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Increased GPR35 expression in human colorectal and pancreatic cancer samples: A preliminary clinical validation of a new biomarker

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Advances in Clinical and Experimental Medicine, ISSN 1899–5276 (print), ISSN 2451–2680 (online) *Adv Clin Exp Med*. 2023

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Funding sources
This research was funded by the Medical University of Lodz, Poland (grant No. #503/1-156-04/503-11-001-19 to JF).

Conflict of interest
None declared

Acknowledgements
TM participates in the program "Doktorat wdrożeniowy" of the Polish Ministry of Science and Education.

Received on June 24, 2022
Reviewed on July 8, 2022
Accepted on December 9, 2022

Published online on January 13, 2023

Cite as
Mackiewicz T, Włodarczyk J, Zielińska M, et al. Increased GPR35 expression in human colorectal and pancreatic cancer samples: A preliminary clinical validation of a new biomarker [published online as ahead of print on January 13, 2023]. *Adv Clin Exp Med*. 2023. doi:10.17219/acem/157291

DOI
10.17219/acem/157291

Abstract

Background. G protein-coupled receptor 35 (GPR35) is involved in carcinogenesis; however, limited experimental data are available on its actual expression in patients with colorectal cancer (CRC) and pancreatic adenocarcinoma (PDAC).

Objectives. We aimed to measure the relative expression of GPR35 in samples from patients with CRC or PDAC.

Materials and methods. Using real-time polymerase chain reaction (RT-PCR), we have examined GPR35 expression in surgery samples from 40 CRC and 17 PDAC patients, and performed analysis of the results.

Results. The analysis of GPR35 expression in patients with CRC revealed correlations between relative GPR35 mRNA expression and several tumor characteristics, with statistical significance for higher American Joint Committee on Cancer (AJCC) stages, T stages and histological grades. GPR35 expression was significantly higher in tumor samples compared to the paired healthy samples collected from the same patient. Similar, although not statistically significant trends were found in PDAC tumor samples for sex (lower expression in women) and for samples with no nodal involvement (lower expression). Samples with higher tumor T stages and higher histological grades or considered inoperable had higher GPR35 expression.

Conclusions. We have identified correlations which confirm our expectation of high GPR35 expression in CRC and PDAC. Our findings suggest the prognostic value of GPR35 testing in patients with an increased risk of CRC or PDAC development, and warrant further clinical confirmation.

Key words: colorectal cancer, biomarker, pancreatic adenocarcinoma, GPR35, G protein-coupled receptor 35

Background

Due to late diagnosis and limited effective treatment options, colorectal cancer (CRC) and pancreatic ductal adenocarcinoma (PDAC) are cancers with poor prognosis and low overall survival (OS); this remains especially critical for PDAC, which is metastasizing early.

Colorectal cancer is the 3rd most commonly diagnosed cancer and 2nd cause of death from cancer worldwide. The 2020 incidence of CRC was 1,931,590 and mortality was 935,173; these trends are increasing as there were 1,850,000 cases and 880,000 deaths cases in 2018.[1] The overall 5-year survival rate in CRC is 65%; however, rates vary between 91% for localized and 15% for metastatic stage.[2]

The peak incidence of PDAC is in patients between 60 and 80 years of age, and there were 495,773 new PDAC cases and 466,003 deaths in 2020,[1,3] with trends also increasing. Approximately 80–85% diagnoses are made as locally advanced (laPDAC) or distant metastatic disease (mPDAC) with limited therapeutic options, and only a minority of patients (15–20%) are eligible for surgical resection.[4,5]

The current surveillance for CRC includes colonoscopies and histological testing of biopsied mucosa, whereas an endoscopic ultrasound (EUS) with fine-needle aspiration biopsy is the recommended diagnostic method for PDAC. We need less invasive methods for early diagnosis, and an extensive research for easily accessible biomarkers is ongoing worldwide; however, no clinically relevant biomarkers for detection of CRC or PDAC have been established so far.

