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Research paper

The oncogenic and druggable hPG80 (Progastrin) is overexpressed in multiple cancers and detected in the blood of patients


ABSTRACT

Background: In colorectal cancer, hPG80 (progastrin) is released from tumor cells, promotes cancer stem cells (CSC) self-renewal and is detected in the blood of patients. Because the gene GAST that encodes hPG80 is a target gene of oncogenic pathways that are activated in many tumor types, we hypothesized that hPG80 could be expressed by tumors from various origins other than colorectal cancers, be a drug target and be detectable in the blood of these patients.

Methods: hPG80 expression was monitored by fluorescence immunohistochemistry and mRNA expression in tumors from various origins. Cancer cell lines were used in sphere forming assay to analyze CSC self-renewal. Blood samples were obtained from 1546 patients with 11 different cancer origins and from two retrospective kinetic studies in patients with peritoneal carcinomatosis or hepatocellular carcinomas. These patients were regularly sampled during treatments and assayed for hPG80.

Findings: We showed that hPG80 was present in the 11 tumor types tested. In cell lines originating from these tumor types, hPG80 neutralization decreased significantly CSC self-renewal by 28 to 54%. hPG80 was detected in the blood of patients at significantly higher concentration than in healthy blood donors (median hPG80: 4.88 pM versus 1.03 pM; p < 0.0001) and shown to be correlated to GAST mRNA levels in the matched tumor (i.e., lung cancers, Spearman r = 0.8; p = 0.0023). Furthermore, we showed a strong association between longitudinal hPG80 concentration changes and anti-cancer treatment efficacy in two independent retrospective studies. In the peritoneal carcinomatosis cohort, median hPG80 from inclusion to the post-operative period decreased from
Physiologically, hPG80 (progastrin), an 80 amino acid protein, is the precursor of the gastrointestinal hormone gastrin. It is synthesized by gastric antrum G cells, and then processed into gastrin by multiple enzymatic processes [1]. In pathological conditions, the GAST gene, which encodes hPG80, was shown to be over-expressed in human colorectal tumor cells, leading to hPG80 accumulation [2,3]. As hPG80 is either unprocessed or poorly processed into gastrin, it is released from the tumor cells and becomes detectable in the blood of colorectal cancer (CRC) patients [4,5].

Initially believed to be biologically inactive, hPG80 was shown to participate in some features of a tumor, such as the disruption of cell–cell junctions [6], cell proliferation [7], inhibition of apoptosis [8], regulation of cancer stem cells [9], and angiogenesis [10]. In addition, our team demonstrated that targeting hPG80 with a specific antibody promotes apoptosis, decreases proliferation and migration/invasion of human colorectal cancer cells, and inhibits self-renewal of colon cancer stem cells (CSC), as well as Wnt-driven tumorigenesis in mice [5]. Interestingly, GAST is a direct target of the Wnt/ß-catenin/Tcf4 oncogenic pathway [3]. Since this pathway is activated in many other cancers and plays a major function in cancer stem cells survival [11], we hypothesized that hPG80 might be expressed by other types of cancers, and would be present in the blood of patients with tumors different from colorectal cancers.

In the present study, we examined the in situ expression of hPG80 in the tissues obtained from the surgical resections of patients (n = 19) with eight tumor types. We compared hPG80 expressions in the tumor cells with those of normal adjacent tissues. Given the critical role of hPG80 as a pro-oncogenic factor, we evaluated the anti-oncogenic efficacy of anti-hPG80 antibodies on in vitro cultured cell lines from 7 cancer types, with survival and sphere formation assays. Furthermore, the blood samples from 1546 patients with 11 different types of cancers (breast, uterus, ovarian, prostate, kidney, colorectal, pancreas, esophagus/stomach, liver, skin melanoma and lung) and from 557 healthy blood donors were assayed for hPG80. Finally, the hPG80 longitudinal kinetics during treatments in cancer patients were assessed in 2 retrospective studies to investigate the relationships between cancer activity and hPG80 titers.

### 2. Materials and methods

#### 2.1. Tumor tissue samples for immunohistochemistry and GAST mRNA expression analyzes

Human tissue samples, obtained by surgical resection, were collected from a tissue bank (Tissue bank, Tumorothèque, CHRU Brest, France). They involved 19 patients with the following cancer origins: colon, kidney, pancreas, liver, breast, ovary, lung, and stomach. The tissue samples were preserved at –80 °C until sectioned with a cryostat, and stored at –40 °C. Paired samples of tumor and adjacent tissues were assessed for hPG80 expression. Confocal images from 19 patients are shown in the figures of this paper. The clinical characteristics and tumor types of these 19 patients are presented on Table S1. For 10 of them, the adjacent tissues were also available.

In addition to the abovementioned tissues, some additional tissues were collected to assess the GAST mRNA expression levels in independent cohorts, including: the primary tumor samples from 12 colorectal cancer (CRC) patients matched with metastasis tumor tissues (Tissue bank, Tumorothèque, ICM Montpellier, France); the primary tumor samples from 14 patients with hepatocellular...
cancer (HCC) tissues (Tissue bank, Tumorothèque, Institut Cochin, France); the primary tumor samples from 15 patients with lung cancers, including 11 with matched tissues and blood samples (Tissue For Research Ltd, Spectrum Health System, Grand Rapids, Michigan, USA).

