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Original Article

The developmental transcription factor Gata4 is overexpressed in pancreatic ductal adenocarcinoma

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Abstract: GATA4 is a transcription factor that plays a role in regulating the normal development of many mesoderm and endoderm derived tissues, including the pancreas. Silencing of GATA4 mRNA expression by promoter methylation has been implicated in carcinogenesis of the ovary, lung and colorectum. By contrast, GATA4 mRNA expression is upregulated in pancreatic cancer cell lines and tissues. To further clarify the relationship of GATA4 to pancreatic cancer, we immunolabeled 90 samples of pancreatic ductal adenocarcinoma using a GATA4 specific monoclonal antibody. Both the intensity and percent of labeling was recorded for each carcinoma and correlated to the clinicopathologic features available for each patient. Samples of normal adult (n=26) and fetal pancreatic tissue (n=8) were also immunolabeled for comparison to expression patterns in pancreatic carcinoma tissues. Immunolabeling for GATA4 indicated robust nuclear expression in developing acini in fetal pancreatic tissues, consistent with the role of GATA4 in embryologic development, and in mature pancreatic acinar epithelium. Immunolabeling for GATA4 was also noted within normal duct epithelial cells, although it was always lesser in intensity than for acinar cell nuclei in the same section. Positive GATA4 immunolabeling was seen in 61/90 (68%) infiltrating pancreatic cancers of which 27/90 (30%) showed strong positive labeling. While there was no relationship among GATA4 and patient age, race or pathologic features, we did find a significant association among strong positive labeling and female gender (p=0.01). These findings support previous studies implicating GATA4 in pancreatic cancer and offer new avenues for investigation into this aggressive tumor type.

Key words: Pancreatic cancer, transcription factor, embryology, development

Introduction

The GATA family of proteins are known structurally related zinc finger transcription factors that bind to DNA at the consensus sequence (A/T)GATA(A/G), the GATA motif [1]. GATA proteins differ based on their sequence homology and expression patterns. For example, GATA1-3 are expressed mainly in hematopoetic cells, whereas GATA4-6 are expressed in mesoderm and endoderm derived tissues [2]. With exception of GATA5, all GATA transcription factors are believed essential for mammalian development since disruption of these factors causes embryonic lethality in mice [3, 4].

During early mouse development, GATA4 and GATA6 are expressed in the extraembryonic endodermal lineages including the visceral and parietal endoderm [5]. *GATA4* null mice die by embryonic day E9 due to defects in heart morphogenesis and *GATA6* null mice die by embryonic day E7.5 due to defects in visceral endoderm formation [3, 4]. The critical role of GATA4 and GATA6 in differentiation of extraembryonic endoderm has also been demonstrated by the forced overexpression of either GATA4 or GATA6 in ES cells. Expression of these factors was sufficient to induce proper differentiation towards extraembryonic endoderm and blockade of stem cell identity as evidenced by the upregulation of the endoderm marker gene Hnf3β, upregulation of the parietal endoderm marker genes Sparc, Laminin B1, tPA and Dab2, and down-regulation of Oct 4 and Sox2 [6]. In a loss-of-function zebrafish model, both the *GATA4* and *GATA6* genes were found to have distinct and non-redundant functions in cardiac development, as well as in the development of the intestine, liver and pancreas [7]. *GATA4* and *GATA6* gene regulation are also intermingled. For example, analyses of knockout mice indicate

that *GATA6* can be regarded as an upstream regulator of *GATA4*. Deletion of *GATA6* expression results in absence of GATA4 expression and deletion of *GATA4* leads to upregulation of GATA6 expression [3, 8].