G protein-coupled receptors (GPCRs) and corresponding kinases play a key role in many human diseases and the GPCR family has been proven strongly associated with tumor growth and metastasis.[6,7] There is an increasing evidence of the role of GPR35, a GPCR family member, in carcinogenesis, facilitating cancer growth and metastasis, and GPR35 expression has already been linked to various cancers (gastric, breast, colon or non-small cell lung),[8–11] indicating a role of GPR35 as a clinical tumor biomarker. Based on the literature research and The Cancer Genome Atlas (TCGA) database, the relationship between the GPR35 expression pattern and OS or disease-specific survival (DSS) in patients with CRC was examined. The performed analysis showed a negative association between positive GPR35 expression Z-score and OS in males, which remains statistically significant in advanced stages of CRC, suggesting the prognostic value of early testing of GPR35 in male patients with an increased risk of CRC development.[12]

Objectives

Based on these initial findings for CRC, and reports on the role of GPR35 in carcinogenesis of PDAC,[11,13] we aimed to obtain further clinical confirmation. We have performed laboratory analyses of GPR35 mRNA relative expression in human CRC and PDAC samples, all obtained with patient's permission, during routine surgery which the patients underwent for primary diagnosis of CRC or PDAC. Our objective was to confirm the increased expression of GPR35 in tissues collected from patients with CRC or PDAC, which we planned to be the first step in assessing the potential role of GPR35 as a biomarker for CRC or PDAC.

Materials and methods

Dataset and patient characteristics

Patients recruited to the study were admitted for CRC surgery at the Clinic of General and Colorectal Surgery of the Central Veterans' University Hospital in Łódź and for PDAC surgery at the Clinic of General and Transplantation Surgery, Norbert Barlicki Memorial Teaching Hospital No. 1 in Łódź, both being the hospitals of the Medical University of Łódź, Poland. The project was approved by the Bioethical Committee at the Medical University of Łódź (approval No. RNN/171I19/KE) and all patients provided written informed consent.

Tumor samples were resected with a safety margin during the routine surgeries from the relevant parts of the affected organs for biochemical and histological assessment. In 30 patients with CRC, additional small samples were collected from nearby healthy bowel tissue with a macroscopic margin of approx. 4–5 cm from the tumor. The samples were kept on ice and transferred to the Department of Biochemistry, Medical University of Łódź, where they were stored at –80°C until further analyses. We managed to recruit 57 patients altogether – 40 with CRC and 17 with PDAC.

Detailed characteristics of the study groups are provided in Table 1 for CRC and in Table 2 for PDAC.

RNA isolation and GPR35 analysis with RT-PCR

RNA isolation

The RNA isolation from the colonic tissue samples has been performed with the use of Total RNA Mini Plus kit delivered by A&A Biotechnology (Gdańsk, Poland), and the purity and quantity of the isolated RNA were measured using a Colibri Microvolume Spectrometer (Titertek Berthold; Colibri, Frankfurt am Main, Germany). Total RNA was eluted using diethyl-pyrocarbonate-treated water.

Reverse transcription

The cDNA synthesis was performed with the RevertAid First Strand cDNA Synthesis Kit (Fermentas Canada Inc., Burlington, Canada) in accordance with the manufacturer's

Table 1. CRC study group characteristics (n = 40)

Characteristics	Variable	Value
Sex	male	n = 24
	female	n = 16
Age [years]		54.73 ±8.85
Tumor localization	rectum	n = 13
	sigmoid	n = 10
	descending colon	n = 3
	transverse colon	n = 4
	ascending colon	n = 10
AJCC stage	II	n = 21
	III	n = 13
	IV	n = 6
Lymph nodes	N0 (no regional involvement)	n = 21
	Nx (regional involvement)	n = 19
Tumor stage	T2	n = 9
	T3	n = 24
	T4	n = 7
Histological grade	1	n = 11
	2	n = 18
	3	n = 11

CRC – colorectal cancer; AJCC – American Joint Committee on Cancer.

Table 2. Pancreatic adenocarcinoma (PDAC) study group characteristics (n = 17)

Characteristics	Variable	Value
Sex	male	n = 10
	female	n = 7
Age [years]		65.05 ±12.4
Lymph nodes	N0 (no regional involvement)	n = 5
	Nx (regional involvement)	n = 12
Tumor stage	T1	n = 1
	T2	n = 4
	T4	n = 12
Histological grade	1	n = 4
	2	n = 9
	3	n = 4
Qualification for surgery	operable	n = 5
	inoperable	n = 12

protocol. Total RNA (300 ng) was used in the reverse transcription reaction in a total volume of 20 µL, with the following 4-step incubation: 25°C for 10 min, 50°C for 15 min, 85°C for 5 min, and 4°C or 10 min.

