MUC4-Mediated Regulation of Acute Phase Protein Lipocalin 2 through HER2/AKT/NF-κB Signaling in Pancreatic Cancer

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Abstract

Purpose: MUC4 shows aberrant expression in early pancreatic lesions and a high specificity for pancreatic cancer. It thus has a high potential to be a sensitive and specific biomarker. Unfortunately, its low serum level limits its diagnostic/prognostic potential. We here report that a multifaceted acute phase protein lipocalin 2, regulated by MUC4, could be a potential diagnostic/prognostic marker for pancreatic cancer.

Experimental Designs and Results: Overexpression/knockdown, luciferase reporter and molecular inhibition studies revealed that MUC4 regulates lipocalin 2 by stabilizing HER2 and stimulating AKT, which results in the activation of NF-κB. Immunohistochemical analyses of lipocalin 2 and MUC4 showed a significant positive correlation between MUC4 and lipocalin 2 in primary, metastatic tissues (Spearman correlation coefficient 0.71, \( P = 0.002 \)) from rapid autopsy tissue sample from patients with pancreatic cancer as well as in serum and tissue samples from spontaneous KRASG12D mouse pancreatic cancer model (Spearman correlation coefficient 0.98, \( P < 0.05 \)). Lipocalin 2 levels increased progressively with disease advancement (344.2 ± 22.8 ng/mL for 10 weeks to 3067.2 ± 572.6 for 50 weeks; \( P < 0.0001 \)). In human pancreatic cancer cases, significantly elevated levels of lipocalin 2 were observed in patients with pancreatic cancer (148 ± 13.18 ng/mL) in comparison with controls (73.27 ± 4.9 ng/mL, \( P = 0.014 \)). Analyses of preand postchemotherapy patients showed higher lipocalin 2 levels in prechemotherapy patients [121.7 ng/mL; 95% confidence interval (CI), 98.1–150.9] in comparison with the postchemotherapy (92.6 ng/mL; 95% CI, 76.7–111.6; \( P = 0.06 \)) group.

Conclusions: This study delineates the association and the downstream mechanisms of MUC4-regulated elevation of lipocalin-2 (via HER2/AKT/NF-κB) and its clinical significance for prognosis of pancreatic cancer. Clin Cancer Res; 20(3); 1–13. ©2013 AACR.

Introduction

Pancreatic cancer, with annual incidences of 45,220 and mortality of approximately 38,460, is among the leading causes of cancer-related deaths in the United States. More than 90% of patients with pancreatic cancer, who at the time of diagnosis present with unresectable disease and distant metastasis, result in a 5-year survival rate of <6% (1). Early-stage pancreatic cancer symptoms are generally vague and nonspecific, which contribute to late diagnosis of the disease. The lack of early diagnostic markers and an intrinsic resistance to chemo- and radiotherapies are the major obstacles to successful management of pancreatic cancer at present.

Mucins present themselves as barriers between epithelial cell surfaces and their luminal milieu. Aberrant changes in mucin, both in expression and localization, are commonly observed in inflammatory conditions as well as in premalignant and malignant lesions (2, 3). Emerging evidence indicates that the cytoplasmic tail of mucins regulates the various chronic and acute phase responses, besides providing the well-known barrier functions by forming the epithelial surface covering. The tails act as central points of interaction between extracellular milieu and cellular cytoplasm (2). MUC4, a membrane-tethered mucin, is normally expressed in the luminal epithelial cells of the stomach, lung, trachea, cervix, and prostate, but is not expressed in normal gall bladder, liver (including intrahepatic and extrahepatic bile ducts), or pancreatic cells (4). De novo expression of MUC4 has been observed during pancreatic cancer development and progression (5, 6). Previously, we...
reported that MUC4 can alter behavioral properties of pancreatic cancer cells to potentiate their growth and metastasis (7–9).

Lipocalin 2 is a low-molecular weight glycoprotein that mediates a number of cellular processes, including apoptosis, proliferation, epithelial to mesenchymal differentiation, and matrix metalloproteinase (MMP)-9 stabilization (10–12). High levels of lipocalin 2 expression are observed in renal, breast, ovary, colon, and brain cancer cells (13). The cDNA microarray analyses have revealed that lipocalin 2 expression in pancreatic cancer cells is 27 times higher than in normal pancreatic cells (14), and it is among the top differentially expressed proteins during pancreatic cancer progression (15). Lipocalin 2 expression differentially impacts tumor growth, invasion, and metastasis of pancreatic cancer cell depending upon differentiation status (16, 17). Furthermore, in vitro studies have shown that lipocalin 2 enhances sensitivity of pancreatic cancer cell lines towards gemcitabine (17). Recently, Raffatellu and colleagues observed simultaneous upregulation of lipocalin 2 and MUC4 transcripts in the ileal mucosa of rhesus macaques in response to enteric Salmonella typhimurium infection (18). We observed that MUC4 and lipocalin 2 are also differentially expressed in pancreatic cancer tissues, from premalignant PanINs to invasive pancreatic cancer (2, 6, 15). Furthermore, microarray analyses of MUC4 knockdown cells show multifold decrease in lipocalin 2 expression (unpublished results). Furthermore, Toll-like receptors (TLR) are directly involved in regulation of lipocalin 2 (19). However, the link between mucin and lipocalin 2 has remained obscure.