2.2. Blood samples from cancer patients for circulating titer analyzes

The blood samples from non-fasting 1546 patients with 11 different types of cancers were used for measuring their hPG80 blood concentrations (breast, uterus, ovarian, prostate, kidney, colorectal, pancreas, esophagus/stomach, liver, skin melanoma and lung). The datasets and blood samples originated from various different biobanks. Plasma were sampled at different times of patient disease managements.

Blood samples from CRC patients were obtained from the SIRIC (Site de Recherche Intégrée sur le Cancer) Montpellier Cancer (Montpellier, France), from the French prospective BIG-RENAPE project database (Lyon, France, NCT02823860) and from Tissue For Research Ltd (Spectrum Health System, Grand Rapids, Michigan, USA). The hepatocellular carcinoma (HCC) cohort came from the CHU (Centre Hospitalier Universitaire) Montpellier biobank (BB-0033-00031; the “Liverpool” collection; DC 2014-2328; AC 2014-2335; Montpellier, France). The metastatic kidney cancer cohort and the metastatic hormone sensitive prostate cancer, both came from the Mayo Clinic biobank (Rochester, NY, USA). All other samples were from Tissue For Research Ltd (Spectrum Health System, Grand Rapids, Michigan, USA).

Moreover, the longitudinal changes in hPG80 concentrations in cancer patients managed with different anti-cancer treatments were assessed in two retrospective kinetic studies, as a way of investigating the relationships between hPG80 blood levels and cancer activity. PRO-RENAPE was a cohort of 194 patients with peritoneal involvements from gastrointestinal cancers, treated with peri-operative chemotherapy regimens and cytoreductive surgery. The patients were extracted from the French prospective BIG-RENAPE project database (Lyon, France, NCT02823860). Blood samples were collected at inclusion (n = 194) and between 8 and 24 h after cytoreductive surgery, performed with or without hyperthermic intraperitoneal chemotherapy (HIPEC) (n = 85).

PRO-HCC was a cohort of 84 patients with HCC, managed with local or systemic treatments, including molecular targeted agents (“Liverpool” collection).

The following patient covariates were retrieved from the datasets: age and inflammation status with C-reactive protein (CRP) levels, disease stages according to either the American Joint Committee on Cancer (AJCC) guidelines (7th Edition 2010), or the Barcelona clinic liver cancer (BCLC) for HCC.

All patients (in the cancer and control groups) provided consent for research on their blood samples, in line with international regulations and ICH GCP (International Conference on Harmonization-Good Clinical Practice).

2.3. The control cohort

The blood samples from non-fasting 557 healthy donors, aged 18–70 years, were obtained from the French blood agency (Établissement Français du Sang) to build the control group. Although the agency requires that blood donors attest to be healthy, with no known history of cancer, we could not exclude the possibility that they had a cancer at the time of donation as the main risk factor for developing a cancer is age [12].

2.4. Cell lines

The following human cell lines were used: colorectal, T84; lung, NCI-H358; ovarian, SKOV3; gastric, AGS; esophageal, OE33; hepatoma, Huh7 and breast, MCF7; all of them being obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA). T84 were cultured in DMEM-F12 medium (PAN-Biotec). SKOV3, OE33, NCI-H358 were cultured in RPMI medium (PAN-Biotec). MCF-7 were cultivated in MEM Eagle with EBSS medium (PAN-Biotec). AGS were cultivated in F12-K medium (Mediatech, Inc.) and Huh7 were cultivated in α-MEM medium (Sigma-Aldrich) supplemented with non-essential amino acids (Gibco) and sodium pyruvate (PAN-Biotec). All culture medium were supplemented with 10% foetal calf serum (PAN-Biotec) and 1% v/v penicillin/streptomycin (PAN-Biotec). Absence of mycoplasma infection was confirmed by regular testing.

2.5. Immunofluorescence IHC

Immunofluorescence IHC was performed as previously described [13]. Briefly, human frozen sections were unfrozen, fixed with 4% PFA for 10 min, permeabilized with Triton X100, incubated simultaneously with the primary antibodies, i.e. one of the antibodies directed against hPG80, made in rabbit, and directed against the C-terminal or the N-terminal parts of hPG80, and one antibody directed against heparin sulfate proteoglycans (10E4 epitope, Abnson, Abingdon, UK), then incubated with secondary antibodies (anti-rabbit conjugated to AlexaFluor-647 and anti-mouse IgM conjugated with AlexaFluor-565, both from Merck, Lyon, France). Human tissues autofluoresce through the entire visible spectrum but not in the near infrared wavelength, as exemplified in Fig. S1A–C. This is due to large quantities of elastin in the connective tissues, and of lipofuscin in the lysosomes of macrophages. In order to address this problem, we visualized hPG80 labeling with secondary antibodies conjugated to AlexaFluor-647 (near infrared). The green channel of the confocal microscope was also turned on during recordings, without antibody labeling in this wavelength, to independently record autofluorescence of the tissue samples. Therefore, the autofluorescence appeared with white color whereas the specific hPG80 labeling was visualized with the red color only (Fig. S1D). The sections were counterstained with bisbenzimide to visualize the cell nuclei. Results were recorded with a Zeiss confocal laser scanning microscope at the PIMM facility (Plateforme d’Imagerie et de Mesures en Microscopie) of the University of Brest.

