Glycodelin: A New Biomarker with Immunomodulatory Functions in Non–Small Cell Lung Cancer

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Abstract

Purpose: In recent years, immune therapeutic strategies against non–small cell lung cancer (NSCLC) based on tissue-derived biomarkers, for example PD1/PD-L1 (CD274), have evolved as novel and promising treatment options. However, the crosstalk between tumor and immune cells is poorly understood. Glycodelin (gene name PAEP), initially described in the context of pregnancy and trophoblastic implantation, is a secreted immunosuppressive glycoprotein with an as-yet largely unknown function in lung cancer.

Experimental Design: In this study, we characterized the expression and role of glycodelin in NSCLC through mRNA and protein expression analyses, functional knockdown experiments, and correlations with clinicopathologic parameters.

Results: Glycodelin mRNA expression was significantly elevated in tumors (n = 336) compared with matched normal tissue (P < 0.0001). Overall survival (OS) was significantly reduced in NSCLC with high glycodelin mRNA levels in women but not in men. Glycodelin was detected in the sera of patients, and the levels correlated with recurrence and metastatic disease. Knockdown of glycodelin with siRNAs in NSCLC cell lines resulted in significant upregulation of immune system modulatory factors such as PD1, CXCL5, CXCL16, MICA/B, and CD83 as well as proliferation stimulators EDN1 and HBEGF. Furthermore, decreased migration of tumor cells was observed.

Conclusions: Altogether, the comprehensive characterization of glycodelin in NSCLC provides strong support for its use as a biomarker with immune modulatory function. Clin Cancer Res; 21(15); 3529–40. ©2015 AACR.

Introduction

With approximately 1.6 million cases worldwide, lung cancer is the leading cause of cancer-related death (1). Historically, lung cancer is divided into two groups: non–small cell lung cancer (NSCLC), which accounts for more than 80% of all cases, and small-cell lung cancer (SCLC; ref. 2).

To improve the poor survival of lung cancer patients, chemotherapy treatments are progressively supplemented with precision therapy based on genetic analyses. Sequencing of lung carcinoma demonstrated a high somatic mutation rate of TP53, KRAS, and EGFR (3). First-line and second-line tyrosine kinase inhibitors that target the EGFR or anaplastic lymphoma kinase (ALK) improve progression-free survival (4–6). Surface markers and secreted chemokines of altered cells attract lymphocytes, natural killer cells (NK) or cytotoxic T cells. The tumor microenvironment is strongly defined by extracellular matrix (ECM) reorganization and chemokine secretion of tumor cells that leads to a barrier for lymphocytes (7). Infiltration of NSCLCs with lymphocytes is associated with better disease-free survival (8). Thus, immune therapy has the potential to stimulate the patient’s immune system response and attack cancer cells more effectively. Manipulation of NK cells and natural killer T cells (NKT) is considered to be a useful tool for cancer immunotherapy (9, 10).

Glycodelin (gene name PAEP), initially described as placental protein 14 (PP14) or progesterone-associated endometrial protein (PAEP), is strongly associated with trophoblastic invasiveness (11). Downregulation of glycodelin leads to an increased activation of the maternal immune system and can result in abortion during the first trimester (12). Glycodelin is differentially glycosylated depending on gender and function (13). Glycodelin A, the immunosuppressive form of glycodelin, functions as a proliferation-suppressor and apoptosis inducer of T cells, monocytes, B
Translational Relevance
Glycodelin is well described as an immune system modulator during pregnancy. In this work, we demonstrate the expression and secretion of glycodelin in non–small cell lung cancer (NSCLC), its potential role as an immune system modulator, and the feasibility of using glycodelin as a biomarker for NSCLC. Glycodelin mRNA (gene name PAEP) was overexpressed in approximately 80% of all tumors compared to normal tissue. Knockdown of PAEP resulted in a deregulation of immune system modulators, for example, PD-L1, a current immune therapeutic target. We demonstrate the detection of glycodelin in tissues and sera of NSCLC patients. The fact that the glycodelin mRNA levels correlated with the patients’ response to treatment suggests its use as a biomarker for follow-up of NSCLC. A diagnostic tool for glycodelin quantification in pregnancy is commercially available and might be conveniently used for use in lung cancer diagnoses.

