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 (PC). 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 multi-faceted 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-value=0.002) from rapid autopsy tissue sample from PC patients as well as in serum and tissue samples from spontaneous KRASG^{12}D mouse PC model (Spearman correlation coefficient 0.98, p-value <0.05). Lipocalin 2 levels increased progressively with disease advancement (344.2 ±22.8 ng/ml for 10 week to 3067.2±572.6 for 50 week; p<0.0001). In human PC cases, significantly elevated levels of lipocalin 2 were observed in PC patients (148±13.18 ng/ml) in comparison to controls (73.27±4.9 ng/ml, p-value=0.014). Analyses of pre- and post-chemotherapy patients showed higher lipocalin

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2 levels in pre-chemotherapy patients (121.7 ng/ml, 95% C.I. 98.1–150.9) in comparison to the post-chemotherapy (92.6 ng/ml, 95% C.I. 76.7–111.6, p-value=0.06) group.

Conclusions—The present 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.

Keywords

Lipocalin 2; mucin; MUC4; Pancreatic Cancer; NF-κB; AKT

Introduction

Pancreatic cancer (PC), with annual incidences of 45,220 and mortality of ~38460, is among the leading causes of cancer-related deaths in the United States. More than 90% of PC patients, 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 PC 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 PC 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 indicate 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 PC 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 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 PC progression [15]. Lipocalin 2 expression differentially impact tumor growth, invasion and metastasis of PC cell depending upon differentiation status [16, 17]. Furthermore, *in vitro* studies have shown that lipocalin 2 enhances sensitivity of PC cell lines towards gemcitabine [17]. Recently, Raffatellu *et al* observed simultaneous upregulation of lipocalin 2 and MUC4 transcripts in the ileal mucosa of rhesus macaques in response to enteric *S. 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 multi-fold decrease in lipocalin 2 expression (unpublished results). Further, toll-like receptors 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 PC. 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.

Material and Methods

Cell lines, reagents and treatments

See supplementary section

Quantitative real time-polymerase chain reaction

In order 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/Scr and CaPan1/Scr control cells was reverse-transcribed using a First Strand cDNA Synthesis Kit (Perkin-Elmer, Branchburg, NJ) and oligo-d(T) primers according to the manufacturer's instructions. Quantitative real-time-polymerase chain reaction (QPCR) amplifications were carried out with 100 ng of first-strand cDNA in 10 μl reaction volumes. The reaction mixture was subjected to a 2-step cyclic 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, Foster City, CA) with SYBR Green chemistry as per manufacturer's instructions. The relative fold differences in gene expression were calculated using the $\Delta\Delta C_T$ method with β-actin as a normalization control.

Sandwich enzyme-linked immunosorbent assay

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 PC was approved by the Institutional Review Boards (IRB) of the University of Nebraska Medical Center (UNMC) (IRB number 209-00, IRB #091-01-EP). Samples from PC (n=91), and healthy controls (HC=24) were used to measure the lipocalin 2 amount. In case of PC patients, "post-surgery" cases include samples from patients who have undergone a pancreaticoduodenectomy, a pancreatectomy, or a pancreaticoduodenogastrectomy 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 "pre-surgery". Any sample drawn before the patient had undergone chemotherapy for PC was classified as "pre-chemotherapy". If the patient had ever had chemotherapy for PC, regardless of whether or not that patient was undergoing chemotherapy at the time the sample was taken, the sample was classified as "post-chemotherapy". Standard curves were produced from lipocalin 2 standards provided with the kit and serially (log_2) diluted from 4ng/ml to 15.6pg/ml. ELISA plates were read at 450 nm and collected data were analyzed using the SoftMax Pro software program (Molecular Devices, Sunnyvale, CA). Data were analyzed using SAS statistical software version 9.2 (SAS Institute Inc, Cary, NC). Levels of lipocalin 2 were analyzed as continuous variables, while tumor grade, cancer stage, surgical status and chemotherapy status were considered to be categorical. Patient characteristics were compared between PC and control patients using ANOVA model for continuous variables and chi-square tests or Fisher's 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's method. P-values 0.05 were considered statistically significant.

Confocal immunofluorescence microscopy

To study 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 hrs. For immunostaining of lipocalin 2 and MUC4 cells were washed followed by blocking with 10% goat serum (Jackson Immunoresearch Labs, Inc., West Grove, PA) containing 0.05% Tween-20 for 1 hr. The cells were then washed 5 times with PBS and incubated with the anti-MUC4 and lipocalin 2 antibodies for 60 minutes at room temperature. Next, the cells were washed 5 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 in the dark. Nuclei were stained with 4′-6-diamidino-2-phenylindole and mounted on glass slides in anti-fade VECTASHIELD Mounting Medium (Vector Laboratories). An LSM 510 microscope (Carl Zeiss GmbH, Oberkochen, Germany) was used to perform laser confocal microscopy. Microphotographs of lipocalin 2 and MUC4 staining were taken in different channels.