2.6. Controls of labeling/antibody specificity for IHC

Control experiments were carried out according to common standards to assess the specificity of the hPG80 labeling by IHC [14]. Additional IHC labelings were processed as described above, with either different primary antibodies directed against hPG80 (Fig. S2A–B), omission of the primary antibodies (Fig. S2C–D), omission of the primary and secondary antibodies (Fig. S2E–H), or pre-absorption of the anti-hPG80 antibodies with the recombinant protein hPG80 (Fig. S2I and J). The recombinant hPG80 was produced in E. coli BL21 Star (DE3) strain (Institut Pasteur). A 50-fold excess of the recombinant hPG80 protein was incubated with the antibodies prior to incubating frozen sections (Fig. S2G and H). All tests were successful. The N-terminal and the C-terminal antibodies generated similar labelings, no signal –besides autofluorescence– was detected without primary and/or secondary antibodies, and the hPG80 immunoreactivity was quasi-absent in the pre-absorption experiments (Fig. S2).

2.7. RNA extraction from tissues, reverse transcription, and qPCR

Lysis of tumor samples stored in liquid nitrogen was achieved using GentleMACS Octo Dissociator (Miltenyi Biotec). The mRNA extraction was performed with RNeasy Mini Kit (QiAGEN) according to the manufacturer’s instructions. Retrotranscription was performed on 1 μg mRNA with Primerscript RT Mastermix (Takara) according to the manufacturer’s instructions on a MasterCycler ep Gradient (Eppendorf) machine. Finally, quantitative PCR was performed on
2 μL of cDNA with the Sybr Premix ExTaq kit (Takara) according to the manufacturer’s instructions in white skirted Twin. The sequences of the qPCR primers (Sigma) were: hGAST-F (5'- CCACACCTGTTG-GACAGC-3'), hGAST-R (5'- TCTATCATCCATGACCCGTTC-3'), hGAPDH-F (5'- CATGAGAACCTGACAGC-3'), hGAPDH-R (5'- AGTCCTCACGATACCAAAGT-3'), hHPRT1-F (5'- ATTCATTTAGCCTTCATGCTCCT-3'), hHPRT1-R (5'- TCCAATAACAAACGTCCTGC-3'); hEEF1A1-F (5'- ATGCTGACCATGTTGATAGT-3'), hEEF1A1-R (5'- ATTCAATTTAGCCTTCATGCTCCT-3'); hACTB-F (5'- GATCAAAGCGTTGCTCCT-3') and hACTB-R (5'- TGTCAAAAGGTTGTAAC-3').

2.8. Survival assays in vitro cultured cells

Survival assays were performed as described by Prieur et al. [5]. Briefly, 100,000 (for T84), 300,000 (for NCI-H358) and 120,000 (for SKOV3) single cell suspensions were homogeneously seeded in 6-well plates (NUNC 055426) in complete medium and incubated for 7 h at 37 °C in a 5% CO2 atmosphere. After cell adherence, the medium was removed and the wells refilled with serum-free culture medium for overnight incubation. The treatments then began by exchanging the medium for fresh serum-free medium containing the antibodies of interest at the indicated concentrations. The treatment was renewed once daily (for SKOV3) or twice daily (for NCI-H358 and T84) for 48 h, before dissociating the cells and assaying the concentration of propidium-positive and -negative cells using a C6 Accuri flow cytometer (Becton-Dickinson). Cell counts at the start of treatment (T0) were subtracted from test and control cell counts measured at 48 h.

2.9. Sphere formation assay

The sphere formation assay was performed as described by Prieur et al. [5]. Spheres were cultivated in Ultra-Low Adherence (ULA) polystyrene flasks or plates (Corning) in M11 medium: DMEM/F12 with Glutamax (Gibco 31331-028), 20 μg/mL insulin (Sigma I0908), 1% N2 supplement (Gibco 17502-048), 20 ng/mL EGF (R&D Systems 236-EG), 10 ng/mL bFGF (R&D Systems 233-FL/CF), 3 mg/mL D-glucose (Sigma 49139), and 1% penicillin-streptomycin (PanBiotech P06-07100). Briefly, dissociated cells were seeded with 150 to 700 single cells, for all cell lines tested, per well in 24-well ULA plates with a FACS Aria (Becton-Dickinson). Cells were cultured for 7 to 11 days and treated every 3 to 4 days with antibodies of interest at the indicated concentrations. After 7–11 days at 37 °C in 5% CO2, each well was photographed by bright-field microscopy (Nikon ECLIPSE TS100, image acquisition with NIS-Element F) and the pictures analyzed by ImageJ (NIH) to count the spheres with a mean diameter >20 μM.

