British Journal of Cancer www.nature.com/bjc

ARTICLE

Molecular Diagnostics

Clinical significance and functional role of adhesion G-protein-coupled receptors in human pancreatic ductal adenocarcinoma

Satoshi Nishiwada[1,2,3], Tadanobu Shimura[1], Kensuke Yamamura[1], Kenji Nakagawa[2], Minako Nagai[2], Kota Nakamura[2,3], Taichi Terai[2], Suguru Yamada[4], Tsutomu Fujii[5], Yasuhiro Kodera[4], Masayuki Sho[2] and Ajay Goel[1,3,6]✉

BACKGROUND: The adhesion G-protein-coupled receptors (GPCRs) play crucial roles in tumour pathogenesis, however, their clinical significance in pancreatic ductal adenocarcinoma (PDAC) remains unclear.
METHODS: We analysed 796 PDAC patients, including 331 from public data sets (TCGA, ICGC and GSE57495) and 465 from independent cohorts (training: $n = 321$, validation: $n = 144$). Using in-vitro studies, we confirmed the biological function of the candidate GPCRs.
RESULTS: Analysis of all 33 adhesion GPCRs, led to identify GPR115, as the only significant prognostic factor in all public data sets. The patients with high GPR115 expression exhibited significantly poorer prognosis for OS and RFS, in training ($P < 0.01$, $P < 0.01$) and validation cohort ($P < 0.01$, $P = 0.04$). Multivariate analysis indicated that GPR115 high expression was an independent prognostic factor in both cohorts (HR = 1.43; $P = 0.01$, HR = 2.55; $P < 0.01$). A risk-prediction model using Cox regression by incorporating GPR115 and clinicopathological factors accurately predicted 5-year survival following surgery. In addition, GPR115 silencing inhibited cell proliferation and migration in PDAC cells.
CONCLUSION: We demonstrated that GPR115 has important prognostic significance and functional role in tumour progression; providing a rationale that this may be a potential therapeutic target in patients with PDAC.

British Journal of Cancer (2023) 128:321–330; https://doi.org/10.1038/s41416-022-02057-1

BACKGROUND

Pancreatic ductal adenocarcinoma (PDAC) remains one of the most lethal malignancies worldwide, which is estimated to be the 11th leading cause of new cancer cases, and is projected to become the 2nd leading cause of deaths in the United States by 2030 [1]. In the United States and the 28 countries of the European Union, it has been projected that pancreatic cancer will surpass breast cancer as the third leading cause of cancer death in the future [1, 2]. Despite progress in our understanding of the molecular and genetic basis of this disease, the 5-year survival rates have remained well under 10% [1, 3]. Several advances in treatments including new regimens of chemotherapy and radiotherapy have been introduced in recent years, but the prognosis of patients still remains poor [4–13]. In addition, the use of one of the newest drugs, the immune checkpoint inhibitors for treating patients with mismatch repair-deficient PDAC, have failed to demonstrate sufficient efficacy in patients with metastatic PDAC, and the patient subgroup is quite limited [7, 14–16]. It was thought that surgical resection could offer the only chance for cure or long-term survival; however, most patients with PDAC already have distant metastases [17–19], and less that 20% of patients have a resectable disease at initial diagnosis [11, 20]. In addition, even in this most favourable patient subgroup, up to 80% of patients experience recurrence after surgery with short recurrence-free interval [21–23]. Therefore, identification of novel targets and development of innovative therapeutic approaches is much needed to further improve treatment outcomes in patients with metastatic disease [12–14].

Multidisciplinary treatment strategies for localised PDAC are being actively investigated [21, 24, 25]. Recently, it was reported that 5-year survival rates of patients who received multidisciplinary treatments including curative surgery and adjuvant therapy were about 20–40% [6, 8, 21, 25, 26]. In particular, adjuvant chemotherapy (ACT) has been an established treatment strategy following curative resection for PDAC [6, 8, 27–29]. However, the optimal indication and regimens are still being debated globally. This highlights the need to develop robust prognostic biomarkers for PDAC, and the expectations are that such biomarkers must offer a superior prognostic clinical usefulness compared to the classic clinicopathological factors.

[1]Center for Gastrointestinal Research, Baylor Scott & White Research Institute and Charles A. Sammons Cancer Center, Baylor University Medical Center, Dallas, TX, USA. [2]Department of Surgery, Nara Medical University, Nara, Japan. [3]Department of Molecular Diagnostics and Experimental Therapeutics, Beckman Research Institute of City of Hope, Biomedical Research Center, Monrovia, CA, USA. [4]Department of Gastroenterological Surgery, Nagoya University Graduate School of Medicine, Nagoya, Japan. [5]Department of Surgery and Science, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Toyama, Japan. [6]City of Hope Comprehensive Cancer Center, Duarte, CA, USA. ✉email: ajgoel@coh.org

Received: 9 December 2021 Revised: 26 October 2022 Accepted: 1 November 2022
Published online: 17 November 2022

The G-protein-coupled receptors (GPCRs) consist of five main families in mammals, the largest being the Rhodopsin family, or class A, with about 284 members (plus about 380 olfactory receptors) in humans, followed by the Adhesion GPCR family with 33 members, the Glutamate family (class C), the Secretin family (class B), and the Frizzled family, with 22, 15, and 11 members, respectively [30, 31]. GPCRs and their agonists have been reported to be involved in the growth stimulation of many solid tumours, including small cell lung cancer, colon cancer, prostate cancer, breast cancer, and pancreatic cancer [32]. Interestingly, in fundamental research utilising pancreatic cancer cell lines, GPCRs have demonstrated cancer accelerative effect by interaction with insulin receptors, and mediation of cancer-associated fibroblasts and cancer cells [32–35]. However, their clinical significance and translational potential have not been clarified. The Adhesion GPCRs, one of the most well-known GPCR family, has received increased attention and has been a topic of an inquisitive biomedical research [31]. Many different research groups, including the Adhesion GPCR Consortium, have studied the Adhesion GPCRs with epidermal growth factor domains within their N termini. The adhesion GPCRs have many important functions, such as their role as receptors, interaction with extracellular molecules, cell-signal transduction and various physiological processes; however, the majority of the adhesion GPCRs are orphan receptors [30, 31, 36]. It was recently reported that several GPCR members are related to the progression of cancers and are frequently overexpressed in several human cancers, including glioblastoma, colorectal, and gall bladder cancer [36–43].

Although accumulating evidence indicates that cates GPCRs may play a critical role in tumour biology, the precise role of GPCRs in tumour progression in human cancers, especially PDAC is not fully elucidated [31]. In this study, we performed a comprehensive analysis of GPCR expression in PDAC, as well as undertook a series of systematic studies to better understand the molecular functions of GPCRs in this fatal malignancy.

METHODS

Clinical specimens and data sources

The three independent public gene-expression profiling data sets, the Cancer Genome Atlas (TCGA), International Cancer Genome Consortium (ICGC), and GSE57495, were used to interrogate the expression of GPCRs in PDAC patients. The primary and processed data were downloaded from these data sets in May 2018, along with the associated clinical information [44]. On the cases where patients who had distant metastasis and insufficient survival information were excluded.

Patient cohorts

In the clinical validation phase, two large, independent patient cohorts were analysed to validate the GPCRs identified in the discovery phase. We examined 465 patients in total, which included a training cohort of 321 patients from the Nara Medical University Hospital, and a validation cohort of 144 patients from the Nagoya University Hospital, who underwent curative surgeries for PDAC between January 2000 and December 2016. In this study, 172 (53.6%) patients in the training cohort and 15 (10.4%) in the validation cohort received neo-adjuvant therapy (NAT). Regarding adjuvant chemotherapy (ACT) following surgery, 297 (92.5%) and 99 (68.8%) patients in the training and validation cohorts were mainly treated with 5-Fluorouracil (5-Fu)-based chemotherapy (84.1%). Archived tumour specimens were available for the entire study population. Tumours were classified according to the TNM staging system of the Union for International Cancer Control (UICC) version 7. The patients who had positive peritoneal washing cytology or paraaortic lymph node metastasis without other distant metastases were included in this study [45, 46]. Exclusion criteria included macroscopically incomplete resection or tumour histology other than presence of PDAC. Patients who died of postoperative complications within 30 days following surgery were also excluded. Each tumour tissue specimen was fixed in 10% phosphate-buffered formalin and embedded in paraffin. Patient follow-up was until death or January 2019. A Written informed consent was obtained from all patients, and the study was approved by the Institutional Review Boards of all the participating institutions.