In the present study, we explored lipocalin 2 for its associations and regulation, and examined the clinical significance of elevated lipocalin 2 in pancreatic cancer. Our findings indicated that MUC4 and lipocalin 2 are coordinately expressed during the progression of pancreatic cancer, and also provided evidence for a novel regulatory mechanism of the expression of lipocalin 2 in pancreatic cancer.

Materials and Methods

Cell lines, reagents, and treatments

See Supplementary Section

Quantitative real-time PCR

To assess the transcriptional regulation of lipocalin 2 by MUC4, total RNA (2 μg) from CD18/HPAF/shMUC4 and CaPan1/shMUC4, the MUC4 knockdown cells, and CD18/HPAF/Src and CaPan1/Src control cells was reverse transcribed using a First-Strand cDNA Synthesis Kit (PerkinElmer) and oligo-d(T) primers according to the manufacturer’s instructions. Quantitative real-time PCR amplifications were carried out with 100 ng of first-strand cDNA in 10 μL reaction volumes. The reaction mixture was subjected to a two-step cycle program (95°C for 10 minutes followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute) using an ABI 7500 Sequence Detection System (Applied Biosystems) with SYBR Green chemistry as per manufacturer’s instructions. The relative fold differences in gene expression were calculated using the ΔΔCt method with β-actin as a normalization control.

Sandwich ELISA

DuoSet ELISA Development Kit was used to measure lipocalin 2 levels in supernatants, and plasma of human as well as mouse samples as per manufacturer’s instructions. This retrospective dual center study for biomarkers in pancreatic cancer was approved by the Institutional Review Boards (IRB) of the University of Nebraska Medical Center (UNMC; Omaha, NE; IRB number 209-00, IRB #091-01-EP). Samples from pancreatic cancer (n = 91), and healthy controls (n = 24) were used to measure the lipocalin 2 amount. In case of patients with pancreatic cancer, "post-surgery" cases include samples from patients who have undergone a pancreatectoduodenectomy, a pancreatectomy, or a pancreatectoduodenogastroctomy before the sample was drawn. All other samples, including those from patients who never had surgery during the course of their disease, were classified as "presurgery." Any sample drawn before the patient had undergone chemotherapy for pancreatic cancer was classified as "prechemotherapy." If the patient ever had chemotherapy for pancreatic cancer, regardless of whether or not that patient was undergoing chemotherapy at the time the sample was taken, the sample was classified as "postchemotherapy." Standard curves were produced from lipocalin 2 standards provided with the kit and serially (log2) diluted from 4 ng/mL to 15.6 pg/mL. ELISA plates were read at 450 nm and collected data were analyzed using the SoftMax Pro software program (Molecular Devices). Data were analyzed using SAS statistical software version 9.2 (SAS Institute Inc.). Levels of lipocalin 2 were analyzed as continuous variables, whereas tumor grade, cancer stage,
surgical status, and chemotherapy status were considered to be categorical. Patient characteristics were compared between pancreatic cancer and control patients using ANOVA model for continuous variables and \( \chi^2 \) tests or Fisher exact test wherever applicable. Biomarkers were analyzed on the natural log scale due to the skewed nature of the data. Biomarkers were compared between groups using \( t \) tests and ANOVA models. If significant differences were found in the overall \( P \) values from the ANOVA models then pairwise comparisons were made adjusting for multiple comparisons with Tukey method. \( P \) values \( \leq 0.05 \) were considered statistically significant.

Confocal immunofluorescence microscopy

To study the localization and effect of MUC4 knockdown on lipocalin 2, confocal microscopy was performed on MUC4-expressing cells. CD18/HPAF/shMUC4 and CaPan1/shMUC4, MUC4 knockdown cells, and CD18/HPAF/Scr and CaPan1/Scr control cells were grown on sterilized cover slips for 48 hours. For immunostaining of lipocalin 2 and MUC4, cells were washed following by blocking with 10% goat serum (Jackson Immunoresearch Laboratories) containing 0.05% Tween-20 for 1 hour. The cells were then washed five times with PBS and incubated with the anti-MUC4 and lipocalin 2 antibodies for 60 minutes at room temperature. Next, the cells were washed five times with PBS containing 0.05% Tween-20 and incubated with fluorescein isothiocyanate-conjugated anti-mouse and Alexa-568–conjugated anti-rabbit secondary antibodies for 30 minutes at room temperature. Then, the cells were washed five times with PBS and incubated with 0.05% Tween-20 and incubated with fluorescein isothiocyanate-conjugated anti-mouse and Alexa-568–conjugated anti-rabbit secondary antibodies for 30 minutes at room temperature. The nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) and mounted on glass slides in anti-fade VECTASHIELD Mounting Medium (Vector Laboratories). An LSM 510 microscope (Carl Zeiss GmbH) was used to perform laser confocal microscopy. Microphotographs of lipocalin 2 and MUC4 staining were provided by the Rapid Autopsy Program (RapidAutopsy Program at the University of Michigan and commercial pancreatic tissue array (Accumax). These were subjected to immunohistochemical (IHC) analysis as described previously (15).