2.10. Measuring hPG80 in the blood samples

DxPG80 lab kit (the ELISA assay developed by ECS-Progastrin, Switzerland) was used to measure hPG80 levels in all blood samples according to the manufacturer’s instruction. According to the manufacturer, the limit of Detection (LoD) is at a hPG80 concentration of 1.2 pM and the limit of Quantitation (LoQ) is at a hPG80 concentration of 2.3 pM. The inter- and intra-assay coefficients of variation (CV%) is below 10%.

2.11. Measuring alfa-fetoprotein (AFP) concentrations

In the HCC cohort, blood AFP concentrations were centrally measured using Cobas E411.

2.12. Statistical analyzes

Changes in hPG80 levels over time in the two kinetic cohorts were assessed with the concentrations measured at the main stages of disease management and at relapse/progression if any. Patterns were explored using graphical descriptive figures and statistics. The kinetics of hPG80 and AFP were assessed in the PRO-HCC cohort. Acknowledging the lack of a validated percentage increase to describe blood tumor biomarker progression, a 25% change in hPG80 concentration was arbitrarily selected in the present study. This cut-off was previously reported for prostate-specific antigen (PSA) in prostate cancer [15].

Correlations between covariates and hPG80 levels were measured by Spearman correlation coefficients. Mann–Whitney U -tests were used to compare continuous covariates. Statistical analyzes were performed with two-sided 5% alpha risks, using SAS software (GraphPad Prism version 9.4 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com.)

3. Results

3.1. Detection of hPG80 in colorectal tumors by immuno-fluorescence IHC

Fluorescence IHC was first conducted in sections of colon cancers where hPG80 is known to be expressed [2,9,16]. Dual immunolabelings for hPG80 (in red), heparan sulfates (in green) and cell nuclei counterstaining (in blue) were performed to visualize hPG80 expression and basement membranes (heparan sulfate proteoglycan immunoreactivity, HSPG) in the histological context. Fig. 1(A) shows that hPG80 is primarily expressed in cell clusters within the intestinal glands (arrow and double arrow). These cells were mostly epithelial cells (Fig. 1(A) and (B)), recognizable by their round cell nucleus and by their location within gland-delineating basement membranes (Fig. 1(A), arrowhead). At the subcellular level, hPG80 expression was concentrated in the perinuclear area (Figs. 1(C) and S3), Fig. 1(E) shows that hPG80 was expressed in the cytoplasm revealing a punctuate or granular pattern which suggests the subcellular localization in the organelles. Investigation of hPG80 in other patients with colon cancer confirmed these data (Fig. S3).

Investigation of hPG80 expression in the tissues adjacent to the tumor revealed variations according to the tissue sample. In most patients, adjacent tissue was showing scarce, if any, hPG80 immuno-reactivity (Fig. 1(D) and (F)). hPG80 expression was generally found in the stroma (arrowhead), and to a lesser extent in epithelial cells (arrow) (Fig. 1(B)). The stroma displayed cells with various morphologies (compare arrowhead and double arrow) (Fig. 1(D)).

3.2. Detection of hPG80 in other types of tumors by immuno-fluorescence IHC

To assess whether hPG80 is expressed in tumors different from CRC, we carried out experiments with multiple cancer tissues. First, dual immunolabelings for hPG80 (in red), heparan sulfates (in green) as well as nuclear counterstaining (in blue) were performed in primary tumor samples of pancreas, liver and kidney. hPG80 was detected in all tumor tissues, as illustrated in Figs. 2 and S4. A loss of basement membrane integrity was usually associated with cancer patterns (Fig. 2(A), (C) and (E)). The adjacent tissues displayed the typical basement membranes of differentiated tissues, and scarce or absent hPG80 immunoreactivity (Fig. 2(B), (D) and (F)).

Fig. S5 shows hPG80 expression in both epithelial and stromal cells of stomach adenocarcinomas. Epithelial cells (Fig. S5A, arrow) were typically delineated by a basement membrane (double arrow), whereas stromal cells were located in the other side of the basement.
membrane (double arrowhead). All images indicate that hPG80 is not expressed in the extracellular matrix (Fig. S5A) but in the cytoplasm. This is clearly illustrated in Fig. S5B (three-dimensional confocal reconstruction). Similarly to colon tumors, hPG80 was often detected in trabeculae, i.e. chains of stromal cells (Fig. S5C, arrow) encompassing the tumor mass, and spanning through the adjacent tissues (Fig. S5D, arrow). The adjacent tissue was clearly altered, showing undifferentiated groups of cells without glandular organization (Fig. S5D, double arrow) and basement membranes (Fig. S5D, arrowhead). In patient shown in Fig. S5, the glands of the adjacent tissue showed no hPG80 expression (Fig. S5D, arrowhead) whereas numerous ectopic cells expressed hPG80 in the lumen of the glands within the tumor mass (Fig. S5C, arrowhead).

High hPG80 expression was then confirmed in other tumor tissues, from ovary, breast and lung cancers. Strikingly, all tumors displayed many hPG80 immunoreactive cells in the tumor bulk (arrows, Figs. S6, S7 and S8), whereas adjacent tissues were free of hPG80 (Fig. S6D and F).

