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Prognostic Value of SMAD4 Expression in Pancreatic Ductal Adenocarcinoma and its Correlation with Clinicopathological Parameters and HER2 Status

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ABSTRACT

Background: This research seeks to assess the immunohistochemical (IHC) expression of Mothers against decapentaplegic homolog 4 (SMAD4) in pancreatic ductal adenocarcinoma (PDAC), explore its association with Human Epidermal Growth Factor Receptor 2 (HER2) expression, and to assess its relation to different PDAC prognostic clinico-pathological variables. The association of SMAD4 and HER2 IHC expression with patients' disease-free survival (DFS) and overall survival (OS) is also evaluated.

Methods: This retrospective cohort research had 83 patients who were diagnosed with primary PDAC from surgical resection specimens at the Gastrointestinal Surgery Center (GISC), Faculty of Medicine, Mansoura University, Egypt. SMAD4 and HER2 expression were evaluated by immunohistochemistry (IHC) on PDAC tumor samples. Statistical analysis was carried out with the SPSS version 20.0 to assess significant associations.

Results: SMAD4 was aberrantly expressed in 51.8% of PDACs, while only 8.4% of them were positive for HER2 (score +3). There was a statistically substantial connection between SMAD4 expression and the following variables: tumor site (p=0.05), tumor size (p=0.042), pancreatic safety (PS) margin infiltration (p=0.028) and the presence of lymphovascular invasion (LVI) (p=0.017), and `statistically significant associations between HER2 expression and the presence of LVI (p=0.03) and TNM stage (p=0.049). No substantial association was identified between SMAD4 and HER2. SMAD4 loss was connected with shorter DFS and OS, but with no statistical significance.

Conclusion: SMAD4 loss is associated with a poor prognosis in PDAC patients. SMAD4 and HER2 status could affect the treatment strategies in PDAC patients.

Keywords: SMAD4, Pancreatic cancer, immunohistochemistry, HER2.

INTRODUCTION

PDAC is one of the most fatal malignant neoplasms in the world. It accounts for 2% of all cancers and it is the 7th most major cause of cancer mortality in both sexes worldwide [(1)].

PDAC has a very poor prognosis with a 5-year-survival rate less than 5% and median survival of 6 months if untreated. The 5-year-survival rate could increase to 20% with early detection, radical surgical resection, and [(2)] adjuvant chemotherapy. Early detection of PDAC is very difficult due to the symptoms vagueness and the absence of specific early clinical indicators of PDAC [(3)]. Unfortunately, 80% of patients have advanced illness at time of presentation and are unfit for surgical resection with metastasis or invasion to the celiac trunk or the superior mesenteric artery [(4)].

Mothers against decapentaplegic homolog 4 (SMAD4), or DPC4 (Deleted in Pancreatic Cancer-4) is an important member of the co-mediated SMAD protein group. In normal conditions; it acts as a tumor suppressor gene [(5)]. Aberrant expression of SMAD4, via genetic alteration or homozygous deletion, promotes uncontrolled cell growth and participates in the epithelial mesenchymal transition (EMT) process. That is why SMAD4 is thought to be involved in tumor progression and metastasis [(6)].

Human epidermal growth factor receptor 2 (HER2) is transmembrane growth factor receptor. When it is overexpressed, it acts as an oncogene and considered as an independent adverse prognostic factor in PDACs [(7)].

Therefore, this research seeks to assess the immunohistochemical (IHC) expression of SMAD4 in pancreatic ductal adenocarcinoma (PDAC), explore its association with HER2 expression, and to assess its relation to different PDAC prognostic clinico-pathological variables. The association of SMAD4 and HER2 IHC expression with patients' disease-free survival (DFS) and overall survival (OS) is further evaluated.

MATERIALS AND METHODS

Study Settings and Design:

The 83 primary PDAC patients who were diagnosed from surgical resection specimens at the GISC, Faculty of Medicine, Mansoura University, Egypt, between January 2014 and June 2019 participated in this retrospective cohort research using formalin-fixed, paraffin-embedded (FFPE) tissue blocks. These patients had not received prior chemotherapy or radiotherapy.

The pathologic database of the Surgical Pathology Laboratory at the GISC was used to

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retrospectively retrieve the clinicopathological information of the 83 patients who were included in the study. This information included the patients' age and gender, tumor site and size, nodal metastases, duodenal extension, tumor grade, TNM stage, lymphovascular invasion (LVI), and perineural invasion (PNI).

The follow-up information was gathered by obtaining patient medical records from the faculty of medicine at Mansoura University's clinical oncology and nuclear medicine department. This data included: the number of months since the last follow-up; the existence or absence of relapse, either locally or via distant metastases. Finally, the OS was calculated from the date of main pathological diagnosis till the time of disease-specific death or lost follow-up. DFS was defined as the time from the date of primary pathological diagnosis to the date of a confirmed recurrence.