Quantitative real-time RT-PCR

For the quantification of mRNA expression, we applied the real-time fluorescence detection polymerase chain reaction (RT-PCR) method with FAM dye-labeled TaqMan probes of GPR35 (Hs00271114_s1; Thermo Fisher Scientific, Waltham, USA). Values obtained for studied genes were normalized to the expression of the hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) gene (Hs02800695_m1; Thermo Fisher Scientific) as an endogenous control. The real-time reaction mixture was prepared in a total volume of 20 µL and consisted of 1.0 µL cDNA, 10 µL TaqMan Gene Expression Master Mix, 1.0 µL TaqMan Gene Expression Assays, and 8 µL RNA-free water; this was performed in duplicate. Cycle parameters were as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles of sequential incubations at 95°C for 15 s and at 60°C for 1 min. The initial amount of the template was evaluated as a Ct parameter. The relative expression level normalized to HPRT1 was calculated as $2^{-\Delta Ct}$ The number of cycles linearly correlates with the logarithmic value of RNA quantity.

Statistical analyses

The data were analyzed using Statistica v. 13.1 (StatSoft Inc., Tulsa, USA). A Shapiro–Wilk test was used to determine a normality of distribution; continuous variables were expressed as mean ± standard deviation (M ±SD). The comparisons of the study groups were performed with the Mann–Whitney U test and Kruskal–Wallis test. Multiple groups comparisons were followed by post hoc tests (Dunn's test). Pearson correlation coefficient was used in the correlation analysis. A value of $p < 0.05$ was considered statistically significant.

Results

GPR35 expression in patients with CRC

We found a correlation between relative GPR35 expression and diverse patient and tumor characteristics.

The median expression of GPR35 was the highest in samples with AJCC stage IV and the difference was statistically significant (Fig. 1A). The median expression of GPR35 was higher in samples with higher T stage, with statistically significant relation between T stage 2 and 3 (Fig. 1B). We have also found a similar statistically significant correlation with relative GPR35 expression between tumor histological grade 1 and grade 3 (Fig. 1C). A paired comparison of healthy and tumor samples obtained from CRC patients revealed significantly higher relative mRNA expression of GPR35 in the latter (Fig. 1D).

Analyzing results from CRC samples, we did not find any significant differences in GPR35 expression between women and men (GPR35 relative mRNA expression in CRC tissue regarding to the patient's sex was 14.61 ±32.04 for male sex and 10.31 ±24.61 for female sex; p = 0.943; Mann–Whitney U test). No significant correlation between