RNA isolation and real-time quantitative reverse transcription polymerase chain reaction

Total RNA was extracted from 10-mm-thick formalin-fixed paraffin-embedded (FFPE) specimens. Cancer cell rich areas were identified and tissues from these regions were microdissected [47]. Total RNA was extracted from this tissue using the AllPrep DNA/RNA FFPE Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Synthesis of complementary DNA (cDNA) was conducted using 250 ng of total RNA using the High-Capacity cDNA Reverse Transcription Kit (Invitrogen, Carlsbad, CA, USA). Quantitative reverse transcription polymerase chain reaction (qPCR) analysis was performed using the SensiFAST™ SYBR® Lo-ROX Kit (Bioline, London, UK) on the QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems, Foster City, CA, USA), and the expression levels were evaluated with Applied Biosystems QuantStudio 7 Flex Real-Time PCR System Software. The relative abundance of target transcripts was evaluated and normalised to the expression levels of beta-actin as an internal control using the $2^{-\Delta DCt}$ method; ΔDCt means the difference of Ct values between GPR115 of interest and the normaliser. Normalised values were further log2 transformed [48–50]. The PCR primers used in the current study were as follows; GPR115: Forward primer 5′-CAGGCAACCAT-GATTTGCTGC-3′, Reverse primer 5′-CCAGCTTTTAGGTGAATCTTGGA-3′, Length of product 83 bp, Beta-actin: Forward primer 5′-CCTTTGCCG ATCCGCCG-3′, Reverse primer 5′-GATATCATCATCCATGGTGAGCTGG-3′, Length of product 59 bp.

Cell lines and culture

The human pancreatic cancer cell lines, PANC-1 and MIAPaCa-2 were obtained from the ATCC (Manassas, VA, USA) and cultured in Iscove's Modified Dulbecco's Medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (Gibco, Thermo Fisher Scientific, Waltham, MA, USA). Both cell lines were maintained at 37 °C in a humidified incubator (5% CO_2). All cells were tested for mycoplasma and routinely authenticated by a panel of genetic and epigenetic markers.

Small-interfering RNA (siRNA) transfection of GPR115

For the transfection experiments, 1.0×10^5 cells from the PANC-1 and MIAPaCa-2 cell lines were seeded in 6-well plates, and thereafter transfected either with the 10 nM of Negative control#1 siRNA (Thermo Fisher Scientific, Waltham, MA, USA) or with 10 nM of siRNA specific for GPR115. Transfections were carried out using the Lipofectamine system (Lipofectamine™ RNAiMAX Transfection Reagent, Thermo Fisher Scientific, Waltham, MA, USA) in accordance with the manufacturer's protocol when cells achieved about 30% confluency. The GPR115 siRNA duplexes, generated with 30-dTdT overhangs and prepared by Life Technologies (Carlsbad, CA, USA) were chosen against the following DNA target sequences: (GPR115 target sequence, sense: 5′-GGAAUCCGUAAGAA-CUGCCtt-3′).

Cell viability assay

Each cell line with different rates of confluence (PANC-1, 2×10^3; MIAPaCa-2, 1×10^3) were seeded in 96-well plates. Once adherent, cells were transfected with 10 nM of GPR115 siRNA or the Negative controls, and cultured for 24 h. Cells were subsequently treated with 5-Fu (10 μM, Sigma-Aldrich, Saint Louis, MO, USA) or an equivalent dilution of the Dimethyl sulfoxide (DMSO) vehicle (Control) and incubated for 24, 48, 72, or 96 h. After incubation, cell viability was measured with the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay according to standard protocols [51, 52]. The absorbance at 565 nm was recorded with a 96-well plate reader (TECAN, infinite 200Pro, i-control software). Each experiment was performed in triplicate and the results were obtained from six independent experiments.

Preparation of cell lysates for western immunoblotting analysis

Following 72 h treatment with GPR115 or control siRNA, total cellular protein was extracted and western immunoblotting was performed, as described previously [18, 53]. We electrophoretically resolved the cell lysates in SDS-polyacrylamide gels and transferred them onto polyvinylidene difluoride membranes, which were then blocked in 5% skim milk at

room temperature for 1 h. The membranes were incubated with the indicated monoclonal mouse anti-human GPR115 antibody (MAB5437, 2 μg/mL; R&D Systems, Minneapolis, MN, USA) overnight at 4 °C, and subsequently incubated with horseradish peroxidase-conjugated IgG (Sigma-Aldrich, Saint Louis, USA). β-actin (Sigma-Aldrich, Saint Louis, MO, USA) was used as a reference protein. All protein bands on the membranes were visualised using the ChemiDoc™ MP Imaging System and Image Lab™ Software version 5.2.1 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Cell migration and wound-healing assays

Two days following transfection with GPR115 or control siRNA, migration assays were performed using the BD BioCoat Matrigel Invasion Chambers (BD Biosciences, Franklin Lakes, NJ, USA) with 8 μm pore-size PET membranes. For wound-healing assays, GPR115 siRNA or control siRNA were transfected when cells were more than 90% confluent. The 'wound' was made by scratching cells with a sterile 200 μL pipette tip, and cell migration was observed for up to 24 h. All experiments were conducted in triplicate in four independent experiments.

Statistical analysis

Patients divided into the high or low expression group of GPR115 were classified by median expression values. The overall survival (OS) and relapse-free survival (RFS) were calculated from the date of surgery to the date of death or recurrence, or the last follow-up date. Kaplan–Meier survival calculations and the corresponding log-rank tests were carried out to determine differences in survival rates. Multivariate analysis was performed using the Cox-regression model. We used the tumour status as T factor, nodal status as N factor, and metastatic status as M factor in tumour-node-metastasis classification, respectively. Multivariate analyses were performed including all variables that achieved a P-value of <0.05 in univariate analysis. The chi-square test or Fisher's exact test was used to analyse the significance of the association between the expression of GPR115 and clinicopathological factors in Table 1. Other data were analysed using the Student's t-test or the Mann–Whitney U-test, as appropriate to determine significant differences. A P-value <0.05 was considered statistically significant. 5-year OS prediction models were established by a Cox-proportional hazard model using expression levels of GPR115 and clinicopathological factors [54]. Patients followed less than five years after surgery were excluded. Receiver-operating-characteristic (ROC) curves were established for 5-year OS; Training cohort: $N = 241$ patients (50 survivors with follow-up ≥5 years and 191 death within 5 years), Validation cohort $N = 117$ patients (19 survivors with follow-up ≥5 years and 98 death within 5 years). Area under the curves (AUC) were compared between the models and clinicopathological factors. Statistical analyses were performed using Medcalc statistical software V.16.2.0 (Medcalc Software bvba, Ostend, Belgium), and GraphPad Prism V7.0 (GraphPad Software, San Diego, CA, USA).

RESULTS

Genome-wide expression profiling of adhesion GPCRs led to identify GPR115 as a significant prognostic predictor in PDAC

The entire workflow for this study is depicted in Supplementary Fig. S1A. In order to evaluate the clinical significance of GPCR for pancreatic cancer, we first used public data sets to analyse GPCR expression. In total, data from 331 patients, which included 177 patients from TCGA, 91 patients from ICGC, and 63 patients from the GSE57495, were analysed. First, we analysed TCGA RNA-seq data. Among the total of 33 GPCRs, the expression of 9 genes correlated significantly with patients' prognoses. We next examined the clinical relevance of GPCR expression in the ICGC and GSE data sets, wherein, high expression level of GPR115 was the only significant prognostic factor in all three data sets (TCGA: $P = 0.02$, ICGC: $P = 0.04$, GSE57495: $P = 0.05$, Fig. S1B–D). Based on these results, we selected GPR115 expression for further evaluation.