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Tissue samples were provided by Lung Biobank Heidelberg, a member of the accredited Tissue Bank of the National Center for Tumor Diseases (NCT) Heidelberg, the BioMaterialBank Heidelberg and the Biobank platform of the German Center for Lung Research (DZL). All diagnoses were made according to the 2009 WHO classification for lung cancer by at least two experienced pathologists. Detailed descriptions are provided in the Supplementary Materials and Methods.

Total RNA isolation and cDNA synthesis
Total RNA was isolated from tissue using an RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions. For RNA isolation from cell lines, the AllPrep DNA/RNA Mini Kit (Qiagen) was used. Total RNA was transcribed to scDNA with a Transcriptor First Strand cDNA Synthesis Kit (Roche). Detailed descriptions are provided in the Supplementary Materials and Methods.

Gene-expression analyses
For gene-expression analysis, we used a human genome expression microarray (Affymetrix U133 Plus 2.0, Affymetrix) covering approximately 47,000 transcripts and variants. The microarray experiment was performed with three biologic replicates of control siRNA-transfected and PAEP siRNA pool-transfected samples per cell line. Detailed descriptions are provided in the Supplementary Materials and Methods.

Quantitative Real-Time PCR
Real-time quantitative PCR (qPCR) was performed in accordance with MIQE-guidelines (19) using a LightCycler 480 Real-Time PCR Instrument in a 384-well plate format (Roche). Gene-specific primers and probes (Universal ProbeLibrary; Roche) were used in combination with ABsolute QPCR Mix (Thermo Scientific). Ct values were calculated with LightCycler 480 software version 1.5 using the second derivative maximum method (Roche). Detailed descriptions are provided in the Supplementary Materials and Methods.

Immunohistochemistry
To compare the expression of whole glycodelin and glycodelin A, the polyclonal N-20 antibody (sc-12289, Santa Cruz Biotechnology) and the monoclonal A87-B/D2 (Glycotope) antibody, raised against glycodelin A purified from mid-trimester amniotic fluid, were used. Detailed descriptions are provided in the Supplementary Materials and Methods.

Detection of glycodelin in human sera
Human sera were collected before any disease-specific treatment and stored at –80°C within 2 hours after venipuncture. The NSCLC validation cohort included pretherapeutic sera of 159 randomly selected NSCLC patients (clinical stage I–IV). The chronic obstructive pulmonary disease (COPD) control cohort included 90 randomly selected sera of COPD I–IV patients. The glycodelin levels of the sera were detected using an enzyme-linked immunosorbent assay kit (ELISA BS-20-30; Bioserv Diagnostics) with 50 µL of each serum in two technical replicates. The readout and standard curve were performed with ELISA Reader (Tecan Group Ltd.). The results of ELISA were visualized with GraphPad Prism 5. Detailed descriptions are provided in the Supplementary Materials and Methods.

Statistical analyses
Data of qPCR analyses were statistically analyzed under REMARK criteria (20) with SPSS 22.0 for Windows (IBM). The evaluation of discriminatory values for PAEP expression in tumor that best differentiated between groups of patients with good and poor survival prognosis was performed with the critelevel procedure using ADAM statistical software package (German Cancer Research Center, DKFZ, Heidelberg, Germany; ref. 21). Binary variables were built using these cutoffs. The endpoint of the study was overall survival (OS). Therefore, survival time was calculated from the date of surgery until the last date of contact or death. Univariate analysis of survival data was performed according to Kaplan and Meier (22). The log-rank test was used to test the significance between the groups. A P value of less than 0.05 was considered significant. Multivariate survival analysis was performed using the Cox proportional hazards model. Receiver Operating Characteristic (ROC) analyses were performed to examine the diagnostic accuracy of glycodelin. ELISA data were statistically analyzed with GraphPad Prism 5. The non-parametric Mann–Whitney U test (23) as well as the Kruskal–Wallis test (24) were used to investigate significant differences between the patient groups.
Cell culture

