Neuropeptide Y inhibits cholangiocarcinoma cell growth and invasion

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Abbreviations: Abbreviations: BIIE 0246 = N-[(1S)-4-[(Aminoiminomethyl)amino]-1-[[2-(3,5-dioxo-1,2-diphenyl-1,2,4-triazolidin-4-yl)ethyl]amino]carbonyl]butyl]-1-[2-[4-(6,11-dihydro-6-oxo-5H-dibenzo[b,e]azepin-11-yl)-1-piperazinyl]-2-oxoethyl]-cyclopentanecetamide; cAMP = 3’-5’-cyclic adenosine monophosphate; CGP 71683 hydrochloride = N-[trans-4-[[4-Amino-2-quinazolinyl]amino][methyl][cyclohexyl][methyl]-1-naphthalenesulfonamide hydrochloride; EIA = Enzyme-linked immunoassay; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; IP3, D-myo-inositol 1,4,5-trisphosphate; NPY, Neuropeptide Y; NPYR, Neuropeptide Y receptor; PCNA, proliferating cellular nuclear antigen; PKCα, protein kinase C α; TUNEL, terminal deoxynucleotidyl transferase biotin-dUTP nick end labelling.
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Abstract:

No information exists on the role of neuropeptide Y (NPY) in cholangiocarcinoma growth. Therefore, we evaluated the expression and secretion of NPY and its subsequent effects on cholangiocarcinoma growth and invasion. Cholangiocarcinoma cell lines and non-malignant cholangiocytes were used to assess NPY mRNA expression and protein secretion. NPY expression was assessed by immunohistochemistry in human liver biopsies. Cell proliferation and migration was evaluated in vitro by MTS assays and matrigel invasion chambers, respectively, after treatment with NPY or a neutralizing NPY antibody. The effect of NPY or NPY depletion on tumor growth was assessed in vivo after treatment with NPY or the neutralizing NPY antibody in a xenograft model of cholangiocarcinoma. NPY secretion was upregulated in cholangiocarcinoma compared to normal cholangiocytes. Administration of exogenous NPY decreased proliferation and cell invasion in all cholangiocarcinoma cell lines studied and reduced tumor cell growth in vivo. In vitro, the effects of NPY on proliferation were blocked by specific inhibitors for NPY receptor Y2, but not Y1 or Y5 and were associated with an increase in intracellular IP$_3$ and PKC$_\alpha$ activation. Blocking NPY activity using a neutralizing antibody promoted cholangiocarcinoma growth in vitro and in vivo and increased the invasiveness of cholangiocarcinoma in vitro. Increased NPY immunoreactivity in human tumor tissue occurred predominantly in the center of the tumor with less expression towards the invasion front of the tumor. We demonstrated that NPY expression is upregulated in cholangiocarcinoma, which exerts local control on tumor cell proliferation and invasion. Modulation of NPY secretion may be important for the management of cholangiocarcinoma.
Introduction:

Cholangiocarcinoma is a devastating tumor that is relatively resistant to treatment with chemotherapy or radiation therapy (6, 57). Cholangiocarcinoma arises from the neoplastic transformation of the epithelial cells (i.e., cholangiocytes) that line the intra- and extra-hepatic bile ducts (6, 57). Typically, cholangiocarcinomas are adenocarcinomas and have a poor prognosis and limited treatment options. This is due, at least in part to the late presentation of symptoms and the relative resistance to current treatment options (57, 60). The incidence of this cancer is increasing in western societies and the mortality rates of the intrahepatic cancer are increasing (57). Therefore, research into the mechanisms by which cholangiocarcinoma growth and disease progression are regulated is imperative in an attempt to design more effective treatment options for this cancer.

We have previously demonstrated that cholangiocarcinoma secretes factors capable of regulating its own proliferation (2, 8). For example, cholangiocarcinoma synthesizes and secretes higher amounts of serotonin (2) and dopamine (8), which can be detected in bile (but not serum) of patients with cholangiocarcinoma (2, 8). Both of these neuroendocrine modulators exert growth-promoting effects on cholangiocarcinoma (2, 8), and blocking their synthesis slowed the rate of cholangiocarcinoma tumor growth \textit{in vitro} and in an \textit{in vivo} xenograft model of cholangiocarcinoma.

Neuropeptide Y (NPY) is a neurotransmitter, mainly found in the brain, but also present in neurons throughout the gastrointestinal tract, around the walls (tunica adventitia and tunica media) of hepatic vessels, and in high concentrations in the biliary tree (25, 32). Other studies have shown that immunohistochemical NPY reactivity is present not only in intrahepatic nerve fibers and ganglion cells but also in cholangiocytes (15). Limited information exists regarding the role of NPY in the regulation of biliary function. NPY has been shown to play a role in the neural control of biliary motility and secretion (14,
NPY has inhibitory effects on cancer cell lines of various origins (51) but nothing is known about the effects of NPY on cholangiocarcinoma growth. NPY exerts its many functions through 6 main receptor subtypes (Y1 through Y6) (7). These receptors are a class of G-protein coupled receptors that can either inhibit 3'-5'-cyclic adenosine monophosphate (cAMP) synthesis (22, 35) or increase IP$_3$/Ca$^{2+}$-signaling (1, 39). We have previously shown that both cAMP- and IP$_3$/Ca$^{2+}$-signaling modulate cholangiocarcinoma growth (3, 27). For example, while both the $\alpha_2$-adrenergic receptor agonist, UK14,304, and secretin inhibit cholangiocarcinoma growth by cAMP-dependent mechanism (28, 44), other molecules such as tauroursodeoxycholate, the H3 histamine receptor agonist, RAMH, and gastrin inhibit cholangiocarcinoma proliferation by activation of the IP$_3$/Ca$^{2+}$-dependent PKC$\alpha$ isoform (3, 19, 27). Furthermore, the neurotransmitter, $\gamma$-aminobutyric acid, inhibits the growth of biliary cancer by activation of both cAMP- and IP$_3$/Ca$^{2+}$-dependent signaling (17).