GATA transcription factors are increasingly recognized as playing a role in human cancers. For example, frequent promoter hypermethylation of *GATA4*, *GATA5* and/or *GATA6* was reported in human esophageal, lung, gastric, colorectal and ovarian cancers [9-15]. In colon and gastric cancers, downstream targets of GATA4 and GATA5 were also epigenetically silenced whereas GATA6 was ubiquitously expressed in all of these same cancers. By contrast, *GATA6* deletion has been found in brain cancers [16]. GATA factors have also been implicated in pancreatic cancer. For example, GATA4 was shown to be upregulated along with other known foregut markers, such as GATA5, GATA6, Villin 1, Villin 2, Sox2, HoxA5, and Fkh 6 in pancreatic intraepithelial neoplasia, a precursor of infiltrating pancreatic cancers [17]. Moreover, we have previously reported our observation of 5-fold overexpression of GATA4, but not GATA5, in pancreatic cancer compared with normal duct epithelial cells [18]. GATA6 is also overexpressed in pancreatic cancer, and in some cancers overexpression is due to amplification of the *GATA6* gene on chromosome 18q [19].

The objective of this study was to further clarify to relationship of *GATA4* to pancreatic carcinogenesis. Towards this goal, we performed immunohistochemical labeling for GATA4 protein in fetal tissues for which *GATA4* has been shown to play a role in embryologic development, as well as in normal pancreas and resected pancreatic cancer tissues in which *GATA4* mRNA is overexpressed.

Materials and methods

Fetal and cancer tissues

Paraffin-embedded samples of 90 different pancreatic ductal adenocarcinomas were identified in the Johns Hopkins Pathology Database using the search term "Whipple and infiltrating adenocarcinoma" and used to make five tissue microarrays. Each array contained four 2-mm diameter cores each of 18 different carcinomas, as well as 33 cores of normal tissue from 12 different organ sites. In addition, paraffin-embedded tissues from eight fetuses were obtained. Clinicopathologic data of each patient whose carcinoma tissues were used for the study was collected under the approved guidelines of the Johns Hopkins Institutional Review Board.

Immunohistochemistry

Immunolabeling for GATA4 was performed using a GATA4 specific monoclonal antibody (clone G-4, Santa Cruz Biotechnology) raised against amino acids 328-439 of the human GATA4 protein. Unstained 5-μm sections were cut from each tissue microarray or paraffin block and the slides were deparaffinized in xylene and graded alcohols followed by incubation in 1X sodium citrate buffer (diluted from 10X heat-induced epitope retrieval buffer, Ventana-Bio Tek Solutions, Tucson, AZ) before steaming for 20 minutes at 80°C. Slides were cooled 5 minutes and incubated with anti-GATA4 antibody using a DAKO autostainer. Immunolabeling was detected using a DAKO LSAB+ kit per kit instructions, and each section was counterstained with hematoxylin before coverslips were applied. Both labeling intensity (0-negative, 1-weak, 2-moderate, 3-strong) and the percent positive labeling cells were recorded for each tissue core and used to calculate an H-score (intensity x % positive cells) that ranged from 0 to 300. In the event of heterogeneity in the H-score for different cores taken from the same primary tumor, a mean H-score was calculated for all cores. H scores of <30 were considered negative, 31-100 were considered weak positive, and >100 were considered strong positive.

Western blots

The cell lines AsPC1, BxPC3, MiaPACA2, and Su86.86 cell lines were obtained from the ATCC (Manassas, VA), and cell lines A13A and A6L were created in our own laboratory [18]. The IMIM-PC2 pancreas cancer cell line was generously provided by Dr. Paco Real [20]. The immortalized normal duct epithelium cell lines HPNE and HPDE were previously described [18]. All cell lines were grown at 37°C in DMEM containing 10% FBS, 1% L-glutamine and 1% penicillin-streptomycin in a humidified atmosphere containing 5% CO_2. Protein lysates were prepared from pellets of each line, and aliquots of 30 ug total protein per cell line were separated by SDS/PAGE using 4-12% gels