The cell line 2106T was generated from human squamous cell carcinomas (SCC) as described previously (18) and cultivated in DMEM/Ham’s F-12 (Life technologies) with 10% FCS (PAA, GE Healthcare Life Sciences) for not more than 20 passages. The H1975 cell line was purchased from the ATCC (Wesely, Germany) and cultivated in DMEM/Ham’s F-12 with 10% FCS. MeWo melanoma cell line was purchased from cell lines service (CLS, Eppelheim, Germany) and cultivated with EMEM supplemented with 2 mMol/L L-glutamine, 1% nonessential amino acids, 1 mMol/L sodium pyruvate, and 10% FCS (prefix from CLS). For the indicated experiments, cell lines were cultured for a maximum of 24 hours with complete serum-free growth medium. Detailed descriptions are provided in the Supplementary Materials and Methods.

siRNA experiments

The following siRNAs were used (Qiagen): Hs_PAEP_1, Hs_PAEP_2, Hs_PAEP_3, Hs_PAEP_5 (Gene Solution siRNA, #1027416). For all assays, all four siRNAs were pooled and used with a resulting RNAi concentration of 10 nmol/L. Allstars negative control siRNA (Qiagen, #1027280) was used as a non-silencing control siRNA. Detailed descriptions are provided in the Supplementary Materials and Methods.

Cytokine array

2106T, H1975, and MeWo cells were transfected with control or PAEP pooled siRNA for 72 hours. The medium was replaced by complete serum-free growth medium for 24 hours. Supernatants were collected and used for cytokine arrays (C-series, custom designed, RayBiotech). Most regulated cytokines and chemokines were selected from the Affymetrix gene-expression analyses. The assay was performed according to the manufacturer’s instructions. Signals were evaluated using Image Studio Lite (LI-COR). Experiments were performed using at least two biologic replicates with two technical replicates each.

Migration assay

For the migration assay, 2106T (2.5 \times 10^6) and H1975 (3 \times 10^6) cells were seeded onto a 24-Well plate. The next day, cells were transfected with control siRNA or the PAEP pooled siRNA for 72 hours. Afterwards, cells were serum-starved overnight (16 hours), treated with 10 \mu g/mL mitomycin C (Applichem) for 2 hours and detached with Accutase-solution (Promocell). Approximately 5 \times 10^5 cells were applied with 300 \mu L serum-free DMEM onto ThinCerts Cell Culture Inserts (Greiner bio-one) with a pore size of 8 \mu m. At the bottom side, 500 \mu L of DMEM completed with 10% FBS was prepared. After 24 hours, cells that migrated through the membrane were detached from the underside of ThinCerts with Accutase-solution. Cells were washed with PBS, and LDH activity was determined with the Cytotoxicity Detection Kit (LDH; Roche) as a measure of relative cell number. The relative migration of control siRNA-transfected cells was set to 100%. Experiments were performed three times with at least three biologic replicates each.

Results

PAEP is strongly overexpressed in adenocarcinomas and squamous cell carcinomas

PAEP mRNA expression was investigated in 362 NSCLC samples. Patients’ characteristics are presented in Table 1. PAEP was expressed (Ct value < 38) in 90% of all tumors, but only in 54% of the corresponding normal tissues (Table 2). One hundred forty-four patients (40%) exhibited PAEP exclusively in tumor but not in normal tissue. Twenty-six (7%) patients did not express PAEP either in tumor or in normal tissue and were excluded from further analyses.

The comparison of tumor and normal tissue revealed that PAEP expression levels were significantly upregulated in more than 80% of all SCCs (P < 0.0001) and more than 75% of all adenocarcinomas (ADC; P < 0.0001) compared with matched normal tissue (Fig. 1A and D). No significant gender-specific expression of PAEP was observed between male and female patients in our study. The median expression of PAEP mRNA in ADC was significantly higher than in SCC (P < 0.0001, Fig. 1B). Both cancer types exhibited a high-expression range of PAEP in tumor tissue, ranging from 20-fold downregulation to a more than 8,000-fold upregulation of expression compared with paired normal tissue. Regarding the different stages of patients’ disease, median PAEP expression was upregulated in stage II (P < 0.05 for ADC) and stage III for both ADC and SCC (Fig. 1C). Although PAEP was expressed in a broad range of more than 16 qPCR cycles in tumor tissue, normal tissue exhibited lower expression with less variation (Fig. 1D), suggesting a tumor-specific function of glycodelin.

