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Tumor-associated neutrophils and malignant progression in intraductal papillary mucinous neoplasms: an opportunity for identification of high-risk disease

Eran Sadot, MD[1], **Olca Basturk, MD**[2], **David S. Klimstra, MD**[2], **Mithat Gönen, PhD**[3], **Lokshin Anna, PhD**[4], **Richard Kinh Gian Do, MD PhD**[5], **Michael I. D'Angelica, MD**[1], **Ronald P. DeMatteo, MD**[1], **T. Peter Kingham, MD**[1], **William R. Jarnagin, MD**[1], and **Peter J. Allen, MD**[1]

[1]Department of Surgery, Memorial Sloan Kettering Cancer Center, New York

[2]Department of Pathology, Memorial Sloan Kettering Cancer Center, New York

[3]Department of Epidemiology and Biostatistics, Memorial Sloan Kettering Cancer Center, New York

[4]Department of Medicine, University of Pittsburgh Cancer Institute, Pittsburgh, PA

[5]Department of Radiology, Memorial Sloan Kettering Cancer Center, New York

Abstract

Objective—To evaluate the association of Tumor-associated neutrophils (TAN) with malignant progression in IPMN, and to study the cyst fluid from these lesions for biomarkers of the inflammation-carcinogenesis association.

Background—There is a strong link between TAN and malignant progression. Inflammatory mediators released by these cells may be a measurable surrogate marker of this progression.

Methods—We evaluated 78 resected IPMN (2004–2013). Lesions were divided into low-risk (low and intermediate grade dysplasia: n=48) and high-risk (high-grade dysplasia and invasive carcinoma: n=30) groups. TAN were assessed and categorized (negative, low, high). A multiplexed assay was performed to evaluate 87 different cyst fluid proteins, including cyst fluid inflammatory markers (CFIM), as possible surrogate markers for parenchymal inflammation.

Results—Significant positive correlation between grade of dysplasia and TAN was found. High TAN were identified in 2%, 33%, and 89% of the lesions when stratified by grade of dysplasia into low/intermediate-grade dysplasia, high-grade dysplasia, and invasive carcinoma, respectively ($p<0.001$). Higher grades of dysplasia were also found to have positive correlation with 29 of the measured proteins, from which 23 (79%) were CFIM. Higher levels of TAN correlated with

Corresponding author: Peter J. Allen, MD, Memorial Sloan Kettering Cancer Center, Department of Surgery, 1275 York Avenue, New York 10021, NY, 212 639 5132, allenp@mskcc.org.

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higher levels of 18 CFIM, from which 16 (89%) were also found to be associated with higher grades of dysplasia.

Conclusions—In this study, TAN were strongly associated with malignant progression in IPMN. Measurement of CFIM may be a surrogate marker for IPMN progression and allow for identification of high-risk disease.

INTRODUCTION

Intraductal papillary mucinous neoplasms (IPMN) of the pancreas represent both an opportunity and a challenge. IPMN represent an opportunity, as these lesions are the only radiographically identifiable precursors of pancreatic cancer. These cystic lesions are presumed to evolve from low-grade dysplasia to high-grade dysplasia to invasive carcinoma[1,2]. Resection of lesions prior to the development of pancreatic cancer may prevent the development of an incurable process, as once IPMN progresses to invasive cancer the prognosis may be as poor as resected conventional pancreatic ductal adenocarcinoma (PDAC)[3–5]. Resection of IPMN, particularly in the setting of high-grade dysplasia, is presumed to provide a survival benefit.

IPMN also present many challenges as the identification of high-grade dysplasia and early invasive carcinoma, and the timing and frequency of malignant progression are not yet established[6,7]. Currently, the most accurate test for prediction of high-grade dysplasia or invasive disease is dilation of the main pancreatic duct on preoperative imaging (main duct IPMN: MD-IPMN). Patients who undergo resection for MD-IPMN have an approximate 60% chance of harboring high-grade dysplasia or invasive disease at the time of resection. This high-risk disease is present in approximately 20%–25% of patients who undergo resection in the absence of a dilated pancreatic duct (branch duct IPMN: BD-IPMN)[6–8]. This limited predictive accuracy presents a challenge, as pancreatic resection continues to be associated with a risk of substantial morbidity and mortality. In high volume centers performing pancreaticoduodenectomy, the reported major morbidity rates are approximately 20–30% and mortality rates approximately 2–4%.

Inflammation and malignant progression has become a central premise in cancer research[9]. A strong link between neutrophil infiltration and malignant progression has been described, with inflammatory mediators released by these cells playing a pivotal role in the crosstalk between neoplastic and inflammatory cells[10]. Recently, it was reported that tumorigenesis in the pancreas is associated with significant intra- and peritumoral inflammation and failure of protective immunosurveillance[9]. Investigators from our institution have reported an association between Tumor-associated neutrophils (TAN) and advanced IPMN lesions[11]. Our group has also previously demonstrated significant elevations of a variety of inflammatory mediators in the cyst fluid of patients with high-risk IPMN[12].