Clinical training clarifies the prognostic significance of GPR115 in an independent clinical cohort of PDAC patients

Next, during the testing phase, we assessed the clinical significance of GPR115 gene-expression by qRT-PCR in a clinical training cohort of 321 PDAC patients. Each sample was categorised into two groups according to their median expression levels of GPR115 expression. The expression levels of GPR115 were analysed based on patient demographics and various clinicopathological characteristics (Table 1). While interrogating GPR115 levels and survival data, RFS data were not available for 8 patients; and hence were excluded from further analysis. Interestingly, the patients that expressed high levels of GPR115 in their tumours exhibited significantly worse OS and RFS compared to those with low tumoural GPR115 levels ($P < 0.01$, $P < 0.01$, Log-rank test; Fig. 1a, b). The median overall survival was 25.4 months in the patients with high GPR115 expression and 35.6 months with low GPR115 expression. Postoperative recurrence was 76.2% in the high GPR115 expression group vs. 70.6% in the low expression patient group at the time of analysis.

Next, we carried out univariate and multivariate analyses using the Cox-proportional hazard model in the clinical training cohort. Univariate analysis of OS revealed that high tumoural expression of GPR115 was a significant prognostic factor (HR = 1.55, $P < 0.01$, Table 2). The other factors that correlated significantly with patients' OS were the tumour nodal status, CA19-9 expression levels, and the tumour size. Furthermore, multivariate analysis indicated that tumour GPR115 status was a significant and an independent prognostic factor for OS (HR = 1.43, $P = 0.01$, Table 2). In addition, tumour and nodal status, CA19-9 levels, and the tumour size, all emerged as significant independent prognostic factors for OS (Table 2). Furthermore, GPR115 expression was associated significantly with poor RFS in both univariate and multivariate analyses (Univariate: HR = 1.46, $P < 0.01$, Multivariate: HR = 1.35, $P = 0.02$, Table 3). These results demonstrated that we successfully proved the prognostic impact of GPR115 in patients with PDAC in large, independent in-house clinical cohort.

Successful validation of GPR115 expression in clinical validation cohort highlights its prognostic importance

To confirm the prognostic significance of GPR115, we analysed another, large, independent cohort of 144 PDAC patients as a validation cohort. The high and low expression groups in the validation cohorts were determined using the same statistical model and cutoff thresholds, as obtained in the training cohort. The associations between GPR115 expression and clinicopathological factors are shown in Table 1. Next, we evaluated the clinical significance of GPR115 expression with regards to OS and RFS. Consistent with the results in the training cohort, patients with high tumoural GPR115 expression exhibited poorer OS and RFS, compared to those with low GPR115 expression ($P < 0.01$ and 0.04, respectively) as shown in Fig. 1c, d.

Next, we performed univariate and multivariate analyses using the clinical validation cohort data. Univariate analysis revealed that high GPR115 expression and positive nodal status were significantly associated with poor OS, and high GPR115 expression, nodal status, and larger tumour size (≥30 mm) were significantly associated with worse RFS, respectively (Tables 2 and 3). Based on the results of univariate analysis using the training cohort, the statistically significant five factors, which included the GPR115 expression levels, the tumour status, nodal status, CA19-9 levels, and the tumour size, were also incorporated in the multivariate analysis. Interestingly, high GPR115 expression emerged as an independent and singular prognostic factor for predicting poor OS and RFS (HR = 2.55, $P < 0.01$, HR = 1.89, $P < 0.01$, Tables 2 and 3); which yet again highlights the clinical significance of high tumour GPR115 expression as an important prognostic biomarker in patients with PDAC.

Establishment of a prognostic model that combines GPR115 expression and key clinicopathological factors for predicting survival in PDAC patients

In an attempt to further improve the prognostic significance of GPR115 in PDAC, we next constructed a 5-year OS prediction

Table 1. Comparison of clinicopathological characteristics according to tumour GPR115 expression.

Characteristics	Training cohort				Validation cohort			
	Total	GPR115 expression			Total	GPR115 expression		
	$n = 321$	Low ($n = 160$)	High ($n = 161$)	*P*-value[a]	$n = 144$	Low ($n = 79$)	High ($n = 65$)	*P*-value[a]
Age, years								
<70, *n* (%)	157	87 (54.4)	70 (43.4)	0.06	94	52 (65.8)	42 (64.6)	0.88
≥70, *n* (%)	164	73 (45.6)	91 (56.6)		50	27 (34.2)	23 (35.4)	
Gender								
Male, *n* (%)	189	92 (55.6)	97 (60.9)	0.65	86	42 (53.2)	44 (67.7)	0.08
Female, *n* (%)	132	68 (44.4)	64 (39.1)		58	37 (46.8)	21 (32.3)	
Tumour status								
T1-2	40	28 (17.5)	12 (7.5)	**<0.01**[b]	4	1 (1.3)	3 (4.6)	0.33[b]
T3-4	281	132 (82.5)	149 (92.5)		140	78 (98.7)	62 (95.4)	
Nodal status								
N0	197	102 (63.8)	95 (59.0)	0.38	37	20 (25.3)	21 (32.3)	0.36[b]
N1	124	58 (36.2)	66 (41.0)		107	59 (74.7)	44 (67.7)	
UICC stage (ver. 7)								
IA, IB	34	24 (15.0)	10 (6.2)	0.06	3	0 (0.0)	3 (4.6)	**0.02**
IIA	156	76 (47.5)	80 (49.7)		37	20 (25.3)	17 (26.2)	
IIB	106	47 (29.4)	59 (36.6)		89	55 (69.6)	34 (52.3)	
IV	25	13 (8.1)	12 (7.5)		15	4 (5.1)	11 (16.9)	
CA19-9 (U/mL)								
<100, *n* (%)	206	110 (68.8)	96 (59.6)	0.09	57	37 (46.8)	20 (30.8)	0.06[b]
≥100, *n* (%)	115	50 (31.2)	65 (40.4)		87	42 (53.2)	45 (69.2)	
Tumour size (mm)								
<30, *n* (%)	200	100 (62.5)	100 (62.1)	0.94	70	40 (50.6)	30 (46.2)	0.66
≥30, *n* (%)	121	60 (37.5)	61 (37.9)		74	39 (49.4)	34 (53.8)	
Resectability status								
Resectable	243	122 (76.3)	121 (75.2)	0.47	0	0	0	NA
BR	70	33 (20.6)	37 (23.0)		0	0	0	
Locally advanced	7	5 (3.1)	2 (1.2)		0	0	0	
Not available	1	0 (0.0)	1 (0.6)		144	79 (100.0)	65 (100.0)	
Neo-adjuvant therapy								
Yes	172	94 (58.8)	78 (48.4)	0.06	15	12 (15.2)	3 (4.6)	0.05[b]
No	149	66 (41.2)	83 (51.6)		129	67 (84.7)	62 (95.4)	
Adjuvant therapy								
Yes	297	149 (93.1)	148 (91.9)	0.83[b]	99	51 (70.8)	48 (66.7)	0.71[b]
No	24	11 (6.9)	13 (8.1)		45	21 (29.2)	24 (33.3)	

Statistically significant *P*-values are bold.
UICC International Union Against Cancer, *BR* borderline resectable, *NA* not available.
[a]Chi-square test.
[b]Fisher's exact test.

model using a combination of GPR115 expression data in conjunction with key clinicopathological factors by performing Cox-proportional hazard analyses in the data from the training cohort of PDAC patients. Similar to our previous analysis, all five factors were included in this analysis as well. The censored cases within five years following surgery were excluded, since they did not meet the 5-year OS cutoff threshold. The multivariate analysis revealed that GPR115 was still a significant prognostic factor (Fig. 2a, b). Interestingly however, when we included the data from CA19-9 levels, T and N status and the tumour size along with GPR115 expression data, it resulted in an even superior predictive accuracy for long-term survivors with a corresponding AUC of 0.75 (95%CI: 0.69–0.80, Fig. 2c, d) in the training cohort and 0.84 (95% CI: 0.76–0.90, Fig. 2e, f) in the validation cohort. Furthermore, when we evaluated the distribution of combination risk scores and survival status, the 5-year survivors demonstrated a significantly lower risk score than patients with death within 5 years in both cohorts ($P < 0.01$, Fig. S2A, B). It was quite encouraging that in both of these independent large patient cohorts, we observed consistent results, and our prognostic model accurately predicted 5-year OS in the PDAC patients. In addition, in order for an easier translation of GPR115 expression in the clinic, we next evaluated its predictive performance for long-term survivors along with the clinicopathological factors and established a nomogram. When we included various pathological features and GPR115 expression in the risk- nomogram, we again confirmed that our