Luciferase reporter assay

To assess the activity of the lipocalin 2 promoter in MUC4-expressing and knockdown cells, cells were cultured to 60% confluence in 6-well plate. Following serum starvation for 4 hours, cells were transfected with 1 \( \mu \)g of promoter reporter construct containing either an intact NF-κB–binding site (pGL3-luc/lipocalin 2;−900) or a mutation that prevents NF-κB binding (pGL3-luc/lipocalin 2; NF-κB-mut) and 0.1\( \mu \)g of control Renilla Luciferase plasmid using Lipofectamine 2000 (Invitrogen Life Technologies). Dual-Luciferase Reporter Assay was performed 24 hours after transfection as per manufacturer’s instructions (Promega). For data analysis, firefly luciferase activity was normalized to that of Renilla luciferase.

Immunoblot assay

Various cell types were processed for protein extraction and immunoblotting using standard procedures. Cell lysates were prepared as described previously (21). Protein concentrations in the lysates were determined using the Bio-Rad D/C protein estimation kit. For MUC4, the lyase (20 \( \mu \)g each) were resolved using electrophoresis on a 2% SDS-agarose gel under reducing conditions. For β-actin, lipocalin 2, HER2, p1248HER2, AKT, pAKT, NF-κB, pNF-κB, IK-κB, and pIK-κB, SDS-PAGE (10%–12%) was performed under similar conditions. Resolved proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane, blocked in 5% nonfat milk in PBS for 2 hours, and subjected to standard immunodetection using specific antibodies. For immunodetection of β-actin, which was used as the internal control, an anti-β-actin mouse monoclonal antibody (Sigma) in a 1:2000 dilution of 0.1% PBS-T was used; for MUC4 immunodetection, an anti-MUC4 mouse monoclonal antibody (8G7; diluted at 1:1000 in PBS) was used. For immunodetection of HER2 (Santa Cruz Biotechnology), p1248HER2 (Upstate), AKT, pAKT, NF-κB, pNF-κB, IK-κB, and pIK-κB, the respective rabbit polyclonal antibodies (Cell signaling Technology) diluted 1:1000 in TBS with tween-20 (50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 0.05% tween-20) were used. The PVDF membranes were incubated for overnight at 4°C and then subjected to 10-minute washes (four times) in TBS-T. The membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences) diluted at 1:2000 in PBS for 1 hour at room temperature and washed six times in TBS-T. The blots were processed using an electrochemiluminescence kit (Amersham Biosciences), and signals were detected by exposing the processed blots to X-ray film (BioMax films; Kodak).

Immunohistochemical analyses of MUC4 and lipocalin 2

Formalin-fixed paraffin-embedded (FFPE) pancreatic cancer tissue microarray (TMA) slides for lipocalin 2 and MUC4 staining were provided by the Rapid Autopsy Program at the UNMC and commercial pancreatic tissue array (Accumax). These were subjected to immunohistochemical (IHC) analysis as described previously (15).

Statistical analysis

For statistical analysis, each spot on TMA was considered an individual sample. Lipocalin 2 and MUC4 expression (positive or negative), the tissue type (normal or pancreatic cancer), and pancreatic cancer grade (well, moderately, or poorly differentiated) were considered categorical variables. These variables were compared using the \( \chi^2 \) test. Continuous variables were compared using the two-tailed Student \( t \) test. \( P \) values less than 0.05 were considered statistically significant.

Results

MUC4 regulates lipocalin 2 expression

To explore mucin-mediated regulation of lipocalin 2, the expression of lipocalin 2 and MUC4 was evaluated in MUC4 knockdown cells and vice versa. The regulatory role of MUC4 was assessed by its stable knocking down in CD18/HPAF and CaPan1 pancreatic cancer cells using MUC4 short hairpin (shRNA), which otherwise express high level of MUC4. Resulting stable transfec...
(CD18/HPAF/shMUC4 and CaPan1/shMUC4) were quantitated for the lipocalin 2 and MUC4 expression by immunoblot analyses (Fig. 1). Scrambled shRNA-transfected CD18/HPAF (CD18/HPAF/Scr) and CaPan1 (CaPan1/Scr) cells were used as a controls. The lipocalin 2 expression levels in CD18/HPAF/shMUC4 and CaPan1/shMUC4 cells were markedly lower than those in control cells (Fig. 1A). Because lipocalin 2 is secretory, we also examined lipocalin 2 concentration in the spent media of MUC4 knockdown and control cells. Briefly, $2 \times 10^5$ CD18/HPAF and CaPan-1 MUC4 knockdown cells and their respective controls were cultured in 2 mL of 1% Dulbecco’s Modified Eagle Medium for 48 hours. The amount of lipocalin 2 was measured in spent supernatant using a DuoSet lipocalin 2 ELISA Kit (R&D Systems). MUC4 knockdown significantly reduced the amount of secreted lipocalin 2. C, confocal analysis in MUC4 knockdown and control cells for lipocalin 2 (Alexa 568 staining; red), MUC4 (fluorescein isothiocyanate staining), and nuclei (DAPI staining). MUC4 knockdown significantly reduced the expression and secretion of lipocalin 2 in CD18/HPAF and CaPan1 cells.

