First-in-man Phase 1 Clinical Trial of Gene Therapy for Advanced Pancreatic Cancer: Safety, Biodistribution, and Preliminary Clinical Findings

Louis Buscail[1–4], Barbara Bournet[1–3], Fabienne Vernejoul[5], Gilles Cambois[5], Hubert Lulka[2,3], Naïma Hanoun[2,3], Marlène Dufresne[2,3], Aline Meulle[2,3], Alix Vignolle-Vidoni[1–3], Laetitia Ligat[2,3,6], Nathalie Saint-Laurent[2,3,6], Frédéric Pont[2,3,6], Sébastien Dejean[7], Marion Gayral[2,3], Frédéric Martins[8], Jérôme Torrisani[2,3], Odile Barbey[4], Fabian Gross[4], Rosine Guimbaud[2,3,9], Philippe Otal[2,10], Frédéric Lopez[2,3,6], Gérard Tiraby[5] and Pierre Cordelier[2,3]

[1]Department of Gastroenterology, CHU Toulouse - Rangueil, Toulouse, France; [2]Université Toulouse III-Paul Sabatier, UMR1037 CRCT, Toulouse, France; [3]Inserm, UMR1037 CRCT, Toulouse, France; [4]CIC Biotherapies 511, CHU Toulouse and INSERM, Toulouse, France; [5]Cayla InvivoGen Company, Research Department, Toulouse, France; [6]Inserm, UMR1037 Proteomic Group-CRCT, Toulouse, France; [7]Department of Mathematics, Université Toulouse III-Paul Sabatier, Toulouse, France; [8]Inserm, UMR1048, Toulouse, France; [9]Department of Oncology, CHU Toulouse-Rangueil, Toulouse, France; [10]Department of Radiology, CHU Toulouse-Rangueil, Toulouse, France

This phase 1 trial was aimed to determine the safety, pharmacokinetics, and preliminary clinical activity of CYL-02, a nonviral gene therapy product that sensitizes pancreatic cancer cells to chemotherapy. CYL-02 was administrated using endoscopic ultrasound in 22 patients with pancreatic cancer that concomitantly received chemotherapy (gemcitabine). The maximum-tolerated dose (MTD) exceeded the maximal feasible dose of CYL-02 and was not identified. Treatment-related toxicities were mild, without serious adverse events. Pharmacokinetic analysis revealed a dose-dependent increase in CYL-02 DNA exposure in blood and tumors, while therapeutic RNAs were detected in tumors. No objective response was observed, but nine patients showed stable disease up to 6 months following treatment and two of these patients experienced long-term survival. Panels of plasmatic microRNAs and proteins were identified as predictive of gene therapy efficacy. We demonstrate that CYL-02 nonviral gene therapy has a favorable safety profile and is well tolerated in patients. We characterize CYL-02 biodistribution and demonstrate therapeutic gene expression in tumors. Treated patients experienced stability of disease and predictive biomarkers of response to treatment were identified. These promising results warrant further evaluation in phase 2 clinical trial.

Received 9 December 2014; accepted 18 December 2014; advance online publication 10 February 2015. doi:10.1038/mt.2015.1

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer death in Western countries, with the lowest 5-year relative[1] and 1-year survival rates[2] among commonly diagnosed cancers. PDAC is anticipated to move to the second leading cause of cancer death worldwide by 2020 in the absence of improvements in early detection and/or treatment.[3] Since 1997, gemcitabine is the only approved first-line treatment for patients with advanced PDAC[2]; however, the 5-year survival rate is only 2%,[1] with 1-year survival rates ranging from 17 to 23%.[2] Recently, phase 2 and 3 trials exploring gemcitabine-based combinations with erlotinib[4] or nab-paclitaxel[5] were found to improve overall survival (OS) of patients with a metastatic disease. Thus, the moderate activity of standard gemcitabine and gemcitabine-based regimens strongly encourages new therapeutic research programs such as gene therapy.

We devised in the past decade a highly innovative approach based on therapeutic gene transfer using nonviral vectors to restore *SSTR2* expression (encoding for somatostatin receptor subtype 2) that is lost in 95% of PDAC tumors.[6] We found that SSTR2-based gene therapy induces a strong bystander antitumoral effect that is antiproliferative, proapoptotic, antiangiogenic, and antimetastatic.[7–13] Resistance to gemcitabine is a major cause of unsatisfactory improvement during pancreatic cancer treatment may help identify novel therapeutic target genes to enhance the efficacy of gemcitabine treatment. Deoxycytidine kinase (DCK) phosphorylates gemcitabine to gemcitabine diphosphate in a rate-limiting step. Loss of expression of this key enzyme was recently associated with acquired resistance to gemcitabine in pancreatic cancer cells, in preclinical models,[14] and in patients.[15] We demonstrated that delivering *DCK::UMK* fusion gene, encoding for both DCK and uridylate monophosphate kinase (UMK), using nonviral vectors overcomes PDAC-derived cells resistance to gemcitabine.[16] Thus, as opposed to many trials for PDAC treatment in which new agents are combined with gemcitabine simply because it is a standard of care, there is a strong rationale to deliver *DCK* and *UMK* genes and to treat advanced pancreatic cancer tumors with chemotherapy.

This phase 1 study was conducted to determine the recommended phase two dose of a clinical grade, gene therapy product

Correspondence: Pierre Cordelier, Cancer Research Centre of Toulouse – CRCT, UMR1037 Inserm/Université Toulouse III-Paul Sabatier, ERL5294 CNRS, 2 avenue Hubert Curien, Toulouse Oncopole, CS 53717, 31037 Toulouse Cedex 1, France. E-mail: pierre.cordelier@inserm.fr

combining for the first time *SSTR2*, *DCK*, and *UMK* gene expression, delivered by a nonviral vector, and administered with gemcitabine in patients with advanced pancreatic cancer. We characterized the feasibility, tolerability, and toxicity profile of the regimen and examined preliminary efficacy. Pharmacokinetic and biomarker studies were also performed.

RESULTS

Patient characteristics

A total of 22 patients with advanced pancreatic cancer were consented and received treatment between December 2010 and September 2012. The demographic and clinical characteristics of the enrolled patients are listed in **Table 1**. Thirteen patients were diagnosed with locally advanced disease, while 9 patients had distant metastasis. Four patients with locally advanced disease had received prior chemotherapy, whereas nine had not. All patients with distant metastasis excepting two had received prior chemotherapy. Among the 22 patients included, 20 patients were evaluable for safety evaluation. The gene therapy protocol was stopped for two patients (both receiving 250 µg of CYL-02): one patient received less than two-third of gemcitabine because of rapid progression of metastatic disease; the second patient had only one injection of CYL-02 because of septicemia caused by chronic biliary stent obstruction (these two patients had received FOLFIRINOX as a first treatment for metastatic PDAC).

Table 1 Demographic and baseline characteristics of the patients enrolled in the THERGAP gene therapy clinical trial

Clinical data	
Age (years)	Median: 61.5
	Range: 50–74
Gender: *n* (%)	Male: 15 (68%)
	Female: 7 (32%)
Pancreatic tumor localization: *n* (%)	Head: 11 (50%)
	Body: 9 (41%)
	Tail: 2 (9%)
	None: 13 (59%)
Metastatic cases: *n* (%)	Liver alone: 8 (36.5%)
	Liver and peritoneal: 1 (4.5%)
	None: 12 (54.5%)
First-line treatments: *n* (%)	Chemotherapy[a]: 8 (36.5%)
	Chemoradiotherapy: 1 (4.5%)
Other treatments: *n* (%)	Surgery[b]: 1 (4.5%)
	Biliary stent: 5 (23%)
	Surgical bypass: 4 (18%)

The gene-therapy protocol was stopped for two patients (both receiving 250 µg of CYL-02): one patient received less than two-third gemcitabine infusions because of rapid progression of metastatic disease; the second patient had only one injection of CYL-02 because of septicemia caused by chronic biliary stent obstruction (these two patients had received FOLFIRINOX as a first treatment for metastatic pancreatic cancer). THERGAP, gene therapy for advanced pancreatic adenocarcinoma.
[a]FOLFIRINOX and GEMOX protocols given to, respectively, six and two patients; liver metastasis occurred in the six patients that received FOLFIRINOX. [b]Left pancreatectomy. Among the 22 patients included, 20 patients were evaluable.

Dose escalation process

Patients received two intratumoral injections of CYL-02 using endoscopic ultrasound (EUS) followed by gemcitabine infusions (**Figure 1a**). Injection of escalating doses (125, 250, 500, and 1,000 µg) of CYL-02 was feasible in all cases whatever the size of the primary tumor (mean maximal size measured by EUS at day 1: 36.5 ± 2.3 mm (median: 33, range: 22–54)); the localization of the primary tumor (including uncus, isthmus, or tail in two, three, and two cases, respectively); and previous local treatment (*i.e.*, biliary stenting, biliary or digestive bypass, partial resection). CYL-02 injections resulted in "white cloudy" shapes within the tumor easily tracked by EUS (**Figure 1b–d**). No further escalation was attempted, as 1,000 µg was the highest planned dose of CYL-02. We declared dose level 4 (1,000 µg) as the recommended phase two dose for this study.