Luciferase Reporter Assay

To assess the activity of lipocalin 2 promoter in MUC4 expressing and knockdown cells, cells were cultured to 60% confluence in six-well plate. Following serum starvation for 4 hrs cell were transfected with 1 μg of promoter reporter construct containing either 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 0.1μg of control Renilla Luciferase plasmid using Lipofectamine 2000 (Invitrogen Life technologies, NY). Dual-Luciferase reporter Assay was performed 24 hrs after transfection as per manufacturer's instructions (Promega, WI). 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 lysate (20 μg each) were resolved using electrophoresis on a 2% sodium dodecyl sulfate (SDS)-agarose gel under reducing conditions. For β-actin, lipocalin 2, HER2, p^{1248}HER2, AKT, pAKT, NF-κB, pNF-κB, IK-κB, and pIK-κB, SDS–polyacrylamide gel electrophoresis (PAGE; 10–12%) was performed under similar conditions. Resolved proteins were transferred onto a polyvinylidene difluoride membrane, blocked in 5% nonfat milk in PBS for 2 hrs, 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), p^{1248}HER2 (Upstate, San Francisco, CA), AKT, pAKT, NF-κB, pNF-κB, IK-κB, and pIK-κB immunodetection, the respective rabbit polyclonal antibodies (Cell signaling, USA) diluted 1:1000 in tris-buffered saline with tween-20 (50 mM Tris-HCl, pH 7.4, 150 mM 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, Buckinghamshire, UK) diluted at 1:2000 in PBS for 1 hr at room temperature and washed six times in TBS-T. The blots were processed using an electro-chemiluminescence kit (Amersham Biosciences), and signals were detected by exposing the processed blots to X-ray film (BioMax films; Kodak, Rochester NY).

Immunohistochemical analyses of MUC4 and lipocalin 2

Formalin-fixed paraffin-embedded pancreatic cancer tissue microarray slides for lipocalin 2 and MUC4 staining were provided by the Rapid Autopsy Program at the University of Nebraska Medical Center and commercial pancreatic tissue array (Accumax). These were subjected to immunohistochemical 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-square 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 shRNA, which otherwise express high level of MUC4. Resulting stable transfectant pools (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). Since 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 levels in the culture supernatants of 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 has 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-over-expressing cell lines (MiaPaCa/lipocalin 2 and Panc1/lipocalin 2) and lipocalin 2-knockdown cell lines (AsPC1/sh-lipocalin 2) (Supplementary Fig. 1). In addition to pancreatic cancer cells, we observed a similar phenomenon in lung cancer cell lines (**data not shown**). These findings suggest 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 post-translational 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 of 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 to that in scrambled shRNA-transfected cells (Fig. 2B).

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 reduced, while the levels of expression of total AKT remained unchanged. Since HER2 mediates its effect on AKT through 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 μM) was used, it resulted in a time-dependent decrease in pAKT and lipocalin 2 expression in CD18/HAF and CaPan1 cells (Fig. 2C&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 PC cells, we employed real time quantitative PCR 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 lipocalin 2 promoter by TRESS indicated the presence of a 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 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 to the knock-down 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&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 IκBα 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, supplementary Fig. 2), confirming that MUC4 regulates lipocalin 2 expression in pancreatic cancer cells by stabilizing 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 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 immunohistochemical staining on FFPE (formalin fixed paraffin embedded) 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 while both cytoplasmic and membranous staining was observed for lipocalin 2. These results suggest that lipocalin 2 and MUC4 are concomitantly overexpressed in pancreatic cancer. Further, 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 $Kras^{G12D}$-driven early progression mouse model of PC, which closely mimics the early developmental stages of PC. Interestingly, MUC4 expression in pancreatic tissues in this early progression model (Fig. 4I–N) 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-value=0.045). Furthermore, lipocalin 2 in serum of double transgenic animals increased progressively with disease advancement in comparison to control animals (p<0.0001). On the log scale, on average lipocalin 2 amount in double transgenic animal were 1.43 times (95% CI: 1.01–1.86) than in the controls.

In the absence of a robust diagnostic/prognostic marker for PC, the diagnostic and prognostic significance of lipocalin 2 was assessed by analyzing its concentration between PC and healthy control subjects through sandwich ELISA. We found that PC patients have significantly higher lipocalin 2 levels (148±13.18 ng/ml) than control patients (73.27±4.9 ng/ml, p-value=0.014) (Fig. 5A). The prognostic significance of lipocalin 2 was indicated by the higher pre-chemotherapy levels of lipocalin 2, compared to post-chemotherapy levels (p=0.060) (Fig. 5B, supplementary table 1). Lipocalin 2 levels were also found to be higher in well-differentiated tumor in comparison to poorly differentiated tumors (supplementary Fig. 3). 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 pre-surgical and post-surgical cases (supplementary Fig. 3A, supplementary table 1). Further, pre- and post-operative levels of lipocalin were assessed in individual patients (n=7). Of the seven patients, 3 had a decrease in the mean levels of lipocalin 2 post-surgery while 4 had an increase (supplementary Fig. 3B). The analyses done using this model did not change the performance of lipocalin 2 with regard to surgical status.