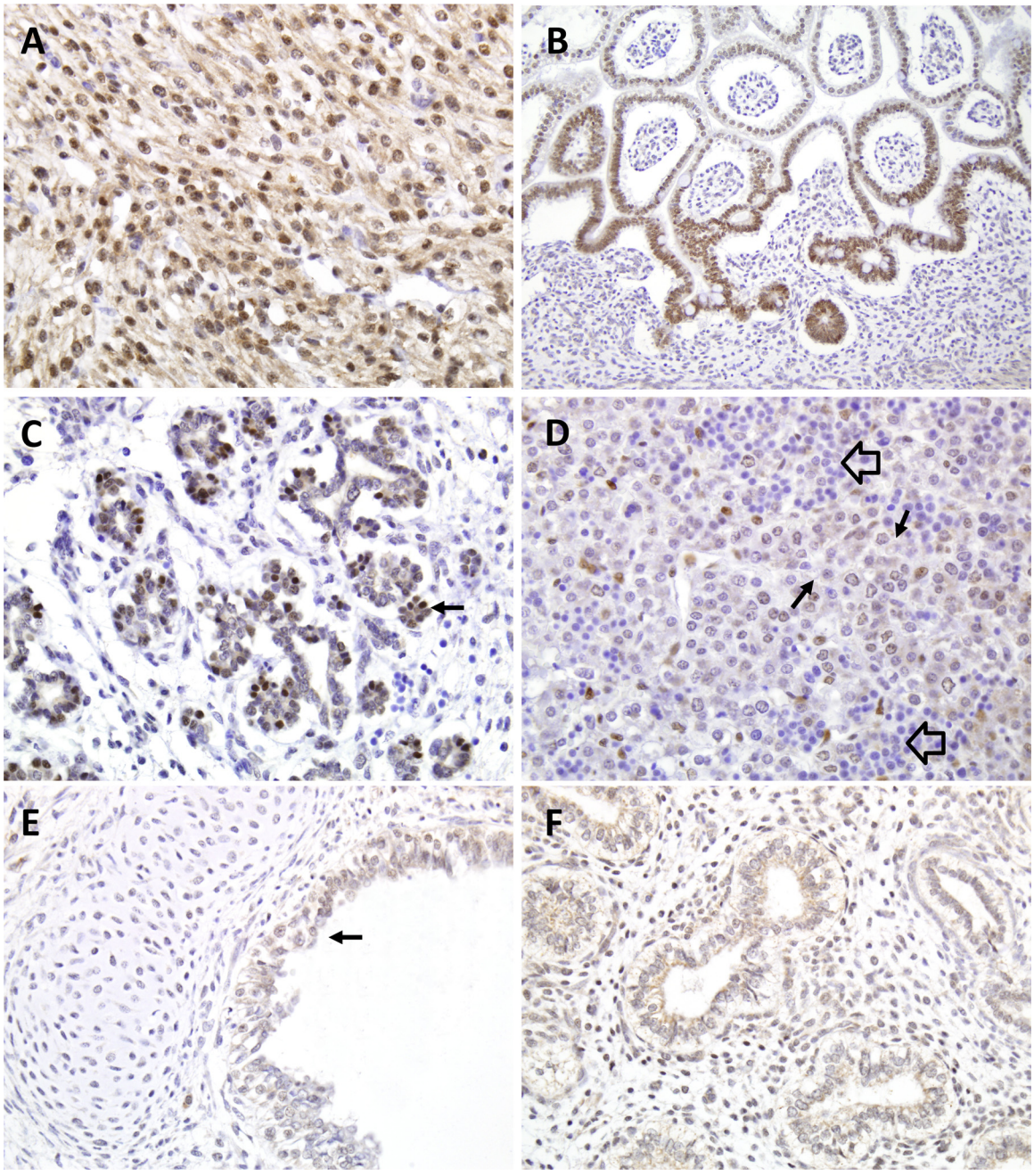

Figure 1. GATA4 expression in fetal tissues. Strong positive nuclear labeling is seen in myocardium (A), small intestine (B) and pancreatic acini (indicated by arrow, C). Moderate intensity labeling is seen in hepatocytes (indicted by small arrows, D), tracheal epithelium (indicated by arrow, E) and alveolar epithelium (F). Small arrows in Panel D highlight nuclear labeling in fetal hepatocytes, and open arrows indicate regions of extramedullary hematopoesis.