Expression of PAE in NSCLC is a prognostic factor in females

Statistical analyses were performed with patients expressing PAEP in NSCLC (n = 336). Patients with SCC and increased mRNA levels of PAEP exhibited a reduced OS rate (Fig. 1E). However, the difference in survival was not significant (P = 0.139). In contrast, the intratumoral expression level of PAEP had no influence on the prognosis of ADC patients (Fig. 1F). The survival of NSCLC patients was strongly associated with gender. Females exhibited a significantly reduced OS rate when expressing a high PAEP level (P = 0.014, Fig. 1G). On the other side, there was no correlation between expression level and survival in male patients (Fig. 1H). Further analyses indicated that lymph node status is a prognostic factor in women (P = 0.016, Supplementary Fig. S1). In a multivariate analysis, including PAEP expression, histology, gender and lymph node status, only gender (female vs. male) and lymph node status (pN2 vs. pN0) were significant prognostic factors (Table 2). High PAEP expression (high vs. low, see Supplementary Materials and Methods) was associated with a moderately increased HR. However, the statistical analysis revealed only a trend (P = 0.19).

Glycodelin as well as immunosuppressive glycodelin A is expressed in NSCLC tissue

To verify the glycodelin protein expression in NSCLC, IHC was performed on formalin-fixed and paraffin-embedded (FFPE) tissue slides derived from patients (n = 13) that were previously shown to have a high glycodelin mRNA expression. Four representative samples (two ADC and two SCC) are presented in Fig. 2. The expression pattern of glycodelin varied between the NSCLC subtypes. In general, glycodelin staining exhibited greater heterogeneity in ADC (Fig. 2A, patient 1 and 2). In contrast, glycodelin was expressed more homogeneously, but at a lower level in almost all tumor cells of SCC (Fig. 2A, patients 3 and 4). Antibody specificity was tested and validated in controls using a blocking peptide (Supplementary Fig. S2A). A monoclonal antibody was used to compare the expression of an immunomodulating form of glycodelin, glycodelin A, with...
that of total glycodeolin in NSCLC. Glycodeolin was expressed by most tumor cells (Fig. 2B, top). However, we observed a various staining pattern with the anti-glycodeolin A antibody (Fig. 2B, bottom). Some signals of both antibodies were completely overlapping. In some cases, glycodelin A expression differed from that of total glycodelin or could not be detected.

Normal lung alveolar cells exhibited low expression of glycodelin (Fig. 2A, third column). All stained tumor tissues were infiltrated by CD8-positive T cells (Fig. 2A, last column). Glycodeolin expression could also be observed in the tumor cells of infiltrated lymph nodes (Supplementary Fig. S2C).

Secreted glycodelin can be detected in the sera of NSCLC patients with primary tumor, recurrence, or metastatic spread. Glycodeolin is known to be a secretory protein. Therefore, we investigated the possibility of detecting the protein in the sera of

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<th>Parameter</th>
<th>% (n)</th>
<th>Median age (65 (38–88))</th>
<th>Gender (362)</th>
<th>Histology (250)</th>
<th>Squamous (112)</th>
<th>Therapy (211)</th>
<th>OP (151)</th>
<th>OP/RT (13)</th>
<th>OP/CT (100)</th>
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Table 2. Patients’ characteristics

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NOTE: Bold values indicate significant events.