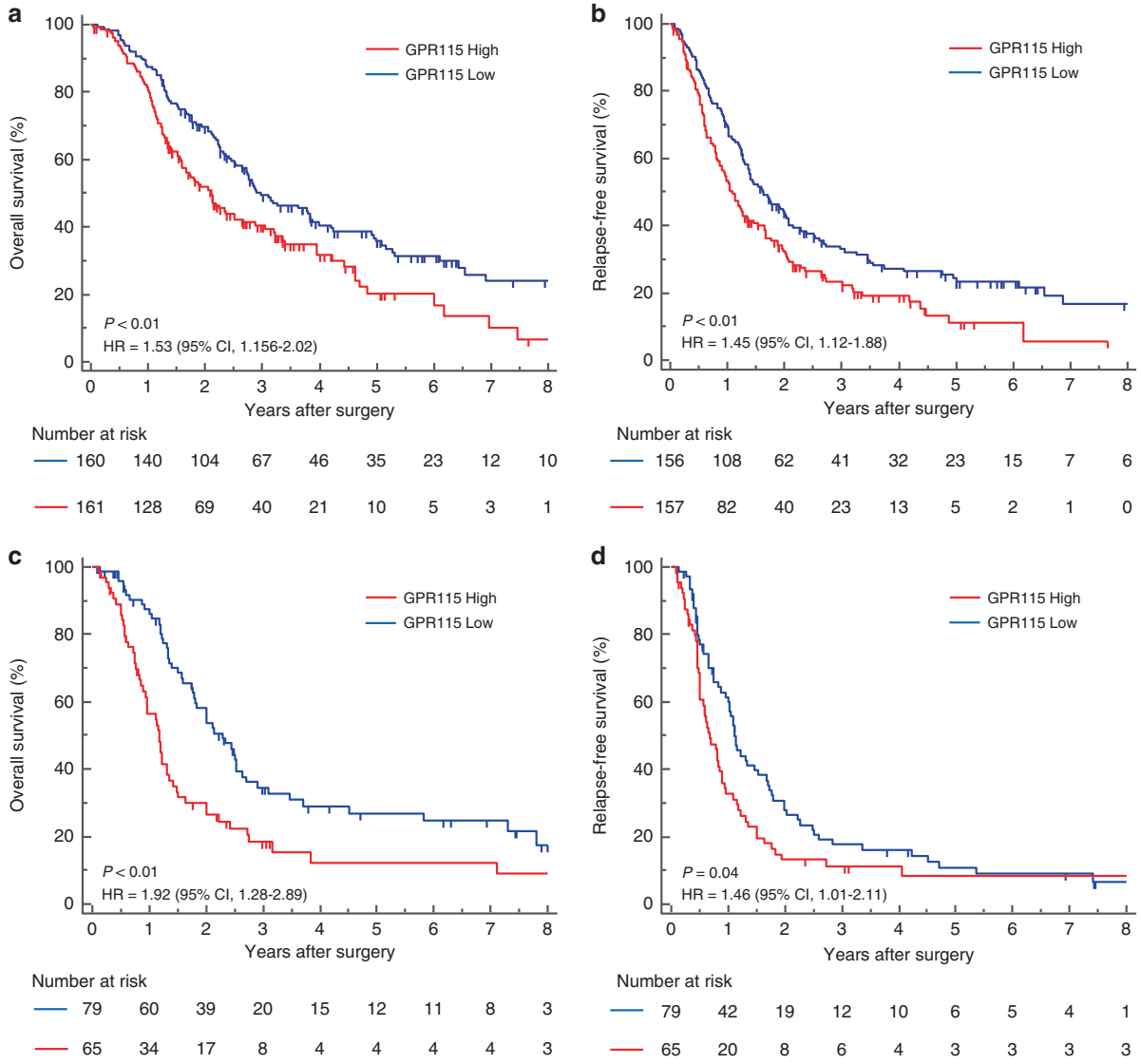

Fig. 1 Overall survival (OS) and Relapse-free survival (RFS) in patients in the training and validation cohorts. Patients with low levels of GPR115 in their tumour had a significantly better OS and RFS than patients with high levels of GPR115. Training cohort: **a** OS: $P < 0.01$, **b** RFS: $P < 0.01$. Validation cohort: **c** OS: $P < 0.01$; **d** RFS: $P = 0.04$.

GPR115 demonstrated the highest weight in this nomogram and emerged as the most significant predictor for 5-year OS in patients with PDAC (Fig. S2C).

The performance of CPR115 for predicting cancer prognosis is applicable to the patient's subgroup according to perioperative adjuvant therapy status

For patients with PDAC, multidisciplinary treatment strategies, including NAT and ACT, are becoming increasingly standard treatment options worldwide. However, NAT can modify genetic profiles in tumour cell and microenvironment. In order to investigate whether there were any associations between the GPR115 expression levels and the NAT status in terms of patient prognosis, we categorised all patients according to the presence or absence of NAT. Interestingly, GPR115 expression once again was a significant prognostic factor regardless of NAT status in the training and validation cohorts (Fig. S3A–D); hence highlighting that the its transrational applicability as prognostic marker to patients with PDAC who received NAT. On the other hand, GPR115 was significantly associated with poor prognosis of RFS in patients who received ACT following surgery, but there was no significant difference in patients who did not receive such treatments (Fig. S4A–D). These data suggest that the clinical significance of tumour GPR115 expression was independent of NAT status and might be related to the effects of ACT in PDAC patients who underwent curative surgery.

Silencing of GPR115 inhibits cell proliferation, migration and would-healing potential in human pancreatic cancer cells

In order to elucidate the underlying function of GPR115 in PDAC cells, we directly examined GPR115 expression dynamics in pancreatic cancer cells by silencing GPR115 function using siRNAs. When transfected with GPR115 or negative control siRNA in human pancreatic cancer cell lines, PANC-1 and MIAPaCa-2, the expression levels of GPR115 mRNA (48 h) and protein (72 h) were substantially reduced (Fig. 3a, $P < 0.01$; Fig. 3b, $P < 0.01$). Next, we were curious to determine the effect of GPR115 on PDAC cell proliferation. We observed that cellular proliferation was significantly suppressed by GPR115 siRNA silencing in these cells as well (Fig. 3c; $P < 0.01$).

Chemotherapy for PDAC, especially for ACT regimens using 5-Fu are the mainstream approach worldwide, and the patients in our clinical cohorts received primarily 5-Fu-based perioperative

Table 2. Univariate and multivariate analysis of OS using Cox-regression model.

	Univariate analysis			Multivariate analysis		
Characteristics	**HR**	**95% CI**	***P*-value**	**HR**	**95% CI**	***P*-value**
Training cohort ($n = 321$)						
Age (≥70 vs. <70)	1.22	0.93–1.60	0.16			
Gender (Female vs. Male)	0.89	0.67–1.18	0.42			
Tumour status (T3-4 vs. T1-2)	3.55	2.02–6.24	**<0.01**	2.47	1.38–4.40	**<0.01**
Nodal status (N1 vs. N0)	1.75	1.33–2.30	**<0.01**	1.37	1.03–1.82	**0.03**
CA19-9 (≥100 U/mL vs. <100 U/mL)	2.26	1.71–2.99	**<0.01**	1.94	1.46–2.58	**<0.01**
Tumour size (≥30 mm vs. <30 mm)	1.49	1.13–1.96	**<0.01**	1.36	1.03–1.80	**0.03**
GPR115 status (high vs. low)	1.55	1.17–2.05	**<0.01**	1.43	1.08–1.89	**0.01**
Validation cohort ($n = 144$)						
Age (≥70 vs. <70)	1.00	0.65–1.52	0.98			
Gender (Female vs. Male)	0.98	0.66–1.45	0.91			
Tumour status (T3-4 vs. T1-2)	2.05	0.51–8.32	0.32	1.64	0.38–7.16	0.51
Nodal status (N1 vs. N0)	2.19	1.38–3.47	**<0.01**	2.61	1.59–4.28	**<0.01**
CA19-9 (≥100 U/mL vs. <100 U/mL)	1.31	0.87–1.96	0.19	1.09	0.72–1.65	0.69
Tumour size (≥30 mm vs. <30 mm)	1.46	0.99–2.16	0.06	1.31	0.88–1.95	0.18
GPR115 status (high vs. low)	1.95	1.32–2.88	**<0.01**	2.55	1.69–3.85	**<0.01**

Statistically significant *P*-values are bold.
HR hazard ratio, *CI* confidence interval.