(Invitrogen) and subsequently transferred to a 0.45-μm nitrocellulose membrane (Invitrogen). The membrane was blocked in wash solution (0.1% Tween 20 in PBS) containing 5% nonfat dry milk. Mouse anti-human GATA4 antibody (clone G-4, Santa Cruz Biotechnology) and rabbit TrueBlot (eBioScience) were used as the primary and secondary antibodies respectively, followed by Lumingen PS-3 as detecting reagent (Amersham). GATA4 protein was visualized using a Bio-Rad Quantity One Imaging Device (Bio-Rad).

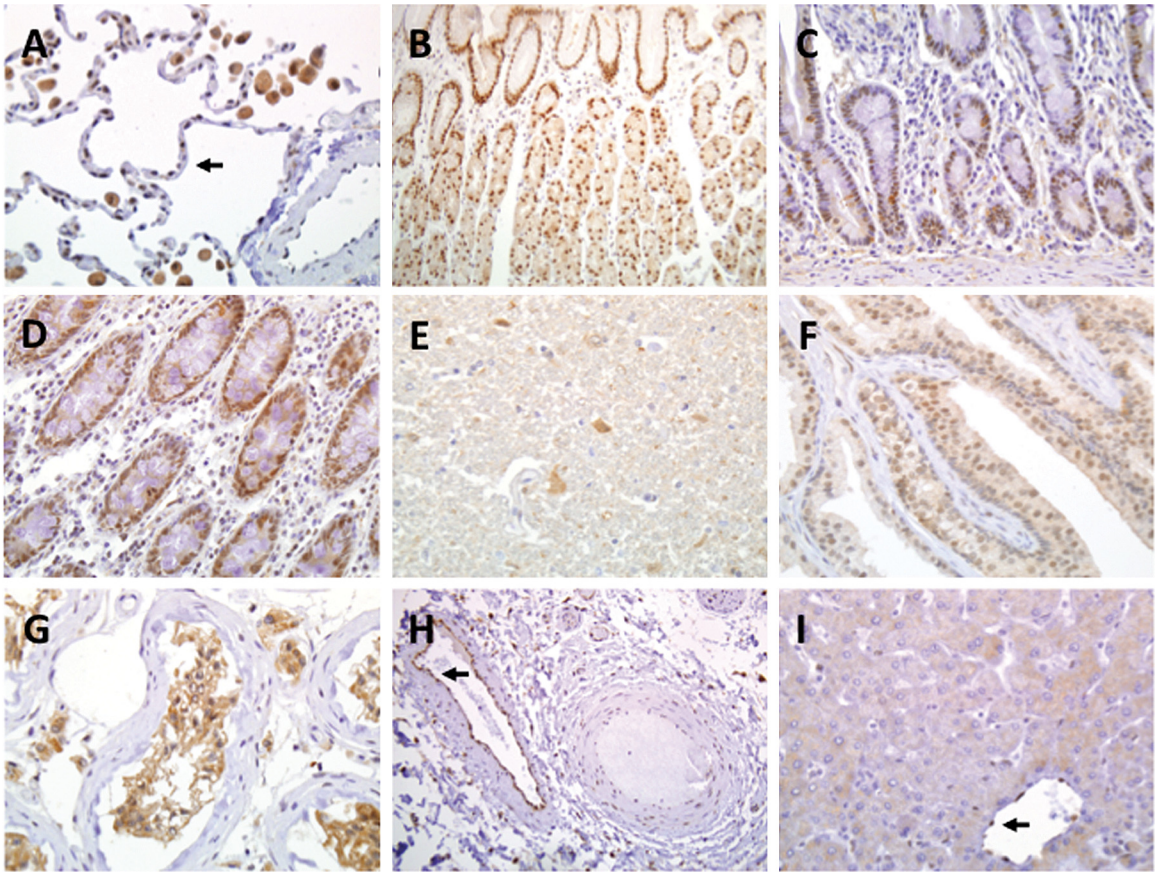

Figure 2. GATA4 expression in developed normal tissues. Positive nuclear labeling is seen in alveolar epithelium (indicated by arrow, A), oxyntic mucosa (B), small intestine (C), colon (D), brain (E), prostate (F), testis (G), and vascular endothelium (indicated by arrow, H). No labeling was seen for GATA4 in hepatocytes, but was seen in central vein (arrow) and sinusoidal endothelial cells.