Table 2. PAEP expression in lung tissue

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<td>Gender (female vs. male)</td>
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NOTE: Bold values indicate significant events.
Figure 1. PAEP is highly overexpressed in lung cancer and leads to a decreased OS. A, PAEP expression in 336 lung tumor and corresponding normal tissues was quantified by qPCR and tumor versus normal tissue expression. B to D, normalized tumor Ct values were visualized with GraphPad Prism. E to H, the percentage of OS is depicted for tumors with normalized Ct values <9.8 or >9.8; (I), median; T, tumor; N, normal; **, P < 0.05; ***, P < 0.0001.
NSCLC and non-cancer patients. In the detection cohort, patients shown in Fig. 1A were divided into subgroups of high ($\Delta C_t < 3$, $n = 30$), average ($6.8 < \Delta C_t < 8.5$, $n = 30$), and low $PAEP$ mRNA tumor expression ($\Delta C_t > 13$, $n = 15$). Serum samples, which were collected before surgery, were tested for glycodelin. Patients with benign hamartomas ($n = 15$) were included as a non-cancer control. The highest amounts of glycodelin were detected in the sera of patients who exhibited high gene expression of $PAEP$ (Fig. 3A), with a median concentration of 8.9 ng/mL and a maximum of 228.4 ng/mL. The glycodelin serum concentrations from high and average $PAEP$ mRNA-expressing patients were significantly increased compared with non-cancer patients. The detection of glycodelin in sera of NSCLC patients was verified in a large cohort of samples randomly picked from the lung biobank of the Thoraxklinik. In addition to patients who were treated by surgery alone or in combination with adjuvant...
chemo-and/or radiotherapy ("early disease cohort"), this cohort also included inoperable tumor stages IIIB–IV treated by chemo- and/or radiotherapy. We then compared the glycodelin serum levels with those of a randomly selected COPD cohort (Fig. 3B and Table 1). The median glycodelin serum concentration was significantly higher compared to that of the COPD control group. Females with affected lymph nodes had significantly higher glycodelin serum concentrations than with unaffected lymph nodes (Supplementary Fig. S3A). This is in contrast with males where no difference in serum concentrations was detected in Figure 3.

Glycodelin is secreted from tumors. A to E, glycodelin concentration was quantified by ELISA. A, patients were grouped related to the qPCR expression level of PAEP and serum level of glycodelin was determined before surgery. B, elevated glycodelin serum levels in NSCLC detection cohort were confirmed in a validation cohort and compared with patients with COPD. C, 10 randomly selected adenocarcinoma and corresponding normal lung tissue pieces were homogenized and glycodelin concentrations were measured. D, serum level was measured before and after excision of tumor. E, glycodelin concentration in serum was quantified before surgery and during follow-up ward rounds. Patient 3 showed a recurrence of primary tumor. An adrenal gland metastasis of Patient 4 was resected after ward round four. F, primary tumor and metastasis of patient 4 was stained for glycodelin; T, tumor; N, normal; R, recurrence; M, metastasis; WR, ward round; +, tumor-dependent death.