Histopathological Evaluation:

Routine, all of the recovered tissue blocks were processed into 3–4 micrometer-thick, microscopic slides that were stained with hematoxylin and eosin (HandE) and analyzed to determine the diagnosis and evaluate the various histological factors.

Tissue microarray construction:

Tissue microarray blocks (TMA) were made utilizing a manual validated technique [8], a hole was made in the recipient TMA paraffin block using a mechanical pencil tip that is about 0.7 mm in diameter. A cylindrical 0.9 mm core sample from the donor block was obtained using another a mechanical pencil tip, and the core was inserted into the recipient TMA block with keeping a suitable distance between each core and the next core. Two to four cores were taken from viable tumor tissue. Finally, five TMA blocks were built up including representative tissues from the studied 83 cases. Multiple cores of normal tissues (pancreas, small intestine, and liver) were inserted according to a pre-designed map to help in orientation and navigation. Normal pancreatic tissue was used as positive control for SMAD4. Similarly, cores of HER2-positive breast carcinoma were utilized as a positive control for HER2.

Immunohistochemistry (IHC):

According to the user's handbook standardized protocol pre-programmed into the autostainer software, IHC was carried out using Autostainer Link 48 with its optimized reagents utilizing pharmDx kits EnVisionTM FLEX Visualization Systems (Link code K8000) and EnVision FLEX Hematoxylin (Link code K8008). Using the 3-in-1 specimen preparation method, FFPE slices were pre-treated by being dewaxed and dehydrated before being subjected to heat-induced epitope retrieval (HIER). These criteria were used to carry out that: Epitope retrieval temperature: 97°C for 20 minutes; cool down to 65°C. Preheating temperature: 65°C. The automated approach employs a universal biotinylated IgG secondary antibody, diaminobenzidine (DAB) substrate, and an indirect biotin-avidin system. The portions were then cleaned, mounted, and dehydrated.

Slides for all the stained immunohistochemical antibodies were viewed under a standard light microscope, and each antibody was then scored using the method that was most appropriate for that antibody. Antibodies SMAD4 (rabbit polyclonal, catalog number: A5657 IgG, diluted 1:100, Biospes) was interpreted as the following. Four kinds of SMAD4 immunoreactivity were discovered, including missing (no staining), trace (poor reactivity relative to adjacent pancreas), localized (two cell population with obviously negative cells), and diffuse (strong staining compared to surrounding pancreas). Only cases with widespread SMAD4 expression were judged positive; cases with absence, focal, or trace SMAD4 expression were classified negative. Background lymphocytes, fibroblasts, and non-cancerous pancreatic tissue served as internal positive controls and often had diffuse SMAD4 expression [9].

The scoring system for biopsy samples of gastric or gastro-esophageal cancer (GC/GEC) employed for the Trastuzumab for Gastric Cancer (ToGA) cohort was applied to the Anti-HER2/neu (4B5) Rabbit Monoclonal Primary Antibody Kit (Ventana/Roche Tissue Diagnostics, catalog number: A0485, ready-to-Use).This scoring system was used when a small cluster of cells (≥5 neoplastic cells) showed a reaction and was applied as follows: negative (score 0), no reactivity, negative (score 1+), faint or barely perceptible membranous reactivity, equivocal (score 2+), weak to moderate complete or basolateral membranous reactivity, and positive (score 3+), strong complete or basolateral membranous reactivity. Cytoplasmic reactivity with no membrane staining was considered as negative (score 0) [10].

Ethical considerations:

The Faculty of Medicine at Mansoura University in Egypt's Institutional Research Board (IRB) gave its approval to this work (Code Number: MD.19.06.191, 2019). For the sake of secrecy and patient anonymity, the pathology code numbers of the paraffin blocks were utilized in place of patient names. Finally, we sent the donor blocks back to the archive for future patient usage or research.

Statistical analysis:

The collected data was processed and analyzed utilizing the (Statistical Product and Service Solutions by International Business Machines Corporation) IBM

SPSS, version 20.0. Statistical tests were used to assess association between SMAD4 and HER2/neu expression and different clinicopathological parameters and patient's survival.

RESULTS

All the 83 PDACs were of the classic histopathological variant, of which 13 tumors were well-differentiated, 66 were moderately-differentiated, and 4 tumors were poorly differentiated. The most frequent TNM stage was stage IIB (53%), followed by stage IB, III, IA, then IA (18.1%, 12% 10.8% and 6% respectively).

