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SMAD4 and TGFβR2 expression in pancreatic ductal carcinoma

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Abstract

Pancreatic ductal carcinoma is the most common type of pancreatic cancer, and currently represents the fourth cause of death by cancer, worldwide. Among classical pancreatic markers that ascertain the histopathology, new emerging targets have been proposed for both diagnostic and prognostic purposes. In the present study, utilizing a group of 28 confirmed resected pancreatic ductal carcinomas, we have assessed the immunoexpression and correlation ratios of mothers against decapentaplegic homolog 4 (*Drosophila*) (SMAD4)/transforming growth factor beta receptor 2 (TGFβR2), and vimentin/cluster of differentiation 105 (CD105). SMAD4 showed an overall increase in tumors *versus* pancreatic control tissue, but a decrease from G1 towards poorly differentiated tumors, while TGFβR2, vimentin and CD105 showed higher expression values in the tumor areas. Vimentin–CD105 colocalization degree decreased in tumor tissues compared to controls, illustrating a desynchronization of these two markers, both of them being negative in the tumor epithelia. Altogether, it is highly plausible that all these key players revolve around the epithelial-to-mesenchymal transition phenomenon, and this itself modulates the clinical outcome of the patient.

Keywords: pancreatic ductal carcinoma, SMAD4, TGFβR2, vimentin, CD105.

Introduction

The incidence of pancreatic cancer varies significantly in the world, the highest incidence rates being reported in Europe (7.7 per 100 000 people) and North America (7.6 per 100 000 people) [1]. The lowest rates (2.2 per 100 000 people) were observed in Africa [2]. In 2018, were registered 458 918 new cases of pancreatic cancer worldwide, representing 2.5% of all cancers. In Romania, in 2013, epidemiological data showed an incidence of 7.9 cases/100 000 habitants [1].

Based on the *GLOBOCAN 2018* estimates, pancreatic cancer causes more than 432 242 deaths per year, ranking as the seventh leading cause of cancer death in both sexes together [3]. In Europe, the number of pancreatic cancer deaths increased with 62% over the past few decades (56 072 deaths in 1992 to 90 591 deaths in 2016) [4]. The highest incidence and mortality rates of pancreatic cancer are observed in developed countries.

Pancreatic ductal adenocarcinoma (PDAC) is the most frequent type of pancreatic carcinoma, with the second most common type, acinar cell carcinoma, representing less than 10% of all cases [5]. For ascertaining the diagnosis of PDAC a large number of immunomarkers have been reported to be useful, including: cytokeratin (CK) 7, CK18, CK19, carcinoembryonic antigen (CEA), cancer antigen 19-9 (CA19-9), mucin (MUC) 1, MUC2, MUC5AC, mammary serine protease inhibitor (Maspin), mesothelin, placental S100, IMP3, von Hippel–Lindau tumor suppressor gene protein (pVHL), and p53 [6].

In addition to the previously markers, novel markers are being evaluated to provide an earlier and a more accurate detection and prediction of pancreatic cancer [7]. Thus, mothers against decapentaplegic homolog 4 (*Drosophila*) (SMAD4), annexin A10, plectin 1, aldo-keto reductase family 1 member B10 (AKR1B10) have been reported to differentiate PDAC from benign or reactive conditions [8].

Recent studies confirmed that the tumor expression and interplay between of transforming growth factor beta (TGFβ)/SMAD4 plays a pivotal role in cancer prognosis and survival of the patients with pancreatic carcinoma [9]. The down-regulation of SMAD4 results in loss of TGFβ/SMAD4 by inducing cell cycle arrest and apoptosis at early stages of tumor formation [10], however the relationship is not clear, nor it is clear how does it is related to tumor grading and disease staging.

In the present work, we aimed to evaluate the expression patterns of SMAD4/transforming growth factor beta receptor 2 (TGFβR2) signaling targets, together with the stromal partners vimentin/CD105, and seek for any putative correlations between their expression levels and the tumor/control statuses, as well as for any stratification depending on the tumor grading.