Statistics

All summary values are expressed as a mean ± standard deviation unless otherwise indicated. For correlation of Gata-4 staining intensity to clinical and pathological features of each patient a Students T-test was used, whereas the Fishers exact test was used for comparison of frequency values. P values less than or equal to 0.05 were considered statistically significant.

Results

GATA4 expression in normal fetal and adult tissues

To determine the patterns of expression of GATA4 in fetal tissues, we immunolabeled paraffin embedded tissues of eight fetuses with an average gestational age of 14.9 weeks (range 13-18 weeks) using a GATA4 specific antibody. In keeping with reports based on mouse fetal development, GATA4 protein was detected in fetal liver, heart, pancreas, gut epithelium, lungs, bladder, and adrenal gland (1,2) (**Figure 1**). In all cases, immunolabeling was detected within the nuclear compartment consistent with the role of GATA4 as a developmental transcription factor [21]. The strongest intensity labeling was seen within the mycocardium, intestinal epithelium and pancreatic acini, whereas moderate intensity labeling was noted within fetal hepatocytes, developing adrenal cortex, urothelium, and lung (both bronchial and alveolar epithelium). No labeling was seen within bone, skeletal muscle, spleen, or thymic tissue.

To further define the expression of GATA4, we also systematically immunolabeled 12 different normal adult tissues. Strong positive

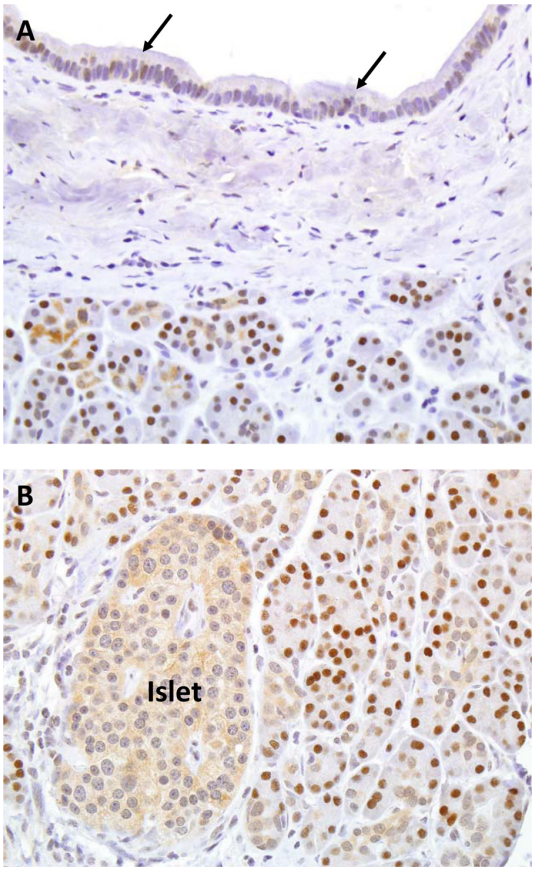

Figure 3. GATA4 expression in normal pancreatic tissue. A. Strong positive nuclear labeling is seen within pancreatic acinar cells. Scattered positive nuclei are also seen within normal ductal epithelium (arrows), although the intensity of labeling is less than for acinar cells within the same section. B. Unlike acinar cells, islets did not show nuclear labeling for GATA4.