Table 3. Univariate and multivariate analysis of RFS using Cox-regression model.

	Univariate analysis			Multivariate analysis		
Characteristics	**HR**	**95% CI**	***P*-value**	**HR**	**95% CI**	***P*-value**
Training cohort ($n = 313$)						
Age (≥70 vs. <70)	1.15	0.89–1.49	0.28			
Gender (Female vs. Male)	0.91	0.70–1.19	0.50			
Tumour status (T3-4 vs. T1-2)	3.03	1.87–4.91	**<0.01**	2.34	1.43–3.84	**<0.01**
Nodal status (N1 vs. N0)	1.67	1.29–2.17	**<0.01**	1.40	1.07–1.83	**0.01**
CA19-9 (≥100 U/mL vs. <100 U/mL)	1.79	1.37–2.32	**<0.01**	1.54	1.17–2.02	**<0.01**
Tumour size (≥30 mm vs. <30 mm)	1.45	1.12–1.88	**<0.01**	1.38	1.06–1.80	**0.02**
GPR115 status (high vs. low)	1.46	1.13–1.89	**<0.01**	1.35	1.04–1.76	**0.02**
Validation cohort ($n = 144$)						
Age (≥70 vs. <70)	0.95	0.65–1.39	0.78			
Gender (Female vs. Male)	0.89	0.61–1.28	0.51			
Tumour status (T3-4 vs. T1-2)	2.80	0.69–11.35	0.15	2.18	0.51–9.35	0.29
Nodal status (N1 vs. N0)	2.19	1.42–3.38	**<0.01**	2.28	1.43–3.64	**<0.01**
CA19-9 (≥100 U/mL vs. <100 U/mL)	1.33	0.92–1.93	0.13	1.06	0.72–1.55	0.78
Tumour size (≥30 mm vs. <30 mm)	1.55	1.08–2.23	**0.02**	1.33	0.92–1.93	0.13
GPR115 status (high vs. low)	1.47	1.02–2.11	**0.04**	1.89	1.29–2.77	**<0.01**

Statistically significant *P*-values are bold.
HR hazard ratio, *CI* confidence interval.

adjuvant therapy. Therefore, we next transfected GPR115 siRNA or control siRNA into these cells and subsequently treated them with 5-FU or control (DMSO) and compared the effects on cell proliferation. Notably, we observed that the combination of GPR115 silencing and 5-FU treatment further elevated the significant reduction in cell proliferation compared to each factor individually (Fig. 3c). Furthermore, we ascertained whether GPR115 inhibition affected the migratory activity of PDAC cells. Transfection with GPR115 siRNA significantly decreased the migration in both PDAC cell lines when measured by the migration and wound-healing assays (Fig. 3d, $P < 0.01$; Fig. 3e, $P < 0.01$). These results suggest that GPR115 plays an important role as a tumour enhancer in PDAC cells, and that by blocking transcription of GPR115 it enhances the anti-tumour effect of 5-FU in this malignancy.

DISCUSSION

In this study, we carried out a comprehensive and systematic investigation on the role GPCRs in PDAC, and firstly report that

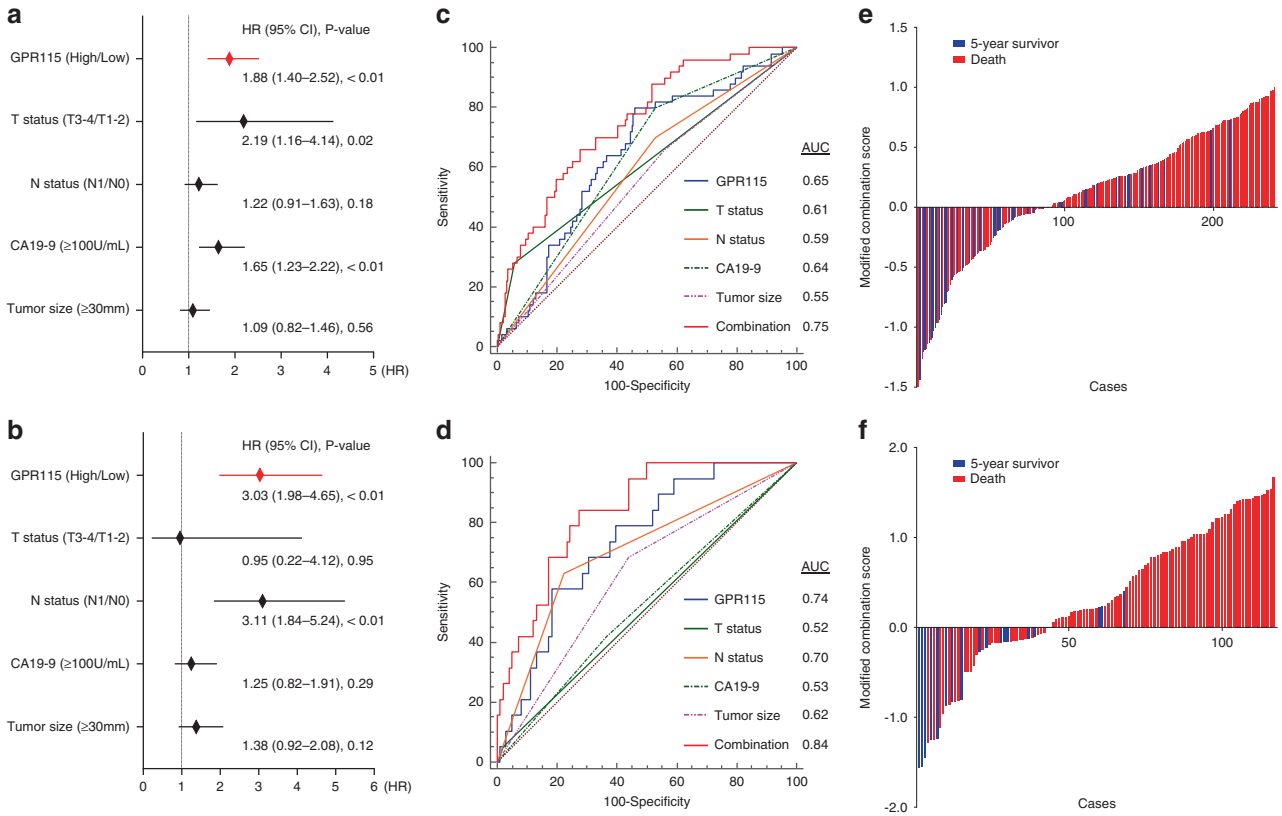

Fig. 2 Expression of GPR115 alone or when combined with CA19-9 levels, T and N status, tumour status, or all of these factors in combination were a superior predictor for 5-year OS in PDAC patients in the training and validation cohorts. a, **b** The Cox-proportional hazard model of GPR115 with all 4 factors along with 5-year OS. HR hazard ratio. **c**, **d** ROC curves constructed with GPR115 alone or in combination with CA19-9, T and N tumour status, and tumour size. The highest AUC values were seen when all factors were used in combination, and an AUC of 0.75 (95% CI: 0.69–0.80) was observed in the training cohort and 0.84 (95%CI: 0.76–0.90) in the validation cohort. **e**, **f** The predictive values of the combination model in each patient (red line: OS < 5 years, blue line: OS ≥ 5 years).

GPR115, has an important clinical significance in PDAC. We evaluated and noted that high expression of GPR115 was associated with poor OS and RFS in patients with PDAC patients, in three public data sets, as well as two large independent clinical cohorts. Evidence from previous reports indicates that GPCRs have diverse physiological and pathological functions in humans [31]. Adhesion GPCRs originally were discovered to play important roles in various physiological conditions including immune responses mediated by large cell surface ligands [55, 56]. Moreover, GPCRs play numerous roles in tumour biology [30–32, 35–39, 43]. However, there are only limited studies on the role of GPCRS in tumour biology [41, 56]. Furthermore, none of the studies have demonstrated its clinical significance or translational potential using independent multiple clinical cohorts of PDAC patients.