Figure 1. Effect of MUC4 knockdown on lipocalin 2 expression in pancreatic cancer cell lines. A, immunoblot analyses were used to assess the expression of MUC4 and lipocalin 2 in CD18/HPAF/shMUC4 and CaPan1/shMUC4 MUC4 knockdown cells and CD18/HPAF/Scr and CaPan1/Scr control cells. Proteins from cell extracts (20 µg) were resolved using electrophoresis on a 2% SDS-agarose and 12% SDS-PAGE gel for MUC4 and lipocalin 2, respectively. Separated proteins were transferred to a PVDF membrane and incubated with an anti-MUC4 monoclonal antibody (8G7) and rabbit anti-lipocalin 2 antibodies. The membrane was then probed with horseradish peroxidase-labeled goat anti-mouse immunoglobulin. β-actin was used as an internal control. B, lipocalin 2 concentration in the culture supernatants of CD18/HPAF and CaPan-1 MUC4 knockdown and control cells (2x10^5 CD18/HPAF and CaPan-1 MUC4 knockdown cells and their respective controls were cultured in 2 mL of 1% Dulbecco’s Modified Eagle Medium for 48 hours). The amount of lipocalin 2 was measured in spent supernatant using a DuoSet lipocalin 2 ELISA Kit (R&D Systems). MUC4 knockdown significantly reduced the amount of secreted lipocalin 2. C, confocal analysis in MUC4 knockdown and control cells for lipocalin 2 (Alexa 568 staining; red), MUC4 (fluorescein isothiocyanate staining), and nuclei (DAPI staining). MUC4 knockdown significantly reduced the expression and secretion of lipocalin 2 in CD18/HPAF and CaPan1 cells.

CD18/HPAF/shMUC4 and CaPan1/shMUC4) were quantitated for the lipocalin 2 and MUC4 expression by immunoblot analyses (Fig. 1). Scrambled shRNA-transfected CD18/HPAF (CD18/HPAF/Scr) and CaPan1 (CaPan1/Scr) cells were used as a controls. The lipocalin 2 expression levels in CD18/HPAF/shMUC4 and CaPan1/shMUC4 cells were markedly lower than those in control cells (Fig. 1A). Because lipocalin 2 is secretory, we also examined lipocalin 2 levels in the culture supernatants of CD18/HPAF/shMUC4 and CaPan1/shMUC4 cells and in controls by sandwich ELISA technique. The lipocalin 2 expression levels in the culture supernatants of CD18/HPAF/shMUC4 and CaPan1/shMUC4 cells were significantly lower than those in the CD18/HPAF/Scr and CaPan1/Scr control cells (Fig. 1B). In addition, confocal microscopy revealed that CD18/HPAF/shMUC4 and CaPan1/shMUC4 MUC4 knockdown cells were significantly lower than those in the CD18/HPAF/Scr and CaPan1/Scr control cells (Fig. 1B). In addition, confocal microscopy revealed that CD18/HPAF/shMUC4 and CaPan1/shMUC4 MUC4 knockdown cells were lower lipocalin 2 expression than CD18/HPAF/Scr and CaPan1/Scr control cells (Fig. 1C). Relative expression of lipocalin 2 was in correspondence with MUC4 expression. However, no alterations in MUC4 expression were observed in lipocalin 2-overexpressing cell lines (MiaPaCa/lipocalin 2 and Panc1/lipocalin 2) and lipocalin 2 knockdown cell lines (AsPC1/sh-lipocalin 2) (Supplementary Fig. S1). In addition to pancreatic cancer cells, we observed a similar phenomenon in lung cancer cell lines (data not shown). These findings suggest that lipocalin 2 is a downstream target of MUC4.

**MUC4 mediates its effect on lipocalin 2 by stabilizing HER2**

We have previously reported that MUC4 interacts with HER2 and regulates its expression via posttranslational mechanisms (22, 23). Furthermore, we found that MUC4 stabilizes HER2 to increase the migratory potential of ovarian cancer cells (24, 25). In breast cancer cells, studies by others have identified lipocalin 2 as a prominent downstream gene of HER2 signaling (26–29). To determine whether MUC4 exerts its regulatory effect on lipocalin 2 in pancreatic cancer cells through HER2, we examined the expressions of HER2, its activated form pHER2, and its downstream signaling proteins in CD18/HPAF/shMUC4 and CaPan1/shMUC4 MUC4 knockdown cells, and in their respective controls (Fig. 2A). To reconfirm that HER2 plays regulatory role in the regulation of lipocalin 2 expression, we analyzed lipocalin 2 expression in the stable HER2 knockdown clones (CD18/HPAF/shHER2 and CaPan1/shHER2). HER2 and MUC4 knockdown showed a significant reduction of lipocalin 2 expression in CD18/HPAF and CaPaN1/shHER2 cells compared with that in scrambled shRNA-transfected cells (Fig. 2B).
MUC4 Regulates Lipocalin 2 through HER2/AKT/NF-κB Pathway

MUC4 mediates its effect on lipocalin 2 expression via the HER2/PI3K/AKT/NF-κB pathway

We analyzed the regulation of MUC4 and HER2 downstream actors in the knockdown clones of CD18/HPAF and CaPan1 cells. Immunoblot analyses of AKT and pAKT expression in CD18/HPAF/shMUC4 and CaPan1/shMUC4 cells revealed that pAKT (Ser473) activation levels were significantly increased, whereas the levels of expression of total AKT remained unchanged. Because HER2 mediates its effect on AKT through the PI3K pathway, we sought to assess the role of AKT in lipocalin 2 regulations in pancreatic cancer cells by treating CD18/HPAF and CaPan1 cells with the PI3K inhibitor LY294002. Immunoblot analyses revealed that when a standard dose of LY294002 (50 μmol/L) was used, it resulted in a time-dependent decrease in pAKT and lipocalin 2 expression in CD18/HPAF and CaPan1 cells (Fig. 2C and D), which confirmed that AKT is involved in MUC4-mediated regulation of lipocalin 2 expression.