To confirm the high hPG80 expression in tumor cells by another method, RT-PCR analysis of GAST, which encodes hPG80, were carried out in a series of primary tumors with matched metastases, surgically removed from patients with CRC, hepatocellular carcinoma (HCC) and lung cancers. We showed the expression of GAST in primary tumors and in the matched metastatic tumors from 12 stage IV CRC patients (Fig. 3A). Depending on the patients, the levels of GAST in the metastatic lesion cells was either lower (42%), equal (16%) or higher (42%) than in the primary tumors, with a maximum of 30 times higher expression. Compared to the expression in T84 CRC cell line, GAST mRNA was also detected in all HCC tumor samples (14/14) with a relative range ranking from 3.95 to 21,277, and 11/15 lung tumor samples with a relative range ranking from 0.06 to 36.37 (Fig. 3B–C).

3.4. Anti-hPG80 antibody decreases cell proliferation in tumor cell lines different from CRC

We assume that aberrant hPG80 expression might be exploited for therapeutic purpose, as already shown in CRC [5] and confirmed...
in Fig. S9 (90% decrease of cell growth in T84 CRC cell line). We tested two cell lines from ovarian and lung cancer origins. Accordingly, treatment of human ovarian (SKOV3) cancer cells with a humanized anti-hPG80 antibody induced a significant 96.1% decrease of cell growth, suggesting that hPG80 was not only released by the human ovarian cancer cells, but was also required for the survival of these cells (Fig. S9B). Cell survival was also significantly decreased in NCI-H358 human lung cell line by 32.5% (Fig. S9C). These results provide evidence that hPG80 is able to decrease the survival of tumor cells from different origins.

3.5. Anti-hPG80 antibody decreases sphere formation from tumor cell lines different from CRC

Cancer stem cells (CSCs) are key drivers of tumor progression [17]. CSCs have the ability to form spheres in vitro when grown in non-adherent serum-free conditions [18]. Sphere formation assays provide therefore a useful tool to assess the growth and propagation of CSCs in cancer cell lines. To evaluate the effects of anti-hPG80 antibodies on sphere formation, cell lines from six different origins were grown as spheres, and incubated with anti-hPG80 antibodies for 7 to 11 days. As shown in Fig. 4(A), a 40% inhibition of spheres number in the T84 CRC cell line was observed in the presence of anti-hPG80 antibodies. Similar results were obtained in the five other cell lines tested, with inhibitions of spheres number by 54.1% for SKOV3 (ovarian), 50.6% for MCF-7 (breast), 37.7% for OE33 (esophageal), 36.7% for Huh-7 (hepatic), and 28% for AGS (gastric) (Figs. 4(B)–(F)), thereby suggesting that hPG80 controls the self-renewal of CSCs originating from various cancers.

3.6. hPG80 was detectable in the blood of patients with various cancer origins

After validating the expression of hPG80 within the tumors, we analyzed the levels of hPG80 in the blood of cancer patients. The cohort was composed of 1546 patients with 11 different cancer origins. These included breast (n = 62); colorectal (n = 305); esophagus/stomach (n = 74); kidney (n = 184); liver (n = 60); skin melanoma (n = 38); ovary (n = 44); pancreas (n = 34); prostate (n = 558); uterus (n = 77) (Table 1). When all these cancer types were combined, the median hPG80 concentration was 4.88 pM (IQR 2.02–11.73 pM) (Fig. 5(A) and Table 1). It was significantly higher than the median hPG80 concentration in the control group composed of healthy blood donors (1.05 pM (IQR 0.00–2.92 pM); p < 0.0001). Median hPG80 concentration across tumor types varied from 2.50 to 11.65 pM (Fig. 5(B), and Table 1). In both healthy donors and cancer patients, the relationship with age existed, but was moderate (Fig. S10). For the cancer group, the Spearman $R^2$ = 0.31; for healthy blood donors, the Spearman $R^2$ = 0.34.

Interestingly, the blood levels of hPG80 correlated with the expression of GAST mRNA in the matched tumors of 11 patients with lung cancers (Spearman r = 0.8; p = 0.0023), thereby suggesting...
the tumoral origin of hPG80 detected in the blood of the patients (Fig. 5(C)).

3.7. hPG80 kinetics in patients receiving anti-cancer treatments

In the two kinetic datasets, explorative and descriptive analyzes of concentration versus time curves were performed in order to analyze the impact of cancer treatments on hPG80 levels, as a way of assessing the links between hPG80 levels and tumor activity.

In the PRO-RENAPE cohort, median hPG80 significantly decreased from inclusion to the post-operative period from 3.08 to 1.57 pM (Fig. 6(A)). The individual curves showed that 74.2% of patients had hPG80 titer declines >25%. The remaining patients experienced stable concentrations (16.1% of patients), or hPG80 level increases >25% (9.7% of patients) (Fig. 6(B)). In Fig. 6(B), we performed an individual analysis on 62 patients before and after surgery using Wilcoxon matched pairs test. Patients were stratified in two groups according to the decrease (n = 46, left part of Fig. 6(B)) and stable or increase (n = 16, right part of Fig. 6(B)). For patients who had hPG80 decrease after surgery (n = 46), the calculated p-value is p < 0.0001. For patients who had stable or increased hPG80 levels (n = 16), the p-value is p = 0.155. For all patients (n = 62), significant differences in median hPG80 levels were observed before and after surgery (5.36 pM versus 3.00 pM; p < 0.0001) (Fig. S11). 16 patients out of 46 patients (34.8%) who had a decreased level after surgery had hPG80 levels below the LoD (1.2 pM). An overall trend for declining hPG80 concentration was found from inclusion (median 3.08 pM; n = 194), through end of peri-operative chemotherapy (median 2.20 pM; n = 23), post-operative time (median 1.57 pM; n = 85), to the end of post-operative chemotherapy (median 1.21 pM, n = 6) (Fig. S12).