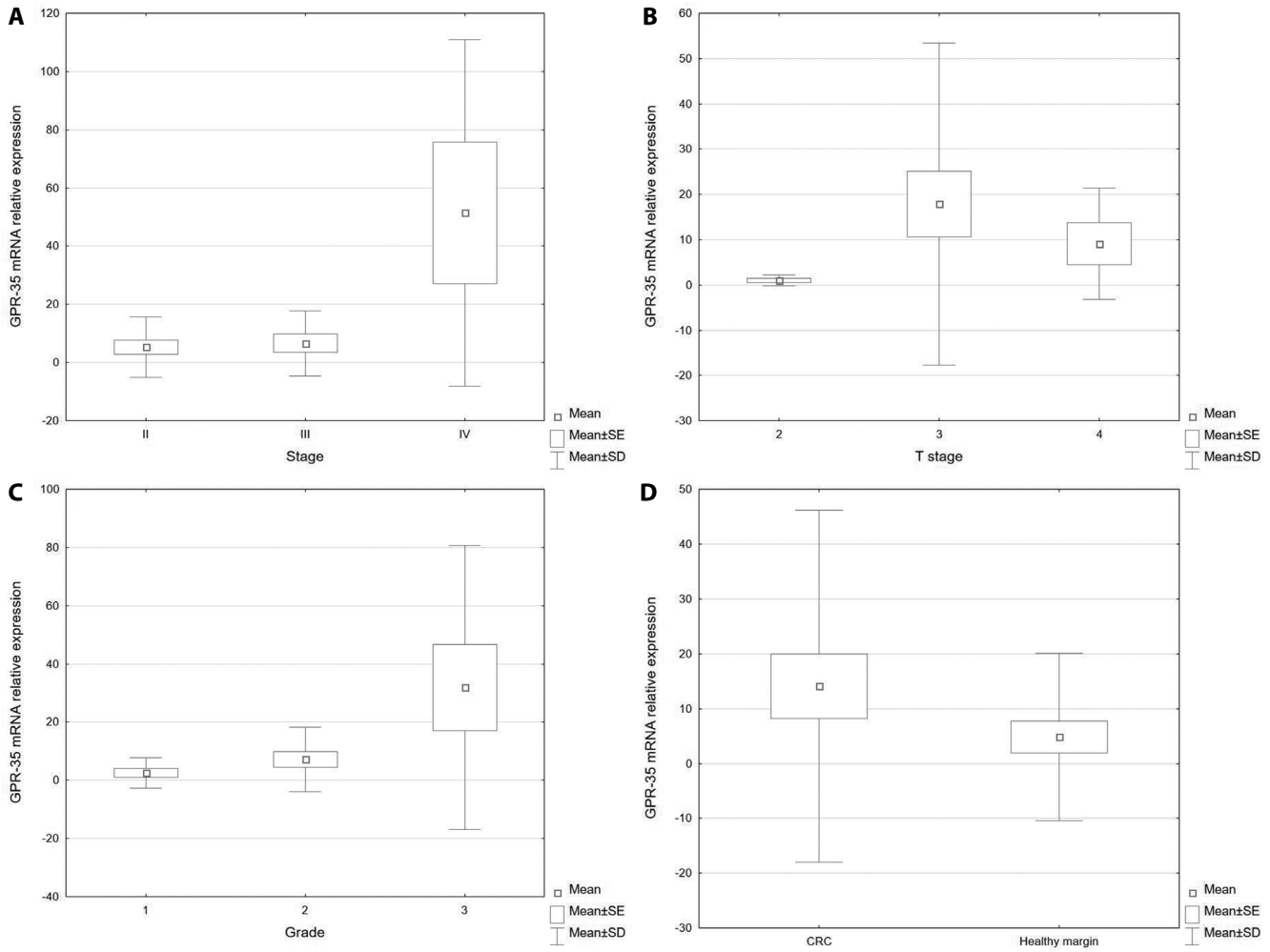

Fig. 1. A. GPR35 mRNA expression in colorectal cancer (CRC) in relation to American Joint Committee on Cancer (AJCC) stage (II: 5.3 ±10.44; III: 6.64 ±11.17; IV: 51.44 ±59.5; p = 0.01; Kruskal–Wallis test followed by Dunn's post hoc test; II compared to IV – p = 0.007; III compared to IV – p = 0.044), n = 40; B. GPR35 mRNA expression in CRC in relation to T stage (T2: 1.02 ±1.15; T3: 17.89 ±35.57; T4: 9.09 ±12.33; p = 0.017; Kruskal–Wallis test followed by Dunn's post hoc test; T2 compared to T3 – p = 0.014), n = 40; C. GPR35 mRNA expression in CRC in relation to tumor grade (G1: 2.52 ±5.28; G2: 7.21 ±11.13; G3: 31.89 ±48.87; p = 0.013; Kruskal–Wallis test followed by Dunn's post hoc test; G1 compared to G3 – p = 0.01), n = 40; D. Expression of GPR35 in healthy margin of colon tissue and in CRC sample (healthy margin: 4.82 ±15.32; CRC: 14.1 ±32.1; p = 0.008; Mann–Whitney test), n = 57.

SD – standard deviation; SE – standard error.

GPR35 mRNA relative expression level and patients' age was found either (r = 0.0277; p = 0.867). We did not find any correlation in relative GPR35 mRNA expression level neither regarding the CRC tumor size (r = –0.1385; p = 0.4) nor the status of nodal involvement (Nx: 15.29 ±34.22 compared to N0: 10.28 ±22.79; p = 0.527; Mann–Whitney U test).