Figure 3.
Glycodelin is secreted from tumors. A to E, glycodelin concentration was quantified by ELISA. A, patients were grouped related to the qPCR expression level of PAEP and serum level of glycodelin was determined before surgery. B, elevated glycodelin serum levels in NSCLC detection cohort were confirmed in a validation cohort and compared with patients with COPD. C, 10 randomly selected adenocarcinoma and corresponding normal lung tissue pieces were homogenized and glycodelin concentrations were measured. D, serum level was measured before and after excision of tumor. E, glycodelin concentration in serum was quantified before surgery and during follow-up ward rounds. Patient 3 showed a recurrence of primary tumor. An adrenal gland metastasis of Patient 4 was resected after ward round four. F, primary tumor and metastasis of patient 4 was stained for glycodelin; T, tumor; N, normal; R, recurrence; M, metastasis; WR, ward round; +, tumor-dependent death.
Knockdown of glycodelin leads to a reorganization of genes involved in tumor immunosurveillance, environment modulation, and cell migration in NSCLC cell lines. A to H, 2106T and H1975 were transfected with a pool of four different PAEP-specific siRNAs for 72 hours. A and B, medium was changed and 24 hours later, supernatants as well as cells were collected and glycodelin was detected by western blot. β-Actin and tubulin were used as loading controls. (Continued on the following page.)
and H1975 were taken after knockdown of three technical repeats each. D, medium was replaced by DMEM different days with at least two biologic replicates each for 24 hours; (Supplementary Fig. S3C). All homogenized tumors exhibit high intratumoral glycodelin concentrations despite low serum levels found in patients 3 and 4. Glycodelin concentrations in the homogenates of randomly selected tumors from 10 ADC patients were significantly higher \( (P = 0.039) \) compared with matched normal tissue (Fig. 3C). To further substantiate the tumor as the source of glycodelin in patient serum, glycodelin was measured in the sera after complete tumor resection by surgery. As presented in Fig. 3D, the relative glycodelin concentrations in the sera decreased significantly \( (P = 0.0005) \) after the resection of the tumor. In a further approach, the glycodelin serum concentration was measured during the clinical follow-up of patients (Fig. 3E and Supplementary Fig. S3D). As demonstrated in Fig. 3C, all four patients exhibited a strong decrease in glycodelin secretion in the second ward round after tumor resection. Glycodelin levels in relapse-free patients 1 and 2 remained stable at approximately 1.5 ng/mL after surgery. In contrast, we observed increasing glycodelin concentrations in patients with recurrence or metastatic spread (patient 3 and 4). IHC of the primary tumor confirmed glycodelin expression in patient 4 (Fig. 3F). Patient 2106, the donor of the cell line used in this study, exhibited an increasing glycodelin serum concentration during follow-up until tumor-independent death (Supplementary Fig. S2D).

The presence of glycodelin in patients’ sera suggests that measurement of glycodelin levels might be a useful tool for disease monitoring and recurrence detection in NSCLC.

Knockdown of glycodelin in cancer cell lines leads to a reorganization of genes involved in tumor immunosurveillance, environment modulation, and cell migration

Glycodelin expression and secretion by lung cancer cells has not been observed so far. To investigate a functional role of glycodelin in NSCLC, PAEP was silenced by siRNA-transfection in two NSCLC cell lines (Fig. 4A and B) and in the MeWo cell line (Supplementary Fig. S4A). Downregulation by RNAi resulted in strongly decreased intracellular and secreted protein levels of glycodelin. Interestingly, culture medium with 2% FCS alone caused an antibody signal (Fig. 4A and B, first lane). This signal resulted from FCS, because culture medium without supplements as well as complete serum-free growth medium did not exhibit this band (Supplementary Fig. S5D). For siRNA transfection, a pool of four different PAEP siRNAs was used. Every single siRNA strongly reduced glycodelin expression (Supplementary Fig. S5E).

MeWo cells exhibited the highest mRNA expression of PAEP (data not shown) and glycodelin secretion of several investigated cell lines (Supplementary Fig. S5F). In addition to 2106T and H1975, we also observed secretion of glycodelin in the supernatant of the large cell carcinoma cell line H460 and the breast cancer-derived cell line MDA-MB-436. No secretion was observed for the monocytic cell lines THP1 and Jurkat or the NK cell line KHYG-1.