Patients, Materials and Methods

This study included 28 patients diagnosed with pancreatic ductal carcinoma (Figure 1), with ages varying between 32 and 75 years old, with an average of 62.32±9.67 years, and normal pancreatic tissue collected from five patients that died of non-pancreatic diseases (54–74 years old, 63.2±8.58 years). Each patient gave a written informed consent approving the participation in the study. The work was also approved by the Ethics Committee of the University of Medicine and Pharmacy of Craiova (No. 123/16.05.2017).

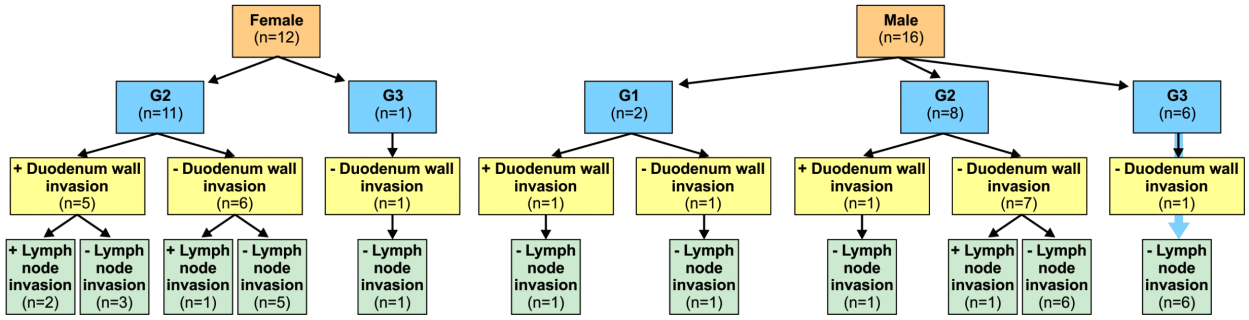

Figure 1 – ***Features of the patients included in this study.***

Selected paraffin blocks have been recut and routinely stained for Hematoxylin–Eosin (HE) for diagnostic ascertaining. Then new slides have been cut and further utilized for immunohistochemistry (IHC). For simple enzymatic IHC, the slides have been de-paraffinized, rehydrated to decreasing ethanol concentrations until distilled water. Next, antigen retrieval was performed by microwaving the slides in 0.1 M citrate buffer, pH 6, for 21 minutes, at 650 W. After cooling down to room temperature, the sections were incubated for 30 minutes in 0.1% water peroxide for blocking the endogenous peroxidase activity, further washed in 1× phosphate buffered saline (1×PBS), and then incubated in 1% skimmed milk for blocking the unspecific antigenic binding sites. Next, the primary antibodies were added on the slides (Table 1) for 18 hours, at 4°C. Next day, the sections were washed in 1×PBS, then a species-specific Horseradish peroxidase (HRP)-labeled polymeric amplification was further performed for one hour (Vector Laboratories, Peterborough, UK), then the signal was detected with 3,3'-Diaminobenzidine (Histofine® DAB-2V, Nichirei Bioscience, Tokyo, Japan). Slides were finally coversliped after a mild Hematoxylin counterstaining.

Table 1 – ***The antibodies utilized for immunohistochemistry***

Name	Species	Clone	Dilution	Antigen retrieval	Producer
CD105	Rabbit	Polyclonal	1:100	Citrate buffer, pH 6	Thermo Scientific
SMAD4	Mouse	4G1C6	1:300	Citrate buffer, pH 6	Antibodies Online
TGFβR2	Rabbit	Polyclonal	1:600	Citrate buffer, pH 6	Bioss Antibodies
Vimentin	Mouse	IgG1k	1:100	Citrate buffer, pH 6	Dako

CD105: Cluster of differentiation 105; SMAD4: Mothers against decapentaplegic homolog 4 (*Drosophila*); TGFβR2: Transforming growth factor beta receptor 2; IgG: Immunoglobulin G.

For fluorescence IHC, after antigen retrieval and unspecific antigenic sites blocking, the sections were incubated with the primary antibody pairs [SMAD4–TGFβR2 or vimentin–cluster of differentiation 105 (CD105)] for 18 hours, at 4°C. Next day, the excess antibodies were washed away and slides incubated for a further one hour with a mixture of anti-mouse and anti-rabbit Alexa 488 and Alexa 596 secondary antibodies (1:300, Thermo Fisher Scientific, Waltham, USA). In the end, the sections were coversliped with a 4',6-Diamidino-2-phenylindole (DAPI)-containing mounting media (Vectashield, Vector Laboratories).

