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The Relationship Among Ki-67, ERBB2, GATA3, STAG2, P53, and YAP1 in Pancreatic Ductal Adenocarcinoma

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Abstract: Pancreatic ductal adenocarcinoma (PDA) is one of the most lethal forms of cancer with a 5-year survival of only 7% for both men and women. Despite substantial progress made in successfully personalizing treatment for other tumors such as breast, prostate, and lung, treatment for PDA remains elusive due, in part, to its unique growth pattern and lack of surveillance tools to detect early lesions. Because most PDA lesions have metastasized at the time of diagnosis and exhibit a heterogeneously infiltrative growth pattern by interdigitating malignant cells among various normal tissue components, decisive, targeted therapies are needed to remove tumor cells while leaving the surrounding benign tissues undamaged. In an effort to identify biomarkers, immunohistochemistry assays were employed to determine the expression of Ki-67, KLK7, YAP1, CK 5, CK 20, CEA, GATA3, XAF1, STAG2, CK 18, ERBB2, and P53 in 42 formalin-fixed, paraffin-embedded PDA samples. Although no statistically significant correlation was associated with tumor aggressiveness as determined by Ki-67 positivity, several pairs of markers demonstrated positive correlations with each other and included ERBB2/STAG2, ERBB/YAP1, ERBB/GATA3, ERBB/P53, GATA3/STAG2, and GATA3/YAP1. Characterization of individual tumors with respect to over- or under-expression of specific proteins may offer dual therapy targets in PDA to potentially improve patient outcomes.

Keywords: Cancer, Pancreas, Therapy

1. Introduction

The probability that a male United States citizen will develop invasive cancer in his lifetime is 43% whereas that for a female is 38% [1]. One of the most aggressive and difficult-to-treat cancers worldwide is pancreatic ductal adenocarcinoma (PDA). It is the 4th leading cause of cancer deaths with only a 7% 5-year survival rate [1], which stems from the lack of surveillance tools to identify early-stage tumors. Pancreatic adenocarcinoma causes very few symptoms initially, most of which are non-specific and therefore eludes detection until the tumor reaches an advanced stage of disease. Only a minority of tumors are candidates for resection, in part because the tumors are not frequently identified before they metastasize, and in part due to their growth pattern.

Contributions made in molecular testing in the last 3 decades have linked multiple genes to an increased risk of tumorigenesis. While mutations causing over- or under-expression in some genes may be found in multiple cancer types, gene expression has also been noted to reverse between tumor types exhibiting overexpression in one cancer but under-expression in another. One of the most ubiquitously employed markers, Ki-67, is a nuclear protein that is expressed in actively mitotic cells and is used to determine the aggressiveness of a tumor [2-7]. The expression of carcinoembryonic antigen (CEA) in PDA has been well documented as a specific and sensitive marker [8]. Similarly, the activity of tumor protein 53 (P53) as a tumor suppressor gene among multiple tumor types is well known [9, 10]. X-linked inhibitor of apoptosis associated factor 1 (XAF1) may also function as a tumor suppressor by inhibiting anti-caspase activity in apoptosis-resistant cancer

cells [11]. Kallikrein related peptidase 7 (KLK7) is a serine protease that is associated with poor outcomes in several types of cancer [12, 13]. GATA-binding protein 3 (GATA3) is a transcription factor for many developmental physiological processes and has been the subject of several recent studies in carcinogenesis [14]. Similarly, Yes 1 associated transcriptional regulator (YAP1) is involved in cell development, growth, and repair and is believed to be active in cancer progression [15]. Stromal antigen 2 (STAG2) is a protein involved in chromatid separation during mitosis and may be partially responsible for aneuploidy in cancer [16] when deficit or mutated. Receptor tyrosine protein kinase erythroblastic oncogene B (ERBB2), also known as HER2, is a member of a family of growth factor receptors and has been substantially documented to express variably in different cancers [17-19]. Cytokeratins 5, 18, and 20 are intermediate filament proteins that lend stability to the cytoskeleton in epithelial cells. Origins of undifferentiated tumors are determined, in part, by the presence or absence of specific cytokeratins, and tumor aggressiveness has been associated with increases or decreases in cytokeratin expression [20]. The purpose of this study was to assay gene products present in PDA, determine if there is a relationship among them, and elucidate whether their presence can be linked to tumor aggressiveness as determined by cell proliferation marker Ki-67.