GPR35 expression in patients with PDAC

We have found a trend in difference in GPR35 mRNA relative expression levels between women and men, with lower GPR35 expression in women (Fig. 2A). GPR35 expression was lower in samples obtained from patients with PDAC and no regional nodes involvement (Fig. 2B). Other analyzed features included tumor T stage, with higher GPR35 expression in T4 (Fig. 2C), and tumor histological grade, where grades 2 and 3 had higher GPR35 expression than grade 1 (Fig. 2D). However, all these differences were not statistically significant.

Another interesting trend observed in GPR35 expression regarded inoperable and operable tumors; however, it was not statistically significant either (Fig. 3). Moreover, we have not found any significant differences in GPR35 expression related to patients' age (r = 0.343; p = 0.178), PDAC tumor size (r = 0.094; p = 0.721) or CA 19-9 level (r = –0.201; p = 0.439).

Discussion

Despite progress in developing diagnostic tools and treatment, the overall 5-year survival rate in PDAC remains below 10%.[14] The current biomarkers used for diagnosis or monitoring treatment progress, such as carbohydrate antigen 19-9 (CA19-9) and carcinoembryonic

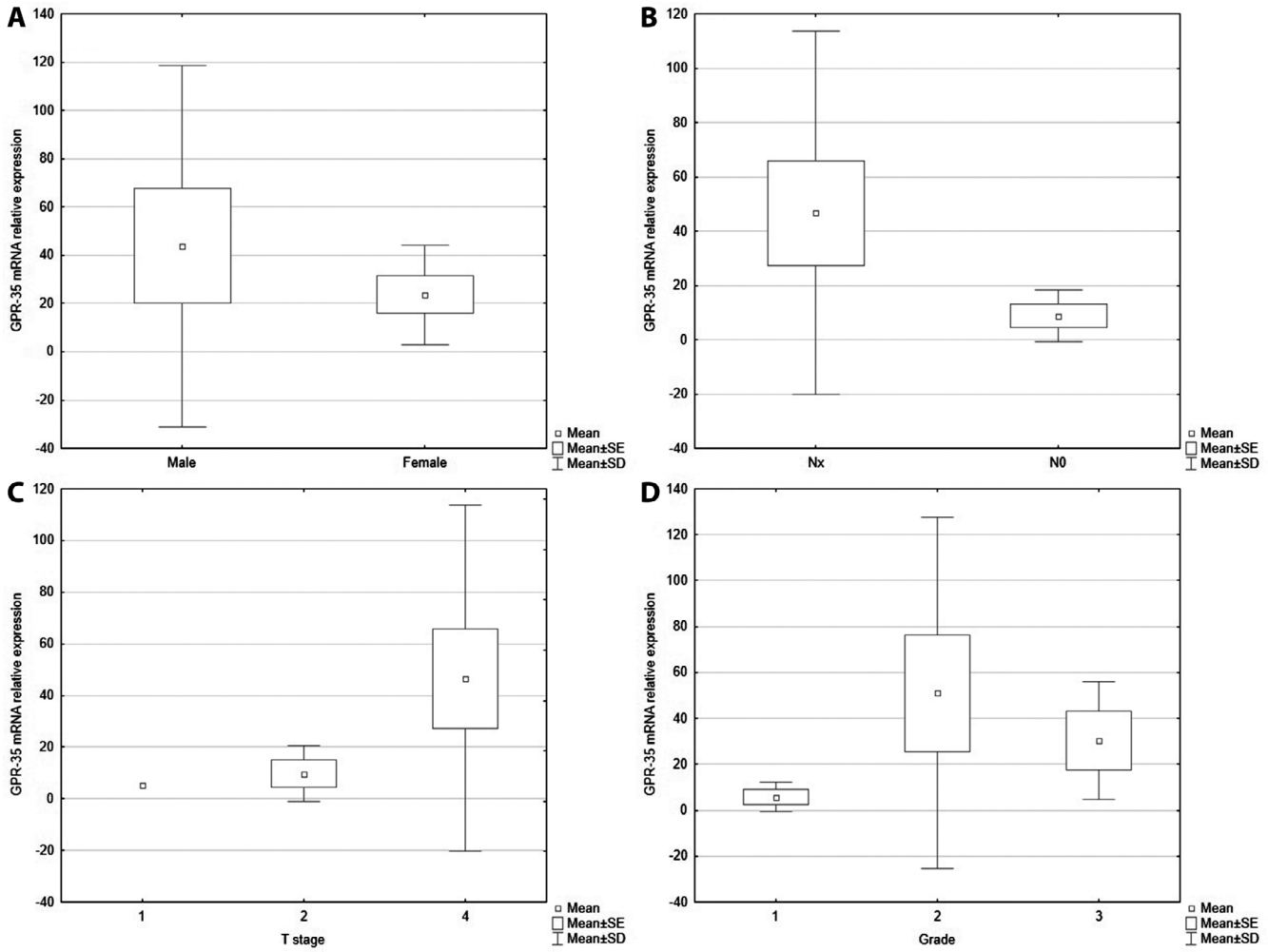

Fig. 2. A. GPR35 mRNA expression in pancreatic tissue in relation to the patient's sex (male: 43.83 ±74.83 compared to female: 23.65 ±20.67; p = 0.464; Mann–Whitney U test), n = 17; B. GPR35 mRNA expression in pancreatic tissue in relation to the nodal metastases status (Nx: 46.65 ±66.89 compared to N0: 8.81 ±9.63; p = 0.126; Mann–Whitney U test), n = 17; C. GPR35 mRNA expression in pancreatic tissue in relation to T stage (T1: 5.05; T2: 