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RESEARCH ARTICLE

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Differential expression of aquaporin-3 and aquaporin-5 in pancreatic ductal adenocarcinoma

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Background and Objectives: Aquaporin-5 (AQP5) and −3 (AQP3) are protein channels that showed to be up-regulated in a variety of tumors. Our goal was to investigate the expression pattern of AQP5 and AQP3 in pancreatic ductal adenocarcinomas (PDA) and correlate with cell proliferation, tumor stage and progression, and clinical significance.

Methods: 35 PDA samples in different stages of differentiation and locations were analyzed by immunohistochemistry for expression of AQP5, AQP3 and several markers of cell proliferation and tumorigenesis.

Results: In PDA samples AQP5 was overexpressed in the apical membrane of intercalated and intralobular ductal cells while AQP3 was expressed at the plasma membrane of ductal cells. AQP5 was also found in infiltrative cancer cells in duodenum. Simultaneous overexpression of EGFR, Ki-67, and CK7, with decreased E-cad and increased Vim that characterize epithelial mesenchymal transition, tumor formation and invasion, strongly suggest AQP3 and AQP5 involvement in cell proliferation and transformation. AQP3 overexpression is reinforced in late and more aggressive PDA stages whereas AQP5 is related with tumor differentiation, suggesting it may represent a novel marker for PDA aggressiveness and intestinal infiltration.

Conclusions: These findings suggest AQP3 and AQP5 involvement in PDA development and the usefulness of AQP5 in early PDA diagnosis.

KEYWORDS
aquaporins, biomarker, duodenum, expression pattern, pancreas, pancreatic cancer

1 | INTRODUCTION

Pancreatic cancer (PC) is the seventh leading cause of cancer mortality in the world and the fourth cause of cancer-related death in the US, with an estimated 5-year prevalence of 4.1 per 10 000 people.[1–3] More than 85% of the pancreatic tumors are invasive ductal adenocarcinomas (PDA) and more than 60% of PDAs are located in the head of the pancreas.[4,5] Depending on the degree of differentiation PDA may show, embedded in a desmoplastic stroma, well to poorly formed glands or individual infiltrating cells forming sheets.[4,5] In contrast to other solid organ malignancies for which early diagnosis and target therapy improvements were made in recent years, PDA mortality rates are actually increasing, being projected that in 2030 pancreatic cancer will be the second leading cause of cancer mortality.[2,6] Due to the retroperitoneal location of the pancreas, the early stages of this type of cancer do not usually produce symptoms. The disease is generally advanced when it is diagnosed and more than 50% of patients have distant metastasis and so the 5-year survival rate from all stages of PC is approximately 6%.[3]

Histological evaluation based on morphologic criteria and on protein markers is currently the best procedure to distinguish between a benign chronic pancreatitis and PDA.[7] It is known that most PDAs express proteins such as cytokeratins 7, 8, 13, 18, and 19,

Abbreviations: AJCC, America Joint Committee on Cancer; AQP, aquaporin; CK7, cytokeratin 7; E cad, e-cadherin; EGFR, epidermal growth factor receptor; EMT, epithelial mesenchymal transition; FIS, final immunohistochemistry score; H_2O_2, hydrogen peroxide; PC, pancreatic cancer; PDA, pancreatic ductal adenocarcinoma; PI3 K, phosphatidylinositol-3-kinase; PPC, percentage of positive cells; pTNM, pathological tumor/node/metastasis; SI, staining intensity; Vim, vimentin.

carcinoembryonic antigen (CEA), mesothelin, carbohydrate antigen 19–9 (CA 19–9), B72.3 (TAG-72), CA 125, DUPAN 2, MUC1 (a pan-epithelial mucin), MUC3, MUC4, and MUC5AC.[7] Yet, these protein markers lack the desirable sensitivity and specificity and the establishment of useful biomarkers for PDA detection at early stages of the disease is an urgent demand.

It is well established that tumor growth, development, invasion, and metastasis depend on tumor microenvironment and metabolism[8] and it is also known that water balance and glycerol metabolism are essential to maintain cell function, including in malignant cells.[9,10] Aquaporins (AQPs), a family of 13 (AQP0-12) integral transmembrane channel proteins play an important role in transcellular water movement in response to osmotic gradients; they may also facilitate tumor growth, local infiltration, and metastasis, by enhancing cell migration, angiogenesis, cell-matrix adhesion, glycerol uptake, and by interacting with oncogenes.[9,10]

Recently, an increasing number of reports showed that AQP3 and AQP5 are abundantly expressed in different tumors such as squamous cell carcinoma of the skin,[11] colon cancer,[12–15] breast cancer,[16,17] lung cancer,[14,18,19] ovarian cancer,[20] cervical cancer,[21] and prostate cancer,[22] playing key roles in cell proliferation and migration.[11,23,24] In tumors, cells divide more frequently, thus needing more energy to maintain their rapid growth. It is known that glycerol transported by AQP3 is necessary for lipid biosynthesis and can be metabolized to generate ATP, which is essential for cell division and migration.[9,10] In addition, AQP3[25] and possibly AQP5[26] are also capable of transporting extracellular hydrogen peroxide (H_2O_2), a reactive oxygen species that acts as a signaling molecule in signal transduction and may promote many aspects of tumor progression.[27] Phosphorylation of AQP5, in turn, activates the RAS/MAPK pathway involved in cell proliferation and survival[28,29] and this isoform may facilitate cancer cell motility due to its preferential polarization in the leading edge of migrating cells and by facilitating lamellipodium formation.[18,20,22,23,30,31] It has been reported that both AQP3 and AQP5 expression are upregulated by epidermal growth factor receptor (EGFR) signaling pathway.[18,24,32] EGFR is frequently overexpressed in tumors, including pancreatic cancer,[33] and its activation leads to the transcription of genes involved in cell growth and proliferation. EGFR expression is also associated with poor prognosis and increased invasiveness in PDA.[34,35] In addition, AQP3[36] and AQP5[23] have been implicated in epithelial mesenchymal transition (EMT), a process in invasive tumors that contributes to invasion and metastasis and by which cells lose their epithelial characteristics, such as cell-cell adhesion and lack of motility, and acquire migratory mesenchymal properties. During this process expression levels of epithelial adhesion molecules, such as E-cadherin (E-Cad) are decreased whereas mesenchymal cell markers, like vimentin (Vim) are increased.[8]

Despite the recognized expression of AQP5 at the apical membrane of intercalated and intralobular pancreatic ductal cells[37,38] and of AQP3 in pancreatic islet cells[39] in normal conditions, little is known about their expression in PDA. This prompted us to investigate the expression pattern of AQP5 and AQP3 in PDA and its clinical significance. To evaluate the potential of these two isoforms as protein markers for PDA we performed immunohistochemical studies in human PDA samples, in comparison with adjacent non-neoplastic pancreatic tissue of the same patient (used as control), and evaluated the relationship between the expression of AQP5 and AQP3 and the expression of Ki-67 (a proliferative cell marker), cytokeratin (CK7, a PDA marker), E-Cad (an epithelial cell marker), Vim (a mesenchymal cell marker), as well as of EGFR (a relevant receptor concerning AQP5 and 3 expression and signaling cascades).

2 | MATERIALS AND METHODS

2.1 | Patients

Formalin-fixed and paraffin embedded tumor samples and matched adjacent non-neoplastic samples were obtained from 35 patients who underwent curative surgery for PDA at Hospital Curry Cabral, Centro Hospitalar de Lisboa Central, Lisbon, Portugal, between November 2012 and March 2015. Head, body and ampulla region tumor locations were considered in this study, and adjacent duodenum was also analyzed. Tissue was obtained and used in a manner compliant with the Declaration of Helsinki, as revised in 1983. The patients' clinicopathological features are summarized in Table 1. All patients were classified according to the pathological tumor/node/metastasis (pTNM) system and based on the 7th edition of America Joint Committee on Cancer (AJCC); the pathologic stages after surgical resection were stages I and II. Tumor histological differentiation was classified from moderately to poorly, corresponding the poorly differentiated to the most aggressive forms. Clinicopathological features were reviewed for gender, histological differentiation, tumor dimension, tumor location, lymph node metastasis, perineural, vascular, and intestinal invasion. This study was approved by the ethics committee of Centro Hospitalar de Lisboa Central.

2.2 | Immunohistochemistry

Immunohistochemistry was performed on 4 μm paraffin sections of pancreas. A 3% hydrogen peroxide solution was used to inhibit endogenous peroxidase activity (30 min at room temperature) and a 0.5% Triton X-100, 3% bovine serum albumin solution was used for blocking and permeabilization (30 min at room temperature). The specific conditions and antibodies used for each parameter are summarized in Table S1 (Supplementary files). Briefly, antigen retrieval was achieved by heat-mediated treatment with citrate buffer pH 6.0. All antibodies were diluted in 0.5% Triton X-100, 3% BSA solution. An additional post-incubation step was required for Ki-67 immunostaining. Sections were incubated with SuperPicture™ Polymer Detection Kit (Invitrogen, Carlsbad, CA), followed by development with 3,3′-diaminobenzidine tetrahydrochloride and counterstaining with haematoxylin. Negative controls with omission of primary antibodies were performed to exclude nonspecific binding or cross reactivity.