Discussion

The differential expression of trans-membrane mucin MUC4 is observed during pancreatic cancer progression. The present study investigated the molecular mechanisms of lipocalin 2 regulation by the MUC4 mucin in PC, 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 PC cells but not 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 *et al.* found that the upregulation of lipocalin 2 expression in neoplastic cells was 27 times higher than that in normal pancreatic cells [14]. Terris *et al.* identified lipocalin 2 as a marker of intraductal papillary mucinous neoplasm, a precursor lesion for invasive carcinoma [32]. Moreover, Iacobuzio-Donahue *et al.*, 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 *et al* 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 PC tissues revealed that the expression of these genes was elevated during PC development [15, 35].

To examine the pathobiological roles of MUC4 and lipocalin 2 in pancreatic cancer, we sought to determine whether MUC4 regulates expression of lipocalin 2 or *vice-versa* in the present study. Paradoxically, knockdown studies revealed that MUC4 had a strong downregulatory effect on lipocalin 2 in cell lysates and spent culture medium. Further, 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 PC 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 intra-membrane 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 (pY^{1248}-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) [35].

As HER2 is highly expressed in PanINs (82% of PanIN IA, 86% of PanIN IB, and 92% of high-grade PanIN II cases) [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. Since 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 CD18/HPAF and CaPan1 cells 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. Quantitative PCR (qPCR) analyses confirmed that MUC4 regulates lipocalin 2 expression at the transcriptional level. In addition, CD18/HPAF and CaPan1 HER2-knockdown and MUC4-knockdown cells had lower levels of IK-κB phosphorylation than did the cell lines transfected with scrambled shRNA. These findings showed that MUC4 regulates lipocalin 2 expressions via HER2/AKT/NF-κB. Studies by Iannetti *et al.* 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 lipocalin 2 promoter revealed the presence of a cis-binding element for NF-κB [42]. In addition to MUC4, other factors including HER2, EGFR and KRAS are also involved in lipocalin 2 regulation in PC by impacting NF-κB activity [16]. Taking a cue from the previous studies that elucidated the anti-apoptotic role of lipocalin 2, it appears to be a plausible factor responsible for imparting Gemcitabine resistance to pancreatic cancer cells.

Immunohistochemical 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 KRAS[G12D] 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 cyclooxygenase (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 *et al*, observed higher levels of lipocalin 2 in pre-operative cases compared to post-operative cases [47]. Similarly, clinico-pathological analyses of Lipocalin 2 in the present study showed higher levels of lipocalin 2 during pre-operative and pre-chemotherapy patient group. Higher level of lipocalin 2 in pre-chemotherapy, pre-surgical cases indicated positive association of lipocalin 2 levels with the tumor burden. Accordingly, we contemplate in pre-surgical cases 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 [45–48]. Interestingly, upon tumor resection *i.e.* in post-surgical cases, immune cells (neutrophils and macrophages) and other cells of inflammatory system become primary source for its production. In this regard, Xu *et al.*, observed that lipocalin 2-negative group show overall poor survival compared to the positive group and proposed PC gene 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 PC, 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 deepening upon cell type, microenvironment as well as tumor burden. Accordingly, it can be suggested that various assets of lipocalin 2 *i.e.* multifaceted role, unique regulation (MUC4, Ras, EGF, IL-10 and TLRs) and various sources (produced by both tumor and immune cells) impacts it effects on tumor growth, progression, invasion, and metastasis in multiple ways [45–48]. Accordingly, the data need

to be interpreted carefully prior to its clinical use in oncolytic viruses harboring cancer targeting genes [49]. Further, the finding from the present study will be of high impact in upper respiratory (oral, oesophageal and lung) infection where MUC4 is heavily expressed and might be involved in regulating in body's response to harmful changes in extracellular milieu.

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-pathological studies suggested lipocalin 2 may be a potential biomarker for PC. 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Translational Relevance