As seen in table (1), SMAD4 was positive in 40 PDACs (48.2%) (Figure 1a, 1b), and showed abnormal expression (negative) in 43 PDACs: 24 tumors were focally positive (figure 1c, 1d), 14 showed positivity and 5 were completely negative. There was a statistically substantial connection between SMAD4 expression and patient's sex (p=0.004) as abnormal (negative) expression was more frequent in males (83.7%) than females (16.3%). There was a statistically substantial connection between SMAD4 expression and the following prognostic variables: tumor site (p=0.05), pancreatic safety margin (PS) infiltration (p=0.028), presence of LVI (p=0.017), and tumor size (p=0.042). Tumors with aberrantly expressed SMAD4 revealed higher frequencies of infiltrated PS margin (42.9%) and LVI (57.1%) and were larger in size (46.5%). Patients with negative SMAD4 expression had higher percentages of both death and relapse than those with retained SMAD4 expression (86%, 44% versus 72%, 42% respectively). However, there was no statistical substantial connection between SMAD4 expression and clinical outcomes including death and relapse.

As regard HER2 IHC expression as seen in table (2), seven cases were considered positive (8.4%; score+3) (figure 2a, 2b), while 59 cases were score zero (71.1%) , 10 cases were score +1 (12%) (Figure 2e, 2f) and seven cases were equivocal (8.4%) score +2 (figure 2c, 2d). There was a statistically substantial connection between HER2 expression and the presence of LVI (p=0.03) and TNM stage (p=0.049). Tumors with positive HER2 expression showed more frequently LVI (57.1%) and were of higher stages (stage IIB, III) (table2).

Concerning combined SMAD4/HER2 expression, two tumors showed both positive HER2 and SMAD4 (2.4%), 38 tumors (45.8%) showed negative both SMAD4 and HER2, 38 tumors showed positive SMAD4 and negative HER2 (45.8%) and five tumors showed positive HER2 and negative SMAD4 (6%). There was no substantial connection between aberrant HER2 and SMAD4 expression (p=.278).

The median follow-up duration was 14.00 (1-99) months. During the follow-up period, 66 patients (79.5%) died. Meanwhile, 36 patients (43.4%) developed relapse in the form of local recurrence or distant metastasis. The median period for DFS was 11.00 (1-65) months. Disease relapse (either recurrence or metastasis) occurred in 36 patients (43.4%).

In univariate analysis (table 3), a statistically substantial connection was found between tumor grade and DFS (P=0.034). Patients with higher grades (grade III) had shorter DFS than those with grade I and II. There was no statistically substantial connection between DFS and the remaining variables; however, patients with higher TNM stages had shorter DFS (20 months) than those with lower stages (26 months).

Patients, whose tumors showed lost SMAD4, had shorter DFS and OS than those with preserved SMAD4 expression (20 months and 13 months versus 26 months and 17 months respectively). In addition, Patients whose tumors showed positive HER2 had shorter DFS (14.43 months) than those with negative HER2 expression (23 months). However, there was no statistically substantial connection between DFS or OS and either SMAD4 or HER2 aberrant expression.