Slide imaging was performed utilizing a Nikon 90i microscope equipped with a stage scanner, piezoelectric motorized stage, plan apochromatic objectives, a 16 Mp Nikon DS-Ri2 color complementary metal–oxide semiconductor (CMOS) camera, and the Nikon NIS-Elements AR image analysis software. Transmitted light microscopy images were utilized for exemplification purposes only, while fluorescence images were used for semiquantitative analysis. Briefly, in each image, the green and red color channels have been analyzed for total signal areas, as well as colocalization percentages, then the values from each patient have been averaged. In the end, all the values of the patients from each group of interest have been averaged (tumors *vs.* controls, different tumor grading groups), and reported as average ± standard deviation (SD). Colocalization values have been reported as the overlap coefficient. Moreover, in order to be able to consider the tumor epithelium only, the same images have been first manually processed by cutting away the stroma utilizing a hand lasso tool in Adobe Photoshop CS2 (Adobe Inc., USA). All data have been exported and analyzed in Microsoft Excel utilizing Student's *t*-testing (paired comparisons) or analysis of variance (ANOVA) testing (multiple groups comparison). In all statistics, $p<0.05$ was deemed significant.

Results

After ascertaining the pathology grading once more according to the *World Health Organization* (WHO) guidelines [6], we have first characterized the expression patterns of SMAD4 and TGFβR2 in both control pancreas tissue and ductal adenocarcinoma (ADK) cases ranging from well-differentiated tumors (G1) to moderate (G2) and poorly differentiated forms (G3) (Figure 2, A and B).

SMAD4 was expressed at a low level in the acinar cells of the control pancreatic tissue (both nuclear and membranous), and more intense in the cytoplasm/nuclei of the cells of Langerhans islets (Figure 2C). The staining was diffuse, and with a nuclear/cytoplasmic pattern. In ADK cases, the staining seemed to be less intense, sometimes mostly membranous in the epithelial cells, with a great variability between different tumor areas, a fact that made intensity more heterogeneous (Figure 2D). For both control and tumor cases, there was no stromal staining for SMAD4, so the overall staining data was only originating in the epithelia. TGFβR2 had a much more diffuse and intense staining, in both the epithelia and stroma of both control (Figure 2E), and tumor cases (Figure 2F). In ADK cases, however, TGFβR2 seemed to be more intense in both the stromal and epithelial areas, compared to controls.

