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Promoter hypermethylation of SFRP1 as a prognostic and potentially predictive blood-based biomarker in patients with stage III or IV pancreatic ductal adenocarcinoma☆

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ABSTRACT

Background: Pancreatic ductal adenocarcinoma remains one of the major causes of cancer-related mortality globally. Unfortunately, current prognostic biomarkers are limited, and no predictive biomarkers exist. This study examined promoter hypermethylation of secreted frizzled-related protein 1 (phSFRP1) in cfDNA as a prognostic biomarker and predictor of treatment effect in patients with metastatic FOLFIRINOX-treated PDAC and locally advanced PDAC.

Methods: We performed methylation-specific PCR of the SFRP1 genes' promoter region, based on bisulfite treatment. Survival was assessed as time-to-event data using the pseudo-observation method and analyzed with Kaplan-Meier curves and generalized linear regressions.

Results: The study included 52 patients with FOLFIRINOX-treated metastatic PDAC. Patients with unmethylated (um) SFRP1 (n = 29) had a longer median overall survival (15.7 months) than those with phSFRP1 (6.8 months). In crude regression, phSFRP1 was associated with an increased risk of death of 36.9% (95% CI 12.0%–61.7%) and 19.8% (95% CI 1.9–37.6) at 12 and 24-months, respectively. In supplementary regression analysis, interaction terms between SFRP1 methylation status and treatment were significant, indicating reduced benefit of chemotherapy. Forty-four patients with locally advanced PDAC were included. phSFRP1 was associated with an increased risk of death at 24-months

Conclusions: This indicates that phSFRP1 is a clinically useful prognostic biomarker in metastatic PDAC and possibly in locally advanced PDAC. Together with existing literature, results could indicate the value of cfDNA-measured phSFRP1 as a predictive biomarker of standard palliative chemotherapy in patients with metastatic PDAC. This could facilitate personalized treatment of patients with metastatic PDAC.

1. Introduction

Pancreatic ductal adenocarcinoma (PDAC) is globally a leading cause of cancer death [1,2]. With increasing incidence- and mortality rates, and a 5-year survival of only 11%, there is a need for progress in developing new treatment modalities and monitoring of disease. Unfortunately, most patients diagnosed with PDAC present with metastatic disease and can only be offered palliative

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chemotherapy [1,2]. Patients in good performance status (PS) are often treated with combination triple chemotherapy FOLFIRINOX (5-FU, irinotecan, oxaliplatin), leading to a median overall survival (mOS) of 11 months in a randomized controlled trial [3]. Real-world data from a Danish population of patients with PDAC receiving palliative chemotherapy from 2011 to 2016 demonstrated a mOS of 10.0 months for FOLFIRINOX, 8.5 months for combination of gemcitabine plus nab-paclitaxel and 6.0 months for gemcitabine monotherapy [4].

Approximately 6% of patients with PDAC have germline PALB2 or BRCA1/BRCA2 mutations and respond well to platinum-based therapies [5]. Unfortunately, remaining patients are left with no useful predictive biomarkers to estimate response to standard chemotherapy. The only clinically useful biomarker carbohydrate antigen 19-9 (CA19-9) is neither cancer specific nor predictive [6,7].

A new paradigm is necessary to predict treatment response and prognosis. One increasingly promising tool is detection of tumor-specific alterations in plasma cell-free DNA (cfDNA) [8]. Aberrant methylation of DNA promoter regions is detectable in cfDNA and linked to both progression and development of cancer [9]. DNA promoter hypermethylation leads to silencing of tumor suppressor genes, which have been proposed as possible biomarkers for PDAC [6,10–12].

Secreted frizzled-related protein 1 (SFRP1) is a tumor suppressor gene inhibiting the oncogenic Wnt/B-catenin pathway of which expression is primarily regulated by DNA methylation [13]. Both promoter hypermethylation and low levels of SFRP1 RNA in tumor tissue have been associated with worse prognosis in several forms of cancer, including PDAC, nasopharyngeal cancer, ovarian cancer, kidney cancer, and glioblastoma multiforme [13,14]. High frequencies of promoter hypermethylation of SFRP1 (phSFRP1) have been demonstrated in PDAC tissue [15]. However, reliance on tumor tissue for analysis can be a detriment. Unfortunately, little research has been conducted into phSFRP1 as a blood-based biomarker in PDAC.

We previously demonstrated the utility of phSFRP1, measured in cfDNA, as a blood-based prognostic biomarker for survival in gemcitabine-treated patients with stage IV PDAC [16]. Patients with unmethylated SFRP1 (umSFRP1) had a mOS almost three times as long as patients with phSFRP1 [16]. Despite a significantly better PS, gemcitabine-treated patients with phSFRP1 only marginally outlived patients who received best supportive care (BSC). This raises questions about the benefits of palliative chemotherapy in this patient group and could imply the utility of phSFRP1 as a biomarker able to predict the effect of chemotherapy. The previous study was limited to patients treated with gemcitabine. We hypothesized that the effects of phSFRP1 may also be present in patients with metastatic PDAC receiving the more intensive chemotherapy regimen FOLFIRINOX, and in patients with locally advanced PDAC.

