples in which the tumor is positive for β_6 expression. It is not clear whether $\alpha_v\beta_6$ is expressed in normal basal cells or if the expression is in response to the tumor environment or represents premalignant cells. Lung tissues from healthy subjects were unavailable for study. However, even for the β_6-positive basal cells, the staining intensity is dramatically less than that observed within the tumor.

Discussion

Unbiased phage panning on intact cells allows for the isolation of highly specific cell targeting peptides even for cells in which the cell surface is undefined. Once identified, these peptides can be used as "fishing hooks" to identify unique cellular receptors. Thus, in addition to yielding cell-targeting reagents for drug delivery, this approach is useful in identifying features present on the cell surface. In such a manner, we have identified the cellular target of an adenocarcinoma lung cancer–targeting peptide to be the integrin $\alpha_v\beta_6$.

Our results show that the peptide binds specifically to $\alpha_v\beta_6$ over other RGD-binding integrins. Integrin-binding specificity is determined by the sequence flanking the RGD domain, especially the sequence to the carboxy terminal side of the RGD (7, 24, 25). This is supported by the observation that strains of FMDV that have the sequence RGDLXXL use the $\alpha_v\beta_6$ integrin with the highest efficiency (26). Binding peptides have been isolated by panning a linear 12-mer–peptide library on recombinant truncated $\alpha_v\beta_6$ (27). From this selection, two classes of peptides were observed: those containing the RGD motif and a second group with the sequence DLXXLX. Interestingly, 9% of the peptides selected had the sequence RGDLXXL, which corresponds to the sequence of the TP H2009.1 peptide. However, the predominant clone was in the DLXXL family. We have also used H2009 cells as bait to pan two other phage-displayed peptide libraries. Although these libraries were obtained from other sources, peptides containing the RGDLXXL motif were selected.[7] We did not observe any DLXXL motif peptides, suggesting that different peptides can be selected when the target receptor is embedded in the cell membrane and perhaps the RGD is required for internalization. Although peptide ligands for $\alpha_v\beta_6$ have been previously isolated, the unbiased selection on cells in culture allowed for this integrin to be identified as a candidate for targeted therapies for lung cancer. Furthermore, the selection isolated a ligand that mediated uptake into the target cells. Thus, biopanning has the advantage that both cell-targeting reagents with the desired properties and information about the cell surface of the target cell can be obtained.

The role of $\alpha_v\beta_6$ in lung cancer progression has not been explored. Expression of $\alpha_v\beta_6$ has been shown to be up-regulated during wound healing (28) and has been reported on other epithelial-derived malignancies, including oral, colon, and ovarian cancers (28–32). Our data show that $\alpha_v\beta_6$ expression is also seen in a significant number of human NSCLC. Expression is observed in early stage tumors and is not restricted to later stages of the disease. However, it should be noted that the sample set available is skewed toward early-stage lung cancers due to the availability of tumor samples. More studies are needed to determine the role of $\alpha_v\beta_6$ during the progression of the disease. Importantly, our data show that presence of $\alpha_v\beta_6$ is an independent prognostic factor for poor patient survival in NSCLC, and patients with $\alpha_v\beta_6$-positive tumors are associated with reduced survival times. Consistent with these observations, increased $\alpha_v\beta_6$ expression is correlated with enhanced cell migration and increased secretion of matrix metalloprotease 9 *in vitro* (33–36). Furthermore, β_6 expression increases during the epithelial-mesenchymal transition in a colon carcinoma model, and β_6 expression is predictive of reduced survival time for patients with colorectal cancer (37). Thus, $\alpha_v\beta_6$ may be a potential therapeutic target to prevent metastasis. Further work needs to be done to understand the biological role of $\alpha_v\beta_6$ in lung cancer.

Surprisingly, $\alpha_v\beta_6$ integrin expression is correlated with smoking status. The biological reason for this observation is unknown. However, nonsmoking lung cancer patients are more likely to have mutations in the epidermal growth factor (EGF) receptor (EGFR) and EGFR amplification (38). Whereas the EGFR mutation status and expression level have not been determined for this patient set, it is possible that the correlation arises from this effect. Additionally, EGF has been shown to increase β_6 expression in primary human airway epithelial cells in culture, suggesting that signaling via the EGFR pathway can regulate expression of this integrin (39). We are exploring the possibility that $\alpha_v\beta_6$ expression is correlated with EGFR amplification or activation.

For $\alpha_v\beta_6$ to be a marker for diagnosis and targeted therapy, it must be present on the cancer cell but not on normal tissues. Expression of the β_6 subunit has been reported to be restricted to epithelial cells, and its expression is low or undetectable in most normal adult primate tissues (40). Relevant to lung cancer, expression of $\alpha_v\beta_6$ has not been detected in the proximal airway and normal bronchial epithelium of nonsmoking patients (23, 41). However, up-regulation of $\alpha_v\beta_6$ has been detected in the proximal airways of a subset of cigarette smokers. It is not clear if this is a response to cigarette smoke, chronic inflammation, or development of neoplasia (41). Our data reveals a clear difference in the expression in the tumor compared with surrounding normal tissue. Additionally, no expression was observed for commercially available TMA containing tumors and matched normal lung samples, although the tissue represents mostly lung parenchyma with alveolar structures distant from the tumor site (data not shown). Although expression of $\alpha_v\beta_6$ is significantly reduced in the normal tissue, we did observe expression in a subset of the basal

[7] SL, LW, and KCB, unpublished results.

cells found along the basement membrane. As patient information is unavailable for these samples, the smoking history is unknown. Further studies need to be done to determine the extent of β_6 expression in the lungs. However, the differential expression of this integrin indicates that $\alpha_v\beta_6$ may be exploited for targeted delivery of therapeutics or imaging agents.

Conflicting with the *in vivo* expression patterns is the affinity of the TP H2009.1 peptide for the "normal" control, SAEC. Interestingly, $\alpha_v\beta_6$ expression can be induced on primary bronchioepithelial cells in culture as a response to the production of autocrine factors by cells in culture (41) or the addition of growth factors (39). It is possible that TP H2009.1 is binding SAEC because of $\alpha_v\beta_6$ expression induced by the *in vitro* environment. Such change in integrin expression has not been observed for lung cancer cells *in vitro* (42). This stresses the importance of identification of the cellular receptors responsible for peptide binding. Important leads could be discounted otherwise. Furthermore, it highlights the need for better control bronchial epithelial cell lines (43).

In conclusion, we have identified $\alpha_v\beta_6$ as the cellular receptor for a lung adenocarcinoma–targeting peptide. The expression of this integrin is widespread in early stage NSCLC, and its expression is associated with poor patient survival. Furthermore, the restricted expression profile of this integrin suggests that it may be a good receptor for targeted therapies for lung cancer as well as other cancers in which $\alpha_v\beta_6$ plays a role. We are currently exploring the use of this cell targeting as a homing ligand for NSCLC.

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