Safety

The toxicity profile observed with the study combination was similar to that reported for gemcitabine alone. All patients received at least one dose of treatment and were evaluable for toxicity. Grade I or higher treatment-related adverse events are summarized in **Table 2**. There was one death at dose 500 µg (patient #18), that was not considered as related to the study treatment due to the temporal relationship, while the patient was showing indications of stable disease (SD). Grade III–IV nondose-limiting treatment-related toxicities included fever in two patients (9%) treated with 250 and 500 µg of CYL-02. We conclude that the intratumoral delivery of anticancerous genes by EUS using nonviral vectors is feasible, well tolerated, and safe in patients with pancreatic cancer.

Pharmacokinetics and tumor analysis

We characterized the biodistribution of CYL-02 following intratumoral injection in patients. We first assessed CYL-02 dissemination in urine and blood. Therapeutic DNA was not detected in the urine of treated patients with the exception of patient #13 in whom the tumor had invaded the right kidney, strongly suggesting a direct leakage of CYL-02 into the urine flow following intratumoral injection, rather than active excretion by the kidneys. On the other hand, CYL-02 DNA levels peaked in the blood of patients following each of the two rounds of gene therapy. **Figure 2a** illustrates CYL-02 dissemination at dose = 1,000 µg, corresponding to 0.02 ± 0.01 and 0.06 ± 0.04% of the injected dose, respectively. In addition, the amount of CYL-02 detected in the blood after the first injection tended to be proportional to the dose administrated (P = 0.057, data not shown). There was no difference in the pharmacokinetic parameters of CYL-02 between day 1 and day 28.

CYL-02 DNA was detected in fine-needle aspiration (FNA) microbiopsies collected from tumors, 1 month following gene transfer. Tumors from patients #2 and #3 were too small to be sampled. We found that 0% (0/4), 75% (3/4, mean copy number $3.18 \pm 2.3 \times 10^2$), 66.6% (4/6, mean copy number $5.88 \pm 4.29 \times 10^3$), and 100% (4/4, mean copy number $1.05 \pm 1.02 \times 10^6$) of EUS-guided tumor biopsies were positive for CYL-02 following injection with 125, 250, 500, and 1,000 µg of CYL-02, respectively. CYL-02 DNA levels were statistically elevated in tumors receiving 1,000 µg as compared to

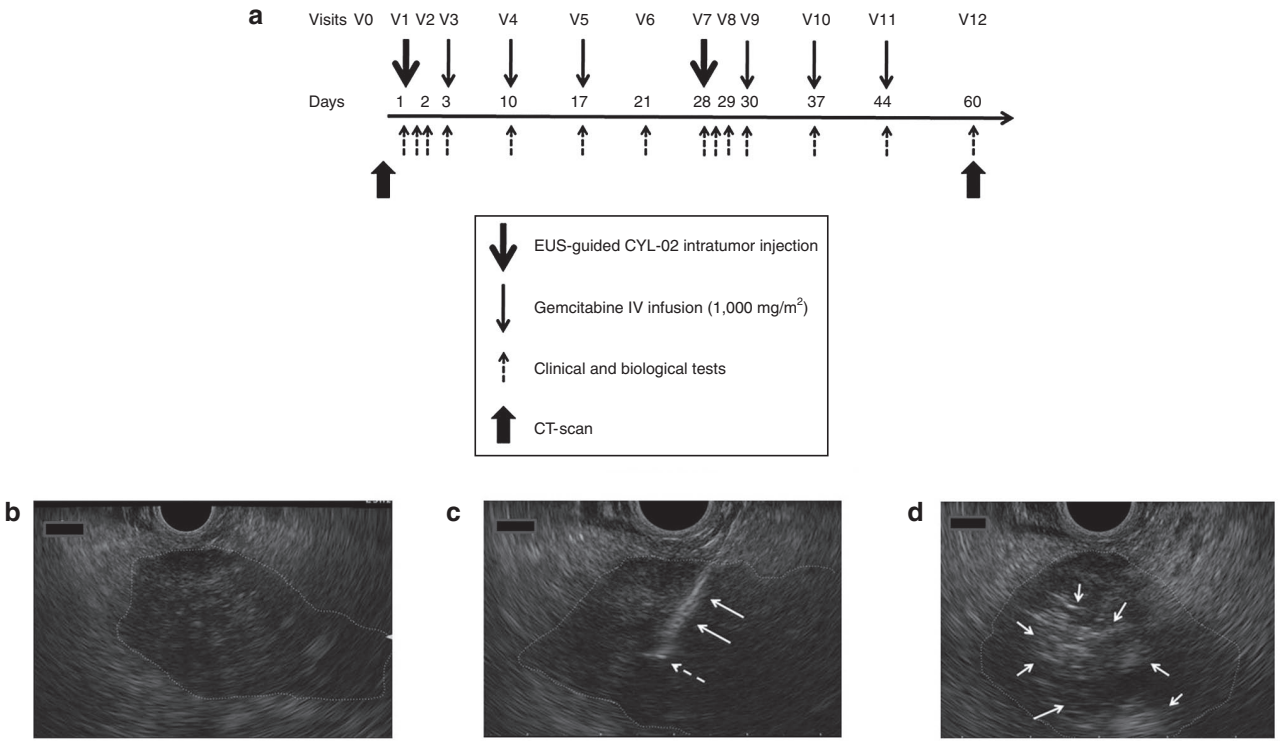

Figure 1 Clinical trial flowchart and injection of the gene therapy product in patients. (**a**) Flowchart of the THERGAP trial for advanced pancreatic cancer. Patients received two intratumoral injections of CYL-02 using endoscopic ultrasound (EUS) followed by gemcitabine infusions. Complete clinical examinations and biological assessments were performed during each visit and twice on the day of the CYL-02 injections (days 1 and 28, at 1 hour before and 6 hours after CYL-02 injections). Blood samples were obtained from patients during each visit (twice on the day of the CYL-02 injection: before and 6 hours after) and were processed for serum and plasma (EDTA-treated tubes) preparations. Urine was collected before and at 24 and 48 hours after CYL-02 injection. The tumor marker CA 19-9 was quantified before (visit 0 and day 1) and at 2 months (day 60) following treatment. V: visit. For intratumoral gene transfer, lyophilized CYL-02 was reconstituted by adding 2.5 ml of sterile water 10 minutes before starting EUS. Gene therapy was performed under general anesthesia. (**b**) Pancreatic carcinoma of the body. The tumor is delineated with a white dashed line. The biopsy needle was then positioned at the center of the tumor. (**c**) Needle (with arrows) using EUS guidance within the tumor (dashed arrow indicates the hyperechoic needle tip) and, after removing the stylet, CYL-02 was slowly injected using backward and forward movements, including a fanning technique of the needle within the tumor under ultrasound control. (**d**) Pancreatic carcinoma of the body immediately following CYL-02 injection showing a white cloud within the tumor (delineated by the arrows). At the end of the procedure, 1.5 ml of 5% glucose (w/v) solution was injected within the tumor to empty the needle. CT, computed tomography; EDTA, ethylenediaminetetraacetic acid; THERGAP, gene therapy for advanced pancreatic adenocarcinoma.

Table 2 Adverse events related to combined treatment of CYL-02 and gemcitabine during the THERGAP gene therapy clinical trial

Dose (patients)	125 µg (*n* = 6)	250 µg (*n* = 4)	500 µg (*n* = 6)	1,000 µg (*n* = 4)
Grade	1–2/3–4	1–2/3–4	1–2/3–4	1–2/3–4
Neutropenia	2/0	1/0	2/0	1/0
Anemia	3/0	1/0	1/0	2/0
Thrombocytopenia	1/0	1/0	2/0	1/0
Fever	4/0	1/1	3/1	1/0
Anorexia/nausea	4/0	1/0	4/0	4/0
Abdominal pain	2/0	1/0	1/0	0/0
Acute pancreatitis	0/0	1/0	0/0	0/0
Pruritus	2/0	1/0	0/0	0/0
Foot and hand syndrome	0/0	0/0	1/0	0/0
Hyperlipasemia	1/0	0/0	0/0	1/0
Increased ASAT	0/0	0/0	4/0	2/0
Increased ALAT	1/0	0/0	4/0	2/0

Adverse events that may be related to the combination of CYL-02 and gemcitabine were mostly of grade 1–2, such as increase in serum alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT) levels. Two patients had significantly increased serum lipase levels (>3 N), but were free of abdominal symptoms. With the exception of a single event of transitory increase in blood pressure during CYL-02 injection, the gene therapy protocol was well tolerated by patients. Two patients experienced tumor pain immediately after gene transfer and were successfully treated by a morphine injection. Transitory fever (<12 hours) occurred at day 1 after the first injection of CYL-02 in six cases (regardless of the dose of CYL-02 administered). A case of acute pancreatitis occurred in one patient (Balthazar A-grade) and led to a 24-hour delay in gemcitabine infusion. Adverse grade 3–4 events were recorded in only two patients (fever in each case) after gemcitabine injection. THERGAP, gene therapy for advanced pancreatic adenocarcinoma.