The primary goal of this study was to evaluate the association between IPMN progression and the inflammatory microenvironment, as reflected by TAN and cyst fluid inflammatory markers (CFIM) from the same patients. The secondary goal was to identify CFIM that are associated with high-risk lesions.

METHODS

Patients

The prospectively maintained pancreatic database was queried for patients who had undergone resection for IPMN with and without associated invasive carcinoma between January 2004 and January 2013. During this time period there were 278 patients who underwent resection. Within this group, 78 patients (28%) were identified who had undergone resection, and had both adequate tissue and cyst fluid available for study. Patients with conventional pancreatic carcinoma lacking an IPMN component were excluded. All patients within this study were consented to our Institutional Review Board approved tissue banking protocol. A waiver of authorization for clinical study was obtained prior to any review.

Demographic data, presenting symptoms, radiographic, and endoscopic characteristics were also queried from the database. An independent review by a single radiologist (R.K.G.D.) was performed on all preoperative imaging, blinded to pathologic findings, and lesions were classified into MD-IPMN and BD-IPMN. Mixed lesions were categorized as MD-IPMN for the purposes of this study. The following imaging features were assessed: presence and size of a mural nodule (defined as a mural based soft tissue component projecting into the cyst) and presence and size of a solid component (defined as an area of abnormal attenuation adjacent to the cyst).The presenting symptoms of weight loss and abdominal pain were defined as a >10 pound loss of weight and upper abdominal and/or back pain, respectively.

Histopathologic assessment

Histopathologic assessment of resected specimens was performed and the samples were divided into low-risk (low-grade and intermediate-grade dysplasia) and high-risk (high-grade dysplasia and invasive carcinoma) groups. Lesions were categorized by histopathologic sub-type (intestinal, pancreatobiliary, oncocytic, or gastric), and degree of dysplasia. Grade of dysplasia was defined as the highest degree of dysplasia identified within the given specimen. Low-grade dysplasia was characterized by basal nuclei, and intracellular mucin. Intermediate-grade dysplasia was characterized by full-thickness nuclear pseudostratification with mild to moderate nuclear atypia. High-grade dysplasia was distinguished by significant cytologic atypia, complex disorganized architecture, and tufting.

Tumor-associated neutrophils (TAN) were identified by 4× screening as previously described[11]. For each tumor, 20 non-overlapping high-power fields (40× objective; magnification 400×; 0.08mm^2) including both tumor cells and neutrophils on two separate slides with most prominent TAN (i.e., a total of 40 fields per neoplasm) were examined. The final number of TAN for each tumor was then determined by calculating the mean value of the 40 high-power fields. Areas with ≤10 neutrophils/100 tumor cells were considered negative and areas with >10 TAN/100 tumor cells were considered as positive for TAN. Positive areas were then further subdivided into two groups. Those with 11–15 TAN/100 tumor cells were designated as ‘low’ while those with >15 TAN/100 tumor cells were regarded as ‘high’.

Cyst fluid analysis

Resected specimens were immediately transported to the tumor procurement facility where cyst aspiration was performed. Cyst fluid samples were aspirated with an 18 to 21 gauge needle, divided into 250 μL aliquots, and stored at −80°C. The time between resection and freezing was recorded, and only samples that had been frozen within 60 minutes of resection were used for this study. None of the samples had undergone any freeze-thaw cycles before analysis.

Multianalyte analysis (Luminex) of the cyst fluid was performed using an antibody microsphere array panel that has been previously designed and developed for pancreatic cancer[13]. This array consists of a variety of inflammatory markers, cell surface proteins, and tumor markers, many of which have been shown to be differentially expressed in pancreatic cancer (Table S1). Cyst fluid analysis on the Luminex platform was performed as per standard protocol[13,14]. In brief, antibody-coated bead concentrates were initially vortexed and diluted with wash solution. All microtiter wells then received 25 μL of diluted antibody coated beads. Standard wells received 100 μL of serially diluted standards and sample wells received 50 μL of diluent and 50 μL of sample. Wells were then zeroed with assay diluent and placed on an orbital shaker in the dark for 2 hours. Biotinylated detection antibody was then added (100 μL), and the samples then incubated for an hour. The beads were then filtered, washed, mixed with 100 μL streptavidin-RPE, and incubated for 30 minutes. The solution was then filtered, washed, resuspended in wash solution and loaded into the Luminex platform. Standard curves were created and the sample concentrations then measured and recorded. Serial dilution of the primary fluid was performed as indicated (mucinous fluid). Cyst fluid inflammatory markers (CFIM) were defined as any protein measured in this study, which has been previously reported to be over/under- expressed in an inflammatory process or proteins that are known to be mediators of an inflammatory cascade.