We first examined associations between the expression of all GPCRs and cancer prognoses using public data sets, and observed that expression of GPR115 was the only GPCR that served as a significant prognostic factor in all three independent data sets. Based on these results, we selected the GPR115 for further evaluation, and focused on deciphering its clinical significance in PDAC. Interestingly, patients with high GPR115 expression exhibited significantly poorer prognoses vs. those with low GPR115 expression. More importantly, the multivariate analysis demonstrated that tumoural GPR115 expression was a significant ahnd independent prognostic factor for PDAC patients in two, large, independent clinical patient cohorts. Ozer et al. reported that GPR115 was altered significantly with methylation changes in four cancers (breast cancer, thyroid cancer, colon adenocarcinoma and prostate adenocarcinoma) from public data sets [41]. Recently, GPR115 has been implicated in cancer invasion, metastasis and poor prognosis in lung cancer [57, 58]. However, the function and clinical significance of GPR115 has yet to be well verified in these and other cancers, especially in PDAC.

PDAC is a lethal malignant cancer, but with the development of multidisciplinary therapy, nowadays, in some instances 20% of patients can survive 5 years or longer after curative surgery [6, 8, 25, 26]. These results demonstrate a need for more accurate prognostic biomarkers to determine which patients might benefit from multi-modality therapy. The results of our study indicate that GPR115-low patients had about a 30% 5-year OS in both groups, and when we combined GPR115 expression levels with key clinicopathological factors, we were able to illustrate that this combined model was a superior prognostic predictor with an AUC of >0.75 in both groups. This value for determining patient prognosis was not affected by the neo-adjuvant therapy (NAT) status and GPR115-low patients always possessed a significantly better OS than those with GPR115-high tumours. Additionally, there was no difference in GPR115 expression between patients who did or did not receive NAT. On the other hand, adjuvant chemotherapy (ACT) was a standard of care following curative resection for PDAC [6, 8, 28, 29]. In our study, GPR115 was significantly associated with poor prognosis of RFS in patients who received ACT, but there was no significant difference in RFS in patients high or low GPR115 levels in the absence of ACT. Although further studies are required, this could be due to the numbers of patients that were in the ACT-positive vs. ACT-negative groups, or GPR115 expression might be related to the effect of ACT in PDAC patients with or without the tumour present.

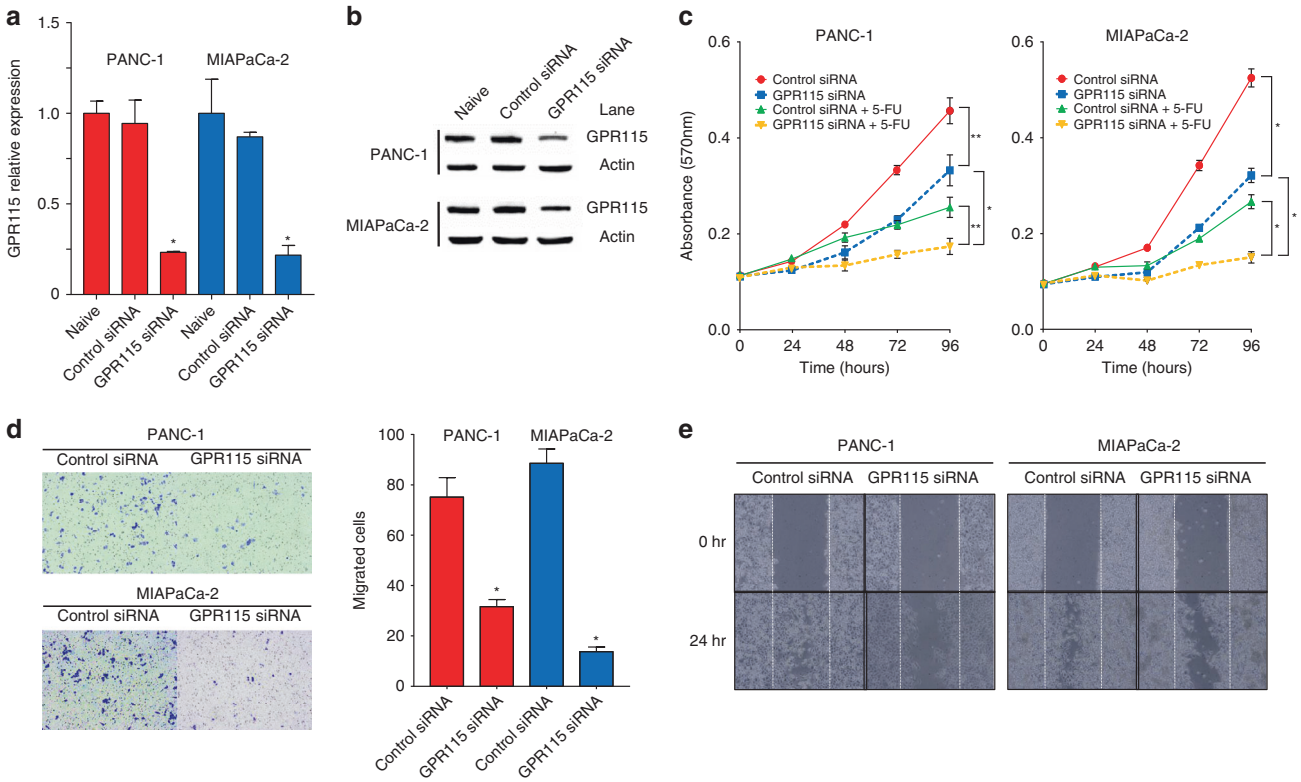

Fig. 3 Inhibition of GPR115 expression by gene silencing decreases cell proliferation in pancreatic cancer cell lines. a, **b** PANC-1 and MIAPaCa-2 cells were either not transfected (naive) or transfected with 10 nM of GPR115 siRNA or control siRNA. The relative expression of GPR115 was reduced significantly in both cell types ($P < 0.01$) when transfected with GPR115 siRNA and incubated for up to 48 h for mRNA and 72 h for protein, as determined by qPCR and Western blot analyses, respectively ($n = 4$ replicates for each subgroup). **c** Use of GPR115 siRNA inhibited cell proliferation compared to cells treated with Control siRNA as determined by MTT assay, in the presence or absence of 5-FU chemotherapy ($P < 0.01$; $n = 6$ replicates for each subgroup). **d** Transfection of cell lines with siRNA for GPR115 inhibited cell migration compared to cells transfected with control siRNA when measured by a migration assay ($P < 0.01$; $n = 6$ for each subgroup). **e** When these same cells were transfected with GP115 siRNA, wound healing was inhibiting compared with cells transfected with control siRNA measured by a wound-healing assay (3 for each subgroup). Cell monolayers were scratched with a pipette tip and imaged 0 h and 24 h after wound formation. *$P < 0.01$, **$P < 0.02$ (Student's *t*-test).

We then investigated the potential function of GPR115 in PDAC and noted that GPR115 may be involved in the proliferation and migration of PDAC cells. This was demonstrated by data showing that the siRNA knockdown of GPR115 significantly inhibited the proliferation and migration of human PDAC cell lines. In addition, GPR115 inhibition enhanced the anti-tumour effect of 5-FU. We interpret these results to suggest that there may be an interaction between the effects of chemotherapy and GPR115 expression. Moreover, this finding was consistent with our data that observed an association between GPR115 expression and prognosis of patients who receive ACT after surgery. Further studies are needed to reveal the molecular mechanisms of GPR115 in association with chemo-sensitivity of pancreatic cancer cells, in order to develop new therapeutic strategies.

Although our results provide clinical significance of GPR115 expression in PDAC, we acknowledge that there were some inherent limitations to this study. First, this was a retrospective study with the associated risks of bias. Second, our database lacked information with regard to additional treatments for recurrence after surgery. Third, in this present study, we did not adequately investigate the potential therapeutic target of GPR115 utilising treatment model. Effective therapeutic agents and/or antibodies are essential to establish a therapeutic model, but unfortunately, there are currently no established therapeutic agents for GPR115. Finally, in order to further increase the clinical significance, it is necessary to predict prognosis before patients undergo surgery. Thus, it may be ideal perform a prospective study to verify GPR115 expression of endoscopic fine needle aspiration biopsy samples.

CONCLUSIONS

We identified that GPR115 might serve as a prognostic indicator in patients with PDAC, especially those who undergo curative resection. In addition, GPR115 may contributes to tumour progression, thus, our study provides a rationale for developing novel therapies targeting GPR115 for PDAC.