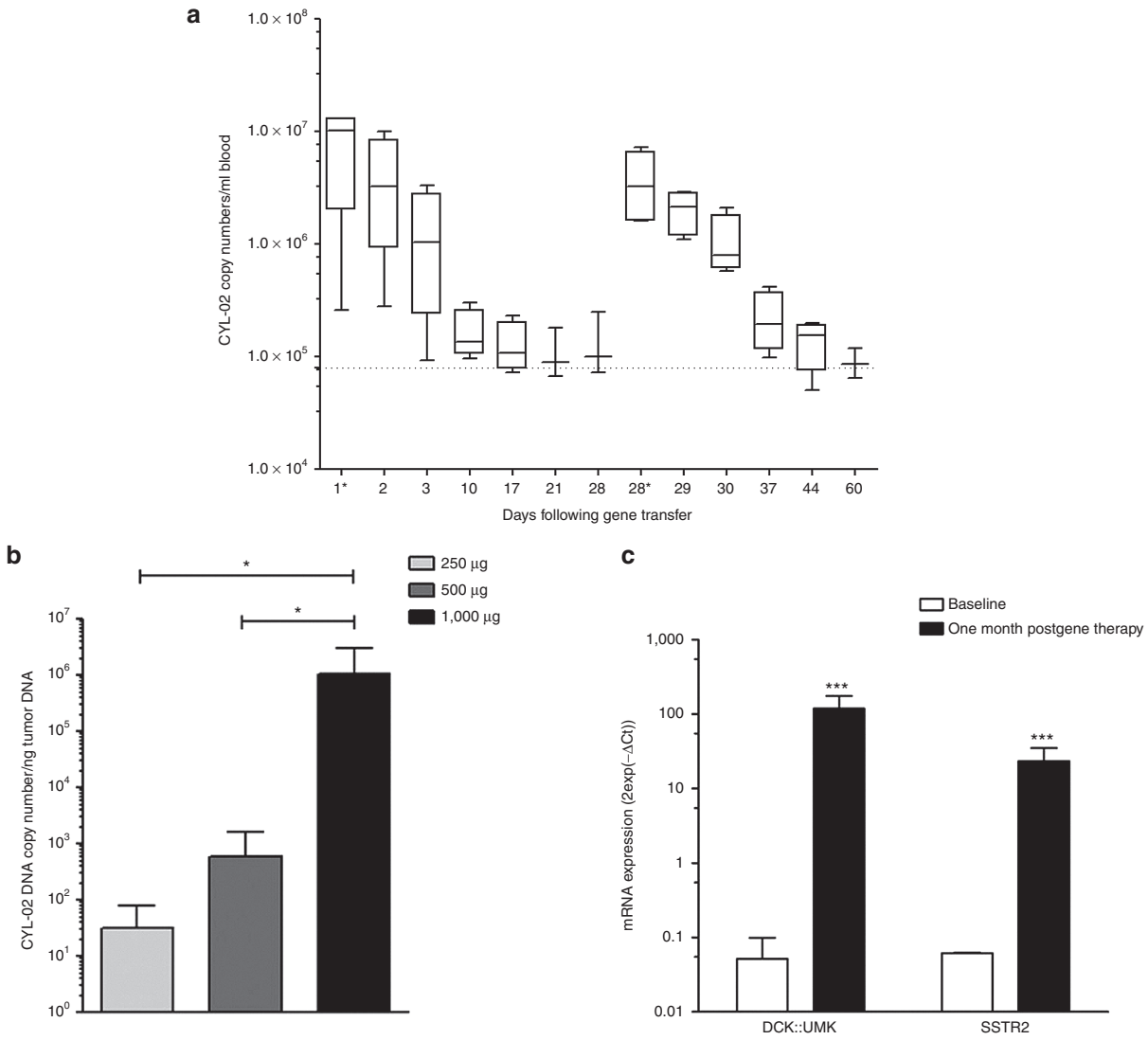

Figure 2 Biodistribution and expression of the gene therapy product. (**a**) CYL-02 was detected by qPCR at the time indicated in the blood of patients receiving 1,000 µg of the gene therapy product. *Indicates 6 hours postintratumoral injection of CYL-02. Data are means ± SD of four biological replicates per group with three experimental replicates and expressed as copies per ml of blood. Experimental threshold: 10 copies/ml of blood; experimental background in blood: $7.8 \pm 0.2 \times 10^4$ copies per ml of blood. (**b**) CYL-02 DNA was detected by qPCR in the tumors of patients at 1 month following gene therapy. Data are means ± SD of four (patients receiving 250 and 1,000 µg of CYL-02) or six (patients receiving 500 µg of CYL-02) biological replicates per group with three experimental replicates and expressed as copy numbers of CYL-02 per ng of tumor DNA. For statistical comparison of two experimental groups, the bilateral Student's *t*-test was used (*$P < 0.05$). Experimental threshold: 10 copies/ng of DNA; experimental background in tumors: 0 copies/ng of DNA. (**c**) *DCK::UMK* and *SSTR2* genes expression were measured in tumors before and 1 month following gene therapy with 1,000 µg of CYL-02. Data are means ± SD of four (patients receiving 1,000 µg of CYL-02) biological replicates per group with three experimental replicates and expressed as arbitrary units ($2^{-\Delta Ct}$ with ΔCt = CT(*DCK::UMK* or *SSTR2*) − CT(*18S*)). For statistical comparison of two experimental groups, the nonparametric Wilcoxon test was used (***$P < 0.005$). qPCR, quantitative PCR.

tumors receiving 250 or 500 µg of the gene therapy product ($P < 0.05$, **Figure 2b**). Therapeutic messenger RNA expression was detected in 75% (3/4, mean relative expression 1.017 ± 0.83), 100% (4/4, mean relative expression 0.89 ± 0.87), 66.6% (5/6, mean relative expression 0.16 ± 0.12), and 100% (4/4, mean relative expression 52.2 ± 28.2) of patients' tumor injected with 125, 250, 500, and 1,000 µg of CYL-02, respectively. **Figure 2c** indicates DCK::UMK and SSTR2 messenger RNAs expression in tumors before and following gene therapy. Thus, we demonstrate that the intratumoral injection of CYL-02 resulted in successful therapeutic DNA delivery to tumors and long-term anticancerous gene expression.

Radiological findings and clinical efficacy

SD by Response Evaluation Criteria In Solid Tumors (RECIST) was observed in 19 patients (95%, **Table 3**), 2 months following gene therapy and gemcitabine treatment. Patients with metastatic disease at the time of diagnosis did not benefit from intratumoral gene therapy as metastases progressed under treatment in five out of seven patients (71%, **Table 3**). On the other hand, 11 out of 12 patients (91%) diagnosed with locally advanced disease remained free of metastases at the end of the protocol (**Table 3**). We next focused on the potential efficacy of CYL-02 in pancreatic cancer patients with locally advanced disease, as they may better benefit from the intratumoral injection of the gene therapy product. Of

Table 3 Primary tumor and metastatic growth, prior treatment, RECIST criteria, overall survival, and progression-free survival of pancreatic cancer patients enrolled in the THERGAP clinical trial

Dose (µg)	Patient (#)	Metastases	Prior treatment	Primary tumor	% Change	Metastases	RECIST	OS	PFS
125	1	Peritoneal, liver	None	Stable	+13	Stable	SD	385	216
	2	None	None	Stable	0	None	SD	586	232
	3	Liver	Chem.	Stable	+19	Progr.	PD	275	60
	4	Liver	Chem.	Progr.	+32	Progr.	PD	63	60
	5	Liver	Chem.	Stable	+12	Progr.	PD	177	60
	6	None	None	Stable	0	None	SD	848	350
250	7	Liver	None	Stable	0	Stable	SD	210	197
	9	None	None	Stable	0	None	SD	374	243
	10	None	None	Stable	−10	Progr.	PD	343	178
	12	None	None	Stable	0	None	SD	795	339
500	13	None	Chem.	Stable	0	None	SD	170	170
	14	None	Resection	Stable	0	None	SD	736	328
	15	Liver	None	Stable	0	Progr.	PD	90	49
	16	Liver	Chem.	Stable	+17	Progr.	PD	222	60
	17	None	None	Stable	+17	None	SD	221	109
	18	None	None	Stable	0	None	SD	56	56
1,000	19	None	None	Stable	0	None	SD	368	157
	20	None	None	Stable	−7	None	SD	412	149
	21	None	Chem.	Stable	0	None	SD	318	160
	22	None	Chem-radio.	Stable	0	None	SD	555	147