2. Materials and Methods

This study was approved by the University of Tennessee Health Science Center (IRB: 21-08209-NHSR). Forty-two pathologist-confirmed formalin-fixed paraffin-embedded PDA tissues were obtained from the University of Tennessee shared tissue resource center. Tissues were sectioned at 4 μm and stained with hematoxylin and eosin (H&E) stain to identify appropriate regions of interest. Selected areas of tumor were removed from paraffin blocks using a 2 mm punch biopsy tool and were assembled into several multi-tissue array blocks. After sectioning arrays and placing tissues on plus-charged slides, the sections were dried in a 60°C oven for 24 hours and stained with H&E [21].

Immunohistochemistry (IHC) assays were performed manually using Bond™ Polymer Refine Detection Kit, (DS9800, Leica Biosystems, USA). Briefly, the sections were deparaffinized with xylenes, and rehydrated through a series of ethanols and water. Epitope retrieval was conducted with either EnVision FLEX Target Retrieval Solution High pH (K800421-2, Dako/Agilent, USA) or low pH IHC Antigen Retrieval Solution (00-4955-58, Invitrogen, USA) using a Biocare Decloaking Chamber (Biocare Medical, USA) for 5 minutes at 120°C. After sections cooled to room temperature, the retrieval solution was replaced by Envision™ Flex Wash buffer, Tris-Buffered Saline (TBS) (DM831, Dako/Agilent, USA) pH 9.0. Between each IHC step, TBS was used as the wash reagent. First, endogenous peroxidase activity was quenched using the kit hydrogen peroxide for 5 minutes. Then the tissue sections were incubated for 20 minutes at room temperature with the primary antibody (Table 1) followed by an 8-minute application of Post Primary reagent. An 8-minute Polymer reagent was followed by diaminobenzidine chromagen for 2 minutes. After a deionized water rinse, a dilute hematoxylin kit solution was added to the sections for 5 minutes, and then TBS was used to blue the sections. Thereafter, the sections were rinsed with water, allowed to air dry, and were mounted with a coverslip using xylene and a resinous mounting medium.

Table 1. *Antibodies used on pancreatic adenocarcinoma tissues. AR = Antigen retrieval solution pH.*

Antibody	Company	Catalog	Clonality	Dilution	AR
CEA	Dako (Agilent)	M7072	Mouse monoclonal	1:100	High
CK 5	Leica Novacastra	PA0468	Mouse monoclonal	RTU	High
CK 18	Zeta Corp	Z2044	Mouse monoclonal	1:75	None
CK 20	Leica Novacastra	PA0022	Mouse monoclonal	RTU	Low
GATA3	Leica Novacastra	PA0798	Mouse monoclonal	RTU	Low
ERBB2	Roche/Millipore Sigma	790-2991	Rabbit monoclonal	RTU	Low
Ki-67	Roche/Millipore Sigma	790-4286	Rabbit monoclonal	RTU	High
KLK7	Santa Cruz Biotechnology	sc-514447	Mouse monoclonal	1:100	High
P53	Zeta Corp	Z2029	Mouse monoclonal	1:300	High
STAG2	Santa Cruz Biotechnology	sc-81852	Mouse monoclonal	1:100	Low
YAP1	Santa Cruz Biotechnology	sc-101199	Mouse monoclonal	1:50	High

For Ki-67 evaluation, a minimum number of 500 cells from each PDA lesion was counted, and the positively-labeled cells were calculated as a percentage. The other markers were scored on a scale of 0-3, where 0 = no labeling of cells, 1 = weak or rare labeling of cells, 2 = moderate labeling, and 3 = marked labeling. Occasionally, half steps were used (0.5, 1.5, or 2.5) when either the quantity or intensity of cells labeled precluded clear categorization. All IHC markers were evaluated by a minimum of 2 investigators and discrepant results were resolved together at the microscope. Final values are presented as investigator averages. Images were captured using an Olympus BX45 light microscope (Olympus Corp, Japan) and CellSens software (Olympus Corp). Statistical analysis used was Pearson r correlation coefficient with a 95% confidence interval and was performed using GraphPad Prism version 9.5.0 for iOS (GraphPad Software, USA). A p-value of <0.05 was considered statistically significant.