DATA AVAILABILITY

All data are available within the article.

REFERENCES

1. Siegel RL, Miller KD, Fuchs HE, Jemal A. Cancer Statistics, 2021. CA Cancer J Clin. 2021;71:7–33.
2. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, et al. Global Cancer Statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2021;71:209–49.
3. Puleo F, Nicolle R, Blum Y, Cros J, Marisa L, Demetter P, et al. Stratification of pancreatic ductal adenocarcinomas based on tumor and microenvironment features. Gastroenterology. 2018;155:1999–2013.e1993.
4. Von Hoff DD, Ervin T, Arena FP, Chiorean EG, Infante J, Moore M, et al. Increased survival in pancreatic cancer with nab-paclitaxel plus gemcitabine. N Engl J Med. 2013;369:1691–703.

5. Okada KI, Shimokawa T, Hirono S, Kawai M, Sho M, Satoi S, et al. Effect of neoadjuvant nab-paclitaxel plus gemcitabine therapy on overall survival in patients with borderline resectable pancreatic cancer: a prospective multicenter phase II trial (NAC-GA Trial). Oncology. 2017;93:343–6.
6. Neoptolemos JP, Palmer DH, Ghaneh P, Psarelli EE, Valle JW, Halloran CM, et al. Comparison of adjuvant gemcitabine and capecitabine with gemcitabine monotherapy in patients with resected pancreatic cancer (ESPAC-4): a multicentre, open-label, randomised, phase 3 trial. Lancet. 2017;389:1011–24.
7. Le DT, Durham JN, Smith KN, Wang H, Bartlett BR, Aulakh LK, et al. Mismatch repair deficiency predicts response of solid tumors to PD-1 blockade. Science. 2017;357:409–13.
8. Uesaka K, Boku N, Fukutomi A, Okamura Y, Konishi M, Matsumoto I, et al. Adjuvant chemotherapy of S-1 versus gemcitabine for resected pancreatic cancer: a phase 3, open-label, randomised, non-inferiority trial (JASPAC 01). Lancet. 2016;388:248–57.
9. Ohkawa S, Okusaka T, Isayama H, Fukutomi A, Yamaguchi K, Ikeda M, et al. Randomised phase II trial of S-1 plus oxaliplatin vs S-1 in patients with gemcitabine-refractory pancreatic cancer. Br J Cancer. 2015;112:1428–34.
10. Ueno H, Ioka T, Ikeda M, Ohkawa S, Yanagimoto H, Boku N, et al. Randomized phase III study of gemcitabine plus S-1, S-1 alone, or gemcitabine alone in patients with locally advanced and metastatic pancreatic cancer in Japan and Taiwan: GEST study. J Clin Oncol. 2013;31:1640–8.
11. Von Hoff DD, Ramanathan RK, Borad MJ, Laheru DA, Smith LS, Wood TE, et al. Gemcitabine plusnab-paclitaxel is an active regimen in patients with advanced pancreatic cancer: a phase I/II trial. J Clin Oncol. 2011;29:4548–54.
12. Conroy T, Desseigne F, Ychou M, Bouche O, Guimbaud R, Becouarn Y, et al. FOLFIRINOX versus gemcitabine for metastatic pancreatic cancer. N. Engl J Med. 2011;364:1817–25.
13. Moore MJ, Goldstein D, Hamm J, Figer A, Hecht JR, Gallinger S, et al. Erlotinib plus gemcitabine compared with gemcitabine alone in patients with advanced pancreatic cancer: a phase III trial of the National Cancer Institute of Canada Clinical Trials Group. J Clin Oncol. 2007;25:1960–6.
14. Puleo F, Nicolle R, Blum Y, Cros J, Marisa L, Demetter P, et al. Stratification of pancreatic ductal adenocarcinomas based on tumor and microenvironment features. Gastroenterology. https://doi.org/10.1053/j.gastro.2018.08.033 (2018).
15. Marabelle A, Le DT, Ascierto PA, Di Giacomo AM, De Jesus-Acosta A, Delord JP, et al. Efficacy of pembrolizumab in patients with noncolorectal high microsatellite instability/mismatch repair-deficient cancer: results from the phase II KEYNOTE-158 study. J Clin Oncol. 2020;38:1–10.
16. O'Reilly EM, Oh DY, Dhani N, Renouf DJ, Lee MA, Sun W, et al. Durvalumab with or without tremelimumab for patients with metastatic pancreatic ductal adenocarcinoma: a phase 2 randomized clinical trial. JAMA Oncol. 2019;5:1431–8.
17. Katagiri R, Goto A, Nakagawa T, Nishiumi S, Kobayashi T, Hidaka A, et al. Increased levels of branched-chain amino acid associated with increased risk of pancreatic cancer in a prospective case–control study of a large cohort. Gastroenterology. https://doi.org/10.1053/j.gastro.2018.07.033 (2018).
18. Yoshida K, Toden S, Ravindranathan P, Han H, Goel A. Curcumin sensitizes pancreatic cancer cells to gemcitabine by attenuating PRC2 subunit EZH2, and the lncRNA PVT1 expression. Carcinogenesis. 2017;38:1036–46.
19. Ferrone CR, Kattan MW, Tomlinson JS, Thayer SP, Brennan MF, Warshaw AL. Validation of a postresection pancreatic adenocarcinoma nomogram for disease-specific survival. J Clin Oncol. 2005;23:7529–35.
20. Paulson AS, Tran Cao HS, Tempero MA, Lowy AM. Therapeutic advances in pancreatic cancer. Gastroenterology. 2013;144:1316–26.
21. Groot VP, Gemenetzis G, Blair AB, Rivero-Soto RJ, Yu J, Javed AA, et al. Defining and predicting early recurrence in 957 patients with resected pancreatic ductal adenocarcinoma. Ann Surg. https://doi.org/10.1097/SLA.0000000000002734 (2018).
22. Groot VP, Rezaee N, Wu W, Cameron JL, Fishman EK, Hruban RH, et al. Patterns, timing, and predictors of recurrence following pancreatectomy for pancreatic ductal adenocarcinoma. Ann Surg. 2018;267:936–45.
23. Parikh AA, Maiga A, Bentrem D, Squires MH 3rd, Kooby DA, Maithel SK, et al. Adjuvant therapy in pancreas cancer: does it influence patterns of recurrence? J Am Coll Surg. 2016;222:448–56.
24. Takahashi H, Ohigashi H, Ishikawa O, Gotoh K, Yamada T, Nagata S, et al. Perineural invasion and lymph node involvement as indicators of surgical outcome and pattern of recurrence in the setting of preoperative gemcitabine-based chemoradiation therapy for resectable pancreatic cancer. Ann Surg. 2012;255:95–102.
25. Takahashi H, Akita H, Tomokuni A, Kobayashi S, Ohigashi H, Fijiwara Y, et al. Preoperative gemcitabine-based chemoradiation therapy for borderline resectable pancreatic cancer: impact of venous and arterial involvement status on surgical outcome and pattern of recurrence. Ann Surg. 2016;264:1091–7.
26. Conroy T, Bachet J-B, Ayav A, Huguet F, Lambert A, Caramella C, et al. Current standards and new innovative approaches for treatment of pancreatic cancer. Eur J Cancer. 2016;57:10–22.
27. Conroy T, Hammel P, Hebbar M, Ben Abdelghani M, Wei AC, Raoul JL, et al. FOLFIRINOX or gemcitabine as adjuvant therapy for pancreatic cancer. N Engl J Med. 2018;379:2395–406.
28. Oettle H, Neuhaus P, Hochhaus A, Hartmann JT, Gellert K, Ridwelski K, et al. Adjuvant chemotherapy with gemcitabine and long-term outcomes among patients with resected pancreatic cancer: the CONKO-001 randomized trial. JAMA. 2013;310:1473–81.
29. Liao W-C, Chien K-L, Lin Y-L, Wu M-S, Lin J-T, Wang H-P, et al. Adjuvant treatments for resected pancreatic adenocarcinoma: a systematic review and network meta-analysis. Lancet Oncol. 2013;14:1095–103.
30. Civelli O, Reinscheid RK, Zhang Y, Wang Z, Fredriksson R, Schioth HB. G protein-coupled receptor deorphanizations. Annu Rev Pharm Toxicol. 2013;53:127–46.
31. Hamann J, Aust G, Arac D, Engel FB, Formstone C, Fredriksson R, et al. International union of basic and clinical pharmacology. XCIV. Adhesion G protein-coupled receptors. Pharm Rev. 2015;67:338–67.
32. Hao F, Xu Q, Zhao Y, Stevens JV, Young SH, Sinnett-Smith J, et al. Insulin receptor and GPCR crosstalk stimulates YAP via PI3K and PKD in pancreatic cancer cells. Mol Cancer Res. 2017;15:929–41.
33. Thakur G, Kumar R, Kim SB, Lee SY, Lee SL, Rho GJ. Therapeutic status and available strategies in pancreatic ductal adenocarcinoma. Biomedicines. 2021;9:178.
34. Insel PA, Sriram K, Wiley SZ, Wilderman A, Katakia T, McCann T, et al. GPCRomics: GPCR expression in cancer cells and tumors identifies new, potential biomarkers and therapeutic targets. Front Pharm. 2018;9:431.
35. Wiley SZ, Sriram K, Liang W, Chang SE, French R, McCann T, et al. GPR68, a proton-sensing GPCR, mediates interaction of cancer-associated fibroblasts and cancer cells. FASEB J. 2018;32:1170–83.
36. He Z, Wu H, Jiao Y, Zheng J. Expression and prognostic value of CD97 and its ligand CD55 in pancreatic cancer. Oncol Lett. 2015;9:793–7.
37. Hilbig D, Dietrich N, Wandel E, Gonsior S, Sittig D, Hamann J, et al. The Interaction of CD97/ADGRE5 With beta-catenin in adherens junctions is lost during colorectal carcinogenesis. Front Oncol. 2018;8:182.
38. Ji B, Feng Y, Sun Y, Ji D, Qian W, Zhang Z, et al. GPR56 promotes proliferation of colorectal cancer cells and enhances metastasis via epithelialmesenchymal transition through PI3K/AKT signaling activation. Oncol Rep. 2018;40:1885–96.
39. Cherry AE, Stella N. G protein-coupled receptors as oncogenic signals in glioma: emerging therapeutic avenues. Neuroscience. 2014;278:222–36.
40. Gerber PA, Hevezi P, Buhren BA, Martinez C, Schrumpf H, Gasis M, et al. Systematic identification and characterization of novel human skin-associated genes encoding membrane and secreted proteins. PLoS ONE. 2013;8:e63949.
41. Ozer B, Sezerman U. Analysis of the interplay between methylation and expression reveals its potential role in cancer aetiology. Funct Integr Genomics. 2017;17:53–68.
42. Wu J, Lei L, Wang S, Gu D, Zhang J. Immunohistochemical expression and prognostic value of CD97 and its ligand CD55 in primary gallbladder carcinoma. J Biomed Biotechnol. 2012;2012:587672.
43. Ward Y, Lake R, Faraji F, Sperger J, Martin P, Gilliard C, et al. Platelets promote metastasis via binding tumor CD97 leading to bidirectional signaling that coordinates transendothelial migration. Cell Rep. 2018;23:808–22.
44. Nishiwada S, Sho M, Banwait JK, Yamamura K, Akahori T, Nakamura K, et al. A microRNA signature identifies pancreatic ductal adenocarcinoma patients at risk for lymph node metastases. Gastroenterology. 2020;159:562–74.
45. Satoi S, Murakami Y, Motoi F, Uemura K, Kawai M, Kurata M, et al. Reappraisal of peritoneal washing cytology in 984 patients with pancreatic ductal adenocarcinoma who underwent margin-negative resection. J Gastrointest Surg. 2015;19:6–14.
46. Sho M, Murakami Y, Motoi F, Satoi S, Matsumoto I, Kawai M, et al. Postoperative prognosis of pancreatic cancer with para-aortic lymph node metastasis: a multicenter study on 822 patients. J Gastroenterol. 2015;50:694–702.
47. Nishiwada S, Sho M, Cui Y, Yamamura K, Akahori T, Nakagawa K, et al. A gene expression signature for predicting response to neoadjuvant chemoradiotherapy in pancreatic ductal adenocarcinoma. Int J Cancer. 2021;148:769–79.
48. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001;25:402–8.
49. Nishiwada S, Cui Y, Sho M, Jun E, Akahori T, Nakamura K, et al. Transcriptomic profiling identifies an exosomal microrna signature for predicting recurrence following surgery in patients with pancreatic ductal adenocarcinoma. Ann Surg. https://doi.org/10.1097/SLA.0000000000004993 (2021).
50. Ozawa T, Kandimalla R, Gao F, Nozawa H, Hata K, Nagata H, et al. A microRNA signature associated with metastasis of T1 colorectal cancers to lymph nodes. Gastroenterology. 2018;154:844–8.e847.