Tumor progression and RECIST criteria (version 1.1) were measured to compare CT performed before (V0) and at day 60 following gene therapy; the radiologists were not aware of the patients' treatments (dose). At the end of the protocol, the symptoms, disease progression, Karnofsky and OMS statuses, and comorbidities were analyzed, and a multidisciplinary decision was made on the follow-up treatment according to the French National Guidelines for Digestive Cancers. All patients were followed-up monthly and CT evaluation was performed every 2 months. We recorded any new clinical events, CA 19-9 levels, and progression of the disease after CT, and the date and cause of death. Overall survival and progression-free survival rates were calculated from the date of the first injection of CYL-02 until the date of death and the date of documentation of disease progression, or death of patients without disease progression. All patients died from cancer. Chem, chemotherapy; Chem-radio, chemotherapy + radiotherapy; CT, computed tomography; OS, overall survival; PD, progresive disease; PFS, progression-free survival; SD, stable disease; THERGAP, gene therapy for advanced pancreatic adenocarcinoma.

the 13 evaluable patients with locally advanced pancreatic cancer, 10 patients had SD as best response. Minor response (defined as −10 to −30% by RECIST) was observed in two patients (**Table 3**). **Figure 3a** is representative of tumor stabilization in patient # 10. Decrease in carbohydrate antigen (CA) 19-9 (>50%) was observed in 6 of 12 evaluable patients (**Figure 3b**). Patient #22 was not evaluable (Lewis A blood group). For metastatic patients (n = 7), CA 19-9 levels increased 1.83 ± 1.42-fold (data not shown). As previous treatments could have influenced the therapeutic outcome in this study, we selected locally advanced patients receiving CYL-02 + gemcitabine as a first-line treatment (n = 9, **Table 1**). When analyzed according to the dose of CYL-02 received, no trend was observed in efficacy (median progression-free survival (PFS) and median OS). All patients were diagnosed with SD (n = 9), and were further treated with gemcitabine for 4 months after completing the gene therapy program. The PFS and OS rates reached 5.9 (1.8–11.5) and 12.6 (1.8–27.8) months, respectively, in this subgroup of PDAC patients (**Table 3**).

Biomarkers discovery

We performed high-throughput proteomic studies from the plasma of patients diagnosed with locally advanced PDAC, before treatment by gene therapy. Six patients were eligible for this study. Proteomic data successfully classified patients in two groups, high responders (patients #6, 9, 12, median PFS = 11.1 ± 2.04 months) and low responders, (patients #17, 19, 20, median PFS = 4.2 ± 1.52 months) to gene therapy (**Figure 4a**). Interestingly, RECIST analysis 6-month postgene therapy revealed that patients #17, 19, and 20 had progressive disease, while patients #6, 9, and 12 were still stable. We validated that statistical analysis of differences between groups was strictly related to biological parameters (data not shown). We identified a set of 14 proteins (**Figure 4b**) with a significant Mascot score (**Supplementary Table 1**), that discriminate the two groups of patients and validated that α2-magroglobulin is elevated prior to treatment in patients that will better respond to gene therapy (**Figure 4c**). We also identified a set of 12 microRNAs (miRNAs) (miR-378, miR-145, miR-150, miR-185, miR-21, miR-484, miR-625, miR-378, miR-122, miR-185, miR-21, miR-320, miR-335, miR-365, and miR-625), that are significantly elevated in the plasma of high responders to treatment (**Figure 4d**).

DISCUSSION

Pancreatic cancer is a highly lethal human cancer in which incidence and mortality are almost identical. Reasons for this are (i) a late diagnosis because pancreatic cancer provides no signs and/or symptoms until the disease has become advanced with metastases

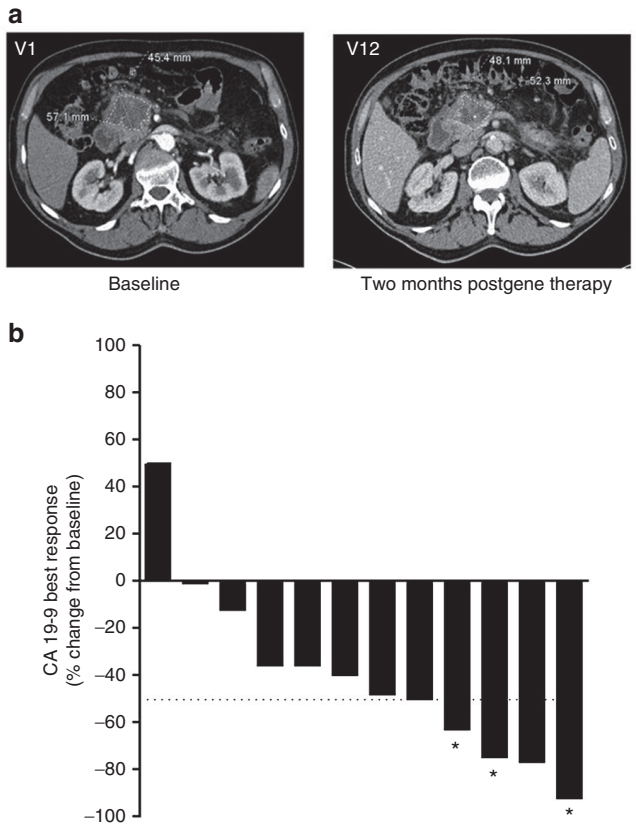

Figure 3 Radiological findings and clinical efficacy of gene therapy. (**a**) Representative CT-scan pictures before (V1 = baseline) and 2 months after treatment (V12). (**b**) CA 19.9 was measured in the blood of 13 patients with locally advanced pancreatic cancer before and at the completion of the gene therapy protocol. Data are expressed as % change from baseline. Dotted line indicates 50% inhibition threshold. *Indicates prior treatment. CT, computed tomography.

and (ii) the lack of curative treatment for advanced disease.[17] Pancreatic cancer has a sophisticated network of biological activities that maintains self-sufficiency in growth signals, is resistant to endogenous antiproliferative signals, evades apoptosis, has limitless replicative potential, and undergoes tissue invasion and metastasis.[18,19] This heterogeneity stems for the unchallenged resistance of pancreatic cancer to conventional therapeutic approaches (chemotherapy, radiotherapy…) and targeted biotherapies. The outlook for patients with advanced pancreatic cancer remains dismal, as these patients are still not cured with conventional therapies, and there remains an urgent need for new approaches, such as gene therapy.

We have devised over the past few years a highly innovative approach based on anticancerous intratumoral gene transfer, which does not rely on a specific genetic and/or cellular background to inhibit the growth of the pancreatic tumors. We designed and produced in this study a plasmid-based gene therapy product, namely CYL-02. The data presented herein from the preclinical and the phase 1 studies of CYL-02 support the following conclusions. From preclinical studies, CYL-02 gene therapy combined with gemcitabine treatment strongly inhibits the growth and the metastatic spread of experimental pancreatic tumors. The study concept is based on an experimental preclinical model which shows that the combination of chemosensitizing (*DCK* and *UMK*), that demonstrate superior antitumoral activity than either protein alone (unpublished observation), and antitumoral (*SSTR2*) genes delivered by a nonviral vector (Jet-PEI) with gemcitabine results in the inhibition of cancer cell proliferation of greater magnitude than either agent alone and correlates with superior antitumor responses.

The phase 1 clinical study supports that gene therapy, administrated in tumors using EUS, and chemotherapy can be given safely to patients with advanced, treatment refractory pancreatic cancer. This is in marked contrast to many conventional options in which the toxicities can be cumulative, and impairment in quality of life has to be weighed against potential benefit. For example, the three-drug combination of fluorouracil (5-FU), oxaliplatin and irinotecan (FOLFIRINOX) showed improved survival compared with gemcitabine monotherapy in patients with good performance status, but safety is less favorable.[20] While EUS can eventually lead to several complications,[21] injecting CYL-02 in the pancreas did not induced morbidity, such as severe acute pancreatitis. In addition, the coadministration of CYL-02 with gemcitabine did not significantly potentiate the hematologic impact of gemcitabine alone. We conclude that CYL-02 nonviral gene therapy is safe and tolerable in subjects. We demonstrate a dose-dependent augmentation of systemic and tumoral CYL-02 DNA, with long-term expression of therapeutic messenger RNAs in tumors. The presence of increasing levels of CYL-02 DNA in the blood suggests systemic leakage of the product in the vasculature or release by necrotic tumor cells. With both biologic and targeted agents, dose selection can be complex as the usual drug development philosophy of using MTD may not be relevant. The maximum dose may not be the most biologically effective dose. While patients receiving the highest dose of CYL-02 had significantly more copies of therapeutic DNA, there does not appear to be a dose-dependent augmentation of therapeutic gene expression in tumors. This may be due to either a greater magnitude of target amplification by quantitative reverse transcription polymerase chain reaction (q(RT)PCR) versus PCR, and/or accelerated RNA degradation in a very hostile tumor microenvironment. Unfortunately, while we demonstrated in experimental models that the gene therapy product could transfect almost one-fourth of the tumor cells, we could not perform immunochemistry to assess the efficacy of gene transfer during the clinical trial because of the paucity of the material collected following FNA. We conclude that the recommended phase two dose for CYL-02 on days 1 and 28 is 1,000 µg when combined with gemcitabine 1000 mg/m^2 on days 3, 10, 17, 30, 37, and 44.