TABLE 1 Clinicopathological features of the 35 patients analyzed

Case	Sex	Histological differentiation	Stage	TNM	Dimension (cm)	Tumor location	Lymph node metastasis	Perineural invasion	Vascular invasion	Intestinal invasion
1	F	Moderately	II	pT3 N0 Mx	3 × 3.2 × 2.4	Head	No	No	No	Yes
2	M	Moderately	I	pT2 N0 Mx	1,5 × 1.5 × 2	Ampulla	No	No	No	Yes
3	M	Moderately	II	pT3 N1	2.5 × 1 × 0.3	Head	Yes	Yes	No	No
4	F	Moderately	II	pT3 N1	2.5 × 2.5 × 2	Head	Yes	Yes	No	No
5	M	Poorly	II	pT3 N1 Mx	0.5 × 1 × 0.4	Head	Yes	Yes	Yes	Yes
6	F	Moderately	II	pT3 N0 Mx	3 × 2.5 × 2.5	Head	No	Yes	No	Yes
7	F	Moderately	II	pT2 N1	1.5 × 1	Ampulla	Yes	No	No	Yes
8	M	Poorly	II	pT3 N1	3.5 × 2.5 × 2	Head	Yes	Yes	Yes	Yes
9	M	Moderately	I	pT2 N0 Mx	4.5 × 3 × 2	Ampulla	No	No	No	Yes
10	M	Poorly	II	pT3 N0 Mx	4 × 3 × 2.5	Head	No	Yes	No	No
11	F	Moderately	II	pT3R1, N1	4.2 × 4 × 2.5	Head	Yes	Yes	No	No
12	M	Moderately	II	pT3 N1 Mx	1 × 1.5 × 1	Head	Yes	No	Yes	No
13	M	Poorly	II	pT3 N1 Mx	3 × 2 × 2	Head	Yes	No	No	No
14	F	Moderately	II	pT3 N0 Mx	2 × 1.2 × 1	Head	No	No	No	Yes
15	M	Poorly	II	pT3 N1 Mx	4 × 3.5 × 3	Head	Yes	Yes	Yes	Yes
16	F	Poorly	II	pT3 N1 Mx	0,4	Head	Yes	Yes	Yes	Yes
17	M	Moderately	II	pT3 N1 Mx	3 × 2.5 × 2.5	Head	Yes	Yes	Yes	Yes
18	M	Poorly	II	pT3 N1 Mx	1.5	Head	Yes	Yes	Yes	No
19	F	Moderately	II	pT3 N1 Mx	2.5 × 1.5 × 3	Head	Yes	Yes	Yes	Yes
20	F	Moderately	II	pT3 N1 Mx	2.7 × 2 × 2.2	Head	Yes	Yes	Yes	No
21	F	Moderately	II	pT3 N1 Mx	3.5 × 3 × 2.5	Head	Yes	Yes	No	Yes
22	F	Poorly	II	pT3 N1 Mx	4.5 × 3.5 × 4	Head	Yes	Yes	Yes	Yes
23	M	Moderately	II	pT3 N1 Mx	2	Head	Yes	No	No	Yes
24	M	Moderately	II	pT3 N1 Mx	4 × 3.5 × 2.5	Ampulla	Yes	No	No	Yes
25	F	Poorly	II	pT3 N1 Mx	3.5 × 3.3 × 3	Ampulla	Yes	Yes	No	No
26	M	Poorly	II	pT3 N1 Mx	2.5 × 2.5 × 2.2	Head	Yes	No	No	Yes
27	F	Poorly	II	pT2 N1 Mx	2.5 × 3 × 2.5	Head	Yes	No	No	No
28	M	Moderately	II	pT3 N1 Mx	2.5	Head	Yes	Yes	Yes	No
29	F	Moderately	II	pT3 N1	4 × 2.7 × 2.5	Head	Yes	Yes	Yes	Yes

(Continues)

TABLE 1 (Continued)

Case	Sex	Histological differentiation	Stage	TNM	Dimension (cm)	Tumor location	Lymph node metastasis	Perineural invasion	Vascular invasion	Intestinal invasion
				Mx						
30	M	Moderately	II	pT3 N1 Mx	2 × 2 × 0.4	Head	Yes	Yes	No	No
31	M	Moderately	I	pT2 N0 Mx	2 × 1	Ampulla	No	No	No	Yes
32	M	Moderately	II	pT3 N1 Mx	3 × 2	Head	Yes	No	No	Yes
33	F	Poorly	II	pT3 N0 Mx	2 × 1.7 × 1.5	Head	No	Yes	No	No
34	M	Moderately	I	pT2 N0 Mx	2.5 × 1.5	Head	No	No	No	Yes
35	F	Moderately	II	pT3 N0 Mx	2 × 2.5	Body	No	Yes	No	No

F, female; M, male; pTNM, pathological tumor/node/metastasis.

2.3 | Scoring of immunohistochemistry

The immunohistochemical analysis was graded based on the staining intensity (SI) and percentage of positive cells (PPC) by one investigator that was blinded to the clinicopathological variables. The SI was scored on a scale of four grades: 0, no staining; 1, weak staining; 2, moderate staining and 3, strong staining. The PPC was graded on a scale of 3 grades: 0, <10% stained cells; 1, ≥10% <50% stained cells and 2, ≥50% stained cells. Expression of AQP5, AQP3, Ki-67, CK7, EGFR, E-cad, and Vim were defined as final immunohistochemical score (FIS) based on the sum of the SI and PPC. AQP5, AQP3, Ki-67, CK7, EGFR, E-cad, and Vim expression in each PDA sample was considered increased or decreased when FIS was higher or lower, respectively, than the mean FIS value for non-neoplastic (control) samples.

2.4 | Statistical analysis

The baseline clinicopathological features and results of the immunohistochemical staining (individual FIS values) were compared using a Fisher's exact test. Mann-Whitney test was used to compare the expression levels (mean of FIS values for each group) between non-neoplastic (control) pancreatic tissues and moderately and poorly differentiated tumors. The correlation between AQP5, AQP3, Ki-67, CK7, EGFR, E-cad, and Vim expression in non-neoplastic, moderately, and poorly differentiated PDA tissues was assessed using individual FIS values and tested with a Spearman rank correlation.

Overall survival (OS) was calculated from the date of the surgical procedure and the date of death or last follow-up. Survival rates were analyzed using the Kaplan-Meier method, log-rank test. Receiver operating characteristic curve (ROC) analysis was used as a screening measure to evaluate AQP3 and AQP5 sensitivity and specificity. Statistical analysis was performed using SPSS 21.0 software (SPSS, Inc., Chicago, IL) and $P < 0.05$ was considered significant.

3 | RESULTS

3.1 | AQP5 expression in PDA and non-neoplastic samples

In normal pancreatic tissue AQP5 is expressed in the apical membrane of intercalated and intralobular ductal cells (Fig. 1A,B). In PDA AQP5 is expressed all over the plasma membrane and becomes to diffuse intracellularly (Fig. 1C-F). Moderately differentiated PDAs show a stronger AQP5 immunoreactivity when compared with poorly differentiated ones. This isoform is also expressed in apical membrane of Brunner's glands cells in the duodenum (Fig. 1G, arrow). AQP5 was also detected in goblet cells (Fig. 1H, triangles). Moreover, AQP5 expression was detected in the plasma membrane of some scattered cells in duodenal epithelium (Fig. 1H, arrows), which suggest that these cells may be infiltrative tumoral cells since it is known from previous studies that epithelial cells from duodenum do not express AQP5 in normal conditions.[40]

Analysis of the SI revealed that tumor samples have higher values for strong staining when compared with non-neoplastic samples and that moderately differentiated tumors have greater values of strong staining than poorly differentiated tumors (Fig. 1I). As shown in Fig. 1J, the PPC was <10 in all the non-neoplastic samples, whereas in tumors the PPC was always ≥10. Accordingly, FIS values (Fig. 1K) range between one and three in non-neoplastic cases, between two and five in moderately differentiated tumors and between three and five in poorly differentiated ones.

3.2 | AQP3 expression in PDA and non-neoplastic samples

Although it is known from previous studies that AQP3 has a major role in cancer cell metabolism in different types of tumors, little is known about its expression in PDA and in non-neoplastic pancreatic tissue. After immunohistochemical staining we found that AQP3

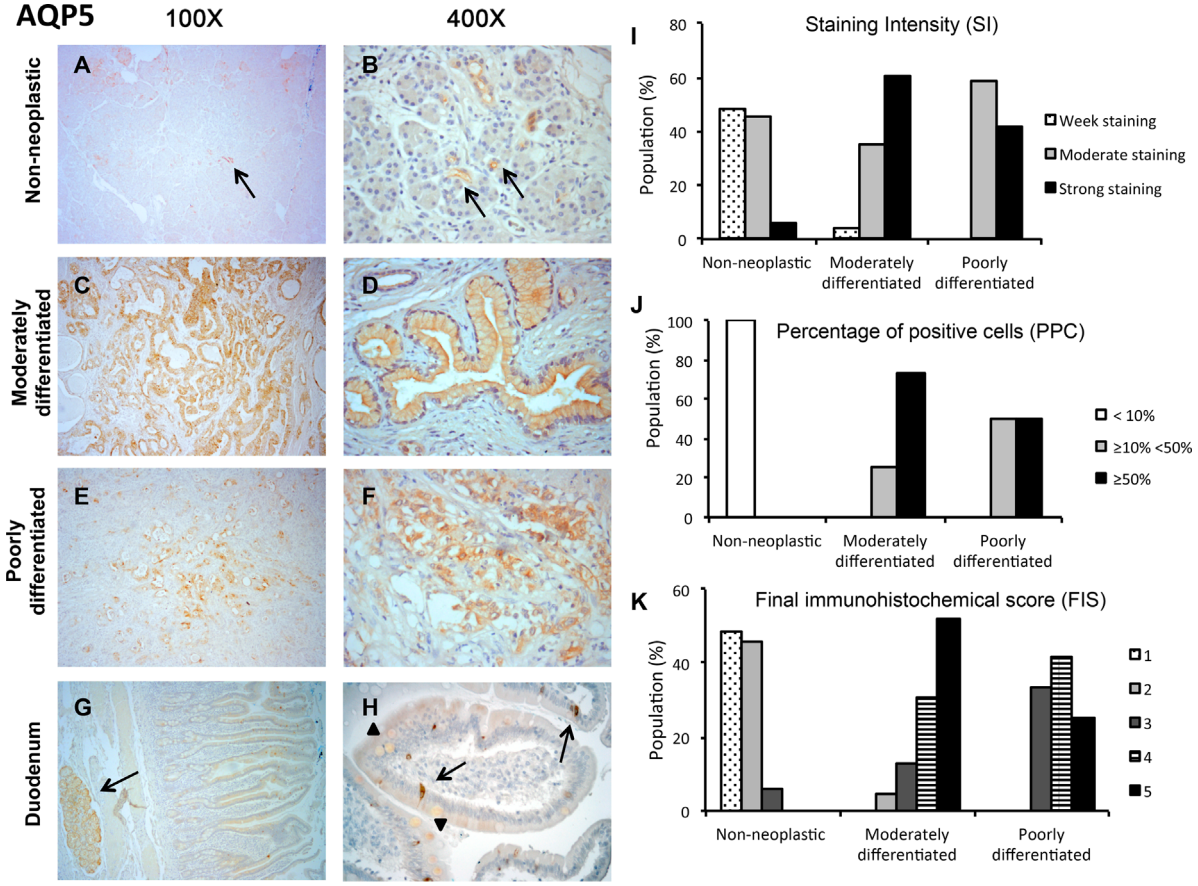

FIGURE 1 Aquaporin 5 (AQP5) expression in non-neoplastic pancreas, in moderately and poorly differentiated pancreatic ductal adenocarcinoma (PDA), and in duodenum samples. AQP5 is expressed in the apical membrane of intercalated and intralobular ductal cells in non-neoplastic pancreas (A,B); AQP5 is expressed in the plasma membrane and becomes to diffuse intracellularly in PDA cells (C-F); AQP5 expression is also detected in Brunner's glands (G, arrow), in some scattered cells in duodenal epithelium (H, arrow) as well as in goblet cells (H, triangles). The evaluation of staining intensity (I) and percentage of positive cells (J), as well as the final immunohistochemical scores (J) are represented as percentages of the total of cases for non-neoplastic (n = 35), moderately differentiated (n = 23) and poorly differentiated samples (n = 12). Strong staining increased from 5.7% in non-neoplastic samples to 60.9% in moderately differentiated tumors, whereas in poorly differentiated tumors was only observed in 41.7%; weak staining decreased from 48.6% in non-neoplastic population to 4.3% in moderately differentiated samples (I). PPC was <10 in all the non-neoplastic samples, 73.9% of the moderately differentiated samples presenting values of ≥50 PPC and an equivalent distribution (50%) of the two highest grades in poorly differentiated tumors (J). More than 50% of the moderately differentiated tumors presented the highest FIS value (5), which was observed in 25% of the poorly differentiated tumors but not attained in any of the non-neoplastic samples; the lowest FIS value (1) was observed in 50% of non-neoplastic cases but not observed in tumors (K)

expression is nearly absent in normal ductal pancreatic cells (Fig. 2A), in contrast to what we observed for AQP5. The expression of this protein was mostly detected in the plasma membrane of some acinar cells (Fig. 2B, arrow), with intracellular expression in some cells also observed.