To identify the downstream mechanisms of MUC4-mediated regulation of expression of lipocalin 2 in pancreatic cancer cells, we used real-time quantitative PCR (qPCR) analyses to measure lipocalin 2 mRNA levels in CaPan1/shMUC4 MUC4 knockdown cells and in scrambled shRNA-transfected control cells. These experiments revealed that lipocalin 2 mRNA levels were higher in the scrambled cells than in the MUC4 knockdown cells (Fig. 3A). A similar trend was observed for MUC4 mRNA (Fig. 3A).

In silico analysis of the lipocalin 2 promoter by TRESS indicated the presence of an NF-κB-binding site (Fig. 3B). To delineate the role of NF-κB in the regulation of lipocalin 2 expression, CD18/HPAF MUC4 scrambled and knockdown cells were transfected with luciferase constructs of the proximal region of the lipocalin 2 promoter with 900 bp of wild-type lipocalin 2 (pGL3-lipocalin 2-267) containing either an intact NF-κB-binding site or an inactivating mutation in the NF-κB-binding site, along with Renilla luciferase. When compared with the knockdown cells, relative luciferase activity was significantly higher in the CD18/HPAF/Scr cells (Fig. 3C). To determine whether MUC4 regulates lipocalin 2 expression through NF-κB, we assessed the levels of total IK-κB and pIK-κB in MUC4 and HER2 knockdown clones of CD18/HPAF and CaPan1 cells, and found that knockdown of either MUC4 or HER2 resulted in decreased IK-κB phosphorylation (Fig. 3D and E).
To further characterize the role of NF-κB in MUC4/HER2-mediated upregulation of lipocalin 2, we treated CaPan1 cells with Bay 11-7082, which inhibits IκB degradation and also blocks the translocation of NF-κB from the cytoplasm to the nucleus. This treatment decreased lipocalin 2 expression levels in a time- and dose-dependent manner (Fig. 3F and Supplementary Fig. S2), confirming that MUC4 stabilizes HER2, which in turn activates NF-κB through the activation of pAKT.

Clinical correlation of MUC4 and lipocalin 2

We observed that MUC4 regulates the expression of lipocalin 2 through the PI3K-AKT-NF-κB pathway. Both
MUC4 and lipocalin 2 show elevated expression during the progression of pancreatic cancer (6, 15, 30). To investigate the association between MUC4 and lipocalin 2 during the pathogenesis of pancreatic cancer, we performed IHC staining on FFPE tissue sections with the mouse anti-MUC4 monoclonal antibody 8G7 and rabbit anti-lipocalin 2 polyclonal antibodies. Interestingly, as can be seen in Fig. 4, both lipocalin 2 and MUC4 showed elevated expression in pancreatic lesions as early as PanIN I and expression, and both increased progressively with disease advancement (Fig 4A–H). We observed no MUC4 staining and only weak lipocalin 2 staining in normal pancreatic tissue. The observed staining for MUC4 was mainly membranous staining, whereas both cytoplasmic and membranous staining was observed for lipocalin 2. These results suggest that lipocalin 2 and MUC4 are concomitantly overexpressed in pancreatic cancer. Furthermore, we analyzed MUC4 and lipocalin 2 staining in primary and metastatic (lung and lymph nodes) pancreatic cancer specimens obtained through a rapid autopsy program. Qualitative analyses revealed a significant positive correlation between lipocalin 2 and MUC4 expression in human and mouse tissue specimen (Table 1). Similarly, the percentages of MUC4-positive metastasis specimens with lipocalin 2 expression (78% for the lungs and 100% for the lymph nodes) were higher than those of MUC4-negative specimens (22% for the lungs and 0% for the lymph nodes). Furthermore, Spearman correlation coefficient analyses indicated significance for coordinated expression of lipocalin 2 and MUC4 in both primary and metastatic cases.

In the absence of a sensitive test to detect MUC4 in the serum for determining the clinical significance of the above observation, tissue expression of MUC4 was compared with serum levels of lipocalin 2. To this end, we examined these molecules in the KrasG12D-driven early progression mouse model of pancreatic cancer, which closely mimics the early developmental stages of pancreatic cancer. Interestingly, MUC4 expression in pancreatic tissues in this early progression model (Fig. 4I–4N) correlated strongly with elevations in the levels of lipocalin 2 in the serum samples at different weeks of disease progression (Fig. 4O; Spearman correlation coefficient 0.98; P = 0.045). Furthermore, lipocalin 2 in serum of double transgenic animals increased progressively with disease advancement in comparison with control animals (P < 0.0001). On the log scale, an average lipocalin 2 amount in double transgenic animal was 1.43 times [95% confidence interval (CI), 1.01–1.86] than in the controls.