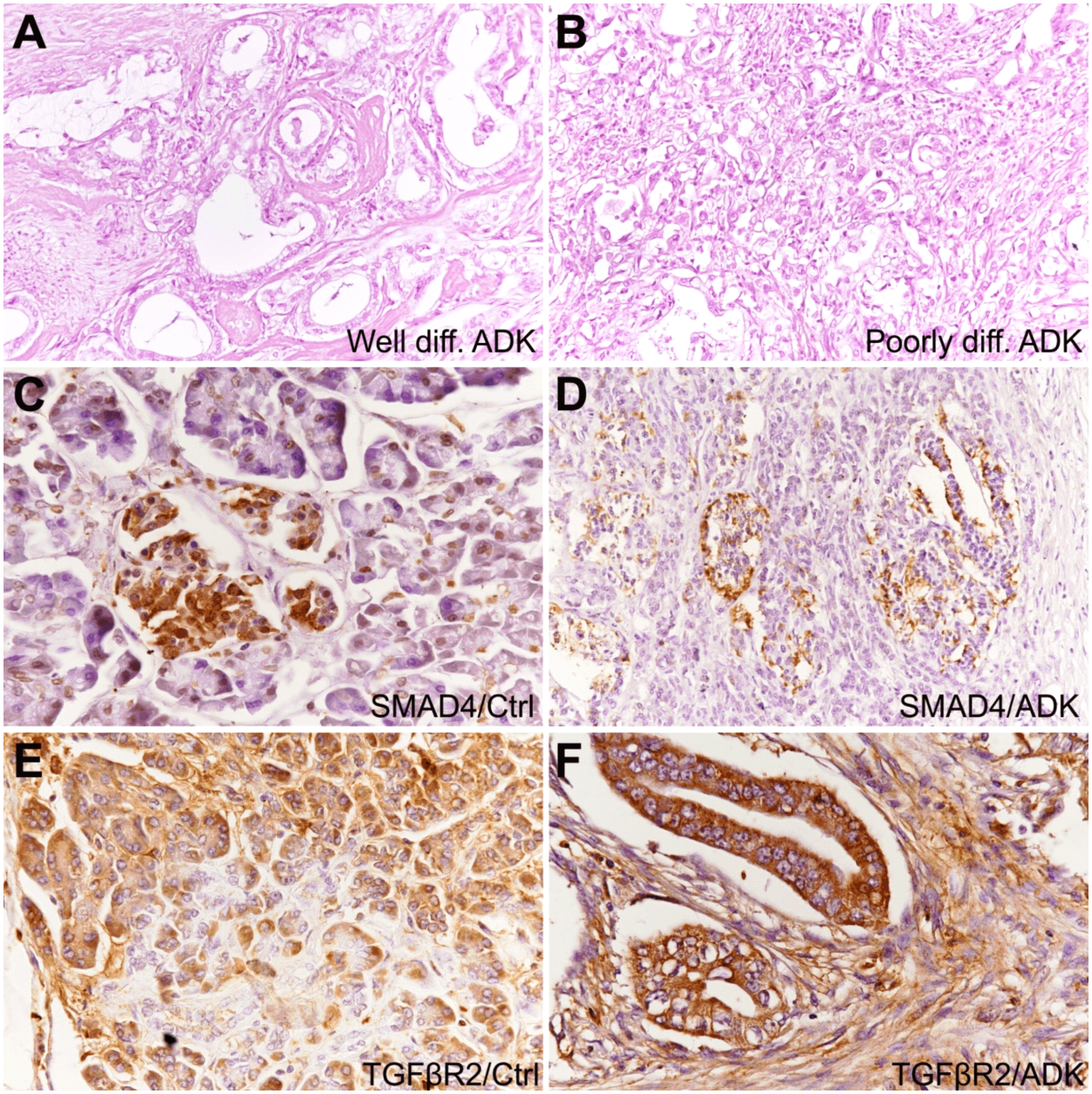

Figure 2 – ***Morphology of well and poorly differentiated ductal ADK (A and B). SMAD4 is expressed mainly in the Langerhans islets (C), while the signal is also present in the glandular epithelia in tumors (D). TGFβR2 is highly expressed in both the stroma and the epithelia, in both tumors and the controls (E and F). Enzymatic immunohistochemistry: (A and B) 200×; (C–F) 400×. ADK: Adenocarcinoma; Ctrl: Control; SMAD4: Mothers against decapentaplegic homolog 4 (Drosophila); TGFβR2: Transforming growth factor beta receptor 2.***

We next wanted to assess semi-quantitatively the expression levels of SMAD4 and TGFβR2, as well as their colocalization degree (Figure 3, A–F). Direct observation revealed that SMAD4 had mostly a membranous expression in tumor ducts, with mostly granular and cytoplasmic expression in control tissue. Since SMAD4 is expressed mainly in the epithelium, we have analyzed here first only tumor epithelia, and did not consider stroma. Overall, both SMAD4 and TGFβR2 signals were higher than for control tissue, the difference being significant only for SMAD4 measurements ($p<0.05$) (Figure 3G). As expected, SMAD4 signal areas were significantly lower compared

to TGFβR2, for both control and tumor cases. We next wanted to see how the two markers are distributed for the G1–G3 tumor gradings (Figure 3H). While TGFβR2 showed a mild decrease from G1 to G3, there was an abrupt and significant decrease (ANOVA) for SMAD4 between G1 and G2–G3 group, with practically no variations in the G2–G3 group for both markers. SMAD4 had constantly lower expression compared to epithelial TGFβR2, however a significant difference was recorded only for the G2 cases.