Interestingly, AQP3 became to be expressed in ductal cells in PDA, however in lower levels than AQP5. Moreover, its expression seems to be heterogeneous, as some tumors highly express this protein while others do not. In moderately differentiated PDAs AQP3 was found to be expressed at the plasma membrane of ductal cells (Fig. 2C,D). In poorly differentiated tumors this isoform seems to be diffused intracellularly (Fig. 2E) and was also detected in some scattered cells in desmoplastic stroma (Fig. 2F, arrows). In the duodenum, AQP3 was also expressed in basolateral membrane and lightly in the cytoplasm of epithelial cells lining the villous tips (Fig. 2G), as previously described[41] and in contrast to what we observed for AQP5, AQP3 was not expressed in goblet cells (Fig. 2H, star). Moreover, we found that AQP3 is extensively expressed in red blood cells (Fig. 2G), as described in previous studies.[42,43]

Analysis of the SI revealed that tumor samples have higher values for strong staining as compared with non-neoplastic samples and that the poorly differentiated tumors have greater values than moderately differentiated tumors (Fig. 2I). As shown in Fig. 2J, the PPC was <10 in almost all of non-neoplastic samples, while in in poorly differentiated tumors the PPC was always defined as ≥10. Accordingly, FIS values (Fig. 2K) range between one and four in non-neoplastic cases, two and five in moderately differentiated tumors and between four and five in poorly differentiated ones. It is worthwhile to point out the different expression profile of AQP3 compared to that of AQP5. In fact, AQP3 expression continuously

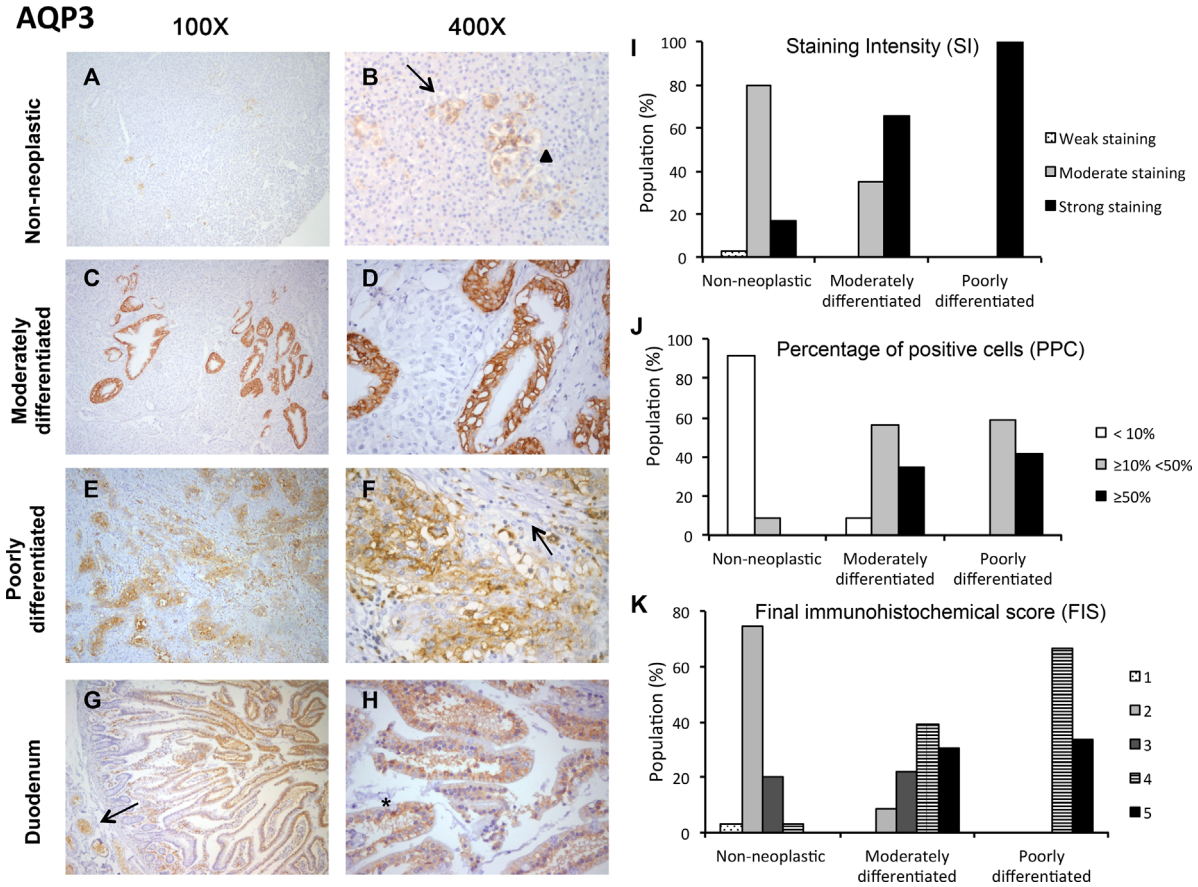

FIGURE 2 Aquaporin 3 (AQP3) expression in non-neoplastic pancreas, in moderately and poorly differentiated pancreatic ductal adenocarcinoma (PDA), and in duodenum samples. In normal pancreatic tissues AQP3 is not evident in ductal cells (A), being expressed in the cytoplasm and in the plasma membrane of some acinar cells (B, arrow); in PDA cells AQP3 starts to be expressed in the plasma membrane of ductal cells and becomes to diffuse intracellularly (C-F) and in poorly differentiated tumors this isoform is also detected in some scattered cells in desmoplastic stroma (F, arrow); in duodenum AQP3 expression is also detected in the basolateral membrane and lightly in the cytoplasm of epithelial cells lining the villous tips, as well as inside blood vessels (G, arrow) but in contrast to AQP5, AQP3 is not expressed in goblet cells (H, star). The evaluation of staining intensity (I) and percentage of positive cells (J), as well as the final immunohistochemical scores (J) are represented as percentages of the total of cases for non-neoplastic ($n = 35$), moderately differentiated ($n = 23$) and poorly differentiated samples ($n = 12$). The percentage of the studied population with a strong staining increased from 17.1% in non-neoplastic samples to 65.2% in moderately differentiated tumors, and 100% in poorly differentiated tumors; moderate staining decreased from 80.0% in non-neoplastic population to 34.8% in moderately differentiated samples (I). PPC was <10 in 91.4% of non-neoplastic samples; in most of moderately and poorly differentiated tumors positive cells ranging from ≥10 to <50 were observed in 56.5% and 58.3% of the studied population, respectively (J). 33.3% of the poorly differentiated tumors presented the highest FIS value (5), which was observed in 30.4% of the moderately differentiated tumors; the lowest FIS value (1) observed in 2.9% of non-neoplastic cases was not observed in tumors (K)

increase from moderately to poorly differentiated tumors in SI and PPC, corresponding to the highest FIS values, whereas AQP5 presented the highest values in the moderately differentiated tumors, decreasing in the poorly differentiated cases.

3.3 | Ki-67 expression in PDA and non-neoplastic samples

To evaluate the possible relationship between AQP5 and AQP3 expression with cell proliferation we performed immunohistochemical analysis of Ki-67, a widely used proliferative cell marker that is expressed in cells in G1 to M phases of cell cycle. We found that Ki-67 nuclear expression is almost absent in normal pancreatic tissue (Fig. 3A) except for some rare mitotic figures as depicted in Fig. 3B. In PDA samples Ki-67 protein levels were very heterogeneous: in some tumors many proliferative cells were observed while in others just a few cells were stained (Fig. 3C-F). As far as duodenum is concerned, the expression of Ki-67 was detected in the base of duodenal crypts (Fig. 3G), where cells responsible for the epithelium renewal are present. Stained cells were also visible in duodenal villi, namely in the apical aspect of cells (Fig. 3H, arrow).

Analysis of the SI revealed that tumor samples have higher values as compared with non-neoplastic (Fig. 3I). The PPC was <10 in all the non-neoplastic samples whereas moderately and poorly differentiated tumors presented values of <10 or of ≥10% <50% PPC (Fig. 3J). Accordingly, FIS values (Fig. 3K) range between 0 and 2 in non-neoplastic cases and between one and four in moderately and poorly differentiated tumors.