The lethality of pancreatic cancer (PC) along with increasing incidence has pushed it to the forefront of the worst malignancies. Differential expression of mucins in PC makes them important diagnostic/prognostic markers. MUC4 expression in very early precursor lesions and its high specificity for tumor lesions makes it quite important for diagnostic/prognostic screening. However, its limited amount in serum diminishes its biomarker potential. To exploit the potential of mucins in the management of PC, we urgently need to explore their mechanisms of action and identify other molecules regulated by them. Herein, we report that the MUC4 mucin expressed *de novo* during early precursor lesions of PC regulates acute phase protein lipocalin 2 by the HER2/AKT pathway. Evaluation of elevated lipocalin 2 in post- chemotherapy and surgical cases revealed its clinical significance. The finding indicates mucins as important sensors for the changing environment in PC that regulates expression of diagnostically/prognostically critical antigens.
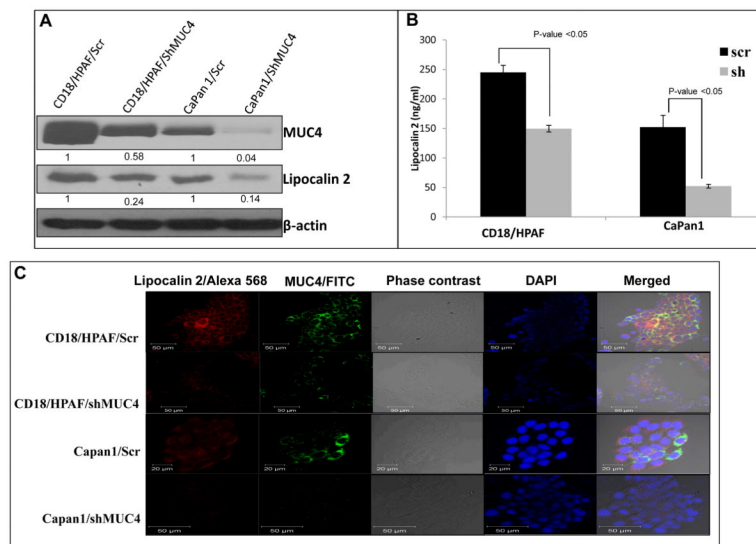
Figure 1. Effect of MUC4 knockdown on lipocalin 2 expression in pancreatic cancer cell lines
A, Immunoblot analyses were employed to assess the expression of MUC4 and lipocalin 2 in CD18/HPAF/shMUC4 and CaPan1/shMUC4 MUC4-knockdown cells and CD18/HPAF/Scr and CaPan1/Scr control cells. Proteins from cell 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 polyvinylidene difluoride 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 spent media of MUC4-knockdown and control cells. Briefly, 2×10^5 CD18/HPAF and CaPan-1 MUC4-knockdown cells and their respective controls were cultured in 2 ml of 1% DMEM for 48 hrs. 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 (4′-6-diamidino-2-phenylindole [DAPI] staining). MUC4 knockdown significantly reduced the expression and secretion of lipocalin 2 in CD18/HPAF and CaPan1 cells.