The role of glycodelin as a regulator of the immune system is well characterized during pregnancy (25) and hormone-related cancer (15). Many studies of glycodelin A have reported immunosuppressive functions. To gain further insight into the potential molecular functions of glycodelin in tumor cells, we performed microarray gene-expression profiles of the three cell lines 2106T, H1975, and MeWo after silencing of PAEP. The highest regulated genes (Supplementary Table S1) were validated by qPCR. Results are illustrated in Fig. 4C and Supplementary Fig. S4B. Knockdown of PAEP led to strong mRNA upregulation of immune system-regulating ligands (MHC class I polypeptide-related sequence proteins [MICA/B], C–C motif chemokine 5 and 16 [CXCL5 and CXCL16], cluster of differentiation 83 [CD83], cluster of differentiation 274 and programmed cell death 1 ligand 2 [CD274 and PDCD1LG2], also known as PDL1/2) and to deregulation of cytokeratin and cell matrix genes [claudin-1 (CLDN1), keratin 17 (KRT17) and keratin-associated protein 2–3 (KRTAP2-3), urokinase-type plasminogen activator (PLAU)], as well as to an increase of signal pathway–regulating proteins [e.g., endothelin-1 (EDN1), prostat glandin G/H synthase 1 and 2 (PTGS1/2), also known as COX1/2] and proheparin-binding EGF-like growth factor (HHEG)]. A cytokine and chemokine array was used to validate the gene-expression data. The secretion of chemokines CCL5, CXCL16, and CXCL5 was increased, whereas secretion of cluster of differentiation 14 (CD14), osteopontin (SPP1), and Ii.11 was downregulated (Fig. 4D and Supplementary Table S1). Matrix metalloprotease-9 (MMP9) expression was strongly decreased in 2106T. Upregulation of PDL1 (in 2106T and H1975) and PTGS2 mRNA (in H1975) after PAEP silencing was confirmed at the protein level by Western blot analyses (Fig. 4E). An example of cytokine array data is presented in Supplementary Fig. S5G. We observed a change in cell structure after knockdown of PAEP (Fig. 4F and Supplementary Fig. S4C) in 2106T, H1975, and MeWo cells. Silencing of PAEP resulted in a significant reduction of migration for 2106T \( (P < 0.0001, \text{Fig.} \; 4F) \) and H1975 \( (P < 0.0001, \text{Fig.} \; 4G) \). MeWo cells did not exhibit any migration in this assay. These data indicate that glycodelin expression is involved in the regulation of tumor immunology.

Discussion

In the present study, we demonstrated the overexpression of glycodelin mRNA (PAEP) in NSCLC in a large patient cohort. For the first time, we reported the expression and secretion of glycodelin in several lung cancer cell lines. Furthermore, we demonstrated an immunomodulatory function of glycodelin in NSCLC.
Knockout of glycodelin in NSCLC cell lines led to the differential regulation of immune system-relevant genes that exhibit potential as targets for NSCLC therapeutics (26), for example, PD-L1 (CD274), which was upregulated after silencing of PAEP. Our findings provide evidence that glycodelin detection in human serum might be useful to monitor the response of NSCLC patients to tumor treatment.

To date, the expression of glycodelin has been well described mainly in hormone-related tumors such as breast and ovarian cancer (15). Zadran and colleagues (27) recently reported that glycodelin mRNA (PAEP) was one of the most differentially regulated genes in a small cohort of lung ADC patients compared with healthy patients. In our study, we demonstrated that glycodelin was highly expressed in a large cohort of NSCLC samples. PAEP mRNA levels in tumors were elevated in approximately 80% of all patients compared with normal tissue and were upregulated in advanced tumor stages. High PAEP expression was associated with shorter survival in SCC. In particular, women seemed to exhibit worse survival, which was observed independently of tumor pathology but was possibly influenced by lymph node status. Because glycodelin A is regulated by progesterone in females during the menstruation cycle and pregnancy (28), there might be a hormone-regulated mechanism that triggers glycodelin expression in NSCLC. We aim to clarify the mechanism of glycodelin regulation in a future study and we will investigate the role of EGFR and KRAS, which are the most common driver mutations in lung cancer (3).

In our immunohistochemical stainings of NSCLC tumors, we observed the inhomogeneous expression of glycodelin in tumors. Glycodelin was highly expressed in tumors that were infiltrated by CD8+ T cells. Two studies reported better survival for NSCLC patients with a high rate of infiltrating macrophages and T cells (8, 29). We were not able to detect PAEP mRNA expression in approximately half of all normal tissues. In the case of a very low expression level, this could be attributed to the glycodelin expression of bronchial epithelial cells. We detected higher glycodelin protein concentrations in the serum samples of NSCLC compared with patients with COPD. In women, glycodelin is normally expressed during the menstrual cycle. Because the median age of lung cancer patients in our large validation cohort was 64, the secretion of glycodelin in the context of menstruation cycle can be largely excluded. A valid AUC can be interpreted as the probability that the classifier will assign a higher score to a randomly chosen positive example than to a randomly chosen negative example. The ROC analyses of NSCLC cohort indicated that glycodelin was only conditionally suitable for early detection of NSCLC. In this context, we observed that a high glycodelin expression in tumor tissues did not necessarily mean that glycodelin was also measurable in patients’ sera. Several factors such as tumor size, position, vascularization, spread, etc., might influence the secretion of glycodelin into the blood. Therefore, the sensitivity of the glycodelin detection in serum was too low for early detection of NSCLC. However, our data demonstrate that the measurement of glycodelin in the serum might be useful for the clinical follow-up of surgically treated patients. We observed not only a correlation between the glycodelin serum level and the tumor response to treatment, but also at recurrence at least in most patients who pretherapeutically expressed glycodelin. Other studies have suggested the use of glycodelin as a serum marker for colon cancer (30) and ovarian cancer (31).