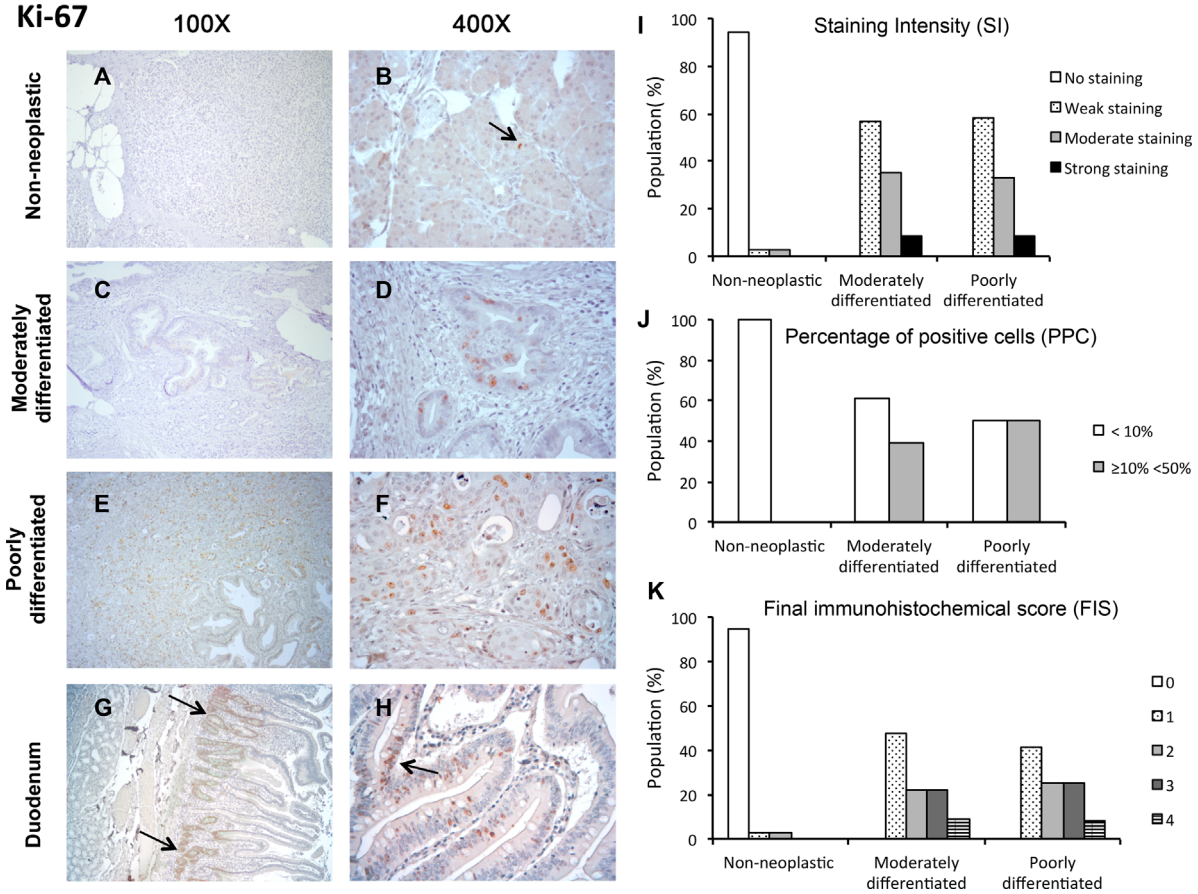

FIGURE 3 Ki-67 expression in non-neoplastic pancreas, in moderately and poorly differentiated pancreatic ductal adenocarcinoma (PDA), and in duodenum samples. Ki-67 expression is almost absent in normal pancreatic tissue (A) except for some rare mitotic figures (B, arrow); Ki-67 nuclear staining is shown in proliferative ductal carcinoma cells (C-F); the expression is also detected in the base of duodenal crypts (G, arrows) and in duodenal villi (H, arrow). The evaluation of the parameters staining intensity (I) and percentage of positive cells (J), as well as the final immunohistochemical scores (K) are represented as percentages of the total of cases for non-neoplastic (n = 35), moderately differentiated (n = 23) and poorly differentiated samples (n = 12). 94.3% of non-neoplastic samples was not stained with Ki-67; weak staining, as well as moderate staining, increased from 2.9% in non-neoplastic population to 56.5% and 34.8% in moderately differentiated samples and 58.3% and 33.3% in poorly differentiated tumors, respectively (I). The PPC was <10 in all the non-neoplastic samples whereas only 60.9% of moderately differentiated tumors and 50.0% of poorly differentiated samples presented values of <10 PPC and the remaining presented values between 10% and 50% (J). 8.7% of the moderately differentiated tumors and 8.3% of poorly differentiated tumors presented the highest FIS value obtained for this parameter (4), which was not observed in any of the non-neoplastic samples; the lowest FIS value (0) was observed in 94.3% of non-neoplastic cases (K)

3.4 | CK7 expression in PDA and non-neoplastic samples

To test the hypothesis that duodenal cells expressing AQP5 were infiltrative PDA cells, we performed immunohistochemical analyses with CK7, a widely used PDA marker. CK7 is known to be expressed in the epithelial cells of the intercalating and large normal pancreatic ducts, but not in the normal small intestine mucosa.[5] CK7 was expressed in ductal cells in normal pancreas (Fig. 4A,B). In PDA cells this cytokeratin was found intracellularly and at the apical plasma membrane (Fig. 4C-F). Interestingly, immunostainning for CK7 was observed in the plasma membrane and intracellularly in some scattered cells in duodenum (Fig. 4G,H, arrows) and in goblet cells (Fig. 4H, triangles), which were also shown to express AQP5 (Fig. 1H).

Analysis of the SI (Fig. 4I) revealed a moderate staining in the majority of the population. However, tumor samples have higher values as compared with non-neoplastic cases. As shown in Fig. 4J, the population with a PPC ≥50 increased from non-neoplastic cases to moderately and poorly differentiated tumors. Accordingly, FIS values range between one and four in non-neoplastic cases, between one and five in moderately differentiated tumors and between three and five in poorly differentiated ones (Fig. 4K).

3.5 | EGFR expression in PDA and non-neoplastic samples

It is known from previous studies that AQP5 up-regulation in lung cancer cells activates EGFR,[18] which in turn triggers the RAS/MAPK as well as phosphatidylinositol-3-kinase (PI3 K)/AKT signaling

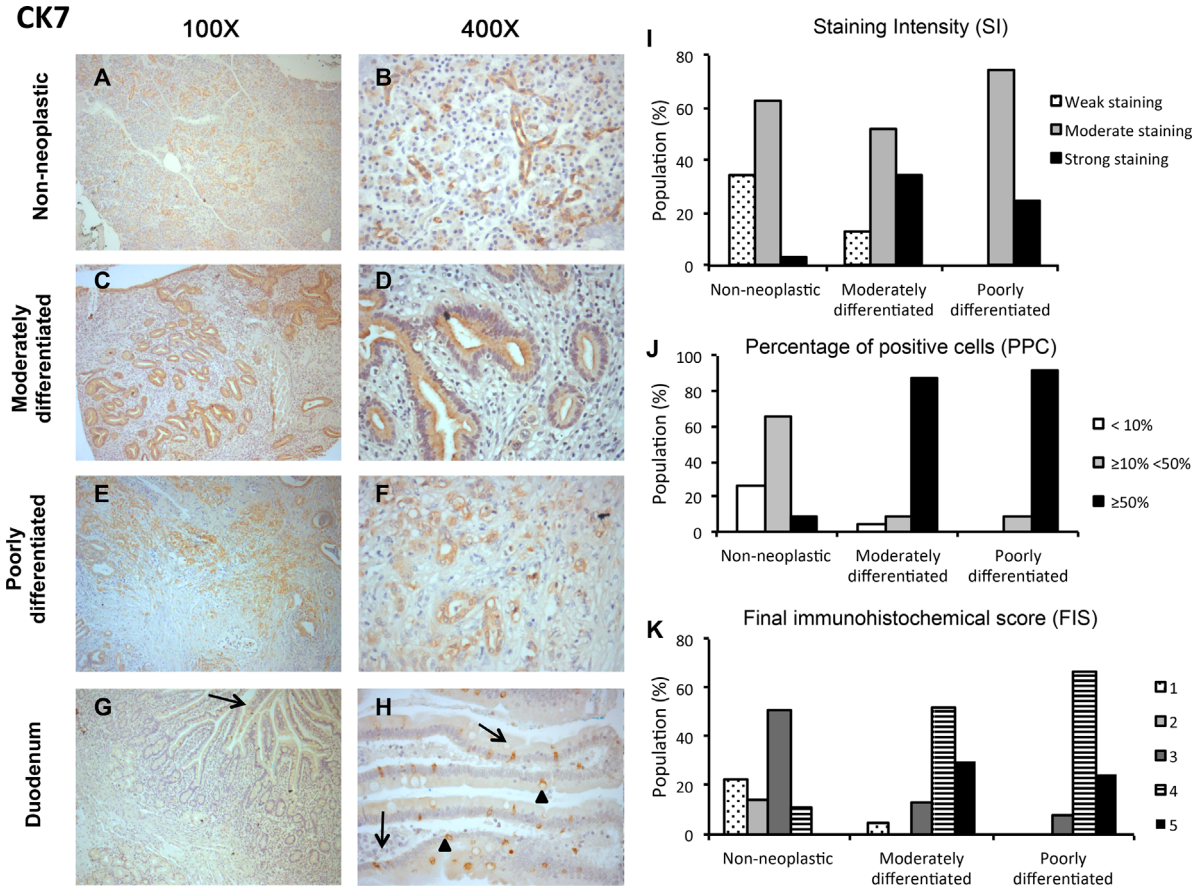

FIGURE 4 Cytokeratin 7 (CK7) expression in non-neoplastic pancreas, in moderately and poorly differentiated pancreatic ductal adenocarcinoma (PDA), and in duodenum samples. CK7 is expressed in ductal cells in non-neoplastic pancreas (A,B); this cytokeratin is also expressed in the apical plasma membrane and intracellularly in PDA cells (C-F); in duodenum (G) CK7 expression is detected in the plasma membrane of some scattered cells (H, arrows), and in goblet cells (H, triangles). The evaluation of the parameters staining intensity (I) and percentage of positive cells (J), as well as the final immunohistochemical scores (J) are represented as percentages of the total of cases for non-neoplastic ($n = 35$), moderately differentiated ($n = 23$) and poorly differentiated samples ($n = 12$). Analysis of the SI revealed a moderate staining in the majority of the population (62.9% of non-neoplastic samples, 52.2% of moderately differentiated tumors and 75.0% of poorly differentiated tumors); weak staining decreased from 34.3% in non-neoplastic population to 13.0% in moderately differentiated samples, whereas in poorly differentiated tumors weak staining was not detected at all (I). The population with a PPC ≥50 increased from 8.6% in non-neoplastic cases to 87.0% in moderately differentiated tumors and 91.7% in poorly differentiated tumors (J). 30.4% of the moderately differentiated tumors and 25.0% of poorly differentiated tumors presented the highest FIS value (5), which was not observed in any of the non-neoplastic samples (K)

pathways.[23,44] Thus, we evaluated the EGFR expression in PDA samples by immunohistochemical analysis. We found that EGFR is almost absent in normal pancreatic tissue (Fig. 5A) except for some light intracellular expression in a few acinar cells (Fig. 5B, arrows). In PDA samples EGFR expression was very heterogeneous: in some tumors a strong EGFR expression was observed in many cells while in other cases just a few cells with light staining were detected (Fig. 5C-F). In PDA samples EGFR localization also alternated from the nucleus to the cytoplasm and plasma membrane of ductal cells. In duodenum, the expression of EGFR was detected in the cytoplasm of several epithelial cells (Fig. 5G,H).