3. Results

The markers Ki-67, KLK7, YAP1, CK 5, CK 20, CEA,

GATA3, XAF1, STAG2, CK 18, ERBB2, and P53 were assayed on 42 PDA using IHC. Ki-67 positivity was found to be an average of 35.7% (range 7.1% - 88.7%) with substantial variability in positivity values both within the same tumor and among different patients (Figure 1). The remaining markers were scored on a scale of 0-3 based on both the overall number of malignant cells staining as well as staining intensity. The two highest-scoring markers were CK 18 with an average score of 2.56 and STAG2 with an average score of 2.31. The other markers, in order of descending scores were CEA (2.03), GATA3 (1.73), P53 (1.51), YAP1 (1.50), ERBB2 (1.47), XAF1 (1.29), KLK7 (0.98), CK 5 (0.31), and CK 20 (0.27) (Figure 2).

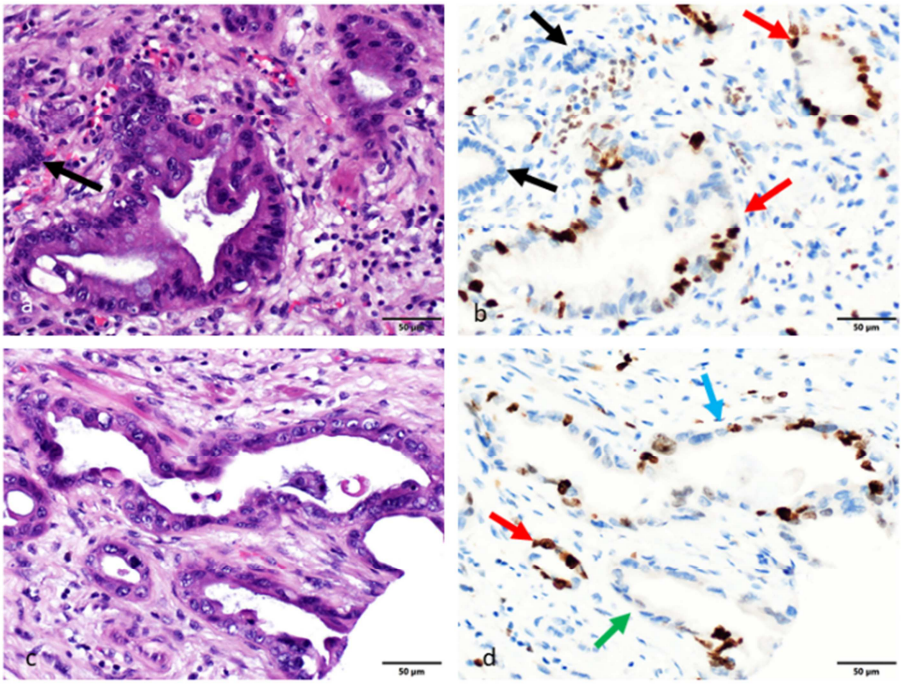

Figure 1. *Pancreatic adenocarcinoma (a, c) stained with hematoxylin and eosin, and (b, d) corresponding serial sections labeled with anti-Ki-67 antibody using an immunohistochemistry assay. (b) Benign pancreatic ducts (black arrows) show negligible labeling of brown chromogen for Ki-67, whereas malignant glands (red arrows) display numerous positively-staining nuclei. (d) Ki-67 labeling exhibits extreme variability with approximately 20% (green arrow), 33% (blue arrow), and 70% (red arrow) positivity in adjacent malignant glands.*