Thus, the aims of our studies were to 1) assess the expression of NPY and the NPY receptors in normal liver and cholangiocarcinoma tissue samples and non-malignant and cholangiocarcinoma cell lines; 2) determine the effects of NPY treatment on cholangiocarcinoma cell growth and elucidate the intracellular mechanism by which this occurs; and 3) evaluate the effects of NPY depletion on cholangiocarcinoma cell growth.

**Materials and methods**

**Materials**

Reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated. The nuclear dye 4,6-diamidino-2-phenylindole (DAPI) was obtained from Molecular Probes, Inc., Eugene, OR. The antibody against proliferating cellular nuclear
The antibodies recognizing the different subtypes of NPY receptors (Y1 to Y6) were purchased from Santa Cruz Biotechnology Inc. The affinity purified goat polyclonal antibody (A-17, against Y1) was raised against a peptide mapping near the N-terminus of Y1 of human origin. The affinity purified goat polyclonal antibody (L-17, against Y2) was raised against a peptide mapping near the C-terminus of Y2 of human origin. The synthetic peptide, corresponding to N terminal amino acids 1-14 of Y3 was purchased from Tocris Biosciences (Ellisville, MO). The affinity purified goat polyclonal antibody (C-20, against Y4) was raised against a peptide mapping at the C-terminus of Y4 of human origin. The affinity purified goat polyclonal antibody (N-20, against Y5) was raised against a peptide mapping within an extracellular domain of Y5 of human origin. The affinity purified goat polyclonal antibody (P-20, against Y6) raised against a peptide mapping within an internal region of Y6 of mouse origin. All the purchased antibodies are recommended for the detection of all NPY receptor subtypes of mouse, rat and human origin by immunoblots and immunofluorescence. The anti-NPY antibody was developed in rabbits using synthetic NPY (porcine) conjugated to KLH as the immunogen (Sigma Chemical Co). The highly selective and potent antagonist of Y1, BVD 10 (4), was purchased from Tocris Biosciences (Ellisville, MO). The potent, selective and competitive non-peptide antagonist for Y2, BIIE 0246 (12), was purchased from Tocris Biosciences (Ellisville, MO). The selective, non-peptide Y5 antagonist, CGP 71683 hydrochloride (13), was purchased from Tocris Biosciences (Ellisville, MO). Specific inhibitors for Y3, Y4 and Y6 are not commercially available. The RIA kits for the measurement of intracellular cAMP ([125I] Biotrak Assay System, RPA509) and IP3 (D-myoinositol 1,4,5-trisphosphate (IP3) [3H] Biotrak Assay System, TRK1000) levels were purchased from GE Healthcare (Piscataway, NJ).
**Cell lines:** The *in vitro* experiments were performed in six human cholangiocarcinoma cell lines (Mz-ChA-1, HuH28, HuCC-T1, CCLP1, SG231 and TFK-1) with different origins. Mz-ChA-1 cells, from human gallbladder (29) were a gift from Dr. G. Fitz (University of Texas Southwestern Medical Center, Dallas, TX). HuH28 cells, from human intrahepatic bile duct (31) and TFK-1 cells, from extrahepatic cholangiocarcinoma (52) were acquired from Cancer Cell Repository, Tohoku University, Japan. These cells were maintained at standard conditions as described (10, 27). CCLP-1 (56) HuCC-T1 (40) and SG231 (58) (from intrahepatic bile ducts) were a kind gift from Dr AJ Demetris (University of Pittsburg, PA) and were cultured as described (40, 56, 58). The human immortalized, nonmalignant cholangiocyte cell line, H69 (from Dr. G.J Gores, Mayo Clinic, Rochester, MN), was cultured as described (21). HIBEC cells were purchased from Sciencell (Carlsbad, CA) and cultured as described (8).

**Real time PCR for NPY and Y receptors:** The mRNA expression of NPY and its receptors were assessed in the selected cell lines by real time PCR (2, 10) using commercially available primers against NPY (accession no NM_000905; Reference position +451; expected product size 93 bp), Y1 (accession no NM_000909; Reference position +1016; expected product size 172 bp), Y2 (accession no NM_000910; Reference position +533; expected product size 164 bp), Y3 (accession no NM_003467; Reference position +192; expected product size 150 bp), Y4 (accession no NM_005972; Reference position +440; expected product size 161 bp), Y5 (accession no NM_006174; Reference position +312; expected product size 116 bp) and Y6 (accession no NR_002713; Reference position +480; expected product size 174 bp; SA Bioscience, Frederick, MD). A $\Delta\Delta$CT analysis was performed (34) using normal cholangiocytes as the control sample and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the
NPY secretion: All cell lines (H69, HIBEC, Mz-ChA-1, HuH28, HuCCT-1, SG231, TFK-1 and CCLP-1) were trypsinized and the resulting cell pellet was resuspended in 1X Hank’s-buffered saline buffer (1 X 10^7 cells/mL). Cells were incubated for 6 hr at 37°C and the amount of NPY released into the media was assayed using a commercially available NPY EIA kit (Bachem Americas; Torrance CA) according to the manufacturer’s instructions. NPY secretion from each cell line was assayed in triplicate and each data point from non-malignant cells or cholangiocarcinoma cells were collated and plotted on a scatter plot.