labeling was noted within gastric oxyntic mucosa, small intestine, colon, lung, testis and vascular epithelium. Moderate intensity labeling was seen in prostate epithelium, brain, whereas no labeling was seen within hepatocytes, spleen or skeletal muscle (**Figure 2**). Moreover, similar to that seen in the fetal pancreas, pancreatic acinar cells showed intense positive labeling for GATA4 protein. In all samples of normal pancreas, GATA4 was also noted within normal duct epithelium although the intensity of labeling was less than that of acinar epithelium (mean labeling intensity 2.4 ± 0.7 in acinar cells versus 1.11 ± 0.9 in ductal cells, $p < 0.0001$) (**Figure 3**). Thus, the GATA4 transcription factor is expressed in human fetal tissues, and in some of these tissues, including the pancreas and tubular gastrointestinal tract, GATA4 expression persists in the developed organ.

Gata-4 expression in pancreatic cancer

We have previously shown that unlike other gastrointestinal cancers, increased GATA4 mRNA levels are a feature of infiltrating pancreatic cancer compared to normal pancreatic ductal epithelium [18]. To further characterize GATA4 in pancreatic cancer, we first performed a western blot to analyze GATA4 protein expression in cell lines for which *GATA4* mRNA levels were previously determined [18]. A single 50 kDa protein form was detected consistent with the predicted size of this protein [22] (**Figure 4**). Moreover, GATA4 protein content was well correlated with the mRNA levels, confirming that GATA4 is expressed in both normal and malignant pancreatic cancer cells.

To determine the relationship of GATA4 expression to infiltrating pancreatic cancer, we next immunolabeled 90 resected pancreatic ductal adenocarcinomas from Whipple (pancreaticoduodenectomy) specimens. The clinicopathologic features of these patients are summarized in **Table 1.** The mean age of all patients was in the 7th decade, 48 patients (53%) were male, and 78 patients (87%) were white. Forty-five carcinomas (50%) were poorly differentiated, and 75 cancers (83%) had lymph node metastases.

Sixty-one pancreatic cancers (68%) showed positive GATA4 labeling, defined as a mean H-score ≥30. Positive cancers were further segregated into those with weak positive labeling (n=34, H-score 31-100) and strong positive labeling (n=27, H-score >100). In all positive cancers, labeling was predominantly nuclear although in some instances both robust nuclear and cytoplasmic labeling was seen (**Figure 5**). Stromal cells were negative for GATA4 expression.

A comparison of GATA4 labeling patterns to clinicopathologic features of resected pancreatic cancer tissues is shown in **Table 2.** There was no relationship among GATA4 labeling and patient age, race, size, tumor size, differentiation, pathologic stage or lymph node status. However, an association was noted between GATA4 labeling and gender. Specifically, pancreatic cancers from female patients more commonly had positive labeling than male patients ($p=0.01$).

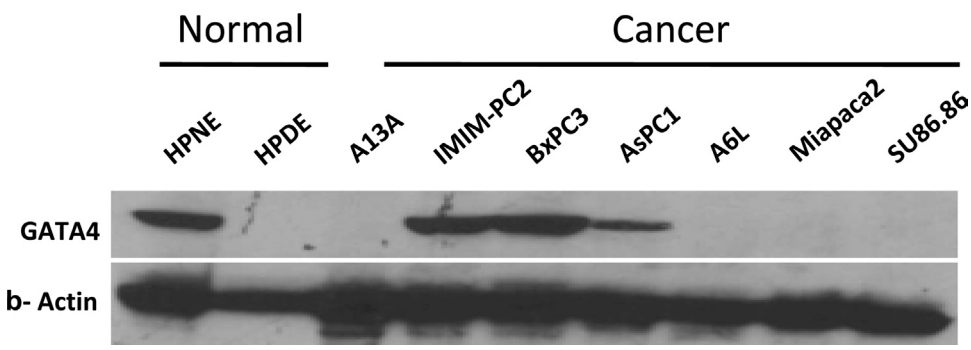

Figure 4. Western blot analysis of GATA4 expression in normal ductal cells and pancreatic cancer cell lines. GATA4 expression is detected in HPNE, an immortalized normal ductal cell line derived from nestin positive precursors, but not in HPDE that was derived from mature ductal epithelial cells. GATA4 expression is also detected within three of seven pancreatic cancer cell lines.