We are encouraged by the antitumor activity of CYL-02 observed in pancreatic cancer patients, as 19 out of 20 subjects did not progress under treatment (95%), according to RECIST criteria. Interestingly, 12 out of 13 patients with locally advanced PDAC at the time of diagnosis remained free of metastasis following gene therapy (92%). The later finding must be pondered because a subset of patients with locally advanced disease never develops metastatic disease. In locally advanced patients, CA 19-9 cancer marker levels decreased significantly following gene therapy combined to gemcitabine treatment. However, there were no

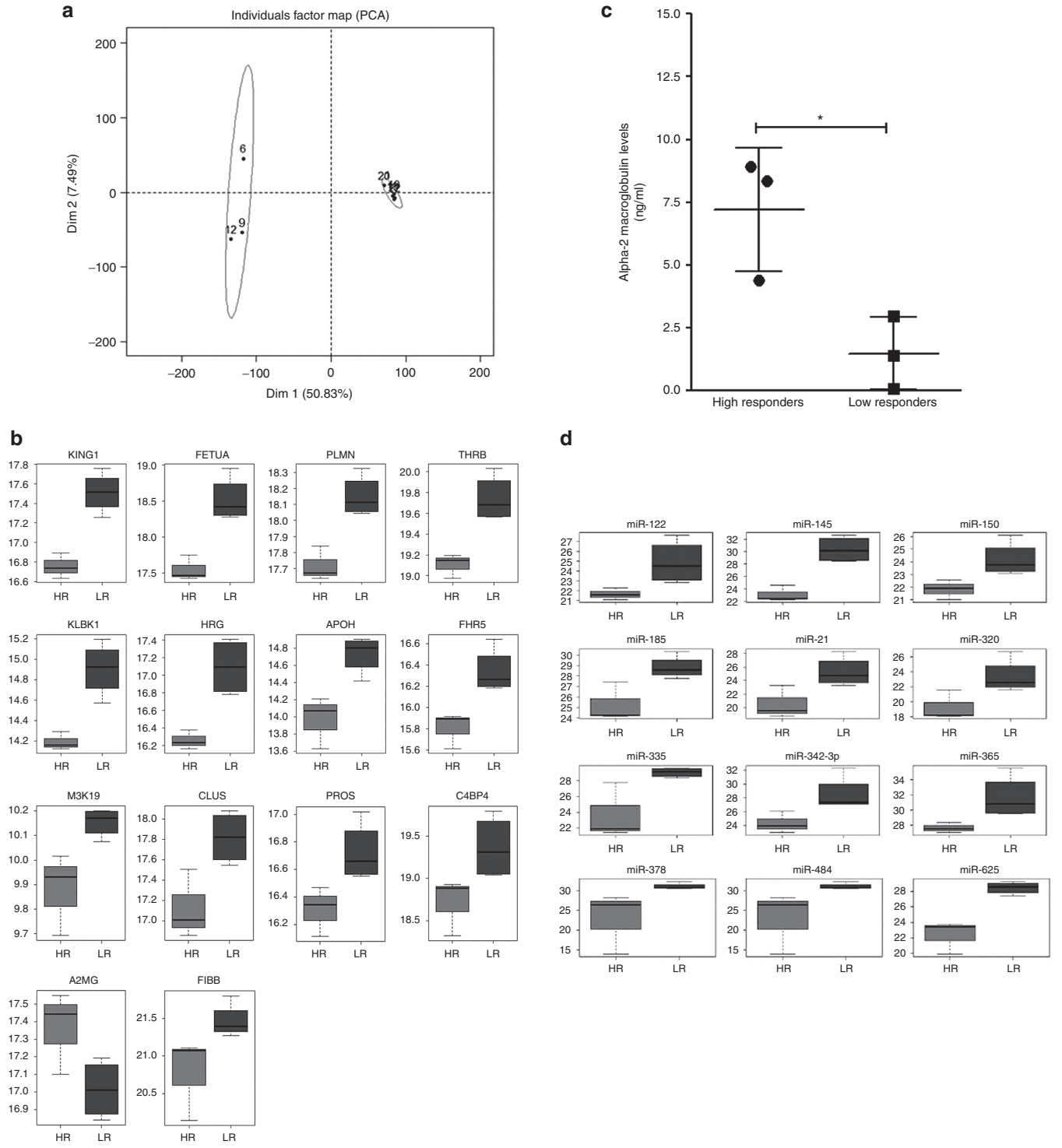

Figure 4 Biomarker discovery. (**a**) Score plots from results of PCA of peptide profiles from plasma patients high ($n = 3$) or low responders ($n = 3$) to treatment according to the first two principal components (Dim 1: 50.63%, Dim 2: 7.49%). Confidence ellipses at 95% around each sample from each group are also represented. Patient number is indicated. (**b**) Individual box plots for the circulating proteins predictive of efficacy identified in this study. Results are expressed as protein frequency in high-responder (HR) versus low-responder (LR) patients. (**c**) Quantification by ELISA of A2MG levels in patient plasma prior gene therapy. Data are expressed as protein level in ng/ml. The bilateral Student's *t*-test was used (*$P < 0.05$). (**d**) Individual box plots for the circulating miRNAs predictive of efficacy identified in this study. Results are expressed as Ct in high-responder (HR) versus low-responder (LR) patients. A2MG, α2-macroglobulin; ELISA, enzyme-linked immunosorbent assay; miRNA, microRNA; PCA, principal component analysis.

significant differences in the efficacies of different dose groups. On the other hand, CYL-02 treatment failed to impact preestablished, distant metastatic growth. Efficacy was documented both in gemcitabine-naive and gemcitabine-refractory patients, supporting the notion that CYL-02-targeted gene therapy could sensitize to and/or reverse acquired gemcitabine resistance.

As previous treatments could have influenced the therapeutic outcome in this study, we restrained the survival rate analysis to patients with locally advanced disease receiving CYL-02 + gemcitabine as a first-line treatment to evaluate CYL-02 therapeutic activity. The 12.6 months of OS, 5.9 months of PFS, and 1-year survival of 66% are longer than that commonly observed with gemcitabine alone.[2] To our knowledge, our study is the first assessment of polyethylenimine (PEI)-based, nonviral gene therapy administrated to pancreatic cancer patients with locally advanced pancreatic cancer. TNFerade (targeted adenoviral vector encoding for tumor necrosis factor-α) was tested in phase 1/2 clinical trial during which clinical efficacy was demonstrated when combined with radiochemotherapy, while dose-limiting toxicities were identified.[22] However, a recent randomized phase 3 multi-institutional study demonstrated that TNFerade combined with standard-of-care was safe, but failed to prolong survival in patients with locally advanced pancreatic cancer, as compared to standard-of-care alone.[23] In another study, naked plasmid DNA encoding for diphtheria-toxin gene was administered intratumorally in subjects with unresectable, locally advanced, nonmetastatic pancreatic cancer patients.[24] This small study conducted in six patients showed evidences of safety and limited efficacy. On the other hand, very promising late-phase vaccine studies are currently ongoing for pancreatic cancer treatment. These studies are based on the use of tumor-associated antigen-specific cytotoxic T lymphocytes, and were proved to be very efficient in experimental models of pancreatic cancer.[25,26] It is tempting to speculate that the antitumoral gene therapy approach described herein may complement cell-based therapies by revealing new tumor-associated antigen to improve the therapeutic response.