Next, we have looked at the colocalization degrees between the two pairs of studied markers (Figure 3I). There was a clear-cut higher colocalization of the two markers for tumor areas for both epithelia only or all tissue pooled together (epithelia and stroma) (p<0.01). Basically, this might be explained by the fact that the bulk of the TGFβR2 is stromal, and thus is not able to alter this ratio in either instance. Both markers were mostly membranous, and besides colocalized areas, seemed to be expressed also in distinct membrane segments.

We also looked at the expression and co-expression of stromal elements known for driving tumorigenesis and epithelial-to-mesenchymal differentiation, *i.e.*, TGFβR2, CD105 and vimentin (Figure 4, A–F). As expected, all three markers had higher expression in tumor areas compared to controls (Figure 4G). The biggest difference was recorded for CD105 and vimentin (p<0.001). However, TGFβR2 had the highest amplitude, with significantly higher values compared for both the other two stromal markers, for both control and tumor areas (p<0.001). In order to assess the vimentin–CD105 colocalization, we have followed only the whole tissue analysis as both markers showed the strongest expression in the stroma. Overall, there was a significant lower colocalization degree for the tumor tissue compared to controls (Figure 4H), a result that might be explained by the fact that although both CD105 and vimentin increase mostly in the tumor stroma (and to a much lesser extent in the epithelia), CD105 remains restricted to the vascular epithelium and vimentin expands more in the surrounding stroma, beneath or even in the tumor epithelia that suffer epithelial-to-mesenchymal transition (EMT). Besides calculating the colocalization degree of vimentin with CD105, morphological expression patterns of the two markers in the stroma showed that in well-differentiated tumors they were mainly expressed in the blood vessels, while in poorly differentiated tumors both signals were also expressed in tumor cells, probably as a result of gaining a mesenchymal phenotype.

There was no significant age-related or grading-related correlation with the expression areas or colocalization coefficients (Pearson's correlation coefficient varying between -0.17 to 0.2) for all markers studied, either due to the relative low number of patients, or to the heterogeneity of the histopathological gradings amongst the tumor cases.