In parallel, NPY secretion was assessed in serum and bile samples obtained from cholangiocarcinoma patients and age-matched controls as described previously (2, 8) by an NPY EIA kit (Bachem Americas; Torrance CA). The human sera and bile samples were obtained from an unidentified tissue bank from the laboratory of Dr. Pietro Invernizzi (co-author in this manuscript, Center for Autoimmune Liver Diseases, IRCCS Istituto Clinico Humanitas, Rozzano, Milan, Italy). The samples were analyzed in a coded fashion in the laboratory of Dr. Invernizzi. The human liver samples were obtained from an unidentified tissue bank from the laboratory of Dr. Eugenio Gaudio (co-author in this manuscript, Department of Human Anatomy, University of the Studies of Rome, La Sapienza, Rome, Italy). The samples were analyzed in a coded fashion in the laboratory of Dr. Gaudio by three board-certified pathologists in a blinded fashion. An informed consent form was obtained from the healthy and the cholangiocarcinoma patients included in the study.
Cholangiocarcinoma tissue array analysis: NPY immunoreactivity was assessed in commercially available Accumax tissue arrays (Isu Abxis Co, LTD, Seoul, Korea) by immunohistochemistry as described (2, 8). The tissue arrays contain 48 well-characterized cholangiocarcinoma biopsy samples from a variety of tumor differentiation grades as well as four control liver biopsy samples. Semi-quantitative analysis was performed by three independent board-certified pathologists, in a blind fashion, using the following parameters. Staining intensity was assessed on a scale from 1-4 (1=no staining, 4=intense staining) and the abundance of positively stained cells was given a score from 1 to 5 (1= no cells stained, 5 = 100% stained). The staining index was then calculated by the staining intensity multiplied by the staining abundance that gave a range from 1 to 20.

Immunoblots for NPY receptors: The expression of NPY receptor subtypes was evaluated by immunoblots in protein (10 μg) from whole cell lysate from the selected normal and cholangiocarcinoma cell lines as previously described (2) using the aforementioned specific antibodies for each receptor subtype.

Immunofluorescence: The expression of NPY receptors was assessed in the normal human cell lines, H69 and HIBEC, and the cholangiocarcinoma cell line, Mz-ChA-1, by immunofluorescence as previously described by us (10, 18) using the aforementioned antibodies to all the NPY receptor subtypes (Y1 to Y6). Pre-immune sera were substituted for the primary antibodies as a negative control. Coverslips were visualized using an Olympus IX-71 inverted confocal microscope (Tokyo, Japan).

MTS cell proliferation assays: To assess the effects of NPY on cell proliferation, all cell lines were stimulated with various concentrations of recombinant NPY (10^-6 to 10^-10
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M) and cell proliferation was assessed by CellTiter 96 Cell Proliferation Assay (Promega Corp., Madison, WI) as described previously (10, 18). Absorbance was measured at 490 nm on a microplate spectrophotometer (Molecular Devices, Sunnyvale, CA). Data were expressed as the fold-change of treated cells as compared to BSA-treated cells. In separate sets of experiments, Mz-ChA-1 cells were treated at 37°C for 48 hr with saline or NPY (10^{-7} M) for 48 hr in the absence/presence of pre-incubation with: (i) BVD 10 (10^{-7} M, a selective and competitive non-peptide antagonist for Y1) (4); (ii) BIIE 0246 (10^{-7} M, a selective and competitive non-peptide antagonist for Y2 (12); or (iii) CGP 71683 hydrochloride (10^{-7} M, a selective, non-peptide Y5 antagonist) (13) before evaluating proliferation by CellTiter 96 Cell Proliferation Assay (Promega Corp.). Conversely, the effects of NPY depletion on cell proliferation were also assessed. Specifically, Mz-ChA-1 cells were treated with a 1:500 or 1:1000 dilution of a neutralizing NPY antibody (26) for 48 hr and cell proliferation was assessed by CellTiter 96 Cell Proliferation Assay (10, 18).

Evaluation of the intracellular mechanisms by which NPY regulates cholangiocarcinoma growth in vitro

Effect of NPY on intracellular cAMP and IP_3 levels

After trypsinization, Mz-ChA-1 cells were incubated at 37°C for 1 hr to regenerate membrane proteins damaged by trypsin (17). Mz-ChA-1 cells (1x10^5 for determination of cAMP levels, and 1x10^6 cells for the measurement of IP_3 levels) (17) were incubated at room temperature with 0.2% BSA (basal) or NPY (10^{-7} M) for 5 (for cAMP evaluations) (17) or 10 (for IP_3 measurements) (17) minutes. Intracellular cAMP and IP_3 levels were measured by RIA as described (17, 19, 20).

Effect of NPY on PKCα phosphorylation and translocation
To begin to assess the involvement of PKCα activation on the antiproliferative effects of NPY, Mz-ChA-1 cells were pretreated with a specific inhibitor for PKCα (Gö6976, 10^{-6} M) (38) for 1 hr prior to the addition of NPY (10^{-7} M). Cells were incubated for a further 48 hr after which time the protein lysates were made as described (2). PCNA expression was used as an indicator of the proliferative capacity of the cells and was assessed by immunoblots (2). The amount of protein loaded (10 μg) was normalized by immunoblots for β-actin (19). Band intensity was determined by scanning video densitometry using the phospho-imager Storm 860 (GE Healthcare) and the Image-Quant TL software, version 2003.02 (GE Healthcare). Furthermore, to assess the phosphorylation of PKCα, Mz-ChA-1 cells were treated with NPY (10^{-7} M) for 2 hr at 37°C. Following stimulation we assessed the expression of the phosphorylated form for PKCα by immunoblotting using a phosphospecific PKCα antibody (Santa Cruz Biotechnology) and expressed the data as a ratio to total PKCα expression (19).