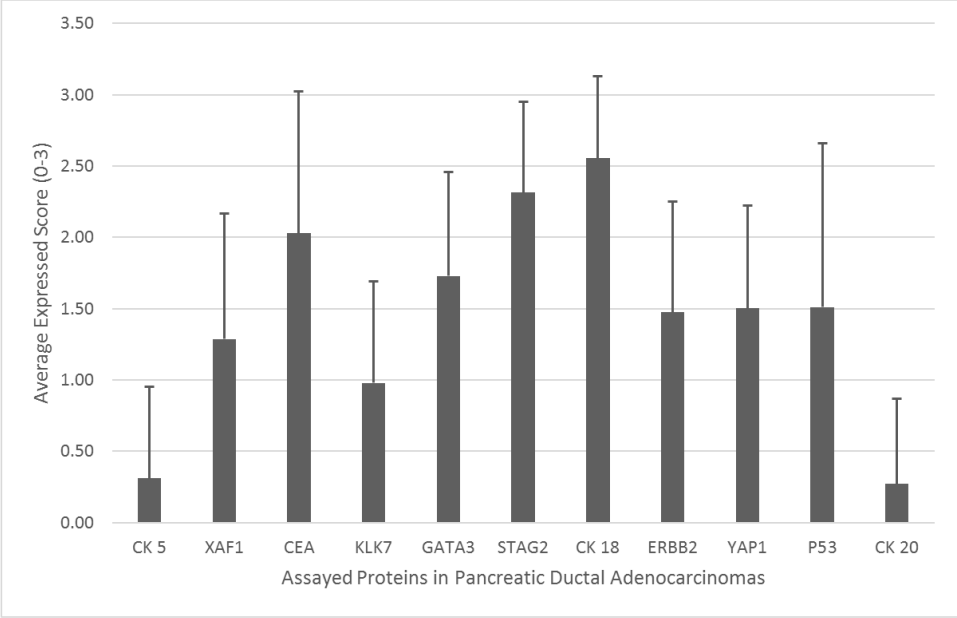

Figure 2. *The average score of protein expression in pancreatic adenocarcinomas as assessed by immunohistochemistry.*

Although no statistically significant correlation was associated with tumor aggressiveness as determined by Ki-67 positivity, select markers did show positive correlations and included ERBB2/STAG2, ERBB/YAP1, ERBB/GATA3, ERBB/P53, GATA3/STAG2, and GATA3/YAP1 (Table 2, Figures 3, 4).

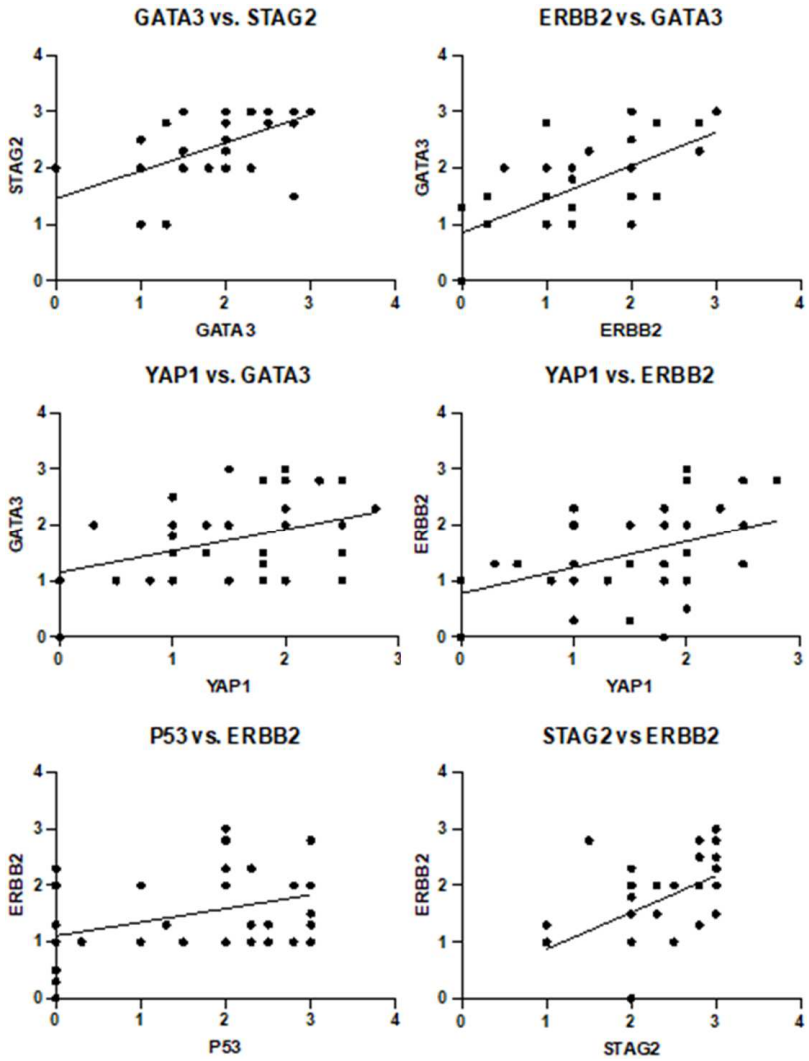

Figure 3. *Among the analyzed 12 genes, only six pairs of gene products were significantly correlated. Each graph shows a simple linear regression analysis between two genes which are significantly different ($p<0.0002$ for GATA3 vs. STAG2; $p<0.0001$ for ERBB2 vs. GATA3; $p<0.0187$ for YAP1 vs. GATA3; $p<0.0070$ for YAP1 vs. ERBB2; $p<0.0282$ for P53 vs. ERBB2; $p<0.0002$ for STAG2 vs. GATA3).*