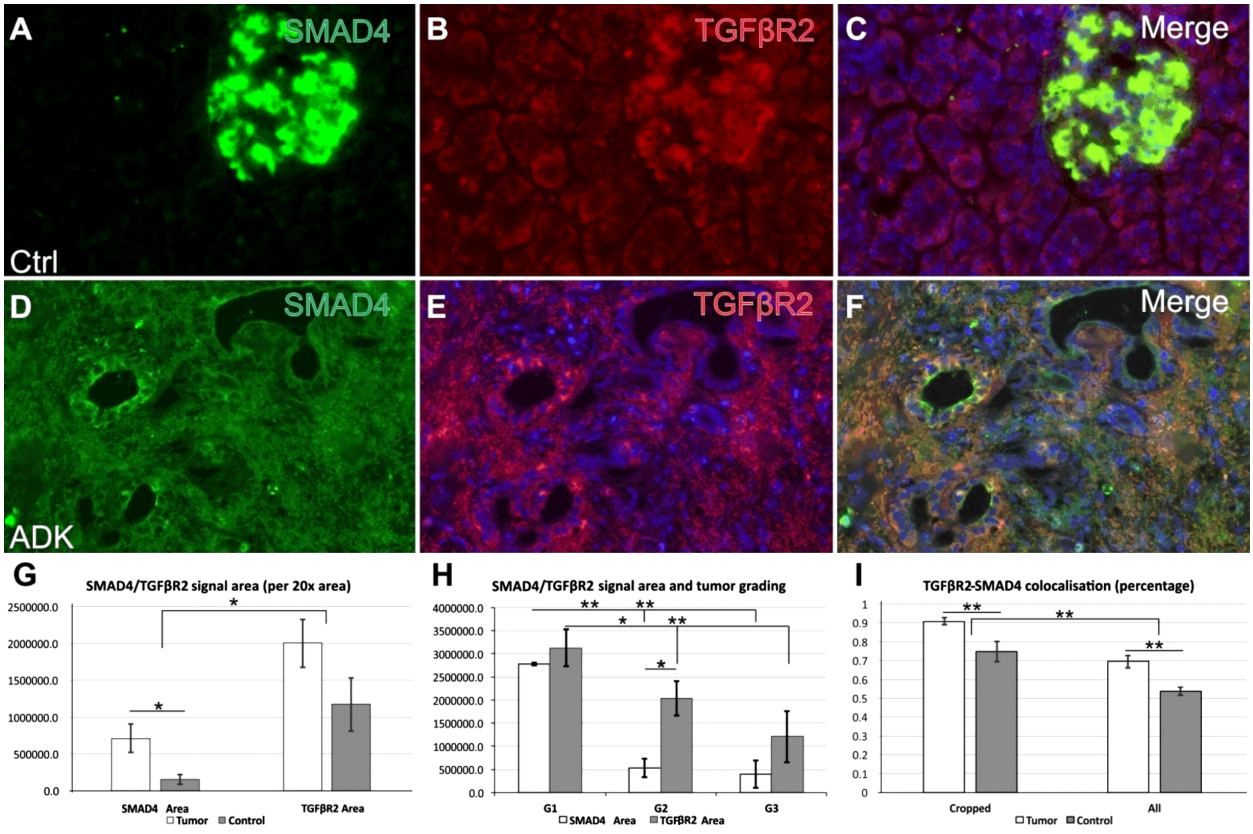

Figure 3 – ***There is a high degree of colocalization between SMAD4 and TGFβR2 in both control tissue (A–C), as well as in the tumor tissue (D–F). Expression areas of both SMAD4 and TGFβR2 are increased in tumors compared to the controls (G), while most of the SMAD4 signal comes from G1 grading tumors (H). When analyzing either epithelia only or epithelia and stroma, analysis of the signals originating in the epithelium only yielded significantly higher colocalization rates (I). (A–F, 400×). Error bars represent standard deviation; *p<0.05, **p<0.001 for Student's t-testing (G–I) and ANOVA testing (H). SMAD4: Mothers against decapentaplegic homolog 4 (Drosophila); TGFβR2: Transforming growth factor beta receptor 2; ANOVA: Analysis of variance.***