In the PRO-HCC cohort, individual concentration versus time curves of hPG80 evolved consistently with disease activity and AFP kinetics in most patients. This is illustrated by seven typical patient profiles (Figs. 7(A), S13 and S14). In particular, Fig. 7(A) illustrates a patient treated with nivolumab. For this patient, hPG80, present before the treatment, was undetectable upon complete response of the patient; upon relapse, hPG80 was again detected. The patients considered to be in remission after disease management had lower hPG80 levels, compared to those with active cancers (median 1.99 versus 15.71 pM, p < 0.0001, n = 63, Figs. S15A and 7(B)). Conversely, 76.0% of the patients whose disease progressed during treatments, had hPG80 increase >25%. Of note, AFP was not informative in 45.7% of patients, because the concentrations were below the most
commonly reported and used cut-off value of 20 ng/ml in clinical practice [19]. In these patients, significant relationships between hPG80 level changes and disease status at the end of treatments were found (median 1.47 pM for patients in remission versus 18.33 pM for those in progression, \( p = 0.018 \), Fig. S15B). We found no link between hPG80 and inflammation status, assessed by CRP concentration in the PRO-HCC cohort (Fig. S16) suggesting that, if any, impact of inflammation is probably limited.

4. Discussion

The data reported in the present paper demonstrate that hPG80 is overexpressed in the tumor, and present in the blood of the patient, not only in colorectal cancers as already known, but also in a wide range of 10 other epithelial and non-epithelial cancers.

There are evidence supporting the tumor origin of this expression. First, IHC revealed strong and specific hPG80 immunoreactivity in all the tumor tissue samples examined, regardless of the tumor origins, suggesting that hPG80 was ubiquitously expressed in all tested cancers. The hPG80-expressing cells were commonly found in the parenchyma, i.e. the epithelium for all organs, and occasionally in the stroma (connective tissue). In contrast, the tissues adjacent to the tumor bulks revealed no or minimum hPG80 expression, and in most cases, this extra-tumoral expression was found in the stroma. These results are in line with the work of Caplin et al. who detected the presence of hPG80 in liver and pancreatic cancers but not in normal liver and pancreas [20,21]. Caplin also showed that hPG80 did not mature into gastrin-NH2, as published for CRC and in agreement with the lack of secretion of gastrin-NH2 by CRC cell lines [20,21]. By contrast, in ovarian cancers, van Solinge et al. detected the biologically active gastrin-NH2 [22]. hPG80 was not detected as such. Thus, it is possible that in ovarian cancers, in addition to the presence and the release of hPG80 that is shown here via IHC and the consequence of hPG80 neutralization by a very specific antibody, part of hPG80 matures into gastrin-NH2 which could also be released. None of these studies however addressed the presence of hPG80 in the blood, described in the present study, nor the consequence of hPG80 neutralization in experimental models originating from these tumors.

Secondly, in vitro studies confirmed that hPG80 exhibited oncogenic functions on cancer stem cells by regulating sphere formations from cell lines originating from these tumors. Moreover, cancer patients had consistently significantly higher levels of hPG80 compared to healthy blood donors expected to be free of cancers even at early stage as exemplified in the HCC cohort, thereby confirming the early role of hPG80 in tumorigenesis, in line with the IHC observations.

Two kinetic studies, where hPG80 was serially measured during cancer managements, confirmed that blood hPG80 level longitudinal changes were related to treatment efficacy and cancer activity. Our data confirm the previous work by Koutser et al., who first showed that blood hPG80 returned to control levels after surgical removal of colorectal tumors [23], but they extend the relevance of hPG80 to other cancer origins.

The ubiquitous overexpression of hPG80 found in the tumor and in the blood of cancer patients, regardless of the tumor origin, can be understood by the regulation of hPG80 expression related to some major oncogenic pathways, such as Wnt/β-catenin and RAS-RAF-ERK pathways. Indeed, the gene GAST, which encodes hPG80, is a target of these pathways, known to be overactivated in CRC and many other cancers, including those tested in the present studies [24–27]. Genetic abnormalities in these pathway genes have been well studied and characterized: APC and CTNNB1 (β-catenin gene) mutations in the Wnt/β-catenin pathway, and KRAS, B-RAF, and EGFR (epidermal growth factor receptor) mutations in the RAS-ERK pathway [28,29]. The correlation found between blood hPG80 levels and tumor GAST mRNA expression in lung cancer patients strengthens this hypothesis.