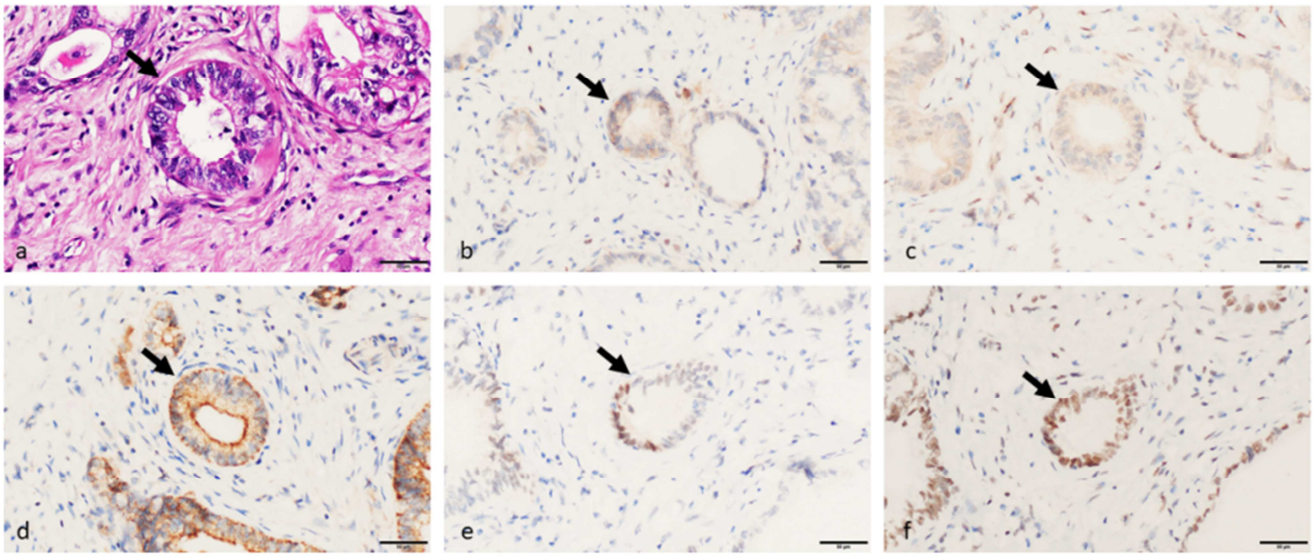

Figure 4. *Pancreatic ductal adenocarcinoma is labeled with (a) Hematoxylin and eosin, (b) ERBB2, (c) YAP1, (d) GATA3, (e) P53, and (f) STAG2 immunohistochemistry markers. Positive correlations were found in the combinations ERBB2/STAG2, ERBB2/YAP1, ERBB2/GATA3, ERBB2/P53, GATA3/STAG2, and GATA3/YAP1. Scale bar = 50 µm.*

Table 2. *Correlation p-values associated with pancreatic markers.*

	Ki-67										
CK 5	0.3037	CK 5									
XAF1	0.2169	0.8494	XAF1								
CEA	0.9019	0.4779	0.1607	CEA							
KLK7	0.1468	0.9163	0.8469	0.7942	KLK7						
GATA3	0.0805	0.4153	0.3598	0.5884	0.7818	GATA3					
STAG2	0.5846	0.3136	0.9046	0.9382	0.4966	0.0002	STAG2				
CK 18	0.4215	0.6284	0.7583	0.9374	0.6612	0.8817	0.4001	CK 18			
ERBB2	0.1566	0.2988	0.7489	0.4490	0.4633	0.0000	0.0035	0.6032	ERBB2		
YAP1	0.1035	0.3870	0.5720	0.4914	0.9543	0.0187	0.5194	0.7546	0.0070	YAP1	
P53	0.8958	0.1727	0.3012	0.5647	0.4455	0.2296	0.7980	0.3010	0.0282	0.2364	P53
CK 20	0.4247	0.8974	0.2227	0.8833	0.7291	0.3681	0.8412	0.7516	0.2411	0.5176	0.0983