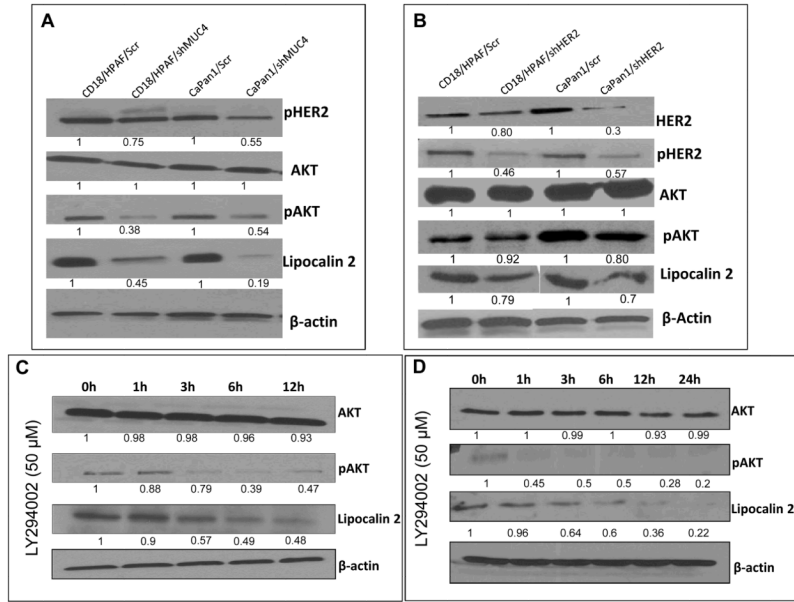
Figure 2. Lipocalin 2 is a downstream target of the HER2/PI3K/AKT pathway
Molecular analyses of MUC4 downstream pathway for the regulation of lipocalin 2
expression in pancreatic cancer. **A,** Equal numbers of MUC4-knockdown and control cells
were seeded in 10-cm petri dishes, and lysates were prepared. Protein from each cell extract
(40 μg) was resolved via SDS–PAGE and then subjected to immunoblot analysis with anti-
HER2, pHER2, AKT, pAKT, and lipocalin 2 in CD18/HPAF/shMUC4 and CaPan1/
shMUC4 MUC4-knockdown cells and CD18/HPAF/Scr and CaPan1/Scr control cells. **B,**
ShRNA technology was used to stably downregulate HER2 expression in CD18/HPAF cells
and CaPan1 cells (CD18/HPAF/shHER2) to confirm the involvement of HER2 in mediating
MUC4 effect on lipocalin 2. Scrambled shRNA-transfected CD18/HPAF (CD18/HPAF/Scr)
and CaPan1 (CaPan1/Scr) cells were used as controls. Lysates of HER2-knockdown and
control cells were resolved on SDS-PAGE gels and immunoblotted with anti-HER2,
pHER2, AKT, pAKT, and lipocalin 2 antibodies. **C** and **D**, MUC4/HER2–stimulated AKT
activity was PI3K-dependent and required for MUC4-induced lipocalin 2 production.
CaPan1 (C) and CD18/HPAF (D) cells were treated with the PI3K inhibitor LY294002 (50
μM) for the indicated times. Immunoblotting was performed to measure pAKT and lipocalin
2 expression levels. Immunoblotting with AKT was performed to measure total AKT
expression levels. LY294002 significantly decreased pAKT expression in CD18/HPAF and
CaPan1 cells. A corresponding decrease in lipocalin 2 expression was observed. β-actin was
used as a loading control.
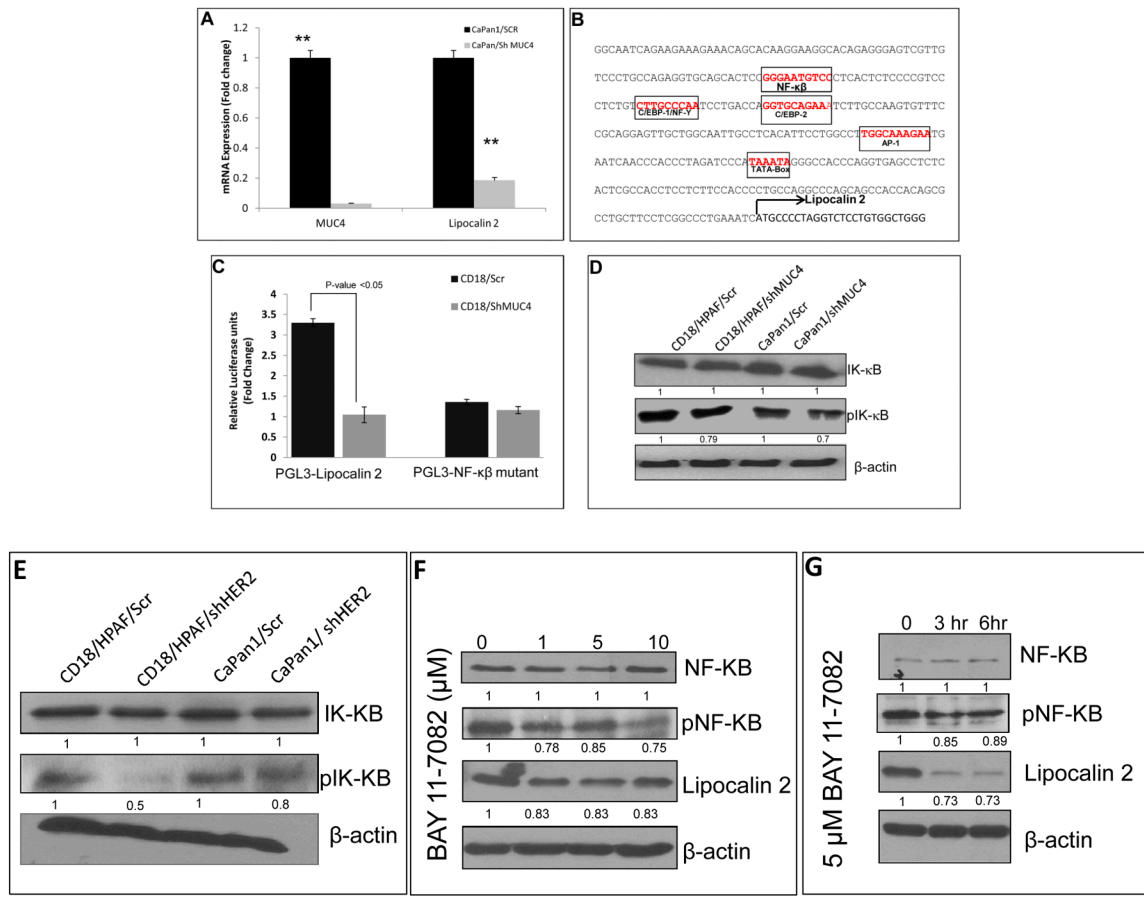
Figure 3. MUC4-mediated regulation of lipocalin 2 *via* AKT/NF-κB pathway