Statistical analysis

Descriptive and comparative statistics were performed using Statistical Software for the Social Sciences (SPSS) version 21 software. Continuous variables were compared using the ANOVA, Student t test, or Mann-Whitney test, as appropriate by the type of distribution. Categorical variables were compared using χ^2 or the Fisher exact test depending on the number of observations. A p-value ≤ 0.05 was considered significant. Each protein was standardized, by subtracting the mean and dividing by the standard deviation, before any regression analysis because of the vast difference in scale of measurements. Logistic regression was used both for univariate screening of proteins and for multivariate modeling. Since the intention was to find protein that will incrementally improve over MD-/BD- IPMN dichotomy used in clinical decision making for identifying high-risk IPMN, our univariate screening for each protein were adjusted for BD-/MD-IPMN. If there are proteins that are highly predictive of high-risk IPMN but also have substantial overlap with MD-IPMN so that their incremental value is small, they will not be identified in this analysis. Following this adjusted univariate screening procedure we planned a multivariate analysis using logistic regression with MD-/BD- subtype, clinical covariates, and the proteins that were incrementally significant in the screening process. The final multivariate model was

evaluated using receiver operating character (ROC) curve analysis. Dependence among proteins was ruled out with Spearman's correlation matrix, accepting correlation coefficients (q value) <0.7 to include in further analysis.

RESULTS

The study included 78 patients who underwent pancreatic resection for IPMN and had their tissue and cyst fluid banked according to protocol. This cohort included 48 patients (62%) with low-risk lesions (10 with low-grade dysplasia and 38 with intermediate-grade dysplasia), and 30 patients (38%) with high-risk lesions (21 with high-grade dysplasia and 9 with invasive carcinoma). The clinical and radiographic characteristics are summarized in Table 1. Patients with high-risk lesions were more likely to be male (67% vs 37%, p=0.01) and have diabetes (37% vs 15%, p=0.02) than low-risk lesions. The following radiological characteristics were more common in the high-risk lesions: MD-IPMN (73% vs 27%, p<0.001), a solid component (17% vs 0, p=0.007), and mural nodule (23% vs 4%, p=0.02).

Initial analysis identified significant correlation between the level of TAN and grade of dysplasia (Table 2). None of the patients with invasive carcinoma were TAN negative, whereas 90% of patients with invasive carcinoma were TAN high. Similarly, 94% of patients with high TAN were found to have high-risk lesions. The majority (96%) of low-risk lesions (n=47) were TAN negative.

Within the group of 87 cyst fluid proteins measured in the multiplex assay there were 29 with differential expression ($p \leq 0.05$) between the high-risk and low-risk lesions (Tables 3 and S2). All the 29 cyst fluid proteins were found to be overexpressed in the high-risk lesions and 23 (79%) of these were CFIM. Similarly, we evaluated the differential expression of cyst fluid proteins according to the level of TAN (Table S3). Higher levels of TAN correlated with higher levels of 21 proteins, from which 18 (86%) were CFIM. Sixteen (89%) of these CFIM were also found to be associated with higher grades of dysplasia as presented in Table 3. Figure 1 illustrates the association between high TAN, CFIM overexpression, and high-risk lesions. The association between selected CFIM and TAN level is demonstrated in Figure 2.

Further analysis of the differential CFIM expression was performed separately for each IPMN sub-type (MD-IPMN and BD-IPMN). In the BD-IPMN group, 5 proteins were differentially expressed between the high-risk and low-risk lesions, from which 4 (80%) were CFIM. The MD-IPMN group demonstrated overexpression of 8 proteins in the high-risk lesions and all of them were CFIM. IL-2R was overexpressed in the BD-IPMN group while its soluble alpha component (sIL-2Rα) was overexpressed in the MD-IPMN group (Table S4).

Within the group of 87 proteins measured in the multiplex assay there were 24 that were not correlated between themselves and were differentially expressed (p<0.1) between the high and low risk lesions. Separate logistic regression for each of the 24 proteins combined with the MD-/BD-IPMN variable identified MMP-9 and CA 72-4 as the strongest predictors for high-risk lesions (data not shown). Multivariate analysis, which included both MMP-9 and

CA 72-4, combined with radiological and clinical variables, which were significant in the univariate analysis identified the following independent predictors for high-risk lesions (table S5): radiographic MD- IPMN (OR=12.4; CI: 3.6-41.9; $p<0.001$) and CA 72-4 (OR=3.5; CI: 1.04-12.1; $p=0.04$). The discriminative potential of this model was compared to radiographic MD-/BD- IPMN variable alone by ROC analysis (AUC=0.796, $p<0.001$; Figure S1).