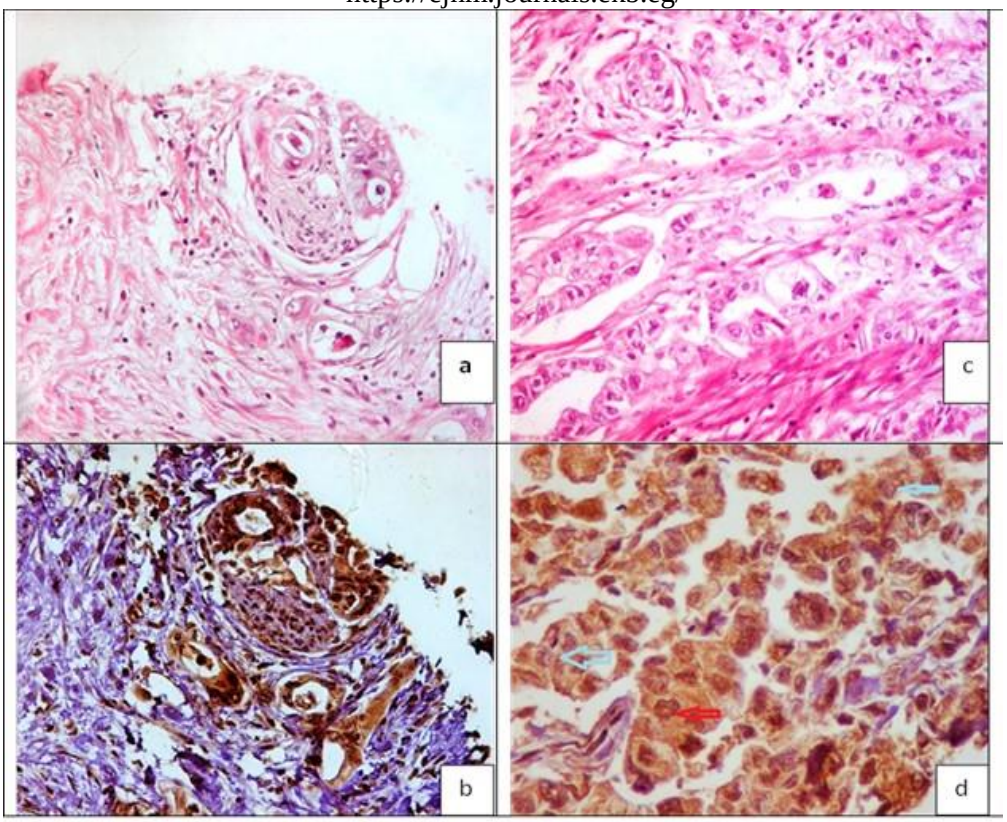

Figure (1): Representative examples of PDAC and different SMAD4 scores (a): TMA core of moderately differentiated PDAC with perineural invasion (H and E; x 200); (b): positive nuclear diffuse SMAD4 staining (DAB; X 200) (c): TMA core of moderately differentiated PDAC (Hand E; X200); (d): focal nuclear SMAD4 stain (DAB; X 400); red arrow: positive nuclei, blue arrow: negative nuclei.

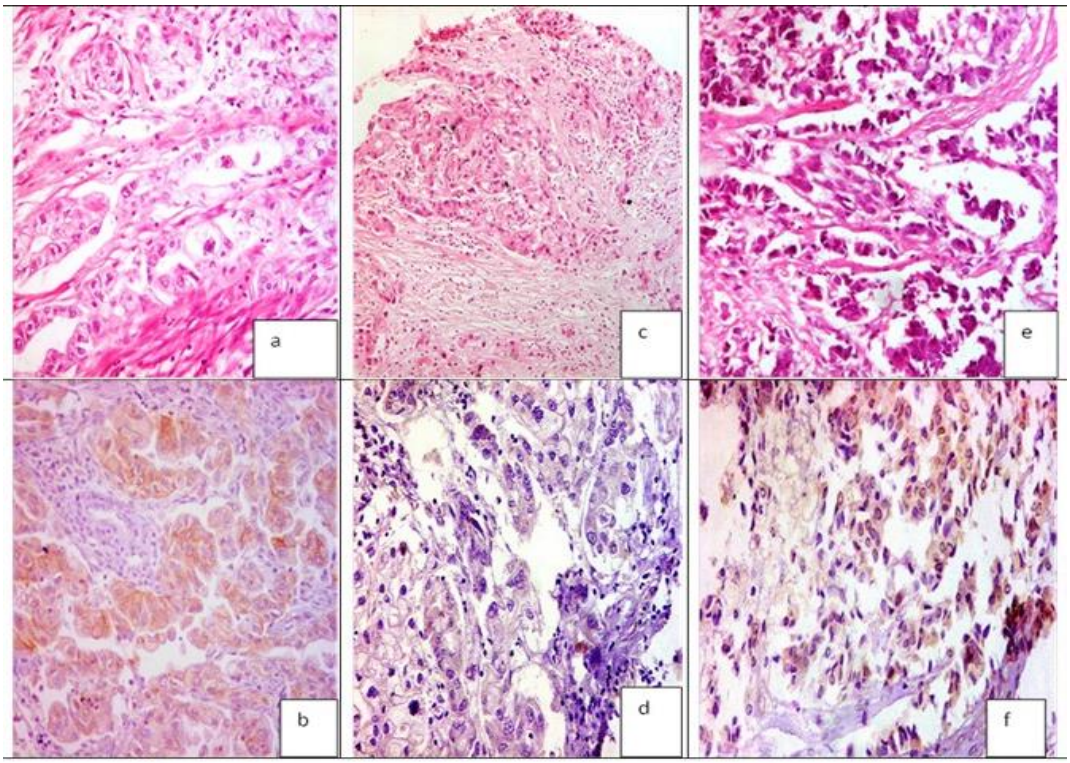

Figure (2): Representative examples of PDAC and different HER2 scores: (a): TMA core of moderately differentiated PDAC (Hand E; X200); (b): positive HER2 (score +3): strong basolateral membrane reactivity (DAB; X 200); (c): TMA core of moderately differentiated PDAC (Hand E; X 200); (d): Equivocal HER2 (Score +2): weak to moderate basolateral membrane reactivity (DAB; X 200); (e): **):** TMA core of moderately differentiated PDAC with mucinous activity (H and E; X200); (f): negative HER2 (score +1): faint or barely perceptible membrane reactivity (DAB; X200).