During the THERGAP (gene therapy for advanced pancreatic adenocarcinoma) trial, PFS and OS rates varied widely when locally advanced patients were treated by CYL-02 and gemcitabine (1.8–11.5 and 1.8–27.8 months, respectively), while these patients received the exact same therapeutic regimen, a combination of gene therapy and chemotherapy for 2 months, followed by gemcitabine alone for 4 months. In other words, patients progressed under treatment (*i.e.*, PFS <6 months), while other patients did not (*i.e.*, PFS >6 months). The identification of any biomarkers that helps refine our therapeutic decision making would be immensely helpful and represents a worthy goal. Gemcitabine requires transporter proteins to cross cell membranes. Low expression of human equilibrative nucleoside transporter-1 (hENT1) may result in gemcitabine resistance in pancreatic cancer. Recent studies have revealed that high levels of hENT1 in pancreatic cancer predict longer survival times in patients treated with adjuvant gemcitabine.[15] In another study, CO-101, a lipid–drug conjugate of gemcitabine, was designed to enter cells independently of hENT1.[27] However, CO-101 was found not superior to gemcitabine in patients with metastatic pancreatic cancer and low tumor hENT1.[27] In addition, metastasis hENT1 expression does not predict gemcitabine outcome.[27] In this study, hENT1 messenger RNA expression at the time of diagnosis did not predict response to treatment (data not shown). Thus, we performed preliminary blood-based proteomic and miRNA expression studies to identify a set of 14 proteins and 12 miRNAs that predict for response to treatment to gene therapy of patients diagnosed with locally advanced pancreatic cancer. These candidates have been already described as noninvasive biomarkers for cancer[28] or involved in oncogenic pathways; we will further refine our approach by investigating whether combination of candidate biomarkers may help stratify patients in forthcoming phase 2 clinical trial.

In summary, CYL-02 plus gemcitabine regimen is well tolerated in patients with advanced pancreatic cancer. There are encouraging evidences of therapeutic gene delivery and expression, and potential clinical benefit with the identification of noninvasive biomarkers for patient selection. To draw broader conclusions, a randomized phase 2 study would be definitely needed. Given the favorable safety profile and the encouraging antitumor activity of the CYL-02 plus gemcitabine regimen, a clinical trial comparing gemcitabine plus CYL-02 to gemcitabine alone has initiated in 80 patients.

MATERIALS AND METHODS

Patient selection. We included patients aged >18 years, who had given their written informed consent, and had histologically or cytologically confirmed PDAC or a solid nonresectable pancreatic mass associated with one or multiple metastases that had been histologically proven as adenocarcinoma. Other criteria for inclusion were no contraindications for general anesthesia, a Karnofsky score of ≥70%, and a primary pancreatic tumor accessible by endoscopic ultrasound. The main exclusion criteria were known intolerance to gemcitabine, pregnancy, a nonmeasurable primary tumor, a tumor eligible for possible neoadjuvant treatment by radiochemotherapy, or a contraindication for EUS-guided FNA.

Treatment plan. CYL-02 is a complex of plasmid DNA and linear polymers of polyethyleneimine (JetPEI 22 kDa from Polyplus Transfection, Illkirch, France), prepared in 5% w/v glucose with a PEI nitrogen to DNA phosphate (N/P) ratio of 8 to 10. The plasmid within the gene therapy product encodes for *DCK::UMK* complementary DNAs (separated by the self-cleaving FMDV 2A peptide), and the human *SSTR2* complementary DNA. Both *DCK::UMK* and *SSTR2* complementary DNAs are under the coordinated transcriptional control of glucose-responsive promoters (GRP78 and GRP94, respectively) that are highly sensitive to stress conditions,[29] which prevails inside pancreatic tumors.[30] The prokaryotic promoter-driven neomycin gene is used for bacterial selection and biodistribution and pharmacokinetic studies. The gene therapy product is assembled and lyophilized by InvivoGen (Toulouse, France), following good medical product guidelines.

The THERGAP protocol was approved by the Ethical Committee (Comité de Protection des Personnes Sud-Ouest et Outre Mer N°1, number 1-10-21) on August 2010 (promoter CHU of Toulouse), and by the AFSSAPS (N° TG.10.05.01) and the HCB (N° 4883) on November 2010 (EUDRACT number: 2006-005317-35-A; Clinical Trial NCT01274455). After signed informed consent, the patients were enrolled (B.B., L.B., R.G.) for a 2-month period to receive two intratumoral injections of CYL-02 followed by gemcitabine infusions (1,000 mg/m^2) starting 48 hours after injection of the gene therapy product and following every week for 3 weeks. We extrapolated the active dose of CYL-02 characterized in hamsters to human equivalent dose (250 μg of complexed DNA per patient) using body surface area normalization method corrected by the animal clearance according to the European Medicines Agency recommendation M3-R2.

Drug administration. Lyophilized CYL-02 was reconstituted in individual CYL-02 flasks by adding 2.5 ml of sterile water 10 minutes before starting EUS. This was performed (L.B., B.B.) under general anesthesia with propofol, using an Olympus GFUCT-140 echoendoscope (Hamburg, Germany) connected to an Aloka Alpha5 ultrasound probe (Landsberg am Lech, Germany). Examination was started by visualization (with a 5–10 MHz ultrasound probe that included power Doppler analysis) and measurement of the primary tumor and possible lymph nodes or metastases (peritoneum, liver). The biopsy needle (22-G EUS-N1 needle; Wilson-Cook, Limerick, Ireland) was then positioned at the center of the tumor and, after removing the stylet, CYL-02 was slowly injected using backward and forward movements, including a fanning technique of the needle within the tumor under ultrasound control. At the end of the procedure, 1.5 ml of 5% glucose (w/v) solution was injected within the tumor to empty the needle. After removing the needle, an examination of the tumor and its surrounding was performed (including Doppler analysis), to assess the spread of the gene therapy product and the absence of local complications. Monitoring (assessment of blood pressure, pulse, oxygen saturation, ventilation frequency each minute) was performed during and for 30 minutes after the CYL-02 injection. EUS examinations were recorded on CD-ROMs for subsequent reviews. Gemcitabine was delivered intravenously at a dose of 1,000 mg/m^2 of body surface area for 30 minutes at 48 hours after the CYL-02 injections. Gemcitabine injections were repeated weekly for 3 weeks at the same dose.

Assessments, follow-up, and monitoring. The Center for Clinical Investigation of Biotherapies of Toulouse (CIC-BT 511) and the promoter (CHU of Toulouse) monitored the clinical trial. Following consent, patients underwent a clinical and physical examination, Karnofsky score evaluation, a complete blood count, hemostasis, biochemical analyses (including hepatic enzymes, lipase, and creatinine), urine analysis, a pregnancy test, and disease assessment by computed tomography on visit 0 and EUS examination on day 1. Complete clinical examinations and biological assessments were performed during each visit and twice on the day of the CYL-02 injections (days 1 and 28, at 1 hour before and 6 hours after CYL-02 injections). The tumor marker CA 19-9 was assessed before (day 1) and at 2 months (day 60) following treatment. Adverse events were graded according to the National Cancer Institute Common Terminology Criteria of Adverse Events (version 3). Patients were only considered evaluable for the phase-1 study if two injections of CYL-02 had been performed plus at least two of the three gemcitabine infusions. RECIST criteria (version 1.1) were used to measure disease response at 2 months. At the end of the protocol, the symptoms, disease progression, Karnofsky and OMS statuses, and comorbidities were analyzed, and a multidisciplinary decision was made on the follow-up treatment according to the French National Guidelines for Digestive Cancers. In case of SD, gemcitabine was maintained for 4 weeks (*i.e.*, weekly for 3 weeks followed by 1 week off), for 4 months. For progressive disease (either after the 2-month protocol or during the subsequent gemcitabine treatment), another protocol of chemotherapy or best supportive care was implemented. All patients were followed-up monthly and computed tomography evaluation was performed every 2 months. We recorded any new clinical events, CA 19-9 levels, and progression of the disease after computed tomography, and the date and cause of death.

Treatment following gene therapy + gemcitabine cycles. Following gene therapy + gemcitabine cycles, patients #4, 8, 11, and 15 received best supportive care. Patient #3 received Xeloda then best supportive care. Patient #5 received Anti kin then best supportive care. Patient #16 received Folfox, then Xeloda, then best supportive care. As mentioned before, patients with locally advanced tumors and SD following gene therapy and chemotherapy cycles were further treated for 4 months with gemcitabine only (patients #2, 6, 9, 10, 12, 13, 14, 17, 19, 20, 21, and 22). As a third line (*i.e.*, second line following gene therapy + chemotherapy), patients received Folfox (patients #12 and 13), radiochemotherapy (patients #14 and 20), or best supportive care (patients # 2, 6, 9, 17, 21, and 22). Patients #12 and 20 received best supportive care as fourth line, while patients #14 and 19 received Folfox or LV5-FU2, respectively, then best supportive care. All patients died from cancer.

Definition of MTD and dose escalation plan. The MTD was defined as the highest dose level of CYL-02 at which one or less of six patients experienced severe acute pancreatitis or died from the experimental treatment, or had major diffusion of CYL-02 into the blood and/or urine during cycle 1. The trial used the standard 6 + 6 dose escalation design. The study drug dose was escalated to the next higher level if none of the six patients developed MTD criteria, as defined above. Dose escalation ranged from 125 to 1,000 µg of complexed DNA (six patients received 125, 250, or 500 µg, and four patients received 1,000 µg). Dose escalations were under the supervision of an independent committee of experts. Nonevaluable patients were not replaced.