DISCUSSION

The challenge associated with identifying high-risk IPMN is evident from the changes in treatment recommendations over the past two decades. Initially, routine resection was recommended as all IPMN were considered to have equal potential to progress to an invasive cancer[15,16]. As more experience developed and a greater understanding of this disease process was achieved, it was realized that many of these lesions had minimal dysplasia at the time of resection, and a non-operative approach was recommended for selected patients with small BD-IPMN that had been incidentally discovered[17,18]. Current recommendations now encourage radiographic surveillance for even larger BD-IPMN[7]. At our institution, the majority of patients presenting with IPMN undergo radiographic surveillance, as the majority of them present with small, incidentally discovered branch duct IPMN[19]. Even in the setting of MD-IPMN, nearly 40% who undergo resection will have low-risk disease. Since the rate and frequency of progression is unknown, it is unclear whether low-grade dysplasia is of clinical significance to patients who are in their 70s and 80s. The ability to better define high-risk disease would aid clinical decision making.

Because cyst fluid cytology is often acellular, a variety of approaches have been taken to evaluate the cyst fluid for alternative markers of dysplasia. These approaches, including genomic approaches (whole exome), glycosylation profiling, and miRNA profiling have shown promise with regards to discriminating between different cystic histopathologic sub-types (serous vs. mucinous) however their ability to discriminate dysplasia in IPMN has not been established[12–14,20–24]. As a result, the recent consensus guidelines[7] recommend radiographic characteristics as the leading predictive parameter for high-risk lesions. At this point, the most accurate predictor of high-risk IPMN is dilation of the main pancreatic duct on preoperative imaging (MD-IPMN).

Previous data from our group has suggested that differential cyst fluid protein expression may allow for discrimination of cyst sub-type[14] (IPMN vs. serous cystadenoma), that dysplasia in IPMN may be linked with an immunogenic/inflammatory microenvironment, and that cyst fluid inflammatory markers may allow for improved identification of high-risk lesions. A recent study from our institution identified an association between pancreatic neoplasms and TAN[11]. In this study, TAN were frequently seen in high-risk pancreaticobiliary sub-type IPMN, and less common in intestinal, oncocytic, and gastric sub-type IPMN. In addition, non-carcinomatous components of these neoplasms failed to demonstrate TAN. Additional work from our group has evaluated the cyst fluid for inflammatory markers as a surrogate for TAN, as TAN cannot be readily identified on preoperative assessment. These initial evaluations identified overexpression of inflammatory markers such as IL-1β, IL-5, and IL-8 in high-risk lesions[12]. In this previous study, cyst

fluid IL-1β remained a significant predictor of high-risk disease on a multivariate analysis that included IPMN sub-type.

In order to further characterize the association between TAN and malignant progression in IPMN, the current study analyzed the level of TAN stratified by grade of dysplasia. The majority (96%) of the low-risk lesions demonstrated no TAN while 89% of invasive lesions revealed high levels of TAN. In addition, 87% of IPMN with no TAN were diagnosed as low-risk lesions and 94% of IPMN with high levels of TAN were high-risk lesions.

The current study expands on our previous work mentioned above as the presented data support the relationship between TAN and dysplasia, (Table 2), and suggests that cyst fluid inflammatory markers may be an excellent surrogate marker for identification of the TAN-dysplasia association. Higher grades of dysplasia were associated with overexpression of 29 of the 87 measured proteins (Table 3, S2) and 79% of these were inflammatory markers. Higher levels of TAN correlated with higher levels of 21 proteins, from which 18 (86%) were CFIM. Sixteen (89%) of these CFIM were also found to be associated with higher grades of dysplasia. These cyst fluid markers encompass an array of factors likely to originate from the developing tumor, the tumor microenvironment, and components of the systemic host inflammatory response to the malignancy (tables S1).

BD-IPMN are known to follow a more indolent course, which is reflected by the lower frequency of high-risk lesions in patients who underwent resection (approximately 25%) for BD-IPMN compared to MD-IPMN (approximately 60%)[7]. Moreover, the 5-year cumulative risk of malignancy for patients who were observed with BD-IPMN was estimated to be 11%[25]. The unique natural history of each IPMN sub-type dictates different management strategy. Acknowledging these distinct entities, we analyzed the cyst fluid proteins in each IPMN sub-type. Both in the MD-IPMN and in the BD-IPMN, the vast majority of the overexpressed proteins in the high-risk lesions were CFIM, 100% and 80%, respectively (Table S4). Interestingly, IL-2R was overexpressed in the BD-IPMN group and its soluble alpha component (sIL-2Rα) was overexpressed in the MD-IPMN group. Therefore, it can be appreciated that the relationship between IPMN progression and the inflammatory microenvironment encompasses both IPMN sub-types with an overlap.