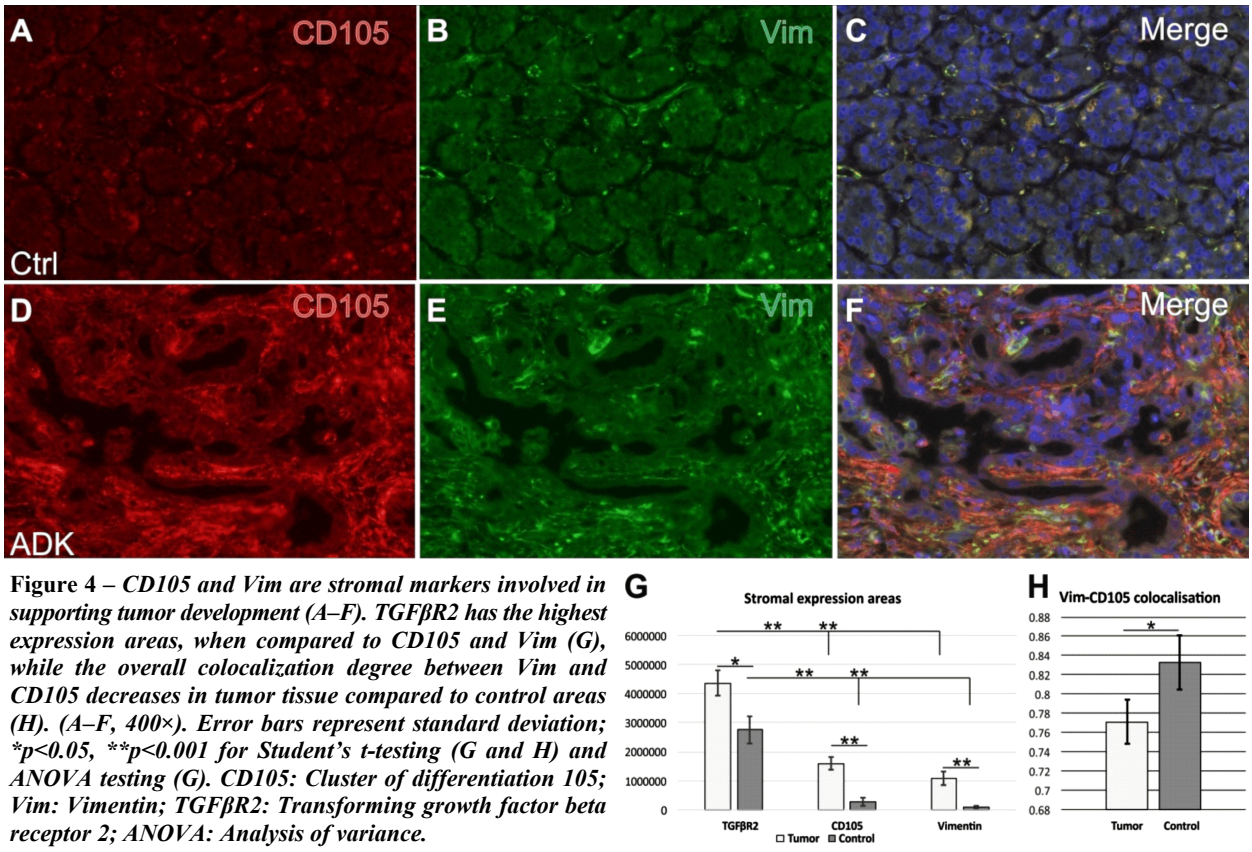

Figure 4 – ***CD105 and Vim are stromal markers involved in supporting tumor development (A–F). TGFβR2 has the highest expression areas, when compared to CD105 and Vim (G), while the overall colocalization degree between Vim and CD105 decreases in tumor tissue compared to control areas (H). (A–F, 400×). Error bars represent standard deviation; *p<0.05, **p<0.001 for Student's t-testing (G and H) and ANOVA testing (G). CD105: Cluster of differentiation 105; Vim: Vimentin; TGFβR2: Transforming growth factor beta receptor 2; ANOVA: Analysis of variance.***

Discussions

PDAC is the most frequent malignancy of the pancreas, gathering more than 90% of all pancreatic tumor types [5]. It represents the fourth most common cause of death related to cancer, and the overall incidence of pancreatic cancer is constantly rising, by 2030 being thought that it will become the second most common cause of death related to cancer in the US, after lung cancer [11].

Many molecular pathways are currently under investigation in PDAC, for both treatment-related perspectives, and also for their prognostic significance. SMAD4 protein is a tumor suppressor in the TGFβ signaling pathway that is inactivated in 55% of pancreatic ADKs, being associated with tumor invasion, metastasis and represents a significant prognostic factor for overall and disease-free survival [12]. In our study, although SMAD4 had, overall, increased expression areas in the tumors compared to the controls, its average value decreased drastically from G1 to G2 and G3 tumors. It is known that patients with pancreatic ADKs that present SMAD4 protein expression tend to have significantly longer survivals compared to those with the loss of the protein [13], and this correlated, in our patients group, that G1 cases showed significantly higher SMAD4 expression values. TGFβ, on the other hand, is involved in the regulation of important cellular functions including tissue differentiation, cell proliferation, migration, apoptosis, wound healing and immune surveillance [14]. TGFβ is one of the most important growth factor in pancreatic cancer, which, in the event of SMAD4 loss, through alternative pathways, supports the tumor growth and the progression [15]. Recent research has shown that the patients with a higher nuclear staining score for TGFβR2 had a relatively shorter survive than those with lower values [16]. In our study, TGFβR2 had overall, increased expression areas in the tumors compared to the controls, and it showed a mild decrease from G1 to G3 grading with marked variability of the signal, but with not such a steep slope like that for SMAD4. Further studies showed that TGFβR2/SMAD4 pathway acts as a tumor suppressor in the early stages of pancreatic ADKs by promoting cell cycle arrest and the apoptosis [10]. The overexpression of TGFβR2 modifies the tumor microenvironment to switch the surrounding signaling pattern from a tumor suppressor to an oncogene pattern. This change could be a result of the inactivation or loss of SMAD4. Downregulation of SMAD4 in the TGFβ signaling pathway switches over the action of cell cycle arrest and apoptosis in epithelial cells. The inactivation of SMAD4 abrogates many functions of TGFβ and its related ligands, such as growth suppression and apoptosis [10]. Javle *et al.* analyzed the nuclear expression of TGFβ and SMAD4 in combination and noted that the patients with lower expressions of TGFβ and higher expressions of SMAD4 had a significantly longer overall survival [16]. In our study, there was a clear-cut higher colocalization of the two markers for tumor areas for both epithelia only or overall tissue (p<0.01). Both SMAD4 and TGFβR2 decreased from G1 to G3 tumor gradings, although SMAD4 showed a more rapid decrease, and this could be explained by the fact that we had a very low number of G3 representative cases. Basically, this might be explained by the fact that stromal TGFβR2 does not co-express significant SMAD4, and thus is not able to alter this ratio.