If the present results establish that the tumor cells from many cancer types overexpress hPG80 and that hPG80 expression is related to cancer cell activity, some findings raise new questions and hypotheses. First, hPG80 was sometimes expressed in the tissues adjacent to the tumor. It is now recognized that regions surrounding tumors can exhibit morphological and phenotypical alterations with respect to non-tumor-bearing healthy tissues [30]. hPG80 expression in the tissues surrounding the tumor might indicate the presence of cancerous or precancerous lesions, that could potentially participate in tumor growth and progression. It would be interesting to examine the clinical outcomes of these patients to better define the clinical significance of hPG80 expression in tissues adjacent to the tumor. Indeed the expression of hPG80 in the tissues adjacent to the tumor might provide relevant information about the risk of subsequent recurrence. Interestingly, hPG80 expression was not restricted to epithelial cells, but was also detected in stromal cells as visualized in Fig. S8.

Table 1

Patient demographic and clinical characteristics

<table>
<thead>
<tr>
<th>Sample Status</th>
<th>No. of Patients</th>
<th>Gender</th>
<th>Median Age, Years (range)</th>
<th>hPG80 Median (IQR), pM</th>
<th>hPG80 Mean (SE), pM</th>
</tr>
</thead>
<tbody>
<tr>
<td>All Cancer</td>
<td>1546</td>
<td>Male</td>
<td>1042</td>
<td>66 (21-93)</td>
<td>4.88 (2.02-11.73)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Female</td>
<td>470</td>
<td>66 (21-93)</td>
<td>12.94 (0.82)</td>
</tr>
<tr>
<td>Breast</td>
<td>62</td>
<td>Male</td>
<td>0</td>
<td>57 (34-70)</td>
<td>2.5 (0.60-6.60)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Female</td>
<td>62</td>
<td></td>
<td>6.35 (1.36)</td>
</tr>
<tr>
<td>CRC</td>
<td>305</td>
<td>174</td>
<td>123</td>
<td>64 (23-93)</td>
<td>4.36 (1.62-10.61)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>124</td>
<td></td>
<td></td>
<td>10.29 (1.11)</td>
</tr>
<tr>
<td>Esophagus/Stomach</td>
<td>74</td>
<td>29</td>
<td>43</td>
<td>65 (21-83)</td>
<td>6.92 (3.00-19.08)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>21.06 (6.25)</td>
</tr>
<tr>
<td>Kidney</td>
<td>184</td>
<td>135</td>
<td>48</td>
<td>64 (25-85)</td>
<td>7.25 (3.21-19.71)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>21.50 (2.96)</td>
</tr>
<tr>
<td>Liver</td>
<td>110</td>
<td>82</td>
<td>23</td>
<td>65 (23-86)</td>
<td>11.65 (3.25-28.24)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>17.76 (1.82)</td>
</tr>
<tr>
<td>Lung (NSCL)</td>
<td>60</td>
<td>39</td>
<td>21</td>
<td>67 (45-85)</td>
<td>5.78 (2.87-13.34)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>10.39 (1.57)</td>
</tr>
<tr>
<td>Skin Melanoma</td>
<td>38</td>
<td>8</td>
<td>12</td>
<td>61 (38-79)</td>
<td>3.65 (1.65-7.62)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5.09 (0.79)</td>
</tr>
<tr>
<td>Ovary</td>
<td>44</td>
<td>40</td>
<td>4</td>
<td>60 (22-86)</td>
<td>3.18 (1.15-9.26)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>10.39 (5.71)</td>
</tr>
<tr>
<td>Pancreas</td>
<td>34</td>
<td>17</td>
<td>17</td>
<td>66 (30-83)</td>
<td>6.47 (5.13-11.98)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>11.24 (2.38)</td>
</tr>
<tr>
<td>Prostate</td>
<td>558</td>
<td>558</td>
<td>0</td>
<td>70 (43-93)</td>
<td>4.59 (2.12-9.79)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>11.58 (1.13)</td>
</tr>
<tr>
<td>Uterus (endometrium/cervix)</td>
<td>77</td>
<td>0</td>
<td>77</td>
<td>60 (29-90)</td>
<td>2.92 (0.90-6.91)</td>
</tr>
<tr>
<td>Healthy blood donors</td>
<td>557</td>
<td>310</td>
<td>247</td>
<td>35 (18-70)</td>
<td>1.05 (0.00-2.92)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.04 (0.11)</td>
</tr>
</tbody>
</table>

* 34 missing gender (2.20%)
** 80 undetermined stage (5.12%)
The tumor stroma hosts many types of cells such as fibroblasts, vascular cells, and immune system cells. In waiting for the findings of further investigations about the origins of the cells that express hPG80 in the stroma, one hypothesis is that hPG80 would be expressed by fibroblasts. Indeed, tumor stroma fibroblasts, also known as cancer associated fibroblasts (CAFs) or myofibroblasts, are known to play a role in tumorigenesis, including tumor initiation and progression [31]. If this hypothesis was confirmed, it would be possible that expression of hPG80 in CAFs could modulate local tumor cell invasion and regulate tumor spreading to metastatic sites. In both tumor parenchyma and stroma, hPG80 was localized in the cytoplasm. In physiological conditions, within the antral G-cell, hPG80 is translated and folded into the endoplasmic reticulum, then transit throughout the trans-Golgi network before being packaged into immature secretory vesicles to undergo further cleavage [32].