Table 1. Clinicopathologic features of 90 patients with pancreatic cancer

Age (years)	67.4±11.0
Gender (M:F)	48:42
Race (W:B:O)	78:10:2
Tumor Size (cm)	3.5±1.9
Tumor Differentiation	
Well/Moderate	45
Poor	45
Tumor Stage	
pT2	4
pT3	83
pT4	3
Lymph Node Status	
Negative	15
Positive	75

Discussion

At a basic level, our findings are in keeping with the many reports of a role of GATA4 in embryologic development of the heart, lungs, adrenal glands, gastrointestinal organs and pancreas [23-28]. We also show that GATA4 is expressed within both acinar cells and duct epithelial cells within the developed pancreas. GATA4 expression within the exocrine compartment of the pancreas has been found in mouse models of pancreatic development, although expression of GATA4 was eventually excluded from the ductal epithelium [24]. Thus, both mouse models and our findings of relatively lesser expression of GATA4 in ductal epithelium compared to acinar cells may reflect, in part, the relatively greater role of GATA4 in maintaining the acinar compartment of the developed pancreas.

These data implicating GATA4, together with our prior findings of GATA6 overexpression in pancreatic cancer [19], also expand upon the developmental processes that may be dysregulated in this tumor type. For example, increased Hedgehog pathway activity has been demonstrated in pancreatic cancer. Moreover, studies using cyclopamine, a specific Hedgehog pathway antagonist, abrogate pathway activity and suppress pancreatic cancer cell growth in vitro and in vivo [29-31]. The Notch pathway has also emerged as a contributory factor in pancreatic carcinogenesis in which Notch pathway activation results in an accumulation of nestin positive precursor cells and formation of metaplastic duct epithelial lesions that are potential precursors for pancreatic cancer [32]. Of interest, both Hedgehog and Notch signaling have also been implicated in cardiovascular and intestinal development, and in some instances to interact with GATA4 [33-35]. Thus, it is conceivable that GATA4 overexpression contributes to pancreatic carcinogenesis by its interactions with one or both of these developmental pathways.

An unexpected finding was the relationship of GATA4 expression to gender in that higher expression was noted within pancreatic cancers in female patients. While the significance of this finding remains to be elucidated, it is worth noting that GATA4 is linked to gonadal development. GATA4 has been shown to be required for testicular steroidogenic cell development in fetal mice and has been identified as a regulator of SRY gene transcription, the genetic determinant of testis development [28, 36, 37]. GATA4 is also important in ovarian development [27]. For example, GATA4 is expressed