4. Discussion

The pancreas is 15-20 cm in length and is composed of four regions, head, neck, body, and tail, which merge imperceptibly into each other. The pancreas contains both exocrine cells which provide digestive enzymes to the small intestine and endocrine cells which, among other actions, govern glucose uptake in the blood (Figure 5a). The pancreas fills a small space in the left abdominal cavity and lies adjacent to the lower stomach, duodenum, jejunum, spleen, aorta, and closely approaches the liver, bile duct, gallbladder, and one of the kidneys with its accompanying adrenal gland. The pancreas does not have a well-defined capsule (Figure 5a), and consequently the indistinct interface between the pancreas and surrounding organs does not prevent the migration of malignant ductal cells into adjacent tissues, thereby confounding accurate diagnosis of tumor origin. Tumor cells infiltrate surrounding tissues multi-directionally and intercalate among benign tissues (Figure 5b). While large collections of malignant ductal cells and corresponding necrosis are uncommon, a desmoplastic response of the pancreas is virtually universal, causing dense fibrosing sheets of collagen to surround individual groups of tumor cells [22] (Figure 6). It has been hypothesized that effective chemotherapeutic and radiation treatments are impeded by this desmoplastic response, as the fibrous tissues shield the malignant cells. Molecular testing is also rendered more challenging due to the heterogeneous nature of pancreatic cancers and the relative deficit of tumor cells compared to benign components [23, 24].

Cytologic changes traversing the spectrum among normal pancreatic ducts, pancreatitis, dysplasia, and invasive tumors can be subtle. Nuclear features of PDA may include irregular membranes, anisonucleosis, altered polarity, and macronucleoli (Figure 7a). Even benign-appearing cells are diagnosed as adenocarcinoma if they are found in unusual locations such as within blood vessels or in the perineurium (Figure 7b). Pancreatic features which favor benign pancreatitis include ducts with rounded contours both internally and externally whereas malignant glands are angulated with pointed corners. [22].

Ki-67 is a proliferation marker often used to determine tumor aggressiveness. Previously in PDA, normal ducts were noted to have a Ki-67 labeling index of 0.41% whereas PDA demonstrated 36.99% positivity [24, 25]. While the 35.7% positivity labeling index of the current study matches that of the literature, high variability in proliferation was seen among and within tumors. In a previous study, patients with a Ki-67 of <5% survived on average 17 months after diagnosis while those with Ki-67 labeling >5% had a 9-month average survival rate [26]. All patients in this study evidenced a Ki-67 rate >5% and therefore low overall survival potential. Similarly, Kim et al. determined that Ki-67 positivity correlated with tumor recurrence, poorly-differentiated tumors, and with lymph node metastases [27]. However, in other studies, no correlation was found between Ki-67 positivity and survival of patients [28-29] but the authors also noted a high degree of intratumoral and intertumoral proliferation variability. [29].

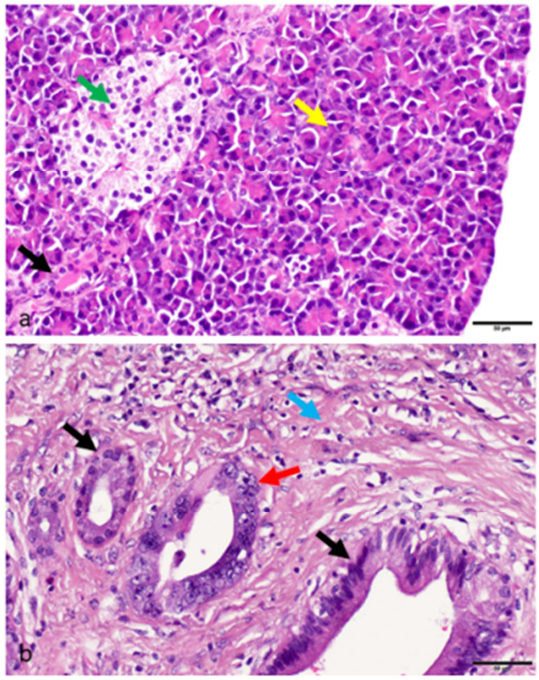

Figure 5. *(a) Normal pancreatic tissue is characterized by acinar exocrine cells (yellow arrow) which secrete digestive enzymes into the small intestine and Islets of Langerhan (green arrow), the endocrine cells which secrete insulin, glucagon, somatostatin, and pancreatic polypeptide. A normal pancreatic duct is seen at the black arrow. (b) Pancreatic ductal adenocarcinoma (red arrow) is situated between two normal ducts (black arrows). In the background, a desmoplastic response (blue arrow) consisting of collagen, fibroblasts, chronic inflammatory cells, and macrophages can be seen which has replaced normal exocrine and endocrine cells. Scale bar = 50 μm.*