In addition to the triangular statistical correlation between TAN-CFIM-Dysplasia, there is an accumulating biological correlation to support the cross talk between TAN, tumor cells, and the differentially expressed CFIM (Tables 2, 3, S2, S3) observed in the current study. Myeloperoxidase (MPO) is a lysosomal peroxidase enzyme most abundantly expressed in neutrophils, from which we observed higher concentrations in IPMN with higher grade of dysplasia and higher level of TAN (Table 3, S3). IL-1β has been previously suggested to contribute to neutrophilia and to the induction of immunosuppressive properties of TAN[26]. Tumor-derived macrophage migration inhibitory factor (MIF) modulates cellular functions of TAN [e.g. secretion of matrix metalloproteinase (MMP)-9][27,28]. MMP-9, which was strongly associated with high-risk lesions, has a pivotal role in carcinogenesis as it was demonstrated to induce tumor progression and prevent apoptosis of tumor cells[29,30]. In addition to the role in carcinogenesis, MMP-9 was shown to potentiate angiogenesis by counteracting antiangiogenic molecules[31].

Thus far, the most commonly used predictor for high-risk lesions is dilation of the main pancreatic duct on preoperative imaging (MD-IPMN)[6–8]. In our study the sensitivity and specificity of the radiologic MD-IPMN alone were 73.3% (22/30) and 72.9% (35/48), respectively. These numbers reflect the prediction performance of this variable as described by most series[7]. The multivariate analysis identified CA 72-4 as an independent predictor of high-risk lesions in addition to the radiologic IPMN subtype. The potential added value of a 2-variable prediction model is its flexibility, in which sensitivity and specificity can be adjusted according to the target population (i.e., low/high risk lesion, comorbidities, age). Accordingly, a 2-variable prediction model can be utilized with high sensitivity for high-risk lesions in low-risk patients, whereas high specificity can be applied to high-risk patients with low-risk lesions. The current prediction model should be regarded as a proof-of-principle and external validation is required.

The retrospective design of the study presents inherent selection bias that cannot be absolutely excluded. IPMN patients who were observed were not included in our study and these patients may have a different cyst fluid protein profile. Moreover, oversampling of high-risk IPMN might have occurred since small lesions are difficult to aspirate. However, the current study presented a BD-IPMN ratio (55%) that is similar to reports[7] from other institutions (approximately 57%) and is in line with a larger cohort (n=219) reported by our group[32] (56%). The rate of high-risk lesions in our study was 19% for BD-IPMN and 63% for MD-IPMN. These percentages are comparable to series from other institutions[7]. Additional limitation is that the current pre-validation study is not powered to establish a robust prediction model, which will discriminate between high-risk and low-risk IPMN. Nevertheless, this study presents proof-of-principle of the utility of a multiplexed evaluation of cyst fluid biomarkers for the identification of high-risk IPMN.