In the absence of a robust diagnostic/prognostic marker for pancreatic cancer, the diagnostic and prognostic significance of lipocalin 2 was assessed by analyzing its concentration between pancreatic cancer and healthy control subjects through sandwich ELISA. We found that patients with pancreatic cancer have significantly higher lipocalin 2 levels (148 ± 13.18 ng/mL) than control patients (73.27 ± 4.9 ng/mL, P = 0.014; Fig. 5A). The prognostic significance of lipocalin 2 was indicated by the higher prechemotherapy levels of lipocalin 2 compared with postchemotherapy levels (P = 0.060; Fig. 5B and Supplementary Table S1). Lipocalin 2 levels were also found to be higher in well-differentiated tumor in comparison with poorly differentiated tumors (Supplementary Fig. S3). For evaluating response to surgery, multiple samples withdrawn from some patients were used to fit mixed effect model. This model allowed the inclusion of all the data (repeated measurements from same patient). However, no significant differences in lipocalin 2 levels were observed in presurgical and postsurgical cases (Supplementary Fig. 3A and Supplementary Table S1). Furthermore, pre- and postoperative levels of lipocalin were assessed in individual patients (n = 7). Of the seven patients, three had a decrease in the mean levels of lipocalin 2 postsurgery, whereas four had an increase (Supplementary Fig. S3B). The analyses done using this model did not change the performance of lipocalin 2 with regard to surgical status.

Discussion

The differential expression of transmembrane mucin MUC4 is observed during pancreatic cancer progression. The present study investigated the molecular mechanisms of lipocalin 2 regulation by the MUC4 mucin in pancreatic cancer, and its potential diagnostic and prognostic significance.

Besides the classical functions performed by mucins, including hydration, lubrication, and protection of exposed surfaces, the transmembrane mucin MUC4 has been indicated to play roles in modulating cell proliferation, apoptosis, morphology, and metastasis of neoplastic cells (7, 9, 22, 24, 31). In previous studies, we observed specific and differential expression of lipocalin 2 in pancreatic cancer cells but not in normal pancreatic cells. In particular, we found low lipocalin 2 expression levels in normal pancreatic tissue and inflamed pancreatic tissue but very high levels in early dysplastic lesions (i.e., PanINs), and it may be a feature even earlier, as studies with PanIN I report (15). Using a high-density cDNA microarray, Han and colleagues found that the upregulation of lipocalin 2 expression in neoplastic cells was 27 times higher than that in normal pancreatic cells (14). Terris and colleagues identified lipocalin 2 as a marker of intraductal papillary mucinous neoplasm, a precursor lesion for invasive carcinoma (32). Moreover, Iacobuzio-Donahue, and colleagues using cDNA microarrays to examine the global gene expression patterns in pancreatic adenocarcinoma cells, also found that lipocalin 2 was significantly overexpressed in pancreatic cancer cells (33). Wang and colleagues used anti-lipocalin 2 antibodies for in vitro and in vivo imaging of human pancreatic cancer cell but its secretory nature limited the utility (34). IHC analyses of MUC4 and lipocalin 2 in pancreatic cancer tissues revealed that the expression of these genes was elevated during pancreatic cancer development (15, 35).

To examine the pathobiologic roles of MUC4 and lipocalin 2 in pancreatic cancer, we sought to determine whether MUC4 regulates expression of lipocalin 2 or vice versa in
Figure 4. Expression of MUC4 and lipocalin 2 during progression of pancreatic cancer. Pancreatic tissue sections were stained for human MUC4 (A–D), lipocalin 2 (E–H) using mouse anti-MUC4 monoclonal antibody (8G7) and rabbit anti-lipocalin 2 polyclonal antibodies, respectively. The MUC4 expression increased progressively [PanIN I < PanIN II (A) < PanIN III (B and C) to invasive pancreatic cancer (D)]. A similar trend was observed for lipocalin 2 expression [expression increased from PanIN I, PanIN II (E) to PanIN III (F and G) to invasive pancreatic cancer (H)]. MUC4 was not expressed in normal ducts, and lipocalin 2 was either low or not expressed in normal ducts. MUC4 and lipocalin 2 were simultaneously expressed in all specimens from PanIN I to pancreatic cancer. Insets show the magnified area (original magnification ×40). Detailed analysis revealed that MUC4 staining was mostly membranous, whereas lipocalin 2 staining was both cytoplasmic and membranous. Similar to human samples, high expression of mouse Muc4 was observed in panINs from 10th (K), 20th (L), 30th (M), and 50th (N) week KrasG12D;Pdx-Cre mice, whereas no staining was observed in the pancreas of 10th (I) and 50th (J) week Pdx-cre control group mice. In correspondence to the increased levels of Muc4 in PanIN lesions, an elevated levels of lipocalin 2 were observed in mice serum samples at different weeks (O).
the present study. Paradoxically, knockdown studies revealed that MUC4 had a strong downregulatory effect on lipocalin 2 in cell lysates and spent culture medium. Furthermore, we found that lipocalin 2 and MUC4 were concomitantly expressed in PanIN1-III lesions. Given the absence of MUC4 from normal pancreatic cells and the de novo expression at very early stages of pancreatic cancer and its suggested roles, including its oncogenic ability (causing oncogenic transformation of NIH3T3 cells), the ability of resisting apoptosis, and the stabilization of the receptor tyrosine kinase HER2, we sought to determine whether lipocalin 2 expressions is regulated by MUC4.