To detect membrane translocation of PKCα, Mz-ChA-1 cells were plated, stimulated with NPY (10^{-7} M) for 2 hr, and processed for immunofluorescence (10, 18) as described above using the antibody for total PKCα. Negative controls were done with the use of pre-immune serum instead of the respective primary antibody. Slides were visualized using an Olympus IX-71 inverted confocal microscope.

**Invasion assays:** The effects of NPY on the invasive properties of cholangiocarcinoma were performed using growth factor-reduced matrigel invasion chambers (BD Biosciences, San Jose, CA) according to the manufacturer’s instructions. Specifically, Mz-ChA-1 cells were plated in the upper chamber and allowed to adhere overnight. Cells were treated with either NPY (10^{-7} M) or anti-NPY neutralizing antibody (1:500 dilution) for 24 hr. The number of cells that had migrated to the underside of the upper
chamber were then counted in 10 fields and expressed as the average (±SEM) no of cells as an indication of the invasion index.

**Nude mice treatment:** The effects of NPY or anti-NPY antibody on cholangiocarcinoma growth were assessed in vivo using a xenograft model of cholangiocarcinoma as previously described by us (9) with prior approval from the Scott & White and Texas A&M Health Science Center IACUC committees. Briefly, Mz-ChA-1 cells (5 x 10^6) were suspended in 0.25 mL of extracellular matrix gel and injected subcutaneously in the flanks of these animals. After the establishment of the tumors, mice received 0.5 μg/tumour/day of NPY, or 50 μL/tumour/day of a 1:500 dilution anti-NPY antibody injected 3 times per week and tumour dimensions were measured using electronic callipers (8, 9). After 60 days, mice were anaesthetized with sodium pentobarbital (50 mg/kg body weight, IP) and sacrificed according to institutional guidelines. Serum was collected and AST and ALT levels were measured using a Dimension® RxL Max Integrated Chemistry system (Dade Behring Inc., Deerfield IL) by the Scott & White Hospital, Chemistry Department.

Tumour tissues were dissected from the flank of these mice. Neoplastic tissues were fixed in formalin, embedded in paraffin, processed for histopathology, stained for H&E for routine examination and Masson’s trichrome for collagen. For immunohistochemistry, glass slides were deparaffinated, and endogenous peroxidase activity was blocked by a 30-minute incubation in methanolic hydrogen peroxide (2.5%). The endogenous biotin was blocked by a biotin blocking system (code X0590; DAKO, Copenhagen, Denmark) according to the instructions supplied by the vendor. Sections were then hydrated in graded alcohol and rinsed in 1x phosphate buffered saline (PBS, pH 7.4) before applying the primary antibody. Sections were incubated overnight at 4°C
with polyclonal antibodies for NPY (E-17, 1:50 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), and proliferating cell nuclear antigen (PCNA) (PC10, 1:100 dilution; DAKO). Samples were then rinsed with 1x PBS for 5 minutes, incubated for 10 minutes at room temperature with secondary biotinylated antibody (LSAB Plus system, DAKO, Milan, Italy), then with DAKO ABC (LSAB Plus system), and finally developed with 3–3’ diaminobenzidine.

To demonstrate the specificity of the immunoreaction, negative controls (the primary antibody was replaced (same dilution) with normal serum from the same species) were performed for all immunoreactions. Apoptosis was measured by quantitative terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) kit (Apoptag; Chemicon International, Inc). Sections were analyzed in a coded manner using BX-51 light microscopy (Olympus, Tokyo, Japan) with a video cam (Spot Insight; Diagnostic Instrument, Inc., Sterling Heights, MI) and processed with an Image Analysis System (Delta Sistemi, Rome, Italy).

NPY immunohistochemistry in human tumor samples

NPY immunoreactivity was assessed in excised tumor samples from cholangiocarcinoma patients, where the invasion front of the cancer was clearly visible. Immunohistochemical staining for NPY was performed as outlined above.

Statistical Analysis: All data are expressed as mean ± SEM. Differences between groups were analyzed by the Student’s unpaired t-test when two groups were analyzed, and by ANOVA when more than two groups were analyzed, followed by an appropriate post hoc test. A p value of less than 0.05 was used to indicate statistical significance.

Results:
**Cholangiocarcinoma express and synthesize more NPY than normal cholangiocytes**

NPY mRNA expression was assessed in six cholangiocarcinoma cell lines and in two non-malignant cholangiocyte cell lines and found to be elevated in all cholangiocarcinoma cell lines studied (Figure 1A). The increase in NPY expression was also reflected by increased NPY secretion from all cholangiocarcinoma cell lines studied compared to non-malignant cholangiocytes (Figure 1B). In addition, immunohistochemical analysis of human liver biopsy samples indicated that there is also increased NPY immunoreactivity in cholangiocarcinoma samples compared to controls as assessed in a coded fashion by three independent board certified pathologists (Figure 1C). Analysis of serum and bile samples from cholangiocarcinoma patients versus age-matched normal controls revealed no significant difference in NPY levels (data not shown), suggesting that the expression and secretion of NPY from cholangiocarcinoma cells is a local event.