9.75 ±10.85; T4: 46.65 ±66.89; p = 0.260; Kruskal–Wallis test followed by Dunn's post hoc test), n = 17; D. GPR35 mRNA expression in pancreatic tissue in relation to tumor grade (G1: 5.75 ±6.46; G2: 51.04 ±76.43; G3: 30.38 ±25.56; p = 0.124; Kruskal–Wallis test followed by Dunn's post hoc test), n = 17.

SD – standard deviation; SE – standard error.

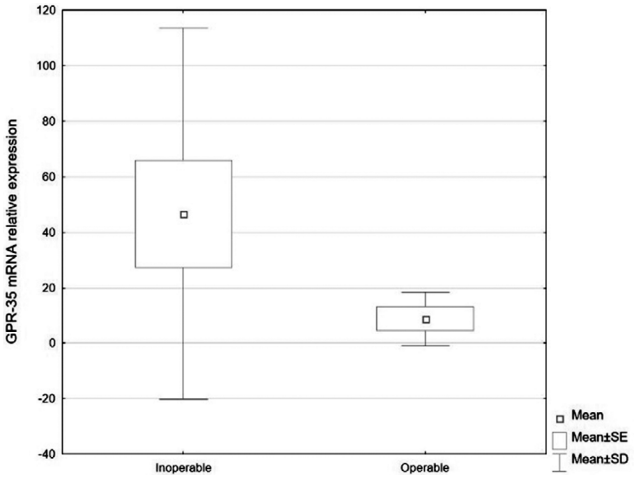

Fig. 3. GPR35 mRNA expression in pancreatic tissue in relation to surgery outcome (inoperable: 46.65 ±66.89 compared to operable: 8.81 ±9.63; p = 0.126; Mann–Whitney U test), n = 17.

SD – standard deviation; SE – standard error.

antigen (CEA), are neither sensitive nor specific enough for detection of PDAC, so biomarkers for early diagnosis are needed. The OS in CRC is around 65%; however, the more advanced the cancer, the lower the survival,[15] so early diagnosis remains crucial.

Several studies confirmed that various tumor cells, including lung, prostate, colon, pancreas, and mesenchyma, express GPCRs in an abnormal manner, including those GPCRs that participate in cell proliferation, migration, invasiveness, and angiogenesis.[16] Consequently, multiple genes, positively correlated with the expression of GPR35 belonging to the signaling pathways involved in CRC pathogenesis, were found using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis tool. These included ribosome and metabolic pathways, glycerophospholipid metabolism, vascular endothelial growth factor (VEGF) or mTOR signaling pathways.[12] GPR35 plays also a role in PDAC pathogenesis, regulating

the anti-proliferation, survival, apoptosis, and autophagy mechanisms, especially via the AKT, ERK and HIF1-α pathways.[13]