Analysis of the SI revealed that tumor samples show higher values when compared with non-neoplastic tissues (Fig. 5I). The PPC was <10 in all non-neoplastic tissues and PPC ≥50 raised with tumor severity (Fig. 5J). As shown in Fig. 5K, FIS values range between 0 and 3 in non-neoplastic cases, between one and five in moderately differentiated tumors and between two and five in poorly differentiated ones.

3.6 | E-cad expression in PDA and non-neoplastic samples

To assess eventual changes in the epithelial phenotype of PDA cells, we next performed immunohistochemical analysis of E-cad, an epithelial cell marker that is expressed in cell-cell junctions. As expected, in non-neoplastic tissues E-cad was widely expressed in intercellular junctions (Fig. 6A,B). The expression of this protein was decreased in PDA with some cells not showing any staining in intercellular junctions (Fig. 6C,D), an effect that was more evident in poorly differentiated tumors (Fig. 6E,F). E-cad was also widely expressed in intercellular junctions of epithelial cells of duodenum (Fig. 6G,H).

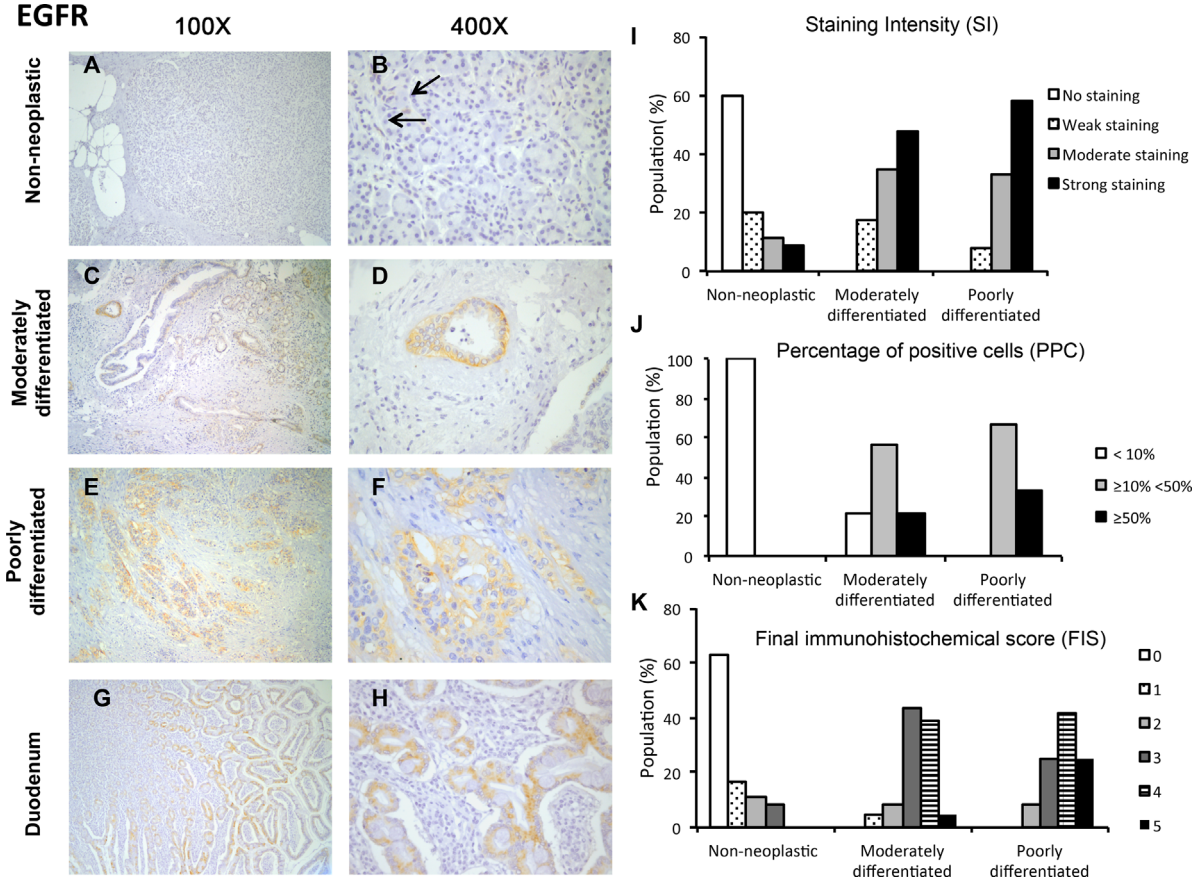

FIGURE 5 Epidermal growth factor receptor (EGFR) expression in non-neoplastic pancreas, in moderately and poorly differentiated pancreatic ductal adenocarcinoma (PDA), and in duodenum samples. In non-neoplastic tissues EGFR expression is almost absent in normal pancreatic tissue except for some light intracellular expression in some acinar cells (A and B); in PDA cells EGFR expression tend to be heterogeneous, varying from the nucleus, to the cytoplasm and plasma membrane (C-F); EGFR is detected in the cytoplasm of some duodenal epithelial cells. The evaluation of staining intensity (I) and percentage of positive cells (J), as well as the final immunohistochemical scores (J) are represented as percentages of the total of cases for non-neoplastic ($n = 35$), moderately differentiated ($n = 23$) and poorly differentiated samples ($n = 12$). The percentage of the studied population with no staining decreased from 60.0% in normal pancreatic tissues to 0 in PDA samples and strong staining was detected in 47.8% and 58.3% of moderately and poorly differentiated tumors, respectively (I). The PPC was <10 in all non-neoplastic tissues and increased to ≥10 <50 in 56.5% of moderately differentiated tumors and to 66.7% of poorly differentiated ones (J). 4.3% of the moderately differentiated tumors and 25.0% of poorly differentiated tumors presented the highest FIS value (5), which was not observed in any of the non-neoplastic samples (K)

Analysis of the SI (Fig. 6I) revealed that the majority of the population exhibited moderate staining, even though tumor samples have lower SI values as compared with non-neoplastic cases. The PPC was ≥50 in all non-neoplastic cases whereas all poorly differentiated tumors and almost moderately differentiated ones presented a PPC within the range ≥10 <50 (Fig. 6J). As shown in Fig. 6K, FIS values range between four and five in non-neoplastic cases, between three and four in moderately differentiated tumors and between two and three in poorly differentiated ones.

3.7 | Vim expression in PDA and non-neoplastic samples

To test if PDA cells display a mesenchymal phenotype, compatible with an EMT, immunohistochemical analyses were performed with Vim, a mesenchymal cell marker. In non-neoplastic samples Vim was expressed in non-epithelial cells such as fibroblasts and was present in the connective tissue surrounding acini and ducts (Fig. 7A,B). In contrast, this protein was abundantly expressed in tumoral desmoplastic stroma (Fig. 7C-F). Vim expression was also found in mesenchymal cells in duodenum, in the connective tissue of duodenal villi (Fig. 7G) and in some scattered cells infiltrated into duodenal epithelium (Fig. 7H, arrow).

Analysis of the SI revealed that tumor samples show higher values when compared with non-neoplastic cases (Fig. 7I). As shown in Fig. 7J, the PPC was ≥10 in in the majority of the cases and all of poorly differentiated tumors present values of ≥50 PPC. Accordingly, FIS values range between one and four in non-neoplastic cases and, between four and five in moderately and poorly differentiated tumors (Fig. 7K).

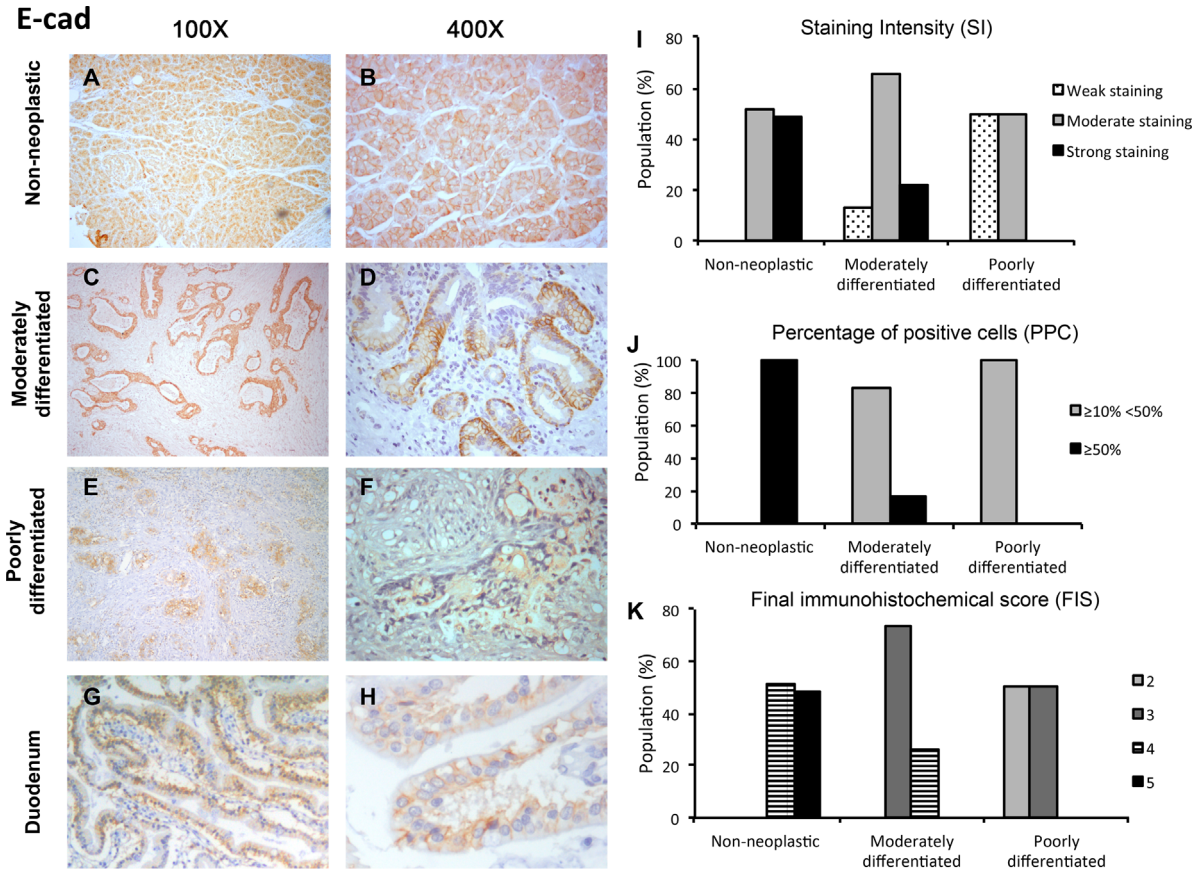

FIGURE 6 E-cadherin (E-cad) expression in non-neoplastic pancreas, in moderately and poorly differentiated pancreatic ductal adenocarcinoma (PDA), and in duodenum samples. E-cad is widely expressed in non-neoplastic tissue in intercellular junctions (A,B); E-cad expression is decreased in intercellular junctions of moderately differentiated adenocarcinoma ductal cells (C,D) and even more decreased in poorly differentiated tumors (E,F); This protein is also widely expressed in intercellular junctions of epithelial duodenal cells (G,H). The evaluation of the parameters staining intensity (I) and percentage of positive cells (J), as well as the final immunohistochemical scores (J) are represented as percentages of the total of cases for non-neoplastic ($n = 35$), moderately differentiated ($n = 23$) and poorly differentiated samples ($n = 12$). Analysis of the SI revealed that the majority of the population exhibited moderate staining (51.4% of non-neoplastic samples, 65.2% of moderately differentiated tumors and 50.0% of poorly differentiated tumors) (I). The PPC was ≥50 in all non-neoplastic cases decreasing to the range ≥10 <50 in 82.6% of moderately differentiated tumors to 100% of the poorly differentiated cases (J). 48.6% of the non-neoplastic samples presented the highest FIS value (5), which was not observed in any of the PDA samples (K)