**Increased local release of NPY decreases cholangiocarcinoma growth and invasion**

We first determined the presence of the NPY receptors in malignant and normal cholangiocyte cell lines. By real time PCR, all NPY receptor subtypes were expressed by non-malignant (H69 and HIBEC) and Mz-ChA-1 cells (Figure 2A) in addition to all the other cholangiocarcinoma cell lines used (data not shown). Similarly, by immunoblots NPY receptors were expressed by Mz-ChA-1 and H69 cells (Figure 2B) and HIBEC (not shown). By immunofluorescence, immunoreactivity for all NPY receptors was predominantly located in the membrane of both H69 cells (non-malignant cholangiocytes) and Mz-ChA-1 cells (cholangiocarcinoma cells; Figure 3) as well as in the other malignant and non-malignant cholangiocyte cell lines studied (data not shown).
Treatment of the non-malignant human cholangiocyte cell lines, H69 and HIBEC, with various concentrations of NPY had no significant impact on cell proliferation (data not shown), whereas treatment of all of the selected cholangiocarcinoma cell lines with NPY (given as a single dose of $10^{-6}$ to $10^{-10}$ M) caused a significant ($p<0.05$) decrease in cell proliferation after 48 hr as demonstrated by cell proliferation assay (Figure 4A). Repeated daily administration of NPY had no additional antiproliferative effects beyond that after a single dose suggesting that the recombinant NPY is stable in these culture conditions (data not shown). In separate sets of experiments, we demonstrated that: (i) NPY decreased the growth of Mz-ChA-1 cells (Figure 4B); and (ii) the antiproliferative effects of NPY ($10^{-7}$M) on cholangiocarcinoma growth was prevented by the specific inhibitor of Y2, BIIE 0246 (12), but not inhibitors for Y1 and Y5 (Figure 4B), suggesting that Y2 are involved in the inhibitory effects of NPY on cholangiocarcinoma growth. Alone, inhibitors of Y1 and Y5 did not affect the growth of Mz-ChA-1 cell growth (not shown). We did not evaluate the effects of Y3, Y4 and Y6 on NPY-inhibition of cholangiocarcinoma growth since these receptor inhibitors are not available.

NPY receptor activation can result in changes to either cAMP levels or to intracellular Ca$^{2+}$/IP$_3$ levels in other cell types (1, 22, 35, 39). Therefore, we assessed the effects of NPY on intracellular IP$_3$ and cAMP. NPY ($10^{-7}$ M) significantly increased intracellular IP$_3$ levels in Mz-ChA-1 cells compared to the basal levels (Figure 5A), and had no effect on intracellular cAMP (data not shown) suggesting the NPY is acting through an IP$_3$/Ca$^{2+}$-dependent signaling pathway.

We have previously demonstrated that one of the major downstream effectors of Ca$^{2+}$ signaling in the regulation of cholangiocarcinoma cell growth is PKC$\alpha$ (3, 19, 20, 27, 28). Pretreatment of Mz-ChA-1 cells with the specific inhibitor of PKC$\alpha$ prior to the addition of NPY ($10^{-7}$ M) prevented the NPY-induced decrease in PCNA protein expression (as a
marker of proliferative capacity; Figure 5B) suggesting that PCKα is involved in the effects of NPY on cholangiocarcinoma growth. Indeed, treatment of Mz-ChA-1 cells with NPY (10^{-7} M) increased the phosphorylation of PKCα (Figure 5C) compared to the basal levels. Furthermore, by immunofluorescence, there was a distinct positive stain for PKCα under basal conditions localized in the cytoplasm (Figure 5D), whereas after NPY stimulation, there was translocation of PKCα from the cytosolic region to the membrane domain of the cells (Figure 5D).

In addition, the effects of NPY on cell invasion were assessed using Mz-ChA-1 cells plated in the Matrigel invasion upper chamber. The average number of cells that invaded the lower chamber in 24 hr under basal conditions was 123.25 ± 1.29, whereas the number of cells that had invaded the lower chamber after 24 hr of NPY treatment decreased to 100.95 ± 1.64 (p<0.05 vs. corresponding basal value).

In support of the in vitro data, treating an in vivo xenograft model of cholangiocarcinoma tumors with NPY significantly suppressed tumor growth (Figure 6A). In addition, the latency of tumor growth (i.e., time taken for tumor volume to increase to 150% of the original size) was increased after NPY treatment compared to vehicle treatment (Figure 6A). Analysis of liver enzymes in the serum revealed that there was no significant difference in AST (Vehicle, 80.67 ± 8.25 U/L vs. NPY, 108.67 ± 21.38 U/L) and ALT levels (Vehicle, 28.33 ± 3.08 U/L vs. NPY, 35.0 ± 2.54 U/L) between NPY-treated and vehicle-treated animals, both of which fell within normal range suggesting that the NPY treatment was well tolerated and did not cause any liver damage. Histological analysis of liver, heart, and kidney also indicated no significant organ damage caused by the chronic NPY treatment (data not shown).

Histological analysis of the excised tumors revealed that all tumor cells within tumors from NPY-treated and vehicle-treated animals were positive for cytokeratin-19, a specific
marker for cholangiocytes (2, 8) indicating biliary phenotypes (data not shown). Using PCNA immunoreactivity as a marker of proliferative capacity, NPY treatment decreased the number of PCNA-positive nuclei per field compared to vehicle treatment (Figure 6B). In parallel, using TUNEL staining as a marker of apoptosis, NPY treatment significantly increased the percentage of TUNEL-positive nuclei per field compared to vehicle treatment (Figure 6B).

**Inhibition of NPY function increases cholangiocarcinoma cell growth and invasion in vitro**

Because NPY expression and secretion increased in cholangiocarcinoma, but then had an apparently contradictive, anti-proliferative effect on cell growth, we designed experiments aimed to demonstrate that blocking NPY activity had growth promoting effects on cholangiocarcinoma. Indeed, treating Mz-ChA-1 cells with an NPY-specific antibody that has neutralizing activity (26), increased cell proliferation as shown by MTS assays (Figure 7A). Furthermore, Mz-ChA-1 cells treated with anti-NPY antibody for 24 hr significantly (p<0.05) increased the rate of cell invasion compared to that seen under basal conditions (basal 123.35 ± 1.29, anti-NPY antibody 162.0 ± 3.25).