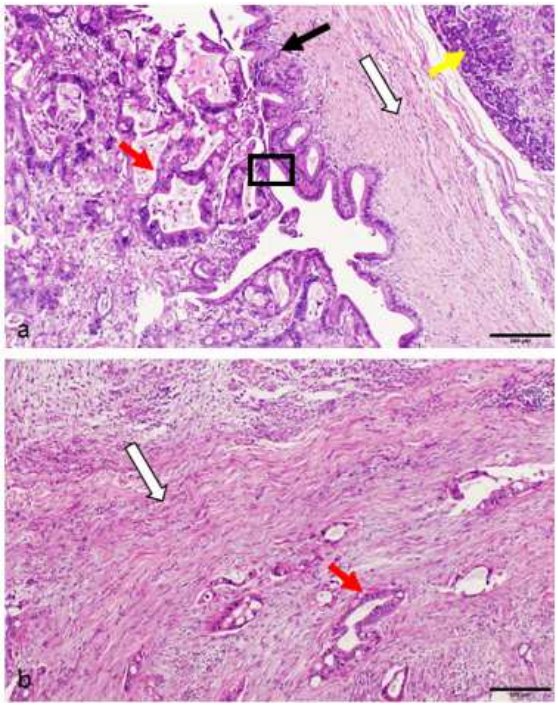

Figure 6. *Examples of pancreatic ductal adenocarcinoma. (a) Benign exocrine cells (yellow arrow), fibrous connective tissue (white arrow), and benign duct (black arrow) abruptly transition to multiple malignant pancreatic ducts (red arrow). See insert enlargement in Figure 7. (b) Extreme desmoplastic response (white arrow) engulfs rare malignant pancreatic ducts (red arrow). Scale bar = 200 µm.*

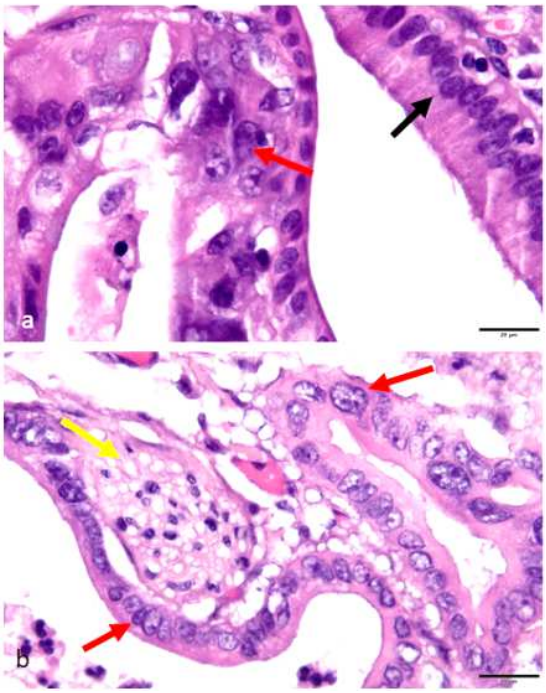

Figure 7. *Pancreatic ductal adenocarcinoma. (a) Inset from Figure 6a. Benign ductal cells (black arrow) form parallel walls with basally-located oval nuclei. The nuclei exhibit bland chromatin in contrast to nuclei from pancreatic adenocarcinoma (red arrow) which are variable in shape, size, polarity, chromatin texture, nucleoli, and have altered nuclear/cytoplasmic ratio. (b) Perineural invasion of adenocarcinoma cells (red arrows). Direct contact of benign-appearing pancreatic ductal cells with peripheral nerves (yellow arrow) is indicative of an invasive process. Scale bar = 20 µm.*