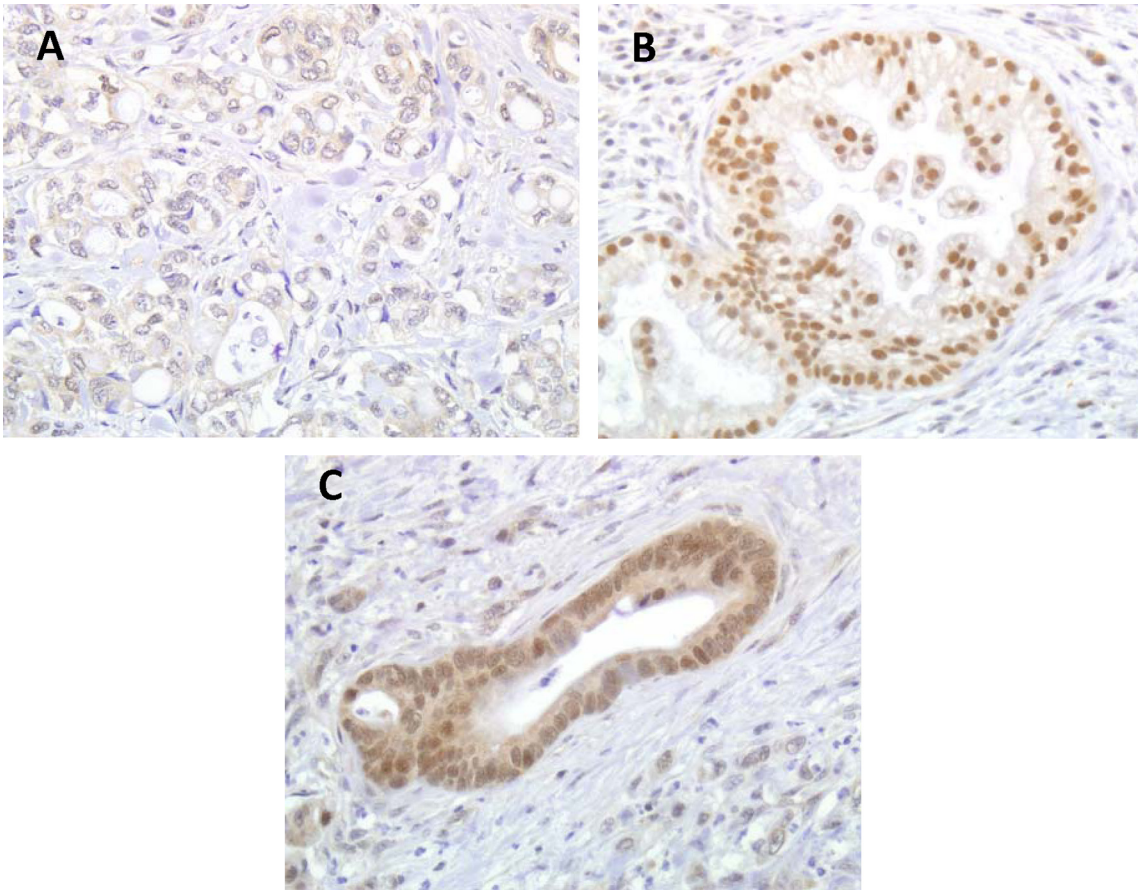

Figure 5. GATA4 expression in pancreatic cancer tissues. A. Weak positive labeling for GATA4 in a poorly differentiated pancreatic cancer. B. Strong positive labeling for GATA4 in a well differentiated pancreatic cancer. The labeling is predominantly nuclear. C. Strong positive labeling for GATA4 in a moderately differentiated pancreatic cancer. In this carcinoma, labeling is seen both within the nuclei and the cytoplasm of the cancer cells.

Table 2. Relationship of Gata4 expression to clinicopathologic features of pancreatic cancer

	Negative (n=29)	**Weak positive (n=34)**	**Strong positive (n=27)**	**P value**
Age (yrs)	66.9±11.3	69.6±12.3	65.3±8.7	0.31
Gender				
Male	22	14	12	**0.01**
Female	7	20	15	
Race				
White	25	29	24	0.91
Black/Other	4	5	3	
Tumor Size (cm)	4.0±2.7	3.3±1.3	3.1±1.5	0.18
Tumor Differentiation				
Well/Moderate	11	16	18	0.09
Poor	18	18	9	
Tumor Stage				
pT2	2	1	1	
pT3	26	31	26	0.73
pT4	1	2	0	
Lymph Node Status				
Positive	22	30	23	0.40
Negative	7	4	4	

in granulosa and thecal cells in the mouse embryo, and gonadotropins regulate GATA-4 expression in these tissues [27]. Thus, to provide one possibility, gender specific regulators may play a role in the overexpression of GATA4 in pancreatic cancer.

Pancreatic cancer is the fourth most common cause of cancer mortality, and claims the life of more than 35,000 people in the United States yearly [38]. Consequently, investigations into pancreatic carcinogenesis are needed to understand the biologic features of this tumor type that may be exploited for early detection and treatment. We now confirm prior reports that GATA4 is aberrantly expressed in pancreatic cancer, and suggest new avenues for study of the GATA family of transcription factors.

Acknowledgments

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