In addition, treating an in vivo xenograft model of cholangiocarcinoma tumors with anti-NPY antibody significantly increased the rate of tumor growth (Figure 7B) and decreased the latency of tumor growth (Figure 7B). Analysis of liver enzymes in the serum revealed that there was no significant difference in AST (Vehicle, 69.66 ± 8.95 U/L vs. anti-NPY, 53.67 ± 13.79 U/L) and ALT levels (Vehicle, 38.67 ± 13.68 U/L vs. anti-NPY, 30.00 ± 8.15 U/L) between anti-NPY antibody-treated and vehicle-treated animals, both of which fell within normal range suggesting that the inhibition of NPY did not cause any liver damage. Histological analysis of liver, heart, and kidney also indicated no significant organ damage caused by the chronic inhibition of NPY (data not shown).
Immunohistochemical analysis of tumor tissue reveals a gradient of NPY expression

The concept that cholangiocarcinoma cells are over-producing NPY, which in turn slows the growth rate of the tumor, appears somewhat counterintuitive. Therefore, we wished to determine if the increased expression of NPY occurs uniformly throughout the tumor. Interestingly, the increased expression of NPY occurred predominantly in the center of the tumor (Figure 8A-B), particularly near the necrotic areas, with considerably less expression towards the periphery of the tumor (Figure 8C). Furthermore, the non-malignant hepatocytes in the normal liver tissue surrounding the tumor appear to also have an increased expression of the anti-proliferative NPY (Figure 8A).

Discussion

The major findings of the study relate to the local regulation of cholangiocarcinoma cell growth and migration by NPY. We demonstrated that cholangiocarcinoma cells express and produce more NPY than normal cholangiocytes, and that there is a gradient of NPY expression within the tumor with the maximum expression occurring toward the center of the tumor, near the necrotic areas. Furthermore, treatment of cholangiocarcinoma cells with NPY in vitro and in vivo decreases both proliferation and migration. In vitro, NPY-inhibition of cholangiocarcinoma growth was prevented by the specific inhibitor of Y2, BIIE 0246 (12), but not inhibitors for Y1 and Y5 (Figure 4B), suggesting that Y2 are involved in the inhibitory effects of NPY on cholangiocarcinoma growth.; inhibitors for Y3, Y4 and Y6 are not available. In addition, we have shown that: (i) NPY-induced inhibition of cholangiocarcinoma cell growth was associated with an increase in intracellular IP₃ and activation of PKCα; and (ii) the antiproliferative effects of NPY are blocked by a specific PKCα inhibitor. Taken together these data suggest that the gradient expression
of NPY in cholangiocarcinoma may be a key regulatory feature of the local regulation of
cholangiocarcinoma growth and progression.
A physiological function of NPY is to regulate food intake and increase fat storage (23).
Furthermore, NPY has been shown to be upregulated in obesity (5), which is a risk
factor for cholangiocarcinoma (59). Therefore, because of the link between obesity and
cholangiocarcinoma, it is conceivable that molecules that are upregulated during obesity,
such as NPY, may also play a role in cholangiocarcinoma initiation, growth and
progression. Indeed, recently we demonstrated that another obesity-related molecule
leptin was involved in the development and growth of cholangiocarcinoma in a
thioacetamide rat model of cholangiocarcinoma (16).
In support of our observation that cholangiocarcinoma cells secrete increased levels of
NPY, this neuropeptide is secreted in a number of other tumors. For example, NPY
immunoreactivity has been shown in approximately 75% of prostate cancer tumor
specimens studied (36). Furthermore, NPY treatment of three different prostate cancer
cell lines stimulated proliferation in one cell line and decreased proliferation in the other
two (49). Both the growth enhancing and anti-proliferative actions of NPY were through
the NPYR1, and the difference between the proliferative and anti-proliferative effects
appears to be via the mechanism by which Extracellular signal-regulated kinase 1/2 is
activated (49). NPY has also been shown to be upregulated in a number of
neuroendocrine tumors, such as pheochromocytomas (11, 24), and neuroblastomas (30,
41), and are thought to be of diagnostic importance; however its contribution to tumor
progression still remains to be clarified. We have demonstrated that
cholangiocarcinoma display typical markers of a neuroendocrine phenotype such as the
expression of chromogranin A and neuron specific enolase (2), therefore it is not
surprising that cholangiocarcinoma also expressed NPY. In the present study, the
diffuse positivity of the neuroendocrine marker, NPY, observed in human ductular
cholangiocarcinoma samples is supported by a number of studies. Indeed, Liu et al. have demonstrated higher expression of NPY in non-aggressive prostate epithelial tumors (33). Moreover, these observations about the non conventional immunolocalization of neuroendocrine proteins are previously discussed in cholestasis and cholangiocarcinoma where the important role of GABA, chromogranin A, glycolipid A2-B4, S-100 protein and neural cell adhesion molecule are demonstrated by us and other groups (17, 37, 46-48) suggesting that the biliary epithelium is a significant target for the neuroendocrine system. A direct outgrowth of our findings will be to study the expression of NPY in hepatocytes and cholangiocytes in cholangiocarcinoma peri-tumoral tissue.