In conclusion, the results of this study suggest that Tumor-associated neutrophils may be important components of malignant progression in IPMN. Measurement of CFIM may be a surrogate marker for IPMN progression and allow for the identification of high-risk disease. Further investigation into the role of inflammation and progression in IPMN is warranted, and an anti-inflammatory strategy should be considered as a possible therapeutic approach.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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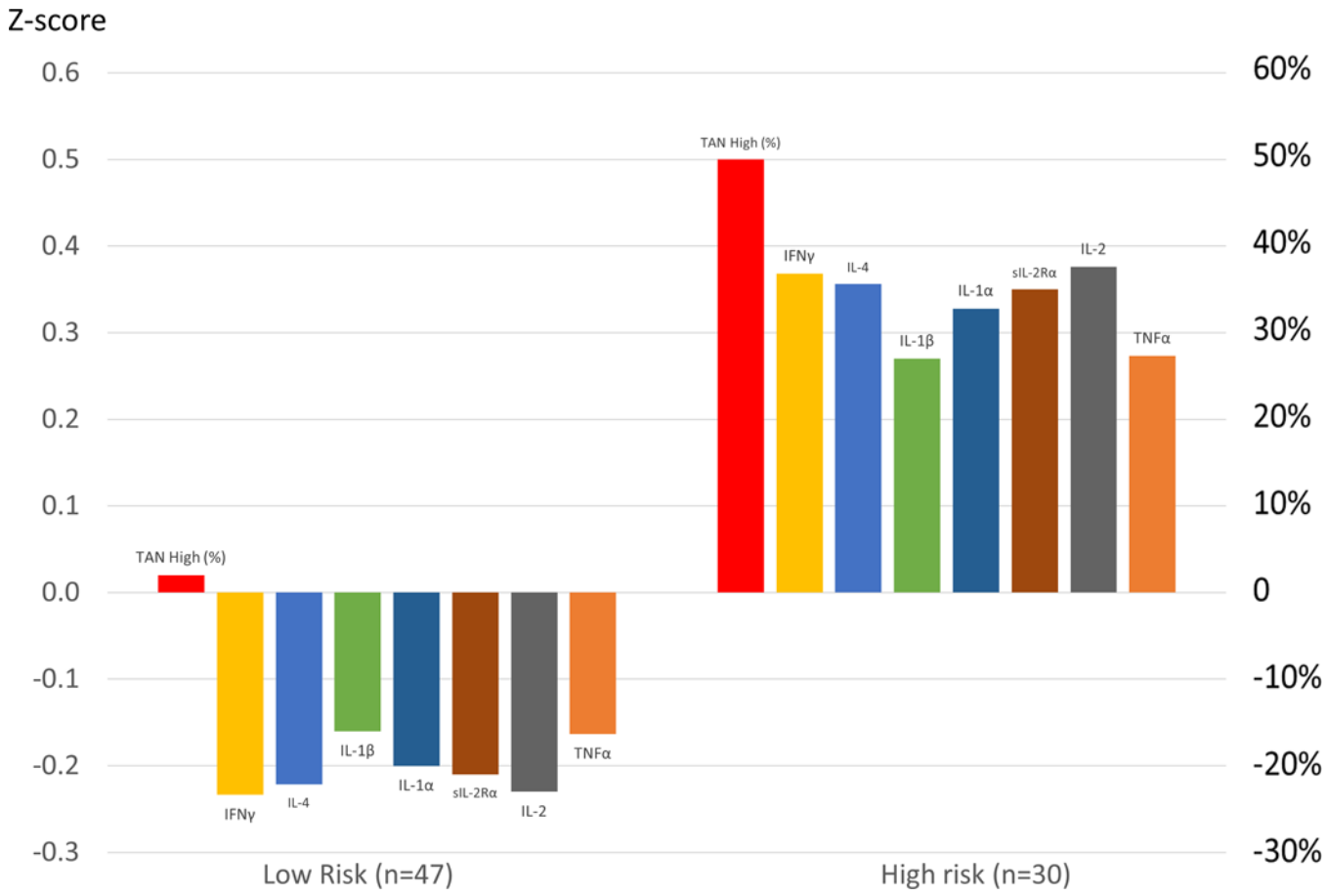

Figure 1. TAN and representative CFIM stratified by low-risk and high-risk groups

Representative CFIM with significant differential expression between the low-risk and high-risk groups were selected. Each CFIM was standardized (z-score), by subtracting the mean and dividing by the standard deviation, because of the vast difference in scale of measurements. Average levels of standardized CFIM are presented. TAN high are presented as percentage of the patients with TAN high in each group.