Table (1): SMAD4 and its association with clinicopathological data, death and relapse

	SMAD4		test of significance
	diffuse	negative / trace / focal	
Age /years			χ^2=2.77 p=0.096
<60	25(62.5)	34(80.0)	
≥60	15(37.5)	9(20)	
Sex			χ^2=8.12 p=0.004*
Male	22(55.0)	36(83.7)	
Female	18(45.0)	7(16.3)	
Tumour site			χ^{2FET}=4.52 p=0.05
Head and uncinate process	36(90)	43(100)	
Body	4(10)	0	
Tumor extension to duodenum			χ^2=0.869 p=0.351
No	15(37.5)	12(27.9)	
Yes	25(62.5)	31(72.1)	
tumor grade			FET=2.89 P=0.242
Grade I , II	40(100)	40(93)	
Grade III	0	3(7)	
PS margin			χ^2=4.83 p=0.028*
Free	37(92.5)	32(74.4)	
Infiltration	3(7.5)	11(25.6)	
LVI			χ^2=5.68 p=0.017*
No	35(87.5)	28(65.1)	
Yes	5(12.5)	15(34.9)	
PNI			χ^2=1.40 p=0.236
No	21(52.5)	17(39.5)	
Yes	19(47.5)	26(60.5)	
T (TUMOR SIZE)			χ^2=6.29 p=0.043*
T1	5(12.5)	9(20.9)	
T2	24(60)	14(32.6)	
T3	11(27.5)	20(46.5)	
<3 cm	30(75)	23(53.5)	χ^2=4.16 p=0.042*
≥3	10(25)	20(46.5)	
N (lymph node infiltration)			χ^2=0.514 p=0.773
N0	15(37.5)	14(32.6)	
N1	22(55)	24(55.8)	
N2	3(7.5)	5(11.6)	
N0	15(37.5)	14(32.6)	χ^2=0.2223 p=0.637
N1,N2	25(62.5)	29(67.4)	
TNM staging:			χ^2=0.055 p=0.814
I (IA, IB, IIA)	14(35)	14(32.6)	
II (IIB, III)	26(65)	29(67.4)	
Death			χ^2=2.34 p=0.127
Alive	11(27.5)	6(14.0)	
dead	29(72.5)	37(86.0)	
Relapse			χ^2=0.024 p=0.877
-VE	23(57.5)	24(55.8)	
+VE	17(42.5)	19(44.2)	

χ^2:Chi-Square test, MC: Monte Carlo test, FET: Fischer exact test , *statistically significant , PS: pancreatic safety, LVI: lymphovascular invasion, PNI: perineural invasion

Table (2): HER2 and its association with clinicopathological data, death and relapse

	HER2		test of significance
	Negative	positive	
Age /years **<60** **≥60**	 53(69.7) 23(30.3)	 6(85.7) 1(14.3)	χ^2=0.796 p=0.372
Sex **Male** **Female**	 55(72.4) 21(27.6)	 3(42.9) 4(57.1)	FET=2.65 P=0.103
Tumor site **Head and uncinate process** **Body**	 72(94.7) 4(5.3)	 7(100) 0	FET=0.387 P=0.534
Tumor extension **No** **Yes**	 26(34.2) 50(65.8)	 1(14.3) 6(85.7)	χ^2=1.159 p=0.282
tumor grade **Grade I , II** **Grade III**	 73(96.1) 3(3.9)	 7(100) 0	FET=0.287 P=1.0
PS margin **Free** **Infiltration**	 65(85.5) 11(14.5)	 4(57.1) 3(42.9)	FET=3.68 P=0.09
LVI **No** **Yes**	 60(78.9) 16(21.1)	 3(42.9) 4(57.1)	χ^{2FET}=4.57 p=0.03*
PNI **No** **Yes**	 37(48.7) 39(51.3)	 1(14.3) 6(85.7)	FET=0.190 P=0.08
T (tumor size) **T1** **T2** **T3**	 13(17.1) 34(44.7) 29(38.2)	 1(14.3) 4(57.1) 2(28.6)	MC=403 P=0.817
<3 cm **≥3**	 48(63.2) 28(36.8)	 5(71.5) 2(28.6)	χ^2=0.190 p=0.663
N (lymph node infiltration) **N0** **N1** **N2**	 28(36.8) 42(55.3) 6(7.9)	 1(14.3) 4(57.1) 2(28.6)	MC=3.78 P=0.151
Negative(N0) **Positive (N1,N2)**	28(36.8) 48(63.2)	1(14.3) 6(85.7)	χ^2=1.43 p=0.23
TNM staging **I (IA, IB, IIA)** **II (IIB, III)**	 28(36.8) 48(63.2)	 0 7(100)	χ^2=3.89 p=0.049*
Death **Alive** **dead**	 15(19.7) 61(80.3)	 2(28.6) 5(71.4)	χ^2=0.307 p=0.579
Relapse **-VE** **+VE**	 42(55.3) 34(44.7)	 5(71.4) 2(28.6)	χ^2=0.682 p=0.409