The objective of the current study was to examine phSFRP1 as a predictor of prognosis and treatment response in patients with stage III and IV PDAC.

2. Methods

2.1. Patients

The study was carried out in accordance with the Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK) and was a retrospective, blinded analysis of prospectively collected samples. Retrospectively, two cohorts were defined: Patients with histologically verified stage IV PDAC treated with 1. line palliative FOLFIRINOX (5-FU, irinotecan, oxaliplatin) and patients with stage III PDAC treated with either BSC, 1. line palliative gemcitabine or 1. line palliative FOLFIRINOX. Serum samples or EDTA plasma samples were received from two Danish biobanks: The BIOPAC study ("BIOmarkers in patients with PAncreatic Cancer (BIOPAC) – can they provide new information of the disease and improve diagnosis and prognosis of the patients"; www.herlevhospital.dk/BIOPAC/; ClinicalTrials.gov ID: NCT03311776) and the GIVTE study ("Venous Thromboembolism and Haemostatic Disturbances in Patients with Upper Gastrointestinal Cancer"; ClinicalTrials.gov registration number NCT00660205).

The BIOPAC study is a Danish nationwide multicenter study, consecutively including patients with PDAC at diagnosis, before treatment with either surgery or palliative chemotherapy. Patients were included between September 2011 and February 2016 at the Department of Surgery, Righospitalet, Denmark (n = 29) or the Department of Oncology, Copenhagen University Hospital - Herlev and Gentofte (n = 54), Denmark. Only patients with PS 0 or 1 were eligible for treatment with FOLFIRINOX. The BIOPAC study protocol was approved by the Danish Data Protection Agency (HGH-2015-027; I-Suite j.nr. 03960; j.nr. 2012-58-0004; and PACTIUS P-2020-834) and the relevant regional ethics committee (VEK ref KA-20060113).

The GIVTE study examined the prevalence of venous thromboembolism at time of diagnosis of upper gastrointestinal cancer [17]. The GIVTE study included patients between February 2008 and February 2011. All patients (n = 13) with stage III PDAC were included in this cohort.

All patients were chemotherapy naïve at inclusion. All methylation analyses were completed before clinical data was received. This study was approved by the Regional Research Ethics Committee of Northern Denmark (approval number: N-2013037). This study was carried out in accordance with Declaration of Helsinki and Good Clinical Practice.

2.2. Analytical methods

Blood samples were centrifuged and serum or EDTA plasma were aliquoted and frozen at −80 °C within 120 min of sampling. In the GIVTE study, blood samples were centrifuged for 20 min at 4.000 rpm and 4 °C. In the BIOPAC study, blood samples were centrifuged for 10 min at 2300 G and 4 °C. Methylation analysis was blinded and carried out by an expert laboratory scientist at the Department of Molecular Diagnostics, Aalborg University Hospital, Denmark.

Extraction of cfDNA and deamination was performed according to the procedure previously described in detail by our group [16,18]. In brief, two rounds of PCR amplification were performed: In round-one the amount of deaminated DNA was expanded. Here, SFRP1 was analyzed using a panel of other genes, with a mix of outer methylation-specific primers (Supplemental Table 1) [16]. Round-two was performed in individual reactions to expand investigated promoter regions in each of the inner methylation-specific primers as well as methylation-specific probes [11,18]. Following the PCR amplifications, SFRP1 promoter methylation status was dichotomized. A sample with no cycle threshold (Ct) value within 45 cycles was defined as a gene with no promoter hypermethylation, while a gene with a positive detection at Ct value was interpreted as being promoter hypermethylated. This procedure has previously been demonstrated to not cause loss of information [19].

2.3. Statistical methods

Patients were stratified according to SFRP1 methylation status and PDAC stage (III/IV). The Kruskal-Wallis test was used to compare continuous variables, while the Pearson Chi-Squared was used for comparison of categorical variables. Survival was calculated from time of pretreatment blood sampling (within 1 month from diagnosis) until death of any cause or end of follow-up. At end

of follow-up (October 8, 2018) all patients had died. Survival was assessed as time-to-event data using the pseudo-observation method and generalized linear regression to calculate absolute risk differences. Survival was visualized with Kaplan-Meier survival curves supplemented with log-rank tests [20]. Risk differences were used to examine the interaction between gene and treatment on an additive scale [21]. This allowed phSFRP1 to be evaluated as a predictor of treatment effect with interaction terms in regression models [16,22]. A series of crude regressions were performed for phSFRP1 and the possible covariates age >65, ECOG PS, sex, and a CA19-9 value above or below the median. This was followed by an adjusted regression, adjusting for age >65, ECOG PS, sex, and CA19-9. ROC analysis was performed to compare the added accuracy of SFRP1 and CA19-9 in predicting mortality at 12 and 24 months. As several potential cutoffs have been proposed for CA19-9, this was performed for three additional cutoffs in addition to the median [23–28].