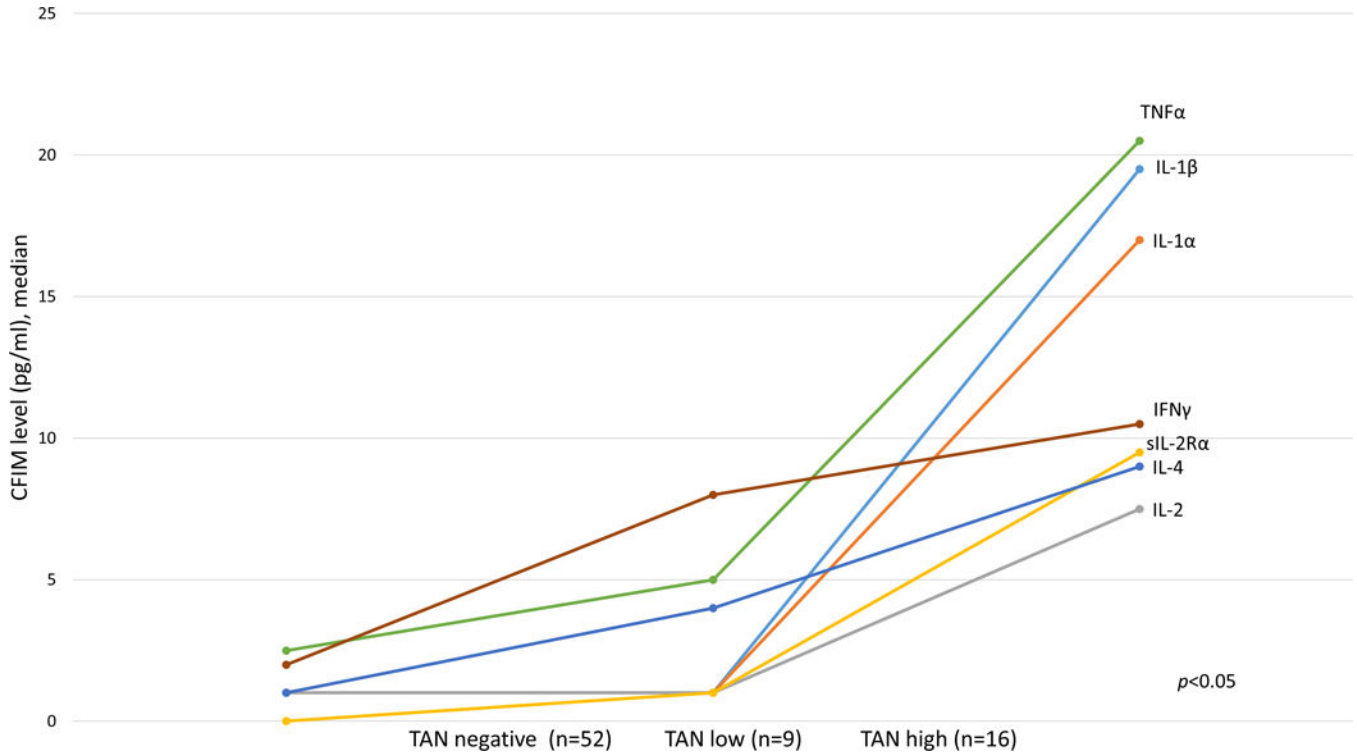

Figure 2. CFIM stratified by level of TAN

Representative CFIM were selected with significant differential expression when stratified by TAN level.

Table 1

Clinical, radiological, and pathological characteristics stratified by low-risk and high-risk groups (n=78).

Characteristics	Low-risk (n=48)	High-risk (n=30)	*p*-value
Age at diagnosis, years	70; 31–89	68; 35–86	0.2
Gender			
Male	18 (37%)	20 (67%)	**0.01**
Female	30 (63%)	10 (33%)	
Radiographic sub-type:			
Main duct	13 (27%)	22 (73%)	**<0.001**
Branch duct	35 (73%)	8 (27%)	
Diabetes	7 (15%)	11 (37%)	**0.02**
Pain	8 (17%)	8 (27%)	0.3
Weight loss	4 (8%)	6 (20%)	0.2
Tumor location			0.1
Body	15 (31%)	3 (10%)	
Head	19 (40%)	19 (64%)	
Tail	8 (17%)	4 (13%)	
Diffuse	6 (12%)	4 (13%)	
Nodule presence	2 (4%)	7 (23%)	**0.02**
Solid component	0	5 (17%)	**0.007**
Histological sub-type[1]:			**<0.001**
Intestinal	6 (13%)	10 (34%)	
Oncocytic	0	2 (7%)	
Gastric	39 (87%)	8 (28%)	
Pancreatobiliary	0	9 (31%)	

Continous variables presented as: median; range. Categorical variables presented as: number of cases (percentage).

[1] Data was not recorded for 4 patients by the time of analysis.

Table 2

Histological sub-type, dysplasia, and cyst fluid inflammatory markers stratified by Tumor-associated neutrophils (TAN) levels (n=77)[1].

	TAN negative (n=52)	TAN low (n=9)	TAN high (n=16)	*p*-value
Low/intermediate-grade dysplasia (n=47)	45 (96%)	1 (2%)	1 (2%)	<0.001
High-grade dysplasia (n=21)	7 (33%)	7 (33%)	7 (33%)	
Invasive carcinoma (n=9)	0	1 (11%)	8 (89%)	
Histology sub-type[2]:				<0.001
Intestinal	8 (16%)	2 (22.5%)	6 (40%)	
Oncocytic	0	1 (11%)	1 (7%)	
Gastric	41 (82%)	1 (11%)	5 (33%)	
Pancreatobiliary	1 (2%)	5 (56%)	3 (20%)	
IL-1α, pg/ml	1; 1–9	1; 1–271	17; 1–760	0.03
IL-1β, pg/ml	1; 1–2	1; 1–235	19.5; 2–952	0.005
IL-2, pg/ml	1; 1–1	1; 1–6.5	7.5; 2–26	<0.001
sIL-2Rα, pg/ml	0; 0–1	1; 1–32	9.5; 1–121	0.004
IL-4, pg/ml	1; 1–2	4; 1.5–19	9; 1–35	0.004
TNFα, pg/ml	2.5; 1–19	5; 1–167	20.5; 3–123	0.02
IFNγ, pg/ml	2; 1–6	8; 2.5–13	10.5; 3–43	0.005

Continous variables presented as: median; interquartile range. Categorical variables presented as: number of cases (percentage).

Representative CFIM were selected with significant differential expression when stratified by TAN level.

Analysis of cyst fluid proteins was performed with Kruskal-Walis 1-way ANOVA. Analysis of dysplasia grade was performed with trend test.