51. Takahashi M, Sung B, Shen Y, Hur K, Link A, Boland CR, et al. Boswellic acid exerts antitumor effects in colorectal cancer cells by modulating expression of the let-7 and miR-200 microRNA family. Carcinogenesis. 2012;33:2441–9.
52. Okada Y, Takahashi N, Takayama T, Goel A. LAMC2 promotes cancer progression and gemcitabine resistance through modulation of EMT and ATP-binding cassette transporters in pancreatic ductal adenocarcinoma. Carcinogenesis. 2021;42:546–56.
53. Shimura T, Kandimalla R, Okugawa Y, Ohi M, Toiyama Y, He C, et al. Novel evidence for m(6)A methylation regulators as prognostic biomarkers and FTO as a potential therapeutic target in gastric cancer. Br J Cancer. https://doi.org/10.1038/s41416-021-01581-w (2021).
54. Ozawa T, Matsuyama T, Toiyama Y, Takahashi N, Ishikawa T, Uetake H, et al. CCAT1 and CCAT2 long noncoding RNAs, located within the 8q.24.21 'gene desert', serve as important prognostic biomarkers in colorectal cancer. Ann Oncol. 2017;28:1882–8.
55. Haitina T, Olsson F, Stephansson O, Alsio J, Roman E, Ebendal T, et al. Expression profile of the entire family of adhesion G protein-coupled receptors in mouse and rat. BMC Neurosci. 2008;9:43.
56. Zhang W, Cui Q, Qu W, Ding X, Jiang D, Liu H. TRIM58/cg26157385 methylation is associated with eight prognostic genes in lung squamous cell carcinoma. Oncol Rep. 2018;40:206–16.
57. Wang Y, Shi M, Yang N, Zhou X, Xu L. GPR115 contributes to lung adenocarcinoma metastasis associated with LAMC2 and predicts a poor prognosis. Front Oncol. 2020;10:577530.
58. Yoon JH, Cho SG. ADGRF4 regulates non-small cell lung cancer cell invasiveness. Anticancer Res. 2020;40:6835–44.

ACKNOWLEDGEMENTS

We thank Tatsuhiko Kakisaka, Takeo Toshima, Raju Kandimalla, Goretti Hernandez, Priyanka Sharma, Raju Adduri, and Souvik Ghatak for discussing the how best to set up the experiments and their analyses. We thank Jasjit Banwait for assisting with the analysis. We thank Naoya Ikeda, Takahiro Akahori, and Tadataka Takagi for collecting clinical samples and patient information.

AUTHOR CONTRIBUTIONS

Study concept and design: SN, MS, AG; Specimen providers: SN, KN, MN, TT, SY, TF, YK, MS; Acquisition of clinical data: SN, KN, MN, TT, SY, TF, YK, MS; Analysis and interpretation of data and statistical analysis: SN, TS, KY, AG; Drafting of the manuscript: SN, MS and AG.

FUNDING

The present work was supported by the CA72851, CA187956, CA202797 and CA214254 grants from the National Cancer Institute, National Institute of Health; In addition, this work was also supported by a pilot research award from the City of Hope Ludwig Cancer Research-Hilton Foundation Partnership award.

COMPETING INTERESTS

The authors declare no competing interests.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All study-related procedures were performed as per the Declarations of Helsinki, wherein a written informed consent was obtained from each patient, and the institutional review boards of all participating institutions involved approved the study.

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41416-022-02057-1.

Correspondence and requests for materials should be addressed to Ajay Goel.

Reprints and permission information is available at http://www.nature.com/reprints

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.