A, Quantitative PCR was used to measure levels of expression of *MUC4* and *lipocalin 2* transcripts in CaPan1/shMUC4 MUC4-knockdown cells and CaPan1/Scr control cells. Briefly, control CaPan1/Scr and CaPan1/shMUC4 MUC4-knockdown cells were seeded in 100 mm petri dishes, and RNA was isolated when cells were 70% confluent. After cDNA synthesis, qPCR was used to measure lipocalin 2 and MUC4 transcription levels. Data were normalized against β-actin. MUC4 knockdown significantly reduced lipocalin 2 transcription. **B,** Putative regulatory consensus binding sites for various transcription factors on lipocalin 2 promoter region. **C,** CD18/HPAF/Scr and CD18/HPAF/shMUC4 cells were cotransfected with pGL3-luc/lipocalin 2 [–900] or pGL3-luc/lipocalin 2 [NF-κB- mut] and pGL4.74 [hRluc/TK] (control for transfection efficiency) for 24 hrs and cell lysate was then used for the luciferase activity assay. Higher luciferase activity was observed in CD18/ HPAF/Scr control cells in comparison to knockdown cells. * represents *p<0.05*. **D,** Immunoblot analyses of IK-κB and pI-κB in MUC4 knockdown and control cells. Equal numbers of MUC4-knockdown and control cells were seeded in 10-cm petri dishes, and lysates were prepared. Protein from each cell extract (40 μg) was resolved via SDS–PAGE and subjected to immunoblot analyses with IK-κB and pI-κB antibodies. **E,** Immunoblot analyses of IK-B and pI-κB in HER2 knockdown and control cells. **F,** Lipocalin 2 levels in CaPan1 cells incubated with BAY11-7082 at different micromolar concentrations. Maximum inhibition of lipocalin 2 was achieved with 5 μM BAY11-7082. **G**, CD18/HPAF cells were treated with 5 μM of BAY11-7082 for the indicated times. BAY11-7082 reduced lipocalin 2 expression in pancreatic cancer cells.
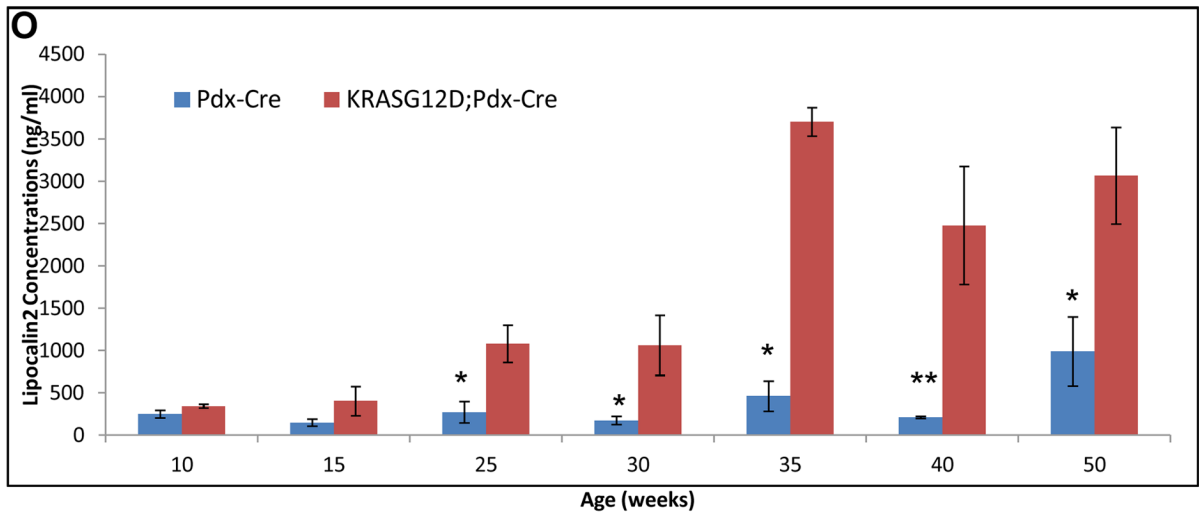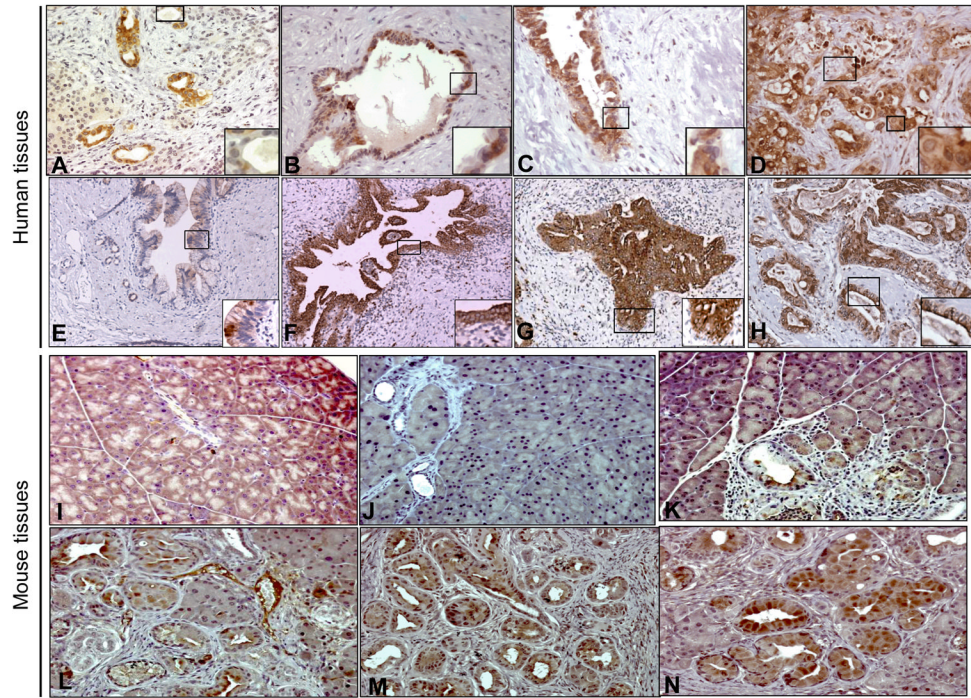
Figure 4. Expression of MUC4 and lipocalin 2 during progression of pancreatic cancer