While the majority of studies into the effects of NPY on cancer growth and invasion demonstrate a growth promoting and invasive effect (45, 50, 55), there are a number of studies that have demonstrated anti-proliferative effects of NPY. As mentioned above, NPY administration to a number of prostate cancer cell lines inhibited the proliferation through an NPYR1-dependent mechanism (49). In addition, NPY administration to colon carcinoma cells in vitro reduced the invasion potential of these tumor cells in a concentration-dependent manner (42). The data that we present here support an anti-proliferative action of NPY on cholangiocarcinoma cell growth in vitro and in vivo and also inhibits cholangiocarcinoma migration and invasion. It is conceivable that the different effects of NPY on cell growth may be due to the number of specific NPY receptors that NPY may exert its effects through. We demonstrated that all receptors are present in cholangiocarcinoma, but due to the lack of reliable specific antagonists for Y3, Y4 and Y6, we were unable to pinpoint the specific NPY receptor that is responsible for the actions of NPY on cholangiocarcinoma and is a limitation of the present study. However, since a specific inhibitor of Y2, BIIE 0246 (12), blocks the in vitro inhibitory effect of NPY on cholangiocarcinoma growth, likely Y2 play an important role in the
inhibitory effect of NPY on cholangiocarcinoma growth. Further experiments (gene silencing of NPY receptors with siRNAs) to pinpoint the NPY receptors involved in NPY effects on cholangiocarcinoma are necessary and will be performed in our future studies.

As mentioned previously, NPY receptor activation can elicit cAMP- or Ca\(^{2+}\)-mediated signal transduction pathways (1, 22, 35, 39). The data presented here supports a role for IP\(_3\)/Ca\(^{2+}\)-mediated PKC\(\alpha\) activation in the antiproliferative action of NPY since NPY increased IP\(_3\) (but not cAMP) levels and induces the activation of PKC\(\alpha\). These findings support the concept that the Ca\(^{2+}\)-dependent PKC\(\alpha\) is a key regulator of the hyperplastic and neoplastic growth of cholangiocytes. Indeed, we have previously shown that the inhibition of cholangiocarcinoma growth (e.g., by gastrin, H3 histamine receptor agonists, a2-adrenergic receptor agonists and the bile salt, tauroursodeoxycholate) is associated with enhanced phosphorylation of PKC\(\alpha\).

In the present study, we demonstrated a gradient of NPY expression in the cholangiocarcinoma tumor, with the highest expression found in the center of the tumor near the necrotic areas. The concept of a gradient of expression to accommodate the different microenvironments within the tumor is not new. Recently, a large-scale expression analysis of melanoma metastases was performed to identify genes that exhibit differential expression between the invasion front and the central tumor areas (54). The authors identified 248 genes that were differentially expressed within the tumor, 97 of which had higher expression within the center of the tumor compared to the invasion front (54). Some of the genes that are expressed higher at the invasion front, generally, had known functions in cell invasion (54); however the function, with respect to cancer progression, of the genes predominantly expressed in the central areas of the tumor were largely unknown (54). In addition, Ohira et al recently demonstrated that a gradient of transforming growth factor-\(\beta\) expression is evident in cholangiocarcinoma
(43) with little to no expression in the invasion front and strong expression in tumors growing in the bile duct lumen (43). Here we speculate that NPY may be expressed higher in the center of the tumor where it exerts growth-suppressive and anti-inflammatory effects to allow for the recruitment of adequate stromal support.

In conclusion, the data presented here demonstrates a role of NPY in the local control of cholangiocarcinoma cell growth. Specifically, NPY appears to be expressed to a greater extent in the center of the tumor where we speculate, it exerts a local anti-proliferative and anti-migratory effect on cholangiocarcinoma cells. Modulation of NPY signaling may be a useful target for the design of therapeutic tools for the treatment of this devastating cancer.

References


26. Ishii T, Muranaka R, Tashiro O, and Nishimura M. Chronic intracerebroventricular administration of anti-neuropeptide Y antibody stimulates


**Figure Legends:**

**Figure 1** Cholangiocarcinoma cells express and secrete increased amounts of NPY than normal cholangiocytes. NPY mRNA (A) expression was assessed in six cholangiocarcinoma cell lines as well as the non-malignant cholangiocyte cell lines H69 and HIBEC by real time PCR (A). (A) Data were expressed as average ± SEM (n=3). Asterisk denotes significance (p<0.05) compared to NPY expression in H69 cells. (B) NPY secretion from the cell lines was assessed by EIA kit. Secretion from each cell line was determined by EIA after 6 hr and was performed in triplicate. Each data point from non-malignant cells or cholangiocarcinoma cells (CCA) were collated and plotted on a scatter plot. Asterisk denotes significance (p<0.05) compared with NPY secretion from cholangiocyte cell lines. (C) NPY levels were also assessed in biopsy samples from 48 cholangiocarcinoma patients and healthy controls by immunohistochemistry. Representative photomicrographs of the NPY immunoreactivity are shown (C; magnification X40). Staining intensity was assessed as described in the methods and expressed as an average ± SEM of all cholangiocarcinoma patients compared to control
samples (C). Asterisk denotes significance (p<0.05) compared with NPY immunoreactivity in control biopsy samples.

**Figure 2** Evaluation of NPY receptors in non-malignant cholangiocytes and Mz-ChA-1 cells by (A) real-time PCR and (B) immunobLOTS. (A) By real time PCR, all NPY receptor subtypes were expressed by H69 and HIBEC and Mz-ChA-1 cells. Data are expressed as average ± SEM (n=3). (B) By immunobLOTS NPY receptors were expressed by Mz-ChA-1 and H69 cells. The comparability of the protein used was evaluated by immunobLOTS for β-actin, the housekeeping.

**Figure 3** NPY receptor localization was assessed by immunofluorescence in H69 and Mz-ChA-1 cells. Specific receptor immunoreactivity was shown in red and nuclei were counterstained with DAPI (blue). Negative controls were performed by substituting the primary antibodies with pre-immune serum. Scale bar represents 20 μm.