[1] One patient was not evaluated for TAN level.

[2] Data was not recorded for 4 patients by the time of analysis.

Table 3

Differentially expressed cyst fluid proteins stratified by low-risk and high-risk groups.

#	Cyst fluid protein	Functional group[1]	N=78	Low-risk (n=48)	High-risk (n=30)	*p*-value
1	MIF, pg/ml	Immune modulator	77	1900; 600–8000 (47)	6600; 3100–21000 (30)	**0.003**
2	MMP-9, pg/ml	Extracellular matrix	78	10000; 1100–66000 (48)	93000; 5500–240000 (30)	**0.03**
3	MPO, pg/ml	Immune modulator	78	73000; 15000–790000 (48)	885505; 85000–2000000 (30)	**0.009**
4	CathepsinD, pg/ml	Protease	78	130000; 55000–490000 (48)	330000; 140000–820000 (30)	**0.01**
5	NCAM, pg/ml	Cell Adhesion	78	1600; 1345–2600 (48)	1841; 1441–16000 (30)	**0.05**
6	OC, pg/ml	Others	76	69; 53–2300 (47)	3300; 70–17000 (29)	**0.01**
7	PTH, pg/ml	Endocrine	76	7; 5–42 (47)	89; 13–365 (29)	**0.001**
8	IFNγ, pg/ml	Chemokines/cytokines	74	2; 1–7 (46)	7; 2–15 (28)	**0.003**
9	IL-1α, pg/ml	Chemokines/cytokines	74	1; 1–9 (46)	6; 1–406 (28)	**0.02**
10	IL-1β, pg/ml	Chemokines/cytokines	74	1; 1–1.25 (46)	3.5; 1–117 (28)	**0.001**
11	IL-1Ra, pg/ml	Chemokines/cytokines	74	34; 1–601 (46)	326; 32–860 (28)	**0.03**
12	IL-2, pg/ml	Chemokines/cytokines	74	1; 1–1 (46)	2; 1–12 (28)	**0.001**
13	IL-4, pg/ml	Chemokines/cytokines	74	1; 1–2 (46)	4; 1–24 (28)	**0.004**
14	IL-5, pg/ml	Chemokines/cytokines	74	0; 0–0 (46)	0.5; 0–1.75 (28)	**0.001**
15	CD40L, pg/ml	Immune modulator	74	13; 2–139 (46)	110; 16–614 (28)	**0.005**
16	sIL-2Rα, pg/ml	Chemokines/cytokines	74	0; 0–1 (46)	1; 0–73 (28)	**0.003**
17	TNFβ, pg/ml	Chemokines/cytokines	74	1; 1–1 (46)	1; 1–7 (28)	**0.01**
18	NGF, pg/ml	Growth factors/cell cycle	63	0; 0–4 (39)	2.5; 0–45 (28)	**0.04**
19	CNTF, pg/ml	Growth factors/cell cycle	71	180; 152–232 (43)	300; 160–2300 (28)	**0.03**
20	TSH, nIU/ml	Endocrine	71	31; 26–36 (43)	39; 27–3500 (28)	**0.02**
21	BDNF, pg/ml	Growth factors/cell cycle	71	42; 32–54 (43)	56; 33–280 (28)	**0.05**
22	Apolipoprotein Apo CIII, ng/ml	Apolipoproteins – acute phase	78	353; 302–421 (48)	390; 345–450 (30)	**0.04**
23	SAP, pg/ml	Other – acute phase	78	215000; 60000–1100000 (48)	640000; 160000–4300000 (30)	**0.03**
24	AFP, ng/mL	Cellular/tumor antigens	73	116; 91–279 (45)	220; 110–1460 (28)	**0.009**
25	Cytokeratin 19, pg/ml	Cellular/tumor antigens	73	110000; 4000–180000 (45)	154000; 90000–540000 (28)	**0.02**
26	ErbB2, ng/mL	Growth factors/cell cycle	73	880; 290–3600 (45)	3811; 860–8700 (28)	**0.01**

#	Cyst fluid protein	Functional group[1]	N=78	Low-risk (n=48)	High-risk (n=30)	*p*-value
27	EGFR, pg/ml	Growth factors/cell cycle	73	471; 103–1990 (45)	2541; 421–9000 (28)	**0.003**
28	TNFα, pg/ml	Chemokines/cytokines	56	3; 3–7 (34)	8; 3–37 (22)	**0.02**
29	IL-2R, pg/ml	Chemokines/cytokines	56	41; 41–87 (34)	80; 47–160 (22)	**0.01**

Analysis of cyst fluid proteins was performed with Mann-Whitney test. Presented as: median; interquartile range (number of patients).

[1] All proteins are CFIM except: OC, PTH, TSH, Apolipoprotein Apo CIII, AFP, Cytokeratin 19.