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, 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, 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 PC. 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 KrasG^{12}D;Pdx-Cre mice while 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**).
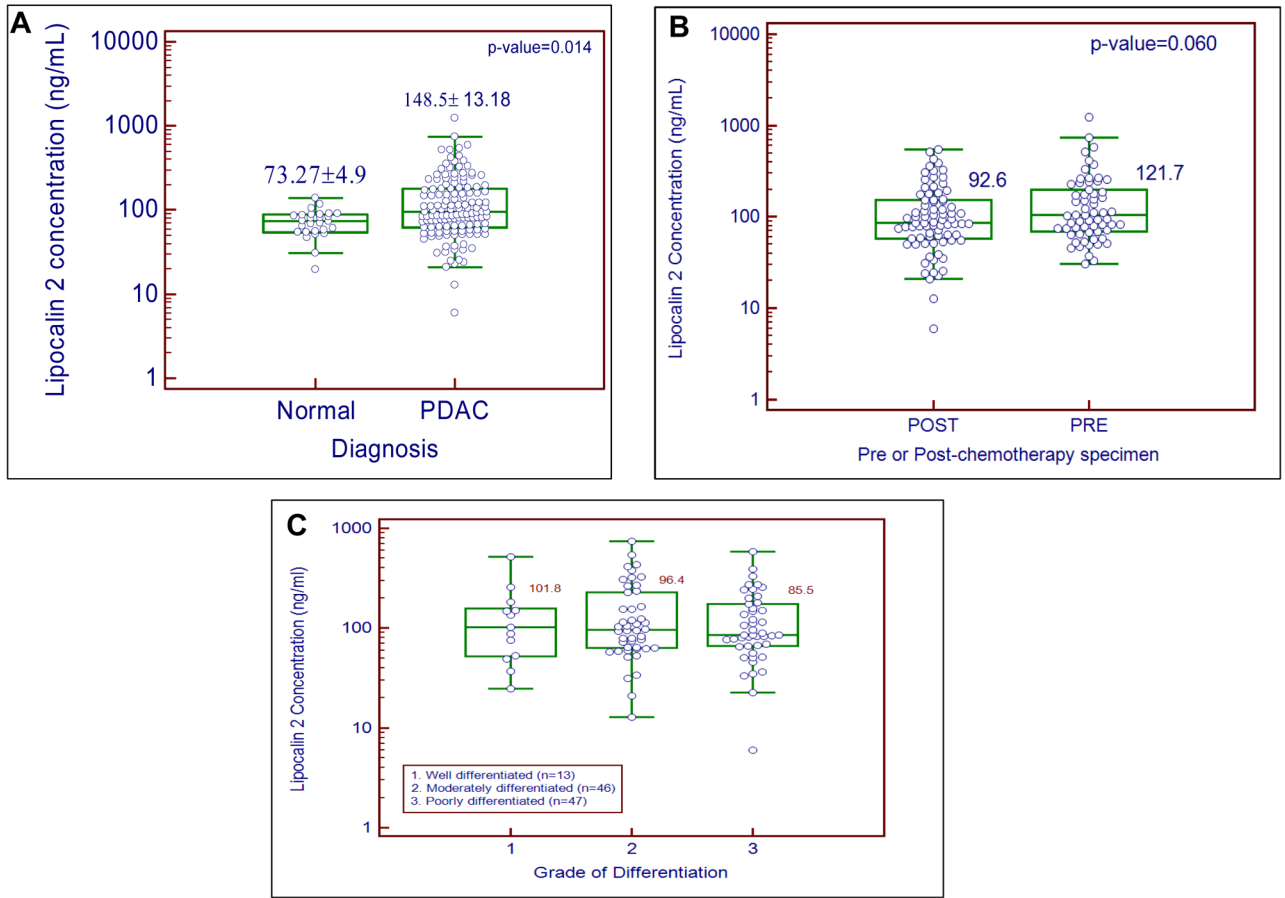
Figure 5. Diagnostic and prognostic efficacy of lipocalin 2 in differentiating PC from normal
The diagnostic and prognostic significance of elevated lipocalin 2 was evaluated by measuring its amount in human samples (PC=91, controls=24) through sandwich ELISA. A, The average amount of lipocalin 2 was significantly higher in PC cases (148.35±14.89) (0.014) than healthy control (73.27±4.9). **B,** Lipocalin 2 levels were compared between pre- (n=62) vs. post- chemotherapy (n=106) samples using mixed-effects models. Pre-chemotherapy levels of lipocalin 2 were found to be higher than post-chemotherapy levels (p=0.060). **C,** Lipocalin 2 levels were marginally decreased with differentiation status of tumor although differences were not significant.
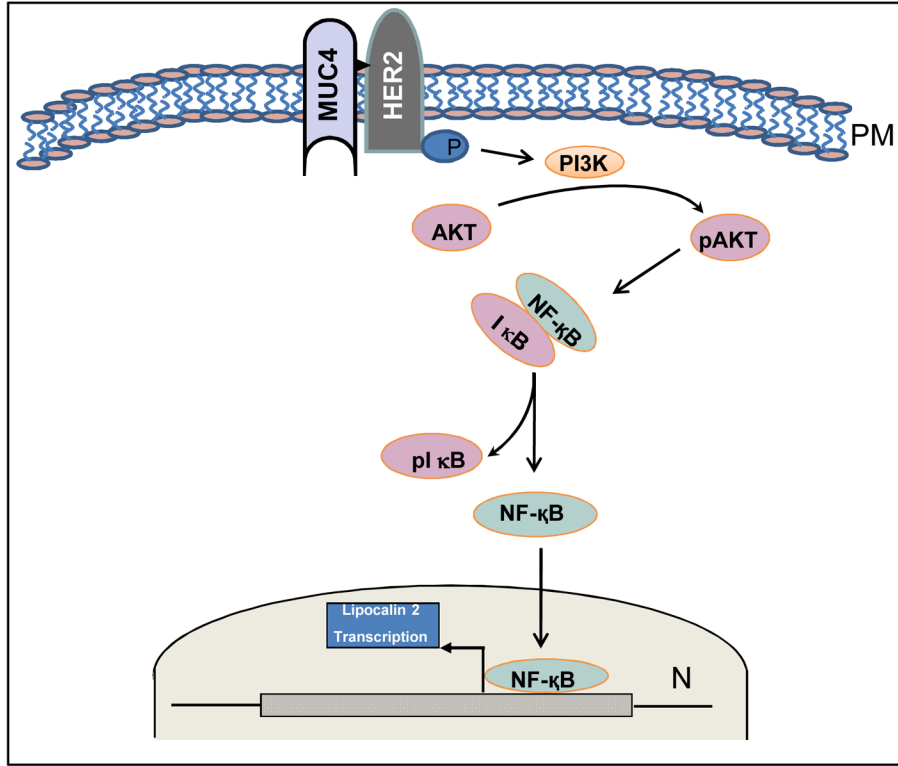