χ^2:Chi-Square test, MC: Monte Carlo test, FET: Fischer exact test , *statistically significant, PS: pancreatic safety, LVI: lymphovascular invasion, PNI: perineural invasion

Table (3): DFS and OS and relation with clinicopathological parameters, SMAD4 and HER2:

	DFS		OS	
	Median survival (95% CI)	Log rank test	Median survival (95% CI)	Log rank test
1-year	69.9%		60.7%	
3-years	30.7%		18.2%	
5-years	5.1%		12.1%	
Age / years				
<60	29(20.01-37.98)	χ^2=1.83	15(12.45-17.55)	χ^2=1.41
≥60	19(11.92-26.08)	P=0.177	13(8.69-17.31)	p=0.234
Sex				
Male	29(14.95-43.05)	χ^2=0.363	14(11.99-16.0)	χ^2=0.101
Female	19(7.64-30.36)	P=0.547	15(11.97-18.03)	p=0.751
tumor site				
Head	23(13.88-32.12)	χ^2=0.0	14(12.18-15.82)	χ^2=0.002
Body	18(9.8-25.14)	P=0.992	22(22-27)	p=0.968
tumor extension				
no	23(7.60-38.39)	χ^2=1.12	12(5.84-18.16)	χ^2=2.90
yes	26(13.62-38.38)	P=0.289	15(12.80-17.19)	p=0.09
tumor grade				
Grade I , II	26(14.72-37.28)	χ^2=4.49	14(11.62-16.38)	χ^2=0.09
Grade III	9(9-9)	P=0.034*	14(0.0-28.40)	p=0.769
PS margin				
Free	23(14.77-31.23)	χ^2=0.166	14(11.57-16.44)	χ^2=0.075
Infiltration	31(0.00-62.27)	P=0.683	13(9.33-16.67)	p=0.785
LVI				
No	23(13.85-32.15)	χ^2=0.293	15(12.12-17.88)	χ^2=0.3.47
Yes	26.23(19.03-33.42)	P=0.589	11(8.08-13.92)	p=0.062
PNI				
No	23(10.11-35.88)	χ^2=0.397	15(10.84-19.16)	χ^2=4.09
Yes	43(Undefined)	P=0.529	14(11.75-16.25)	p=0.043*
Tumor size				
T1	43(40-89.39)		12(9.56-14.45)	χ^2=5.99
T2	19(8.32-29.69)	χ^2=1.92	18(12.61-23.39)	p=0.051
T3	26(15.09-39.91)	P=0.382	12(9.27-14.73)	
<3 cm	23(10.59-36.40)	χ^2=0.115	16(12.20-19.79)	χ^2=3.72
≥3	31(13.16-48.84)	P=0.735	12(9.32-14.68)	p=0.054
N (lymph nodes)				
N0	26(8.48-43.52)		14(12.72-15.27)	χ^2=0.157
N1	20(12.59-27.40)	χ^2=0.451	15(12.45-17.54)	p=0.924
N2	44(44-44)	P=0.798	10(8.66-11.34)	
LN				
-VE	26(8.48-43.52)	χ^2=0.005	14(12.72-15.27)	χ^2=0.137
+VE	20(7.40-32.59)	P=0.946	15(12.35-17.65)	p=0.712
TNM stage				
I	26(8.19-43.80)	χ^2=0.051	14(12.33-15.67)	χ^2=0.202
II	20(7.59-32.40)	P=0.822	14(11.85-16.15)	p=0.653
SMAD4				
Positive	26(15.48-36.52)	χ^2=0.613	17(12.81-21.19)	χ^2=0.60
Negative	20(00.0-40.06)	P=0.434	13(10.65-15.35)	p=0.439
HER2				
				χ^2=0.078
Negative	23(13.81-32.18)	χ^2=0.139	14(11.57-16.43)	p=0.780
Positive	14.43(12.35-16.50)	P=0.710	14(9.89-18.10)	

χ^2:Chi-Square test, MC: Monte Carlo test, FET: Fischer exact test, *statistically significant, PS: pancreatic safety, LVI: lymphovascular invasion, PNI: perineural invasion

DISCUSSION

In this study, the expression of SMAD4 and

HER2 was assessed in 83 PDACs and was evaluated in relation to different clinicopathological variables and patient outcomes. Combined detection of SMAD4 loss and HER2 could be very valuable on prognostic and therapeutic level expression of both markers was associated with worse prognosis in pancreatic and other different neoplasms as well [11].