3.8 | Evidence of AQP3 and AQP5 as potential PDA markers

Mann Whitney test was used to collectively depict the changes in the expression pattern of each of the assayed parameters, in moderately and poorly differentiated PDAs versus control tissue samples and between moderately versus poorly differentiated tumors. Absolute values of FIS, calculated as the mean value of each parameter, were represented together in Fig. 8. Additionally, to assess the relationship between AQPs and the indicators of cell proliferation (Ki-67), EMT (E-Cad and Vim), invasion (CK-7), as well as of the receptor widely involved in signaling transduction pathways (EGFR), the correlation coefficients were also determined with a Spearman rank correlation (using individual FIS values) and the statistically significant ones are represented in Fig. 8. As reported above, the expression levels of AQP5 significantly raised ($P < 0.001$) from non-neoplastic (1.57 ± 0.61) to PDA tissue, with a higher expression in the moderately differentiated tumors (4.30 ± 0.88) than in poorly differentiated ones (3.58 ± 0.67). In line with AQP5 overexpression a significant increase ($P < 0.001$) in AQP3 was also detected, continuously rising from normal pancreatic tissues (2.23 ± 0.55) to moderately (3.91 ± 0.95) and poorly differentiated tumors (4.33 ± 0.49). In poorly differentiated tumors, AQP3 expression was found to be significantly higher ($P < 0.001$) than AQP5.

Although AQP5 and AQP3 expression levels in non-neoplastic samples were significantly different, a positive correlation was found ($r = 0.359$, $P < 0.05$) between these two isoforms, suggesting a dependent role in normal pancreatic physiology. Curiously, no statistically significant correlation was obtained between AQP5 and AQP3 in PDA samples, raising the hypothesis that the overexpression of one is independent from the other in this pathology. Interestingly, in contrast to AQP5, AQP3 expression was not found to be significantly different between moderately and poorly differentiated tumors, suggesting that AQP5 is a better biomarker than AQP3 to study early disease stages.

AQP5 overexpression was accompanied by a significant ($P < 0.001$) elevation of the proliferation cell marker, Ki-67, in PDA

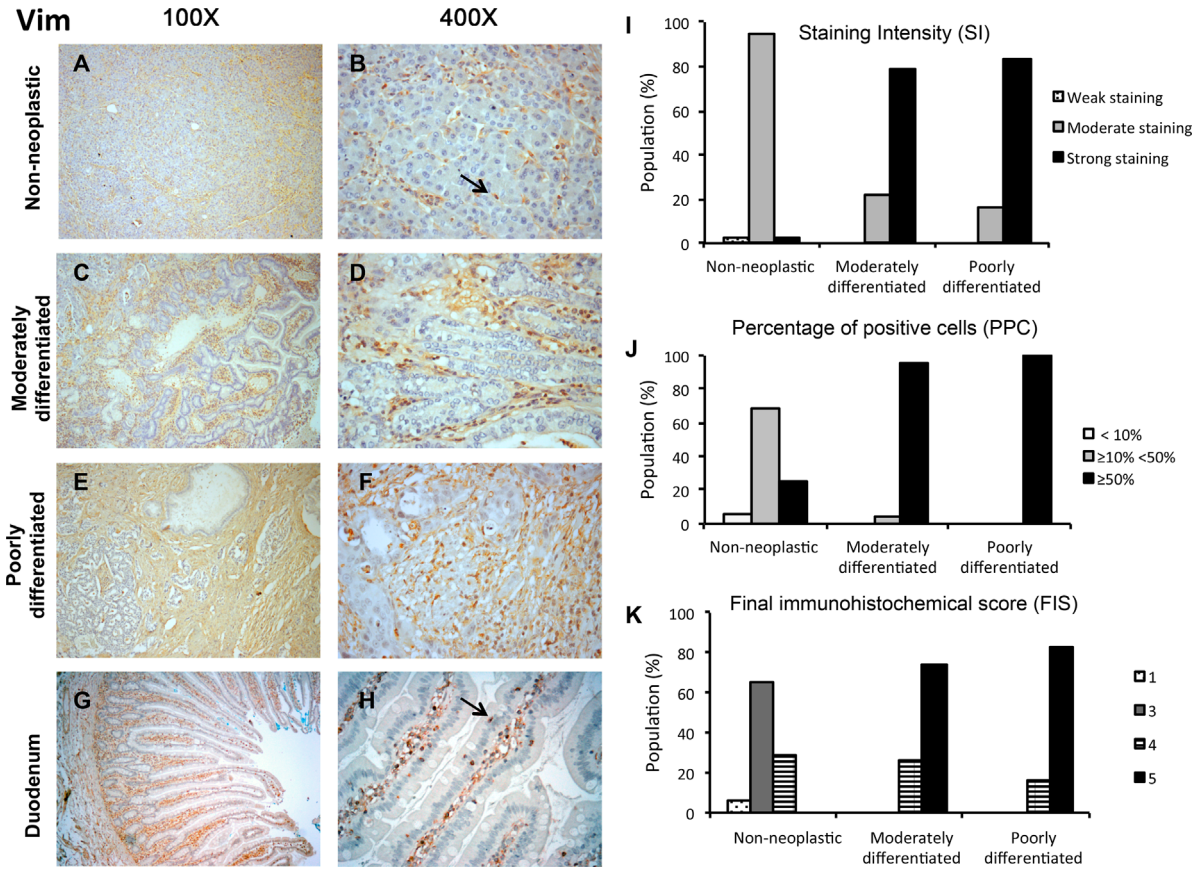

FIGURE 7 Vimentin (Vim) expression in non-neoplastic pancreas, in moderately and poorly differentiated pancreatic ductal adenocarcinoma (PDA), and in duodenum samples. Vim is expressed in nonepithelial cells such as fibroblasts and inflammatory cells (A,B); Vim is abundantly expressed in tumoral desmoplastic stroma (C-F); this protein is also abundantly expressed in connective tissue cells in duodenum (G), in villous stroma, and in some scattered infiltrating cells in duodenal epithelium (H, arrow). The evaluation of the parameters staining intensity (I) and percentage of positive cells (J), as well as the final immunohistochemical scores (J) are represented as percentages of the total of cases for non-neoplastic ($n = 35$), moderately differentiated ($n = 23$) and poorly differentiated samples ($n = 12$). Strong staining increased from 2.9% in non-neoplastic samples to 78.3% of moderately differentiated tumors and 83.3% of poorly differentiated tumors (I). The PPC was <10 in 5.7% of non-neoplastic samples whereas in tumors the PPC was always ≥10, with 95.7% of the moderately differentiated samples and all of poorly differentiated cases presenting values of ≥50 PPC (J). 73.9% of the moderately differentiated tumors and 83.3% of poorly differentiated tumors presented the highest FIS value (5), which was not observed in any of the non-neoplastic samples (K)

samples (1.91 ± 1.04 and 2.00 ± 1.04) in moderately and poorly differentiated tumors, respectively, as compared with adjacent normal pancreatic tissue (0.09 ± 0.38). Accordingly, a significant correlation coefficient ($r = 0.497$, $P < 0.05$) was observed between AQP5 and Ki-67 in moderately differentiated tumors, pointing to the usefulness of AQP5 as an early biomarker of PDA. Regarding CK7, a PDA marker, the mean FIS value significantly ($P < 0.001$) raised from 2.51 ± 0.98 in non-neoplastic samples to 4.04 ± 0.92 in moderately differentiated and 4.17 ± 0.58 in poorly differentiated tumors ($P < 0.001$), similarly to the observed for AQP5. In addition, the expression of CK7 and AQP5 in scattered cells in intestinal epithelium reflects the invasion of the duodenum by PDA positive cells. Therefore, the simultaneous overexpression of CK7 with AQP5 and AQP3 point to these proteins as biomarkers of PDA.