Importantly, the relative GPR35 expression can already be found in the publicly available databases, such as the TCGA PanCancerAtlas, and there are reports available about statistically significant shorter OS in patients with positive GPR35 Z-score based on these data – the recent concerning males with CRC and PDAC.[12]

To confirm the correlation between GPR35 mRNA relative expression and development of PDAC or CRC, we performed laboratory investigation of tumor samples from patients with advanced PDAC or CRC who were undergoing surgery for their primary diagnosis. From a group of patients with CRC, we also collected healthy samples. These assessments of GPR35 expression were our first steps to confirm the relevance of GPR35 in the carcinogenesis of both CRC and PDAC.

Here, we found correlations between a relative GPR35 expression at mRNA level and different tumor characteristics; of note, we observed statistical significance in CRC samples only. In general, the more advanced the tumor, the higher the median GPR35 expression. In CRC, this was confirmed for AJCC stage IV, T stage and histological grade, and also by a higher relative GPR35 expression in tumor samples than in the healthy paired samples collected from the same patient. All these correlations were statistically significant. Correlations regarding patient's sex (lower GPR35 expression in women), age, tumor size, or nodal involvement were not statistically significant.

In PDAC tumor samples, we found similar trends in GPR35 mRNA relative expression levels regarding sex (lower expression in women) and involvement of regional lymph nodes (less involved or not involved at all). Samples with tumor T stage 4 had much higher GPR35 expression than stages 2 or 3; similarly, higher tumor histological grades (2 and 3) had higher GPR35 expression than grade 1. A clear trend was seen between tumors considered inoperable when compared to operable, with much lower GPR35 expression in the latter. Interestingly, there was no correlation in GPR35 expression level regarding CA 19-9 level, which, if paired with GPR35, could play a role in PDAC diagnostics.

GPR35 expression levels are increased in CRC and PDAC tumors, and should be investigated further on larger groups of patients, including patients from the risk groups, to enable conclusions about the role GPR35 could play as a biomarker. In case of CRC, the patients have higher risk if: 1) they suffer from inflammatory bowel disease (IBD), such as Crohn's disease or ulcerative colitis; and/or 2) have a personal or family history of CRC or colorectal polyps, familial adenomatous polyposis (FAP) or Lynch syndrome.[17] GPR35 expression could be measured in them for early CRC diagnosis. Such measurement could be included during screening procedures for CRC, which is usually performed in patients 40–45 years old, and repeated regularly later, especially in those with lifestyle risk factors, such as lack of physical activity, inappropriate diet, overweight or obesity, alcohol consumption, and tobacco use. Confirmation of increased GPR35 expression in samples collected during colonoscopy could suggest increased risk of CRC development and encourage targeted diagnostic procedures.

The assessment of GPR35 expression could have similar importance in early PDAC diagnosis in high-risk populations before they develop the symptoms. These could be patients with genetic conditions (including hereditary breast and ovarian cancer syndrome, Lynch syndrome, familial adenomatous polyposis, Peutz–Jeghers syndrome, familial atypical multiple mole melanoma syndrome, hereditary pancreatitis, cystic fibrosis, and ataxia–teleangiectasia), chronic pancreatitis or those with diabetes mellitus, or patients with modifiable risk factors such as tobacco or alcohol use, diet or obesity.[18] Assessments of GPR35 expression from pancreatic tissues will have to be performed on pancreas samples collected during EUS, which is less reliable than colonoscopy for this purpose.

In summary, our results show the correlation between relative GPR35 mRNA expression and advanced development of CRC or PDAC, and confirm the role of GPR35 in carcinogenesis. Further clinical investigations are needed to confirm whether GPR35 could play a role as an early biomarker for CRC or PDAC.

Limitations of the study

Our analysis has some limitations, related especially to small sample size of our study, which provided us with restricted number of samples from patients with CRC and especially with PDAC. It needs to be underlined that this is only a preliminary study; however, we hope that our encouraging results will motivate researchers to continue investigations on bigger populations, including patients at risk.

Conclusions

Our results suggest the role of GPR35 as a potential biomarker for CRC or PDAC; however, more clinical data from patients at risk to develop these cancers are needed to confirm the suitability of GPR35 as an early biomarker.

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