In this work, SMAD4 was negative in 51.8% of cases. This was close to **Yamada *et al.*** [12] where SMAD4 was inactivated in 59.8% of PDACs. On the contrary, **Hua *et al.*** [13] disclosed negative SMAD4 expression in 23.5% PDACs. This difference could be explained by the different scoring system for SMAD4.

As regard HER2 IHC results, 8.4% of cases were considered positive (score +3), while 71.1% of cases were score zero, 12% of cases were score +1 and 8.4% of cases were equivocal (score +2). Similarly, **Han *et al.*** [10] reported HER2 +3 in 7.3% of PDACs with score +2, +1 and 0 representing 9.1%, 25.4%, and 58.2% of tumors respectively.

In this study, negative SMAD4 expression was statistically associated with male gender (p=.004) but was not associated with patients' age. However, **Yamada *et al.*** [12] **and Shin *et al.*** [14] showed no significant statistic relationship between aberrant SMAD4 expression and patient age or sex. This could be explained by the prevalence of male gender (69.9%) in our study. Similar to **Chou *et al.*** [15] **and Han *et al.*** [10]**,** we didn't report association between HER2 expression and age or sex.

In this study, statistically significant associations were detected when SMAD4 expression was compared to the clinicopathological prognostic parameters including tumor site (p=0.05), pancreatic safety margin infiltration (p=0.028), presence of lymphovascular invasion (p=0.017) and tumor size (p=0.042). Likely, other studies revealed a significant association between SMAD4 expression and the resection margin status [14] and tumor's size. [16]

Concerning LVI, tumors with abnormal SMAD4 expression in this study showed more LVI (57.1%) as compared to those with normal SMAD4 expression (21.1%). This comes in concordance with the studies by **Wang *et al.*** [16] **and Yamada *et al.*** [12] who found a significant association between SMAD4 aberrant expression and the presence of LVI (p=0.029 and 0.033 respectively). Thus, aberrant SMAD4 seems to facilitate EMT and metastasis. As reported by **Shin *et al.*** [14]**,** aberrant SMAD4 expression was not associated with node infiltration, tumor extension to the duodenum, perineural invasion, tumor grade or TNM stage among our patients.

For HER2, we detected a statistically significant association between score+3 HER2 expression and the presence of LVI (p=0.03) and a higher TNM stage (p=0.049). However, it was not associated with any other investigated variables in this work. **Chou *et al.*** [15] **and Li *et al.*** [17] also found no significant associations between HER2 expression and any of the tumor characteristics.

Concerning the relation between clinical outcomes and IHC results, patients with negative SMAD4 tumors had higher frequencies of both death and relapse (86%, 44%) than those with retained SMAD4 expression (72%, 42%), but these differences didn't reach the level of statistical significance. Likewise, **Tascilar *et al.*** [18] reported lost SMAD4 to be linked to deaths compared to retained expression (86% versus 78%), but with no significant statistic association**. Yamada *et al.*** [12] also found more local recurrence and metastasis among SMAD4 negative cases, but with no statistical significance (p=0.318). On the contrary, **Shin *et al.*** [14] showed a statistically substantial connection between relapse and SMAD4 negativity (p=0.04). In addition, **Iacobuzio-Donahue *et al.*** [19]**,** showed a significant association between SMAD4 status and widespread metastasis (p=0.007). This difference could be explained by the different inclusion criteria as the later study included autopsy on 76 cases that died from PDACs.

Regarding HER2, it was not significantly associated with clinical outcome including death and relapse. Likely, **Chou *et al.*** [15] reported a similar finding. On the contrary, **Lei *et al.*** [20] found a significant relation between HER2 expression and patient's death (p<0.05). This could be explained by different sample size and different antibodies used in IHC.

We didn't find any association between SMAD4 and HER2 expression in our PDACs. This finding was difficult to compare as to the best of our knowledge, no other studies combined SMAD4 and HER2 till now.

Regarding DFS, our patients had a median DFS of 11 months. Disease relapse (either recurrence or metastasis) occurred in 36 (43.4%) of cases and this was significantly associated with tumor grade (p=0.034). Patients with higher grades (grade III) had shorter DFS than those with grade I and II. **Bachet *et al.*** [21] also found a significant relationship between DFS and tumor grade (p=0.005). However, there were no statistically significant associations between DFS and the other tested parameters in this work. On the contrary, **Yamada *et al.*** [12] found a significant association between DFS and PNI (p=0.030) and lymph node metastasis (p=0.012) and **Yoon *et al.*** [22] showed that tumor size (p <.001), poor differentiation (p<0.01) and positive lymph node metastasis

(p<0.001) were significantly associated with recurrence. This difference could be explained by the larger sample size included in the later study.