EGFR expression levels were found significantly increased ($P < 0.001$) from non-neoplastic samples (0.66 ± 1.00) to moderately differentiated (3.30 ± 0.88) and poorly differentiated (3.83 ± 0.94) PDAs. Curiously, no statistically significant correlation was obtained between AQP5, AQP3, and EGFR expression in PDA samples, which raises the hypothesis that the overexpression of these two isoforms may be independent of EGFR stimulation, in contrast to the upregulation proposed mechanisms through this receptor for other types of cancer.[18,24]

The changes in AQP5 expression were also accompanied by a down-regulation of E-cad (4.49 ± 0.71 in non-neoplastic tissue, 3.26 ± 0.50 in moderately differentiated and 2.50 ± 0.39 in poorly differentiated tumors, $P < 0.001$ for PDA vs non-neoplastic samples), as well as by an overexpression of Vim (2.50 ± 0.51 in non-neoplastic tissue, 4.74 ± 0.50 in moderately differentiated and 4.83 ± 0.52 in poorly differentiated tumors, $P < 0.001$ for PDA vs non-neoplastic samples). These results indicate that AQP5 overexpression is concomitant with a decreased expression of the epithelial marker and an increased expression of the mesenchymal marker, implying the occurrence of an EMT, which is known as a pivotal event in cell

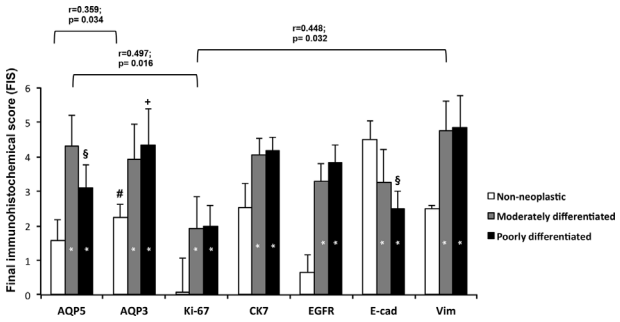

FIGURE 8 Evidence of aquaporins (AQPs) as potential markers of pancreatic ductal adenocarcinoma (PDA). Protein expression levels of AQP5, AQP3, Ki-67, cytokeratin 7 (CK7), epidermal growth factor receptor (EGFR) and vimentin (Vim), evaluated as final immunohistochemistry scores (FIS) were significantly higher in PDA than in adjacent non-neoplastic pancreatic tissues. On the contrary, E-cadherin (E-cad) protein levels were found to be significantly down-regulated. A continuous variation from non-neoplastic to moderately and poorly differentiated pancreatic tissue was observed for all the parameters, except for AQP5 for which the greatest change was observed for moderately differentiated tumor samples. AQP3 FIS values were significantly higher than AQP5 FIS values in non-neoplastic tissues and in poorly differentiated tumors. Positive correlations were found between AQP5 and AQP3 in non-neoplastic samples ($r = 0.359$; $P = 0.034$), as well as between AQP5 and Ki-67 ($r = 0.497$; $P = 0.016$), and between Ki-67 and Vim ($r = 0.448$; $P = 0.032$) expression in moderately differentiated PDAs. $^{*}P < 0.001$ versus non-neoplastic; $^{\S}P < 0.001$ versus moderately differentiated tumors; $\#P < 0.001$ versus AQP5 control; $+P < 0.001$ versus AQP5 in poorly differentiated tumors; Correlation coefficients (r)

malignancy.[8] Supporting this assumption, a significant correlation ($r = 0.448$; $P < 0.05$) was also observed between the proliferation marker, Ki-67, and the mesenchymal marker, Vim.

To evaluate AQP3 and AQP5 sensitivity and specificity, a receiver operating characteristic curve (ROC) analysis was used as a screening measure. Data from the 23 moderately differentiated tumors and from the 12 poorly differentiated tumors were compared with the control samples for AQP3 and AQP5 expression. The area under the ROC curve (AUC) was 0.426 ($P = 0.317$) and 0.920 ($P < 0.001$) for AQP3 and AQP5 detection in moderately differentiated tumors, respectively (Supplementary Fig. S1A). On the other hand, in poorly differentiated tumors, AUC was 0.615 ($P = 0.213$) and 0.700 ($P = 0.030$) for AQP3 and AQP5 detection (Supplementary Fig. S1B). These results indicate that whereas AQP3 may help distinguishing between benign and poorly differentiated PDA, AQP5 has high specificity and sensitivity for moderately differentiated tumors, suggesting it may be a useful and potent biomarker to detect early stages of the disease.

3.9 | Relationship between clinicopathological features and AQP5, AQP3, Ki-67, CK7, EGFR, E-cad, and Vim expression

The clinicopathological features and their relationships with FIS values of AQP5, Ki-67, CK7, EGFR, E-cad, and Vim were compared using a Fisher's exact test and are summarized in Table 2. From the 35 patients analyzed, 19 (54.3%) were males, and 16 (45.7%) were females. Histological examination revealed that ductal adenocarcinomas were predominantly moderately differentiated (65.7%). The pathologic stages after surgical resection were as follows: stage I ($n = 4$; 11.4%) and stage II ($n = 31$; 88.6%). PDAs were predominantly bigger than 2.5 cm in major axis (60.0%) and were located in the head of the pancreas (82.9%), in the body of the pancreas (2.9%) or in the ampulla of Vater region (14.3%). Lymph node metastasis was identified in 71.4% of the patients. Perineural, vascular, and intestinal invasion were positive in 60.0%, 34.3%, and 60.0% patients, respectively. The expression of the studied proteins was not associated with gender, stage, tumor dimensions, perineural, and intestinal invasion. Interestingly, AQP5 and E-cad expression levels were significantly related with tumor differentiation (AQP5, $P < 0.05$; E-cad, $P < 0.001$): AQP5 and E-cad expression were higher in moderately differentiated adenocarcinomas than in poorly differentiated ones, pointing to AQP5 as an early biomarker of epithelial characteristics loss along PDA progression. In turn, Ki-67 expression was significantly correlated with tumor location ($P < 0.05$) in the head of the pancreas. Interestingly, EGFR expression was related with lymph node metastasis ($P < 0.05$) and with vascular invasion ($P < 0.05$), raising the hypothesis that this signaling receptor is involved in the process of PDA dissemination through lymph and blood circulation.

3.10 | Prognostic values of AQP3 and AQP5 in PDA patients

Survival analysis of 34 of the 35 studied patients was performed using information available on clinical follow-up and the actuarial survival rate was used to calculate the overall survival (OS). As shown in Fig. 9A, both AQP3 and AQP5 expression showed to follow the short overall survival of PDA patients. Although the median OS for the groups with higher and lower AQP3 expression (considered as FIS ≥3 or FIS ≤2) was 251 days and 451 days, respectively, the difference was not statistically different from the OS of the total PDA population (246 days) ($P = 0.979$). However, patients with tumors expressing higher levels of AQP3 appear to have a poorer prognosis in an interval of 2 years of follow-up. On the other hand, the OS was 514 days and 251 days, for the patients with high and low AQP5 expression (considered as FIS ≥5 or FIS ≤4), respectively, unveiling a trend towards a different behavior ($P = 0.0653$). Considering that AQP5 was markedly expressed in moderately differentiated tumors, we further analyzed the OS within this sub-group (Fig. 9B). Interestingly, the medians OS of higher and lower AQP5 expressers (649 and 251 days) were significantly different ($P = 0.0181$), with prolonged survival for the highest AQP5 expression, suggesting that AQP5 may have a prognostic significance for the less aggressive PDA stage.

4 | DISCUSSION

In the current study, we analyzed the expression pattern of AQP3 and AQP5, in parallel with that of widely used markers of cell proliferation (Ki-67), PDA (CK7), epithelial cells (E-cad), and mesenchymal cells

TABLE 2 Relationship between Aquaporin 5 (AQP5), Aquaporin 3 (AQP3), KI-67, Cytokeratin 7 (CK7), Epidermal growth factor receptor (EGFR), E-cadherin (E-cad) and Vimentin (Vim) final immunohistochemical scores (FIS) in PDA samples and clinicopathological features

Parameters	*n*	AQP5 *P*-value	AQP3 *P*-value	Ki-67 *P*-value	CK7 *P*-value	EGFR *P*-value	E-cad *P*-value	Vim *P*-value
Gender		0.284	0.470	0.347	0.937	1.000	0.486	0.424
Male	19 (54.3%)							
Female	16 (45.7%)							
Tumor differentiation		**0.030**	0.274	1.000	0,922	0.654	**3.47×10^{-4}**	0.685
Moderately	23 (65.7%)							
Poorly	12 (34.3%)							
Stage		0.586	0.849	0.417	0.619	0.690	0.251	0.553
I	4 (11.4%)							
II	31 (88.6%)							
Tumor Dimensions		0.852	0.686	0.171	0.480	0.563	0.889	0.108
<2.5 cm	14 (40.0%)							
≥2.5 cm	21(60.0%)							
Tumor Location		0.899	0.894	**0.021**	0.124	0.573	0.106	0.466
Head	29 (82.9%)							
Body	1 (2.9%)							
Ampulla	5 (14.3%)							
Lymph node metastasis		0.921	0.102	0.944	0.137	**0.019**	0.540	0.390
Positive	25 (71.4%)							
Negative	10 (28.6%)							
Neuronal invasion		0.070	0.210	0.656	0.514	0.190	0.678	0.108
Positive	21 (60.0%)							
Negative	14 (40.0%)							
Vascular invasion		0.263	0.193	0.685	0.922	**0.014**	0.868	0.402
Positive	12 (34.3%)							
Negative	23 (65.7%)							
Intestinal invasion		0.209	0.897	1.000	0.871	0.634	1.000	1.000
Positive	21 (60.0%)							
Negative	14 (40.0%)							

Data in bold correspond to *P*-values with statistical significance.

(Vim), as well as of a signaling transduction receptor involved in carcinogenesis (EGFR), in 35 human PDA samples and matched adjacent non-neoplastic tissues (in total 70 samples). To the best of our knowledge this is the first study to evaluate AQPs expression and their clinical significance in PDA patients. Since PDA is one of the most intractable and mortal types of cancer, the finding of new biomarkers that could be used to detect early stages of the disease is imperative.

The primary mechanisms supporting AQP3 and AQP5 involvement in cancer development are still under debate, but based on the literature and on our data a proposed model for their possible involvement in PDA tumorigenesis is depicted in Fig. 10. AQP5 is expressed at the apical membrane of tumoral ductal cells facilitating water transport through cell membranes, which is essential for lamellipodium formation and, consequently, for cell migration and spread. On the other hand, glycerol transported by AQP3 can be used for lipid biosynthesis and for the production of ATP, supporting cell processes like migration and proliferation. In addition, the uptake of extracellular H_2O_2 via AQP3 or AQP5 may modulate intracellular signaling pathways. Thus, AQP5 and AQP3 overexpression together with EGFR upregulation may stimulate activation of intracellular transduction cascades, leading to cell proliferation, transformation, and invasion (increase in Ki-67 and CK7 expression), and enhancing EMT (E-cad downregulation and Vim overexpression in PDA samples).