MUC4 is an intramembrane ligand and activator of HER2 and thus facilitates the dimerization of HER2 with other ErbB receptors (9, 36, 37). Researchers have detected the MUC4/HER2 complex in many tissues in which MUC4 is normally expressed (38, 39) and in various tumors and cancer cell lines that aberrantly overexpress MUC4 (22, 23). Silencing MUC4 with shRNA led to the downregulation of HER2 expression and a concomitant decrease in expression of its phosphorylated form (pY1248-HER2; 22). The present study showed that expression of lipocalin 2 was lower in CD18/HPAF/shMUC4 and CaPan1/shMUC4 MUC4 knockdown cells and in CD18/HPAF/shHER2 and CaPan1/shHER2 HER2 knockdown cells than in the controls. Similar to the present study’s findings of HER2-mediated regulation of lipocalin 2, HER2 was earlier reported to induce lipocalin 2 expression in breast cancer cells (28). Moreover, the rat homologue of lipocalin 2 (known as Neu-regulated lipocalin) is a lipocalin that is overexpressed in rat mammary cancer induced by activated neu (HER-2/C-erbB2; ref. 35).

As HER2 is highly expressed in PanINs (82% of PanIN IA, 86% of PanIN IB, and 92% of high-grade PanIN II cases; ref. 40), we investigated lipocalin 2 expression in stable HER2 knockdown clones of pancreatic cancer cells. Similarly to MUC4 knockdown, HER2 knockdown also significantly decreased lipocalin 2 in the CD18/HPAF and CaPan1 cells. Because HER2 can mediate its effects by activating its downstream PI3K/AKT pathway (41), we investigated the effect of MUC4 and HER2 knockdown on AKT phosphorylation. We observed increased AKT phosphorylation in MUC4-overexpressing pancreatic cancer cells. To determine whether AKT has a specific effect on lipocalin 2 expression, we assessed the expression of lipocalin 2 in the presence of LY294002, a PI3K inhibitor. LY294002 clearly decreased lipocalin 2 expression.

Furthermore, the inhibition of NF-κB phosphorylation by BAY-117082 caused significant downregulation of lipocalin 2 expression in both CD18/HPAF and CaPan1 cells. qPCR analyses confirmed that MUC4 regulates lipocalin 2 expression via HER2/AKT/NF-κB pathway. Studies by Iannetti and colleagues showed that lipocalin 2 is a downstream target of NF-κB (12). They found that lipocalin 2 gene expression in the secretome of NF-κB–expressing FRO thyroid carcinoma cells were higher than that in NF-κB null FRO cells (12). Another study of the lipocalin 2 promoter revealed the presence of a cis-binding element for NF-κB (42). In addition to MUC4, other factors including HER2, EGF receptor (EGFR), and KRAS are also involved in lipocalin 2 regulation in pancreatic cancer by impacting NF-κB activity (16). Taking a cue from the previous studies that elucidated the

<table>
<thead>
<tr>
<th>Type of tissue</th>
<th>MUC4 positive (N, %)</th>
<th>MUC4 negative (N, %)</th>
<th>Spearman rank correlation coefficient (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary pancreatic tumor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipocalin 2 positive</td>
<td>14 (100%)</td>
<td>20 (69%)</td>
<td>0.36 (0.02)</td>
</tr>
<tr>
<td>Lipocalin 2 negative</td>
<td>0 (0%)</td>
<td>9 (31%)</td>
<td></td>
</tr>
<tr>
<td>Liver metastasis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipocalin 2 positive</td>
<td>8 (57%)</td>
<td>17 (51.5%)</td>
<td>0.05 (0.72)</td>
</tr>
<tr>
<td>Lipocalin 2 negative</td>
<td>6 (43%)</td>
<td>16 (48.5%)</td>
<td></td>
</tr>
<tr>
<td>Lung metastasis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipocalin 2 positive</td>
<td>7 (78%)</td>
<td>8 (67%)</td>
<td>0.12 (0.58)</td>
</tr>
<tr>
<td>Lipocalin 2 negative</td>
<td>2 (22%)</td>
<td>4 (33%)</td>
<td></td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipocalin 2 positive</td>
<td>12 (100%)</td>
<td>3 (37.5%)</td>
<td>0.71 (0.002)</td>
</tr>
<tr>
<td>Lipocalin 2 negative</td>
<td>0 (0%)</td>
<td>5 (63.5%)</td>
<td></td>
</tr>
<tr>
<td>Spontaneous progression model</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipocalin 2 positive</td>
<td>21 (100%)</td>
<td>0 (0%)</td>
<td>0.902 (0.042)</td>
</tr>
<tr>
<td>Lipocalin 2 negative</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td></td>
</tr>
</tbody>
</table>
antiapoptotic role of lipocalin 2, it seems to be a plausible factor responsible for imparting gemcitabine resistance to pancreatic cancer cells.