Figure 6. Proposed model of MUC4-mediated regulation of lipocalin 2 expression in pancreatic cancer cells
MUC4 stabilizes HER2, leading to the PI3K-dependent activation of AKT and activated AKT enhances the phosphorylation of the NF-κB p65 subunit. The lipocalin 2 promoter has an NF-κB–binding site; activated NF-κB induces 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.

Table 1

Correlation between lipocalin 2 and MUC4 expression in primary and metastatic pancreatic cancer cases.

Type of tissue	MUC4 positive (N, %)	MUC4 negative (N, %)	Spearman's Rank correlation coefficient (p-value)
Primary pancreatic tumor			
Lipocalin 2 positive	14 (100%)	20 (69%)	0.36 (p=0.02)
Lipocalin 2 negative	0 (0%)	9 (31%)	
Liver metastasis			
Lipocalin 2 positive	8 (57%)	17 (51.5%)	0.05 (p=0.72)
Lipocalin 2 negative	6 (43%)	16 (48.5%)	
Lung metastasis			
Lipocalin 2 positive	7 (78%)	8 (67%)	0.12 (p=0.58)
Lipocalin 2 negative	2 (22%)	4 (33%)	
Lymph node metastasis			
Lipocalin 2 positive	12 (100%)	3 (37.5%)	0.71 (p=0.002)
Lipocalin 2 negative	0 (0%)	5 (63.5%)	
Spontaneous Progression model			
Lipocalin 2 positive	21(100%)	0(0%)	0.902(p=0.042)
Lipocalin 2 negative	0(0%)	0(0%)	