Cytokeratin 18 was the marker with the highest positivity (2.56) in the PDA samples analyzed in this study which was similar to the findings of Menz et al. who noted 99% positivity in PDA [30]. Carcinoembryonic antigen was also highly expressed, but neither it nor CK 18 showed corresponding increases or decreases with other expressed proteins in PDA. ERBB2 expression, however, correlated closely with STAG2, YAP1, GATA3, and P53 expression. Perhaps not unsurprisingly, GATA3 expression correlated with that of both STAG2 and YAP1. ERBB2 overexpression is found in 11.8% of breast and 8% of stomach adenocarcinomas [17]. ERBB2 protein and mRNA are found in normal pancreatic acinar and ductal cells [31], but overexpression of ERBB2 in PDA varied among investigators from 1% to 48% [17, 31, 32]. Chronic pancreatitis can closely resemble well-differentiated PDA, particularly in biopsy tissue where the sample size is small and possibly composed of desmoplastic fibrosis, damaged acinar cells, and rare glands with reactive nuclear features. Standop et al. determined that chronic pancreatitis can be reliably differentiated from adenocarcinoma using 2 specific ERBB2 antibody clones [33]. There did not seem to be any correlation between ERBB2 expression and tumor aggressiveness or overall survival [31] in one study while overexpression of ERBB2 was found to correlate with shortened patient survival in another [32].

The relationship among P53, STAG2, and ERBB2 is not novel to this study, and all three genes were found to contain mutations in urinary bladder cancer [34, 35]. YAP1 is a proto-oncogene that is downregulated by Hippo signaling pathway [36]. Blockading of YAP1 may inhibit tumor cell proliferation and reduce immune cell suppression in pancreatic cancer. [37]. In breast cancer, YAP1 functions as a tumor suppressor and is present in normal breast tissue of non-diseased patients. However, in cancer patients, normal breast tissue has reduced amounts of YAP1, with even further suppression observed in cancerous breast cells [38]. In contrast, YAP1 expression was found to be increased in pancreatic ductal cells in the presence of pancreatitis and in cells undergoing malignant transformation [39, 40]. GATA3 expression is associated with improved outcomes in breast cancer [41] and is inversely related to the Ki-67 index and ERBB2 positivity [42], but in the current study on PDA, GATA3 and ERBB2 demonstrated a positive correlation, likely due to the differences in pathophysiologies between breast and pancreatic cancers. Indeed, GATA3 expression has been associated with PDA development [43] but was identified in only 16% of PDA [44].

STAG2 encodes a protein needed during mitosis. Loss of STAG2 expression leads to nondisjunction and aneuploidy and promotes tumorigenesis [45]. Normal tissues expressed STAG2, but there was significant loss in the tumor cells. Upregulation of deficient STAG2 gene could be a potential therapeutic target for PDA [45]. The STAG2 gene was associated with a good prognosis in ER+/ERBB2- breast cancers [46]. Most cases of mammary Paget's disease are associated with positive expression of both ERBB2 and

GATA3 [47]. P53 positivity was not found to correlate with overall survival in PDA. [28]. However, P53 positivity in PDA varied between 40% - 91% based on the population evaluated [24, 27, 48, 49]. P53 was found mutated in 85% of high-grade urothelial carcinomas. Other mutations in high- and low-grade urothelial cancers were found in ERBB2 and STAG2 [50].

5. Conclusions

One major difficulty encountered when evaluating pancreatic disease histologically is the vast heterogeneity observed among the tissues present. Diseased or malignant ductal cells are widely interspersed with fibrous stromal elements, chronic inflammatory cells, blood and lymphatic vessels, peripheral nerves, residual acinar cells, and endocrine cells. Biopsy tissues, specifically due to their small size, may only capture minimal diagnostic cells, precluding definitive diagnosis. Consequently, molecular methods such as gene expression or sequencing efforts employed when evaluating pancreatic biopsies may return equivocal results due to the concealment of the relevant tumor cells by the preponderance of benign elements. The correlations in protein expression between ERBB2/YAP1, ERBB2/GATA3, ERBB2/STAG2, ERBB2/P53, GATA2/STAG2, and GATA3/YAP1 in PDA may offer multi-hit therapeutic opportunities to improve outcomes by providing targeted therapies in affected patients.

Conflicts of Interest

The authors declare that they have no competing interests.

Study Approval

This study was approved by the University of Tennessee Health Science Center (IRB: 21-08209-NHSR).

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