Patients whose tumors showed lost SMAD4 or score+3 HER2 had shorter DFS (20 and 14.43 months) than those with preserved SMAD4 expression or negative HER2 (26 and 23 months). However, there were no statistically significant associations were noticed between DFS and any of SMAD4 or HER2 aberrant expression in this study. In agreement with our results, **Bachet *et al.*** [21] found no significant association between SMAD4 expression and DFS, likely, **Aumayr *et al.*** [23] **and Li *et al.*** [17] showed no association between HER2 expression and DFS. Contrarily**, Yamada *et al.*** [12] **and Shin *et al.*** [14] proved the association between aberrant SMAD4 expression and shorter DFS (p<0.01). This difference could be explained by the larger sample size and different scoring system for SMAD4.

The median OS for our patients was 14 months. Disease related deaths occurred in 66 of cases (79.5%). We had a statistically significant association between the OS and the PNI (p=0.043) as tumors with positive PNI occurred in patients with shorter OS (14 months) than those who are negative for PNI (15 months). **Shin *et al.*** [14] also reported a similar finding (p=.0.002). On the other hand, **Biankin *et al.*** [24] **and Jiang *et al.*** [25] found no significant association between PNI and OS. Using different protocols for tumor gross sectioning and microscopic assessment for PNI in different institutes may contribute to this finding.

There were no associations between OS and patient age or gender in our study.

Ottenhof *et al.* [26] **and Shin *et al.*** [14] showed the same results. Moreover, there were no associations between OS and tumor site, size, grade, stage, duodenal extension, PS infiltration, nodal infiltration and LVI were noticed in this work.

Notable, we didn't report any association between OS and either SMAD4 or HER2. However, patients with negative SMAD4 expression tumors had shorter OS (13 months) than those with positive SMAD4 (17 months).

Bachet *et al.* [21] **and Wang *et al.*** [16] also found no significant association between SMAD4 expression and OS, but also showed that patients with lost SMAD4 expression had shorter OS. On the other side, **Oshima *et al.*** [27] **and Shin *et al.*** [14] proved a significant statistical association between SMAD4 expression and OS (p=0.014 and 0.04 respectively). This difference could be explained by the difference of PDAC stage at inclusion and the different scoring system for SMAD4 adopted by these studies.

Aumayr *et al.* [23] **and Ata *et al.*** [28] also found no significant association between HER2 expression and OS. Meanwhile, **Saxby *et al.*** [29] **and Han *et al.*** [10] reported the reverse (p=0.01, 0.021), as they proved an association between HER2 overexpression and reduced OS. This difference could be explained by the different number of cases and different adopted scoring system.

As we can see, SMAD4 loss is associated with higher percentage of both death and relapse, larger tumor size and more LVI. This could be explained by the fact that SMAD4 performs its function through the TGF-β signaling pathway. In normal physiological conditions, Transforming growth factor beta (TGF) proteins maintain tissue homeostasis and regulate many cell functions as: cell differentiation, proliferation, migration & apoptosis [30].

However, TGF-β is overexpressed in inflammation, fibrosis and tumorogenesis. In tumors, it has a dual function as a tumor suppressor gene & oncogene. The cell cycle inhibitory effect is done via the SMAD dependent pathway. When SMADd4 is lost as in PDAC, it acquires an oncogenic role and share in tumor progression via mediating epithelial mesenchymal transition (EMT), angiogenesis and immune suppression[31].

It mediated EMT via inducing transcription of the Snail protein that enhances EMT and decreases expression of epithelial junction proteins as E-cadherin and occluding [32]. Hypoxia within tumor microenvironment causes TGF-β to enhance expression of vascular endothelial growth factor (VEGF) that acts on endothelial cell proliferation, migration and new capillary formation [33].

In conclusion, the utility of IHC to assess SMAD4 and HER2 in PDACs could be of prognostic and therapeutic importance. In this regard, **Yamada *et al.*** [12] suggested a preoperative strategy depending mainly on SMAD4 status via performing IHC for SMAD4 on the endoscopic- fine-needle aspiration specimens. Local treatment with chemo-radiation could be helpful to SMAD4 positive PDAC patients, while systemic neoadjuvant chemotherapy could be helpful in SMAD4 negative patients [34]. SMAD4-positive carcinomas would benefit more from intensive local control by chemotherapy to prevent metastasis more than those with negative SMAD4 [19]. SMAD4-negative PDAC cells are sensitive to drugs that target the cell cycle as cytarabine and Olaparib as they upregulate the cell cycle-related genes such as CDK1 [35]. Using anti-HER2 drugs (Trastuzumab) for the HER2-positive PDAC patients can increase the conventional chemotherapy efficacy [17].

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