Pharmacokinetic sampling and analytic assay. Blood samples were obtained from patients during each visit (twice on the day of the CYL-02 injection: before and 6 hours after). Venous blood samples were collected, and processed for serum and plasma (ethylenediaminetetraacetic acid-treated tubes). Samples were stored at −80 °C. Urine was also collected before and at 24 and 48 hours after CYL-02 injection. CYL-02 DNA levels were quantified by quantitative PCR. Briefly, total DNA was isolated from whole blood and urine using a QIAamp DNA Mini Kit (Qiagen, Courtaboeuf, France). Nucleic acids were quantified using ND-1000 NanoDrop spectrophotometry (NanoDrop Products, Wilmington, DE). Triplicate qRT-PCR assays were carried out on 50 ng of DNA extracted from urine or blood from patients in a SYBR Green PCR Master Mix (SsoFast; Biorad, Marnes-la-Coquette, France) with primers directed against the neomycin gene (*NeoF*: 5′-CTC CAG CTG AGA AAG TGT CAA-3′; *NeoR*: 5′-GCT GGG TCA AGG GTG TGG-3′), using a StepOne II (Life Technologies, Saint Aubin, France). CYL-02 levels were expressed as copy numbers/ml of blood.

Tumor analysis. FNA biopsies were obtained from tumors before (V0) and 1 month following gene therapy (V7) using a 22-G EUS-N1 needle (Wilson-Cook). FNA biopsies were performed before the second injection of CYL-02. The tissue/cellular materials were divided into two and placed within formalin or RNABle (Qiagen) before performing a histological examination and nucleic acid extraction, respectively. DNA and total RNA were extracted from formalin-fixed, paraffin-embedded tissues from baseline biopsies using RecoverAll Total Nucleic Acid Isolation Kit (Life technologies, St. Aubin, France) following the manufacturers' recommendations. AllPrep DNA/RNA/miRNA Universal Kit (Qiagen) was used to process FNA material dropped in RNALater from patients 1 month after gene therapy. CYL-02 DNA was quantified as described above. For gene expression studies, aliquots of 50 ng of total RNA were used for reverse transcription using a RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Marnes-la-Coquette, France) before specific target amplification to increase target concentration. Triplicate qRT-PCR assays were carried out in a ViiA7 Real-Time PCR System (Life Technologies), in a SYBR Green PCR Master Mix (SsoFast; Biorad) using: pN1R5 (5′-CAG CCA GCT TGA GCA GGT CAA AGT TGA G-3′) and pN1F5-7 (5′-CAA CTT CGA TTA TCT TCA AGA GGT GCC TAT-3′) primers to quantify *DCK::UMK* gene expression, and D6573B04 (5′-TTT TGT GGT CTG CAT CAT TGG-3′) and D6573B05 (5′-AAA GGC AGA CCC AGC ATG AA-3′) primers to quantify *SSTR2* expression, respectively. 18S RNA expression was used as calibrator, as previously described (18). Relative amounts were calculated by the comparative cycle threshold (Ct) method as $2^{-\Delta Ct}$, where ΔCt = Ct(DCK::UMK or SSTR2) − Ct(18S). Samples with Ct(18S)<28 were considered for analysis.

Biomarker discovery. Circulating miRNAs: Total RNAs were extracted from patients' plasma using Trizol LS (Life technologies) following the

manufacturers' recommendations. Plasmatic microRNAs were quantified in 200 ng of total RNA using QuantStudio 12K Flex OpenArray microRNA plates and Megaplex Primer Pools according to the manufacturer's instructions. Each TaqMan OpenArray Human MicroRNA Panel, QuantStudio 12K Flex contains 754 well-characterized human miRNA sequences from the Sanger miRBase v14.

Proteomic studies: ProteoMiner protein enrichment kit was used according to Bio-Rad's instructions on 50 µl of plasma. Proteins were eluted from beads, reduced, alkylated before running on sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Proteins were stained by Coomassie Blue, each lane was cut and each gel piece was washed several times in acetonitrile 100%, ammonium bicarbonate 100 mmol/l, and dried *in vacuo*. Gel pieces were rehydrated with 20 ng/µl trypsin prepared in 100 mmol/l ammonium bicarbonate, and digested overnight at 37 °C. Peptides were then extracted and subjected to mass spectrometry analysis. The peptides mixtures were analyzed by nanoHPLC-chip-MS/MS with a system consisting of a nano-pump, a capillary-pump (G1376A and G2226; Agilent, Massy, France) with two four-channel micro-vacuum degasser (G1379B; Agilent), a microfluidic chip cube (G4240-64000; Agilent) interfaced to an Amazon ETD mass spectrometer (Bruker Daltonics, Billerica, MA). A microfluidic reversed-phase HPLC chip (Zorbax 300SB-C18, 5-µm particle size, 75-µm internal diameter, and 150 mm length) was used for peptide separation. Peptides were eluted and scans MS were acquired on the 300–1500 m/z range in the enhanced resolution mode. For peptide fragmentation, the Amazon was operated in data-dependent acquisition mode with the trap control software. Scans The Bruker data files (.d folder) generated with the Chip-MS technology were loaded to Progenesis LC-MS version 4.0 (Nonlinear Dynamics, Durham, NC). Peak picking was performed to detect features (*i.e.*, ions detected on the mass spectrometer) using automatic parameters for sensitivity and retention time window. Statistical filters were set and only features matching all filters were kept. Filters used was P value <5% (Student's t-test). A csv file was then exported from Progenesis and loaded to R 2.13.2. Descriptive statistics and principal component analysis were performed using mixOmics R Package.22. As potential biomarkers, relevant features selected by Progenesis were exported in.mgf files and the corresponding proteins were identified using the MASCOT software (http://www.matrixscience.com/) and SwissProt database (http://web.expasy.org/docs/swiss-prot_guideline.html). Mascot files were then imported in Progenesis software to select the most relevant identified proteins.

Statistical analysis. All data are presented as mean ± SD. For statistical comparison of two experimental groups, the nonparametric Wilcoxon test was used (*$P < 0.05$; **$P < 0.01$; ***$P < 0.005$) using Graphpad Prism software (Graphpad Software, La Jolla, CA). A level of $P < 0.05$ was considered statistically significant. No statistical method was used to predetermine sample size. The experiments were randomized. During the gene therapy clinical trial, patients were not selected with respect to the dose administered. The investigators were not blinded to allocation during experiments except for tumor growth experiments in preclinical models, DNA and (micro)RNA quantification, and histological examinations. No animals were excluded from the preclinical study. For proteomic studies, descriptive statistics are presented as principal component analysis that allows exploratory data analysis combining samples and proteins. A Tukey test was used to analyze coefficient of variation in order to compare variability between groups and variability within groups. Statistical analyses were done with FactoMineR package for R software (http://cran.r-project.org/web/packages/FactoMineR/).

SUPPLEMENTARY MATERIAL

Table 1. Proteomic profiling of patients with locally advanced pancreatic cancer treated by gene therapy.

ACKNOWLEDGMENTS

The authors thank the following for their help and advice: Severine Joubert, Pascale Duret, Claire Thalamas, and Olivier Rascol from CIC-BT 511 and CIC-P of Toulouse; Jean-José Maoret from Get-TQ transcriptomic facility; Nicolas Ricard, Eric Perouzel, Daniel Drocourt, and Jean-Paul Reynes from InvivoGen; Jean-Pierre Delord, Laurent Alric, and Pascale Olivier who participated in the independent survey committee; Benoît Bourdet, Isabelle Migueres, Marie De Lacroix, Dominique Vigouroux, and Annick Galiana from Department of Anaesthesiology; Pierre Fajadet and Guillaume Canevet from the Department of Radiology; Yara Barreira from the animal facility of UMR INSERM; Hafid Belhadj from the French Society of Toxicology; Nicolas Carrère, Bertrand Suc, Marion Deslandres, and Pascale Rivera for the inclusion of patients; and Pr J Selves for cytopathological evaluation of EUS-guided FNA. Combination gene therapy product is patented (EP2209484 B1). French Ministry of health (PH-RC 2004 N° 62), Région Midi-Pyrénées APRTCN 2006 N° 0401401 and APRTCN 2011 N° 12050667, ANR-RIB 07, INSERM COSSEC, INVIVOGEN, CHU of Toulouse, Thermo Fischer Scientific 2011, and INCa AAP-Translat-2012 DGOS_5687. Study concept and design: L.B., P.C., G.T., F.L., A.M., M.D., F.G., J.T., S.D. Experimental procedures: A.V.-V., G.C., F.V., N.H., O.B., H.L., A.M., P.C., N.S.-L., L.L., F.P., M.G., F.M. Clinical staff: B.B., L.B., R.G., P.O. Data collection: L.B. and P.C. Data analysis: B.B., L.B., P.O., R.G., P.C., J.T., M.D., A.M. Data interpretation: L.B., M.D., A.M., J.T., P.C. Manuscript writing: L.B., P.C. The authors declare that they have no competing interests.