In fact, in non-neoplastic pancreas we found that AQP5 is expressed at the apical membrane of intercalated and intralobular ductal cells while AQP3 is expressed in the plasma membrane of some acinar cells, in accordance with previous studies.[38,39] These two isoforms seem to have an interdependent role in normal pancreatic physiologic processes as supported by the positive correlation found between AQP5 and AQP3. On the other hand, in PDA cells AQP5 is upregulated, being mainly expressed all over the plasma membrane of ductal cells and becoming to diffuse intracellularly in less differentiated tumors, while AQP3 becomes to be overexpressed in ductal cells. In addition, AQP5 expression was found to be related with tumor differentiation. Actually, it seems that moderately differentiated tumors, that recapitulate better the morphological ductal

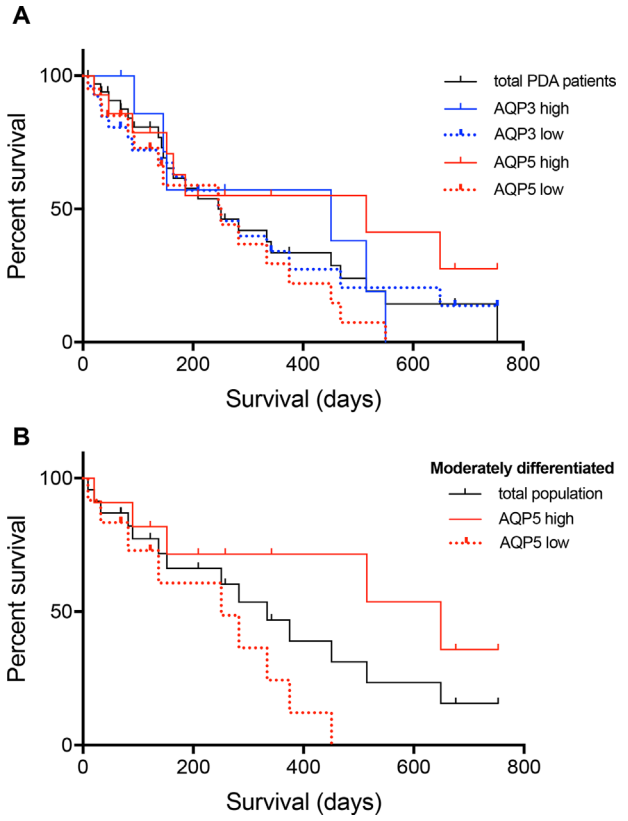

FIGURE 9 Kaplan-Meier survival curves of the PDA patients. Overall survival of PDA patients independently of the tumor stage (black line), and of patients with tumors expressing higher (FIS ≥3) and lower (FIS ≤2) levels of Aquaporin 3 (AQP3) (blue line) and Aquaporin 5 (AQP5) (red line) (A). Overall survival of PDA patients with moderately differentiated tumors (black line), and those expressing higher (FIS ≥5) (red line) and lower (FIS ≤4) (red dotted line) levels of AQP5 ($P < 0.05$) (B)

characteristics of normal pancreas, are higher AQP5-expressors than the poorly differentiated ones. On the contrary, the highest AQP3 expression levels were found in poorly differentiated tumors that are usually more aggressive and later stages of the disease. Since tumorigenesis of PDA occurs in ductal epithelial cells and in moderately differentiated tumors a positive correlation was found between AQP5 and Ki-67, one of the most important markers of active cellular proliferation, this indicates that AQP5 may play an important role in PDA progression and proliferation, as reported for other types of cancer[12–14,20,22,30,45–50] and depicted in our proposed model (Fig. 10). In addition, being a water channel protein, AQP5 facilitates water transport through cell membranes which is essential for lamellipodium formation and, consequently, for tumor cell migration and spread, as described for other types of cancer.[23] Regarding AQP3, its ability to transport glycerol that can be used for lipid biosynthesis and for ATP production may be advantageous for cell proliferation and migration in poorly differentiated tumors. In addition to water and glycerol, AQP3[25] and AQP5[26] also facilitate H_2O_2 permeation that may be required for activation of the EGFR-mediated cell signaling cascade in cancer cells.[32] Our findings indicate that AQP5 overexpression is an early event in PDA progression and is independent of AQP3 overexpression since no statistically significant correlation was found between the expression of these two AQPs isoforms. Moreover, our data, supported by ROC analysis, suggest that AQP5 may be a useful novel sensitive and specific marker for early stages of PDA while AQP3 expression seems to be related with late and more aggressive stages of the disease.

A comparison of actuarial survival curves of low and high expressers of AQP3 and AQP5 showed a differential picture between the overexpression of these two isoforms in PDA. A trend toward a better prognosis for patients with AQP5 overexpressing tumors and a poorer prognosis for patients with AQP3 overexpressing tumors, which are generally poorly differentiated and more aggressive, can be inferred by the analysis of OS in a 2-years follow-up period. In fact, differences between high and low AQP5 expressing tumors were found to be nearly statistically significant when the total PDA cohort was considered. Moreover, focusing on the moderately differentiated group where AQP5 is mostly expressed, a clear and significant difference could be detected. These findings suggest that the overexpressions of these two isoforms are specific events in different stages of PDA development and progression and that AQP5 overexpression in moderated differentiated tumors might be of prognostic value, raising the interest in further validation.

Previous studies reported that both AQP3[24] and AQP5[18] are upregulated by EGFR signaling and that these two isoforms may directly activate this receptor. Our study revealed that the elevation of AQP3 and AQP5 expression in PDA is observed in parallel with an increase of EGFR expression, remaining uncertain whether the upregulation of AQPs leads to the activation of EGFR or the other way around, or if these are unrelated events. In PDA samples the EGFR expression was found to be related with lymph node metastasis and with vascular invasion. These results are in accordance with previous studies in which EGFR activation results in the transcription of genes involved in cell growth and proliferation and, consequently, its expression is associated with poor prognosis and increased invasiveness.[34,35]

Interestingly, we found AQP5 expression in goblet cells in duodenum, which was not previously described for small intestine although it is known that this isoform is expressed in goblet cells of other organs, like lungs, being involved in mucous hyperproduction.[51] We also found AQP5 expressed in the cytoplasm and plasma membranes of some scattered cells in duodenal epithelium only when intestinal invasion was present. These fusiform cells in duodenum also express CK7 that is known to be expressed in almost all stages of PDA,[5] and Vim that is only expressed by mesenchymal cells. It is certain that CK7 is also expressed by primary adenocarcinomas of the small intestine,[5] making it impossible to distinguish these tumors from PDA. However, primary tumors from small intestine are extremely rare,[5] which makes this possibility very unlikely. Since in normal conditions duodenal epithelial cells do not express AQP5,[40] neither CK7[5] or Vim,[52] these observations support the idea that AQP5-duodenal expresser cells are, in fact, PDA migrating cells that escaped from the primary tumor and infiltrated the intestinal epithelium. Therefore, these results suggest that AQP5 could also be used as a sensitive and specific marker to discriminate intestinal infiltration by PDA cells.

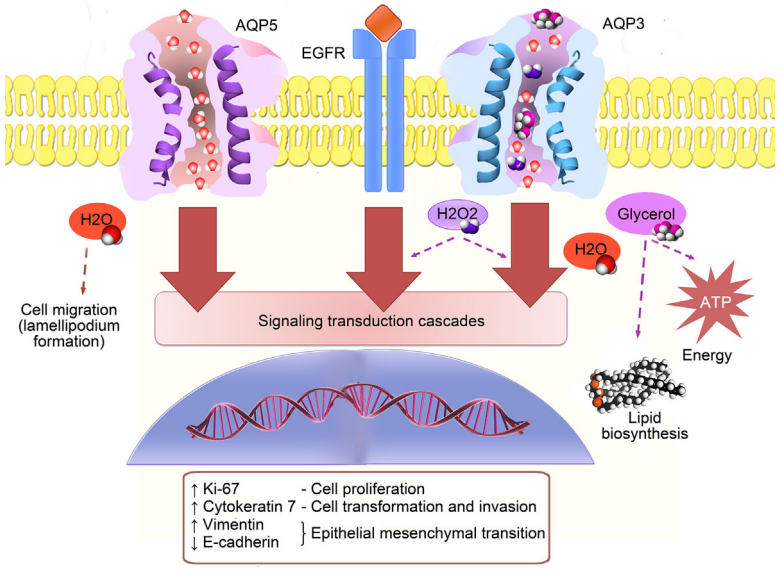

FIGURE 10 Proposed model for AQP3 and AQP5 involvement in pancreatic ductal adenocarcinoma (PDA) development. In PDA cells the overexpression of AQP5, AQP3, and EGFR lead to the activation of signaling transduction pathways known to induce cell proliferation, transformation and invasion, as well as epithelial mesenchymal transition (EMT), events that are supported by the increase in Ki-67, upregulation of CK7 as well as the concomitant decrease in E-cadherin and increase in vimentin expression. Moreover, AQP5 overexpression facilitates water permeation through cell membranes which is essential for lamellipodium formation and, consequently, for cell migration and spread. AQP3 overexpression enhances glycerol uptake to be used for lipid biosynthesis and energy (ATP) production, required for cell division and migration. Additionally, extracellular hydrogen peroxide (H_2O_2) permeation via AQP3 modulates signaling pathways that may contribute to the observed outcomes of PDA

In this study, we also observed that AQP3 and AQP5 overexpression are accompanied by a downregulation of the epithelial adhesion molecule, E-cad, and by an upregulation of the mesenchymal marker Vim. Actually, poorly differentiated tumors that have a less cohesive morphology, being mainly constituted by isolated infiltrative cells and disrupted ducts, showed lower expression levels of E-cad than moderately differentiated ones. This fact, together with the opposite change in Vim expression, translates the occurrence of EMT (Fig. 10), which is pivotal for metastasization. This suggests that AQP5 and AQP3 may participate in the process of EMT in PDA, as described for other tumors.[29,36] The significant correlation found between Vim and the proliferation cell marker, Ki-67, supports the assumption that EMT contributes to tumor invasion and metastasis.[8] Also relevant is the relationship between E-cad expression and tumor differentiation, similarly to the observed for AQP5.

Altogether our results suggest that AQP3 and AQP5 are involved in PDA development and progression. Whereas AQP3 appears related with late and more aggressive stages of PDA, AQP5 emerges as a potential novel histological marker for early stages of PDA. The fact that AQP5 overexpression is related with tumor differentiation independently of AQP3, suggests that AQP5 per se is involved in PDA development and may be a useful therapeutic target. Although the mechanisms underlying AQP3 and AQP5 differential expression in PDA tumorigenesis require further clarification, our findings highlight for the first time the usefulness of AQP5 in early PDA diagnosis and histological detection of intestinal infiltration.

5 | STUDY LIMITATION

Though our work generated important findings that may prove useful for PDA detection and follow-up, a study limitation due to relatively small sample size should be acknowledged. Nevertheless, it is worth mentioning that pancreatic cancer is a rare type of cancer compared to other tumors, accounting for only 3% of the newly diagnosed cancer worldwide. Its incidence ranges from 1 to 10 cases/100 000 inhabitants, being slightly higher in developed countries. Overall survival at 5 years, for all stages, is currently 6%.

Additionally, among a larger number of diagnosed patients in our hospital, only confirmed PDAs were included in the study, which helped reinforcing sample homogeneity but reduced the number of patients analyzed.

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ETHICAL APPROVAL

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or

national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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