We hypothesize that the punctate pattern of intracellular staining represents secretory granules in which neo-synthesized hPG80 is stored before being secreted. Our findings showing that anti-hPG80 neutralizing antibodies decreased in vitro cell proliferation and sphere formation of tumor cell lines confirmed that hPG80 is indeed released from tumor cells. Another possible source of blood hPG80 detected in cancer patients might be the G cells in the antrum of the stomach. One could hypothesize that hPG80 measured in the blood of cancer patients might come from these cells rather than from the tumor cells, emphasizing an endocrine secretion rather than a pathological one. A strong argument against this hypothesis is the fact that the expression of the GAST gene, encoding hPG80, decreases with age, which is not what is observed in cancer patients or even healthy blood donors since, as stated above, the relationship between hPG80 with age, if any, is a positive relationship [33–35].

Fig. 5. hPG80 is detected in the blood of patients with various cancer origins. (A) Comparison of median hPG80 concentrations between healthy blood donors (n = 557) and cancer patients (n = 1546). (B) Comparison of median hPG80 concentrations between all types of cancers tested: breast (n = 62), CRC (n = 305), esophagus/stomach (n = 74), kidney (n = 184), liver (n = 110), lung (n = 60), skin melanoma (n = 38), ovary (n = 44), pancreas (n = 34), prostate (n = 558), uterus (n = 77). (C) Correlation between GAST mRNA tissue expression and hPG80 in the blood from the same lung cancer patient (n = 11).
Of note, if this observation is consistent with the assumption of hPG80 secretion by cancer cells, it also raises therapeutic perspectives. Indeed, since sphere formation indicates the presence of cells with cancer stem cells (CSC) features, hPG80 could be used as a target for future anti-hPG80 therapies. It was previously shown that anti-hPG80 antibodies decrease both in vitro and in vivo cancer stem cell frequency in colorectal cancer cell lines [5]. The present data suggest that similar results could be obtained in 5 other cancers from different origins and further studies will be required to evaluate for each individual type of cancer the risk and benefit of using anti-hPG80 as a treatment. Nevertheless, our data support the potential relevance of CSCs targeting, through hPG80, as a pan-cancer treatment strategy. Indeed, CSCs were identified in multiple malignancies including leukemia and different solid tumors, such as glioblastoma, breast and colon cancers [36], and have frequently been associated with chemoresistance and limited efficacy of standard anticancer therapies [37,38]. This is why therapies that target both CSCs and other more differentiated tumor cells are currently gaining attention within the scientific community. In that context, hPG80 represents an attractive potential therapeutic target due to its role in CSCs feature regulation in various cancer types [9]. A monoclonal humanized therapeutic antibody targeting hPG80 is being developed [5]. Furthermore, it has to be noted that similarly to hPG80, both APC and KRAS mutations promote CSC phenotype and disease dissemination in a mouse xenograft model [39]. This implies that hPG80 may act synergistically with Wnt and Ras pathways to support malignant transformation and activation of CSCs.

Moreover, the data from the kinetic studies support the potential role of hPG80 as a monitoring marker of treatment efficacy. In the peritoneal carcinomatosis cancer patient cohort, hPG80 levels decreased significantly after surgery in 74.2% of the cases. In the remaining patients, the concomitant HIPEC (Hyperthermic Intraperitoneal Chemotherapy) might have induced an increase in hPG80 levels since we previously described that chemotherapy induces hPG80 secretion [5]. Also, as illustrated with a HCC patient treated with nivolumab on Fig. 7(A), hPG80 reliably witnesses response to the treatment and disease evolution. Finally, blood hPG80 monitoring might also be considered as the future companion test for the monoclonal antibody on-development [5].

In summary, the data presented here show the potential value of hPG80 as a novel cancer target with potential strong important applications for cancer management. hPG80 could embody a new ubiquitous circulating tumor biomarker for monitoring disease activity and treatment efficacy in a wide spectrum of human malignancies, including the eleven frequent cancer origins tested here. It also represents a relevant target for a monoclonal antibody meant to improve anti-cancer therapeutic efficacy.
Declarations of competing interest

AP and DJ own shares and are patent inventor in relation with this project.

DJ is a co-founder of the company and a senior scientific advisor.

AP is a co-founder of the company and the Chief Scientific Officer of the company.

All other authors: no conflict of interest.

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ECS-Progastrin.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ebiom.2019.11.035.

Fig. 7. hPG80 kinetics in patients receiving cancer treatments in the PRO-HCC cohort. (A) Illustrative hPG80 longitudinal changes around and during disease management (baseline; remission; progression), with associated imaging obtained at the same times (multifocal liver involvement at baseline; remission after treatment with nivolumab; new liver lesions on ultrasound at progression) in a typical patient. (B) hPG80 levels at different disease stages (focal, n = 23; locally advanced, n = 42; metastatic, n = 19) and at disease remission after treatment (n = 32). In order to simplify the reading of the graph, only statistically significant differences were shown on the graph. All the other comparisons were tested and none of them were significant.

References