Endoglin (also known as CD105) is described as an accessory receptor of TGFβ that localizes on the cell membrane. It is highly expressed in activated vascular endothelial cells, currently being considered an important

angiogenesis marker [17–19]. Most studies report that CD105 is upregulated in pancreatic tumor cells and is associated with poor prognosis [20, 21]. In our study, CD105 had clearly higher expression in tumor stroma compared to controls, but with rare expression only in cancer epithelia. Other studies have also localized CD105 in the pancreatic carcinomatous epithelia, hypothesizing that tumor cells acquiring a CD105+ phenotype might in fact acquire stem cells-like properties and switch towards a more mesenchymal profile, a transition that is also though to occur in this malignancy [21]. Moreover, other studies showed that pancreatic cancer cells expressing CD105 exhibit enhanced migratory properties, explaining why this profile may explain its link with pancreatic cancer extension and metastasizing [21]. It is of great interest to mention, in these lines, that none of the patients included in this study did not present with a distant metastatic disease at the date of surgery, except for peripancreatic fat and lymph nodes involvement. Therefore, the fact that we did not have quantifiable CD105 expression in the tumor epithelia is perfectly conceivable in these conditions. Vimentin is a major constituent of the intermediate filament family of proteins and is highly expressed in normal mesenchymal cells. In the same line, of EMT, the overexpression of vimentin in cancer cells is correlating with an accelerated tumor growth and was significantly correlated with poorly histological differentiation. The high vimentin expression (>10% of cancer cells expressing), margin-positive surgical resection and tumor size (>30 mm) are predictors of a shorter postsurgical survival [22]. In our study, vimentin had higher expression in tumor areas compared to controls. Similar results were found in other studies, where vimentin showed a direct correlation with poor survival, and with increased Ki67 and CD44 expression [23].

As a correlation between vimentin and CD105 should show enhanced EMT properties, we have also assessed the degree of vimentin–CD105 colocalization, and we have followed only the whole tissue analysis, as both markers were mainly stromal targets. Co-expression of vimentin with CD105 in stromal vessels proved active tumor angiogenesis, while their co-expression in other stromal elements might have underlined stromal fibroblasts or mesenchymal stem cells, which might intermediate the invasion process [24, 25]. Overall, there was a significantly lower colocalization degree for the tumor tissue compared to controls, a result that might be explained by the fact that we did not have almost any expression in areas other than the stroma, and the patients showed only a localized disease.

Conclusions

We have assessed here the expression of SMAD4, TGFβ, vimentin and CD105 in a series of patients with localized PDAC of different gradings. We showed that SMAD4 showed indeed a decrease towards the G3 tumors, although overall there was a higher SMAD4 expression in the tumor compared to controls. TGFβR2, vimentin and CD105 showed, as expected higher expression values in the tumor areas, while vimentin–CD105 colocalization degree showed lower values for the tumor compared to controls. Overall, it is highly plausible that all these key players revolve around the EMT phenomenon, and this itself modulates the clinical outcome of the patient.

Conflict of interests

The authors declare that they have no conflict of interests.

Authors' contribution

Ion Alexandru Văduva and Claudiu Mărgăritescu share equal contributions.

Acknowledgments

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