REFERENCES

1. Cancer Facts & Figures 2013. <http://www.cancer.org/research/cancerfactsfigures/cancerfactsfigures/cancer-facts-figures-2013>.
2. Burris, HA 3rd, Moore, MJ, Andersen, J, Green, MR, Rothenberg, ML, Modiano, MR *et al.* (1997). Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. *J Clin Oncol* **15**: 2403–2413.
3. Pancreatic Cancer Action Network. <http://www.pancan.org/>.
4. Moore, MJ, Goldstein, D, Hamm, J, Figer, A, Hecht, JR, Gallinger, S *et al.*; National Cancer Institute of Canada Clinical Trials Group. (2007). 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* **25**: 1960–1966.
5. Von Hoff, DD, Ervin, T, Arena, FP, Chiorean, EG, Infante, J, Moore, M *et al.* (2013). Increased survival in pancreatic cancer with nab-paclitaxel plus gemcitabine. *N Engl J Med* **369**: 1691–1703.
6. Buscail, L, Saint-Laurent, N, Chastre, E, Vaillant, JC, Gespach, C, Capella, G *et al.* (1996). Loss of sst2 somatostatin receptor gene expression in human pancreatic and colorectal cancer. *Cancer Res* **56**: 1823–1827.
7. Carrere, N, Vernejoul, F, Souque, A, Asnacios, A, Vaysse, N, Pradayrol, L *et al.* (2005). Characterization of the bystander effect of somatostatin receptor sst2 after *in vivo* gene transfer into human pancreatic cancer cells. *Hum Gene Ther* **16**: 1175–1193.
8. Vernejoul, F, Faure, P, Benali, N, Calise, D, Tiraby, G, Pradayrol, L *et al.* (2002). Antitumor effect of *in vivo* somatostatin receptor subtype 2 gene transfer in primary and metastatic pancreatic cancer models. *Cancer Res* **62**: 6124–6131.
9. Rochaix, P, Delesque, N, Estève, JP, Saint-Laurent, N, Voight, JJ, Vaysse, N *et al.* (1999). Gene therapy for pancreatic carcinoma: local and distant antitumor effects after somatostatin receptor sst2 gene transfer. *Hum Gene Ther* **10**: 995–1008.
10. Cordelier, P, Bienvenu, C, Lulka, H, Marrache, F, Bouisson, M, Openheim, A *et al.* (2007). Replication-deficient rSV40 mediate pancreatic gene transfer and long-term inhibition of tumor growth. *Cancer Gene Ther* **14**: 19–29.
11. Delesque, N, Buscail, L, Estève, JP, Saint-Laurent, N, Müller, C, Weckbecker, G *et al.* (1997). sst2 somatostatin receptor expression reverses tumorigenicity of human pancreatic cancer cells. *Cancer Res* **57**: 956–962.
12. Guillermet, J, Saint-Laurent, N, Rochaix, P, Cuvillier, O, Levade, T, Schally, AV *et al.* (2003). Somatostatin receptor subtype 2 sensitizes human pancreatic cancer cells to death ligand-induced apoptosis. *Proc Natl Acad Sci USA* **100**: 155–160.
13. Benali, N, Cordelier, P, Calise, D, Pages, P, Rochaix, P, Nagy, A *et al.* (2000). Inhibition of growth and metastatic progression of pancreatic carcinoma in hamster after somatostatin receptor subtype 2 (sst2) gene expression and administration of cytotoxic somatostatin analog AN-238. *Proc Natl Acad Sci USA* **97**: 9180–9185.
14. Ohhashi, S, Ohuchida, K, Mizumoto, K, Fujita, H, Egami, T, Yu, J *et al.* (2008). Down-regulation of deoxycytidine kinase enhances acquired resistance to gemcitabine in pancreatic cancer. *Anticancer Res* **28**: 2205–2212.
15. Maréchal, R, Bachet, JB, Mackey, JR, Dalban, C, Demetter, P, Graham, K *et al.* (2012). Levels of gemcitabine transport and metabolism proteins predict survival times of patients treated with gemcitabine for pancreatic adenocarcinoma. *Gastroenterology* **143**: 664–674.e1.
16. Vernejoul, F, Ghénassia, L, Souque, A, Lulka, H, Drocourt, D, Cordelier, P *et al.* (2006). Gene therapy based on gemcitabine chemosensitization suppresses pancreatic tumor growth. *Mol Ther* **14**: 758–767.
17. Siegel, R, Naishadham, D and Jemal, A (2013). Cancer statistics, 2013. *CA Cancer J Clin* **63**: 11–30.
18. Yachida, S, Jones, S, Bozic, I, Antal, T, Leary, R, Fu, B *et al.* (2010). Distant metastasis occurs late during the genetic evolution of pancreatic cancer. *Nature* **467**: 1114–1117.
19. Jones, S, Zhang, X, Parsons, DW, Lin, JC, Leary, RJ, Angenendt, P *et al.* (2008). Core signaling pathways in human pancreatic cancers revealed by global genomic analyses. *Science* **321**: 1801–1806.

20. Gourgou-Bourgade, S, Bascoul-Mollevi, C, Desseigne, F, Ychou, M, Bouché, O, Guimbaud, R *et al.* (2013). Impact of FOLFIRINOX compared with gemcitabine on quality of life in patients with metastatic pancreatic cancer: results from the PRODIGE 4/ACCORD 11 randomized trial. *J Clin Oncol* **31**: 23–29.
21. Bournet, B, Migueres, I, Delacroix, M, Vigouroux, D, Bornet, JL, Escourrou, J *et al.* (2006). Early morbidity of endoscopic ultrasound: 13 years' experience at a referral center. *Endoscopy* **38**: 349–354.
22. Hecht, JR, Farrell, JJ, Senzer, N, Nemunaitis, J, Rosemurgy, A, Chung, T *et al.* (2012). EUS or percutaneously guided intratumoral TNFerade biologic with 5-fluorouracil and radiotherapy for first-line treatment of locally advanced pancreatic cancer: a phase I/II study. *Gastrointest Endosc* **75**: 332–338.
23. Herman, JM, Wild, AT, Wang, H, Tran, PT, Chang, KJ, Taylor, GE *et al.* (2013). Randomized phase III multi-institutional study of TNFerade biologic with fluorouracil and radiotherapy for locally advanced pancreatic cancer: final results. *J Clin Oncol* **31**: 886–894.
24. Hanna, N, Ohana, P, Konikoff, FM, Leichtmann, G, Hubert, A, Appelbaum, L *et al.* (2012). Phase 1/2a, dose-escalation, safety, pharmacokinetic and preliminary efficacy study of intratumoral administration of BC-819 in patients with unresectable pancreatic cancer. *Cancer Gene Ther* **19**: 374–381.
25. Anurathapan, U, Chan, RC, Hindi, HF, Mucharla, R, Bajgain, P, Hayes, BC *et al.* (2014). Kinetics of tumor destruction by chimeric antigen receptor-modified T cells. *Mol Ther* **22**: 623–633.
26. Chmielewski, M, Hahn, O, Rappl, G, Nowak, M, Schmidt-Wolf, IH, Hombach, AA *et al.* (2012). T cells that target carcinoembryonic antigen eradicate orthotopic pancreatic carcinomas without inducing autoimmune colitis in mice. *Gastroenterology* **143**: 1095–1107.e2.
27. Poplin, E, Wasan, H, Rolfe, L, Raponi, M, Ikdahl, T, Bondarenko, I *et al.* (2013). Randomized, multicenter, phase II study of CO-101 versus gemcitabine in patients with metastatic pancreatic ductal adenocarcinoma: including a prospective evaluation of the role of hENT1 in gemcitabine or CO-101 sensitivity. *J Clin Oncol* **31**: 4453–4461.
28. Schultz, NA, Dehlendorff, C, Jensen, BV, Bjerregaard, JK, Nielsen, KR, Bojesen, SE *et al.* (2014). MicroRNA biomarkers in whole blood for detection of pancreatic cancer. *JAMA* **311**: 392–404.
29. Chang, SC, Erwin, AE and Lee, AS (1989). Glucose-regulated protein (GRP94 and GRP78) genes share common regulatory domains and are coordinately regulated by common trans-acting factors. *Mol Cell Biol* **9**: 2153–2162.
30. Al Saati, T, Clerc, P, Hanoun, N, Peuget, S, Lulka, H, Gigoux, V *et al.* (2013). Oxidative stress induced by inactivation of TP53INP1 cooperates with KrasG12D to initiate and promote pancreatic carcinogenesis in the murine pancreas. *Am J Pathol* **182**: 1996–2004.