Tumors might use various strategies to bypass the immune system. Tumors remodel their microenvironment. In our study, we demonstrated that the knockdown of glycodelin results in the decreased expression of PLAI in 2106T and H1975 and the decreased expression of PAEP in 2106T and H1975. Both proteins are involved in the regulation of the ECM. In this regard, the dense matrix areas around lung tumors have been shown to strongly influence the cell migration (7). Increasing glycodelin concentrations have been linked to deregulated PLAI and MMP9 activity and inhibition of trophoblast invasiveness (11). This finding is supported by observations in melanoma cells, where cell migration and invasion were strongly inhibited after glycodelin silencing (16). In our study, we observed the expression of glycodelin in metastatic lymph nodes and distant metastases. Similarly, we observed that glycodelin knockdown changed cell morphology and led to the decreased migration of NSCLC cell lines. We observed that glycodelin was secreted by breast cancer, melanoma and several lung cancer cell lines. Therefore, glycodelin expression might be a general mechanism by which other cancers regulate tumor immunology.

Tumors escape immunosurveillance through the regulation of chemokine secretion and the presentation of cell surface molecules. Immunoediting is a well-known important factor within this context (32). In our work, we observed that several immune system modulators were upregulated in cell lines after siRNA knockdown of PAEP. MICA and MICB, both MHC-I–related chains (MIC), are ligands for NKG2D, a receptor on the surface of NK cells and CD8+ T cells (33). For lung cancer, MICA and MICB are reportedly recognized by tumor-infiltrating T cells (34). The expression of immune system-defending factors CD83 as well as chemokines CXCL5 and CXCL16 is strongly increased after knockdown of PAEP. CXCL5 and CXCL16 have been shown to be involved in angiogenesis (35, 36) and attracting CD4+ immune cells in NSCLC (37), similar to PTGS2/COX2 (38). CD83 expression influences CD4+ T-cell development by stimulating dendritic cells (39). Altogether, glycodelin knockout leads to the reorganization of immune system-stimulating and immune system-silencing factors and plays an important role in the immune system regulation of NSCLC.

The fetomaternal interface that is present during pregnancy has evolved immunomodulatory properties to allow the fetus to invade the uterus without an attack from the maternal immune system (12). Parallels between cancer and pregnancy have been described in detail (40). As the knockdown of PAEP in our study regulated immune system-relevant genes in particular, we postulate that glycodelin expression and secretion are essential for lung cancer cells to escape from the immune system.

In a prospective study, we plan to analyze glycodelin serum levels in the palliative setting to investigate whether glycodelin might be used as a surrogate for the success of chemotherapy and/or radiotherapy.

In summary, we have demonstrated the expression of glycodelin at the mRNA and protein levels in NSCLC. From our results and the known immune modulating functions of glycodelin, it is feasible to propose an immune system defense mechanism of lung tumors through the expression and secretion of glycodelin. Glycodelin produced by tumor cells might reduce the effect of current therapies using immune-checkpoint inhibitors (e.g., PD-L1–targeted therapies), as it is able to remodel the host immune system and the tumor immune system defense. Targeting glycodelin in NSCLC therapy might be a new mechanism to overcome
tumor immunosurveillance. Our data strongly suggest that the detection of glycodelin in the tumor and serum seems to be helpful in designing tumor-specific immunotherapies and in monitoring the response to treatment during clinical follow-up of NSCLC patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-14-2464

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