IHC analyses of lipocalin 2 and MUC4 expression in pancreatic tissue sections obtained from a rapid autopsy program, as well as those with sera and tissues of the KRASG12D mice, showed a strong correlation between lipocalin 2 and MUC4. Histologically, PanIN I show cuboidal morphology to a columnar phenotype with abundant supranuclear, mucin containing cytoplasm. As the mice ages, higher grade PanIN II with moderate nuclear atypia and loss of polarity are observed. By 36th week, the mice develop significant nuclear atypia and highly progressed PanIN III lesion. Similar to human PanINs, the mouse PanINs express mucins, the epithelial protein cytokeratin-19, and components of signaling pathways including Cox-2, MMP-7, and Hes1 (43, 44). Overall, it seems that MUC4-mediated regulation of lipocalin 2 could be patho-biologically significant in pancreatic cancer.

El-Mesallamy and colleagues, observed higher levels of lipocalin 2 in preoperative cases compared with postoperative cases (45). Similarly, clinicopathologic analyses of lipocalin 2 in the present study showed higher levels of lipocalin 2 during preoperative and prechemotherapy patient group. Higher level of lipocalin 2 in prechemotherapy, presurgical cases indicated a positive association of lipocalin 2 levels with the tumor burden. Accordingly, we contemplate in presurgical cases that lipocalin 2 is produced by tumor cells and regulated by various tumor-derived oncogenes, including MUC4, Ras, and EGF. Its observed functions on tumor growth, progression, invasion, and metastasis may be context dependent in multiple ways (46–48). Interestingly, upon tumor resection, that is, in postsurgical cases, immune cells (neutrophils and macrophages) and other cells of inflammatory system become primary source for its production. In this regard, Xu and colleagues observed that the lipocalin 2–negative group shows overall poor survival compared with the -positive group and proposed pancreatic cancer gene

![Figure 5. Diagnostic and prognostic efficacy of lipocalin 2 in differentiating pancreatic cancer from normal. The diagnostic and prognostic significance of elevated lipocalin 2 was evaluated by measuring its amount in human samples (pancreatic cancer = 91, controls = 24) through sandwich ELISA. A, the average amount of lipocalin 2 was significantly higher in pancreatic cancer cases (148.35 ± 14.89; 0.014) than healthy control (73.27 ± 4.9). B, lipocalin 2 levels were compared between pre- (n = 62) versus postchemotherapy (n = 106) samples using mixed-effects models. Prechemotherapy levels of lipocalin 2 were found to be higher than postchemotherapy levels (P = 0.060). C, lipocalin 2 levels were marginally decreased with differentiation status of tumor although differences were not significant.](image-url)
MUC4 Regulates Lipocalin 2 through HER2/AKT/NF-κB Pathway

In conclusion, the present study provides evidence that MUC4 regulates lipocalin 2 expression in pancreatic cancer cells as it stabilizes HER2 that activates the downstream PI3K/AKT/NF-κB pathway (Fig. 6). The clinic-pathologic studies suggested lipocalin 2 may be a potential biomarker for pancreatic cancer. These results emphasize that mucin covering are not inert layers but active sensors of extracellular environmental changes. Further molecular analyses of their downstream pathway could be expected to identify better disease predictors.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Writing, review, and/or revision of the manuscript: S. Kaur, N. Sharma, S.R. Krishn, M.J. Baine, S.M. Lele, A.R. Sasson, S. Guha, M.A. Hollingsworth, S.K. Batra
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Kaur, N. Sharma, J.M. Anderson, K. Mallya, S.K. Batra
Study supervision: M.A. Hollingsworth

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therapy protocol using an oncolytic virus harboring the lipocalin 2 gene (49).

Intriguingly, lipocalin 2 acts as a survival factor in various malignancies by helping cancer cells resist apoptosis and in turn promotes tumor growth (12, 50, 51). On the other hand in pancreatic cancer, stable overexpression of lipocalin 2 in MiaPaCa-2 and PANC-1 (MUC4-negative cell lines) significantly blocked cell adhesion and invasion and decreased angiogenesis under in vitro conditions in pancreatic cancer cells (16, 30). Herein, we observed oncogene-mediated regulation of lipocalin 2 in the present study. It is plausible that lipocalin 2 impacts are context dependent and an effect varies depending upon cell type, microenvironment as well as tumor burden. Accordingly, it can be suggested that various assets of lipocalin 2, that is, multifaceted role, unique regulation (MUC4, Ras, EGF, interleukin-10, and TLR), and various sources (produced by both tumor and immune cells) impact its effects on tumor growth, progression, invasion, and metastasis in multiple ways (46–48). Accordingly, the data need to be interpreted carefully before its clinical use in oncolytic viruses harboring cancer-targeting genes (49). Furthermore, the finding from the present study will have a high impact in upper respiratory (oral, esophageal, and lung) infection where MUC4 is heavily expressed and might be involved in regulating in body’s response to harmful changes in extracellular milieu.

Figure 6. Proposed model of MUC4-mediated regulation of lipocalin 2 expression in pancreatic cancer cells. MUC4 stabilizes HER2, leading to the PI3K-dependent activation of AKT and activated AKT enhances lipocalin 2 transcription in pancreatic cancer cells. Thus, MUC4-mediated activation of NF-κB via the HER2/PI3K/AKT pathway leads to elevated expression of lipocalin 2 during pancreatic cancer progression.


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MUC4-Mediated Regulation of Acute Phase Protein Lipocalin 2 through HER2/AKT/NF-κB Signaling in Pancreatic Cancer

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