**Figure 4** (A) NPY decreases cholangiocarcinoma cell proliferation in vitro. Cholangiocarcinoma cells were treated with various concentrations of NPY (10^{-10} M to 10^{-6} M) for 48 hr. Cell proliferation was assessed using MTS cell proliferation assays. Data are expressed as fold change in proliferation (average ± SEM, n=7) and the asterisk denotes p<0.05 compared to basal treatment within each cell line. (B) The inhibitory effects of NPY could be prevented by a specific inhibitor of Y2, but not Y1 and Y5. Mz-ChA-1 cells were pretreated with BVD 10, (10^{-7} M; Y1 inhibitor), BIIE0246 (10^{-7} M; Y2 inhibitor) or CPG 71683 hydrochloride, (10^{-7} M; Y5 inhibitor) for 1 hr prior to the addition of NPY (10^{-7} M) for 48 hr. Cell proliferation was assessed using MTS cell
proliferation assays. Data are expressed as fold change in proliferation (average ± SEM, n=7) and the asterisk denotes p<0.05 compared to basal treatment.

**Figure 5**  *In vitro* effect of NPY on (A) IP₃ levels, (B) PCNA, and the (C) phosphorylation and (D) translocation of PKCα in Mz-ChA-1 cells. (A) Mz-ChA-1 cells were treated with 0.2% BSA (basal) or NPY (10⁻⁷ M) for 10 minutes at room temperature before evaluation of IP₃ levels by RIA. NPY increased IP₃ levels of Mz-ChA-1 cells compared to Mz-ChA-1 cells treated with 0.2% BSA. *p<0.05 vs. its corresponding basal value (n = 6). (B) Mz-ChA-1 cells were pretreated Gö6976 (a specific PKCα inhibitor) for 1 hour prior to the addition of NPY (10⁻⁷ M). Pretreatment of Mz-ChA-1 cells with Gö6976 prior to the addition of NPY prevented the NPY-induced decrease in PCNA protein expression. *p<0.05 vs. its corresponding basal value (4 immunoblots from cumulative preparations of cholangiocytes). (C-D) Following treatment of Mz-ChA-1 cells with 0.2% BSA (basal) or NPY (10⁻⁷ M), we evaluated the phosphorylation (C, by immunoblots) and translocation (D, by immunofluorescence in cell smears) of PKCα. (C) Treatment of Mz-ChA-1 cells with NPY (10⁻⁷ M) increased the phosphorylation of PKCα compared to the basal levels. *p<0.05 vs. its corresponding basal value (4 immunoblots from cumulative preparations of cholangiocytes). (D) By immunofluorescence, there was a distinct positive stain for PKCα under basal conditions localized in the cytoplasm, whereas after NPY stimulation, there was translocation of PKCα from the cytosolic region to the membrane domain of the cells. Bar = 50 μm.

**Figure 6**  NPY treatment decreases tumor growth in an *in vivo* xenograft model of cholangiocarcinoma. Mz-ChA-1 cells were injected into the flank of athymic mice. After tumors were established, mice were treated with 0.5 μg/tumour/day NPY (intratumoral),
three days per week for 60 days and tumor volume assessed (A; left panel). Tumor latency was assessed as the time taken for the tumor to grow to 150% of the original size (A; right panel). Data are expressed as average latency (days ± SEM) and the asterisk denotes significance (p<0.05) from vehicle-treated tumors. Immunohistochemistry on tumors from vehicle- and NPY-treated mice was performed using a specific antibody against PCNA (B). Apoptosis was detected by TUNEL staining (B). Representative photomicrographs of the immunoreactivity are shown. (Orig. magn. X40). Semi-quantitative analysis of PCNA immunoreactivity and TUNEL positive nuclei were performed and data were expressed as average (± SEM) positive nuclei per field and the asterisk denotes significance (p<0.05) compared to vehicle-treated tumors.

**Figure 7** Blocking NPY activity promotes cholangiocarcinoma cell growth *in vitro* and *in vivo*. Mz-ChA-1 cells were treated with various concentrations of anti-NPY antibody (1:500 and 1:1000 dilution) for 48 hr. Cell proliferation was assessed using an MTS cell proliferation assay (A). Data are expressed as fold change in proliferation (average ± SEM, n=7) and the asterisk denotes p<0.05 compared to basal treatment within each cell line. (B) Mz-ChA-1 cells were injected into the flank of athymic mice. After tumors were established, mice were treated with 50 μL of a 1:500 dilution anti-NPY antibody (intratumoral), three days per week for 60 days and tumor volume assessed (A; left panel). Tumor latency was assessed as the time taken for the tumor to grow to 150% of the original size (A; right panel). Data are expressed as average latency (days ± SEM) and the asterisk denotes significance (p<0.05) from vehicle-treated tumors.

**Figure 8** Immunohistochemical analysis of NPY expression across human cholangiocarcinoma tumors. Representative photomicrographs of the immunoreactivity
are shown. (Orig. magn. X10, particular X40). The NPY expression, present in all tumor
tissues (A, B), appeared higher near the neoplastic necrotic areas (C). Furthermore, the
non-malignant hepatocytes in the normal liver tissue surrounding the tumor appear to
also have an increased expression of the anti-proliferative NPY (A).
Figure 2

Relative mRNA receptor expression

Y1

Y2

Y3

Y4

Y5

Y6

H69  HIBEC  Mz-ChA-1

H69  HIBEC  Mz-ChA-1

H69  Mz-ChA-1

Y1  45 kD

Y2  54 kD

Y3  45 kD

Y4  42 kD

Y5  57 kD

Y6  42 kD

β-actin  43 kD
Figure 6

A

Tumor volume (mm³)

Days of tumor growth

Time (days)

B

Vehicle

NPY

PCNA

TUNEL

Percentage of PCNA positive nuclei

Percentage of TUNEL positive nuclei

Vehicle

NPY
Figure 7

A

Fold change in proliferation

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48 hrs of incubation

B

Tumor volume (mm$^3$)

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Days of tumor growth

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