The "timeROC" package in R was used to compute the time-dependent AUC curve plots.

We used 95% confidence intervals (CI) where applicable and considered tests with p-values less than 0.05 statistically significant.

All calculations of the study were carried out in either R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/. or in Stata v. 16, StataCorp, LLC, TA, USA.

3. Results

3.1. SFRP1 promoter hypermethylation as a prognostic biomarker in patients with FOLFIRINOX-treated stage IV PDAC

Fifty-two serum samples from patients with FOLFIRINOX-treated stage IV PDAC were received from the BIOPAC study. Characteristics according to phSFRP1 status are presented in Table 1. Twenty-three (54%) patients had phSFRP1. There were no significant differences either in sex, age, weight, BMI, CA 19-9, PS, attempted curative surgery, location of the tumor, or location of metastases according to phSFRP1.

The Kaplan Meier survival curves according to phSFRP1 in patients with FOLFIRINOX-treated stage IV PDAC are presented in Fig. 1A. Patients with phSFRP1 had a mOS of 6.8 compared to 15.7 months in patients with umSFRP1. The 2-year survival rate was 4% among patients with phSFRP1 and 24% in patients with umSFRP1.

There was a trend towards fewer series of chemotherapy in patients with phSFRP1. As the same trend was seen in all cutoffs of CA19-9 as well as ECOG PS, this indicates the cause to be the shorter survival of the phSFRP1 group (Supplemental Table 2).

SFRP1 methylation status showed good discriminatory capacity for differentiating between patients who died at time *t* and patients who lived beyond (Fig. 2A). The accuracy was highest between 0 and 6 months follow-up, with AUC(t) ranging from 0.80 to 0.85 at 6 months. Past 6 months, the AUC(t) stabilized at approximately 0.70. The 1-year concordance was estimated to be C = 0.67.

phSFRP1 was significantly associated with shorter survival in crude regression analysis with an increase in absolute risk of death of 36.9% (95% CI 12.0%–61.7%) and 19.8% (95% CI 1.9–37.6) at 12 and 24-months respectively. Neither age >65, PS, sex, or CA19-9 above the median were associated with survival at either time point (Fig. 3). In an adjusted model, phSFRP1 was also significantly associated with shorter survival with an increase in absolute risk of death of 42.3% (95% CI 18.1–66.5) and 18.2% (95% CI 0.5–35.8) at 12- and 24-months, respectively. At 12-months, age >65 had a protective effect in the adjusted model, reducing risk by 30.6% (95% CI -54.6, −6.7). Neither PS, sex, nor CA 19-9 above the median were associated with survival in the adjusted model (Fig. 3).

Using alternative cutoffs for classifying a methylated sample did not improve the model. Including the Cycle threshold as a

Table 1
Characteristics of stage IV PDAC patients treated with 1. line palliative FOLFIRINOX.

Characteristics	umSFRP1	phSFRP1	All	p-value
Number	29	23	52	
Age, years (mean, range)	63 (46–75)	65 (43–79)	64 (43–79)	0.41[a]
Sex				
Male	15 (52%)	8 (35%)	23 (44%)	0.22[b]
Female	14 (48%)	15 (65%)	29 (56%)	
Weight[1] (mean, range)	74 (51–93)	73 (50–115)	73 (50–115)	0.59[a]
BMI[1]				
<18.5	2 (7%)	1 (4%)	3 (6%)	0.72[b]
18.5–25	14 (50%)	14 (61%)	28 (55%)	
>25	12 (43%)	8 (35%)	20 (39%)	
CA 19–9 (median, range)	603 (3–36900)	2210 (3–171000)	860 (3–171000)	0.45[a]
Curative surgery attempted				
No surgery	28 (97%)	23 (100%)	51 (98%)	0.37[b]
Surgery	1 (3%)	0 (0%)	1 (2%)	
Series of chemotherapy (mean, range)	14 (1–38)	9 (1–27)	12 (1–38)	0.07[a]
Location of the primary tumor				
Caput	13 (45%)	10 (43%)	23 (44%)	0.09[b]
Corpus	11 (38%)	3 (13%)	14 (27%)	
Cauda	5 (17%)	9 (39%)	14 (27%)	
Diffuse	0 (0%)	1 (4%)	1 (2%)	
Location of metastases (%)				
Liver	16 (55%)	18 (78%)	34 (65%)	0.14[b]
Lung	1 (3%)	0 (0%)	1 (2%)	
Liver and Lung	1 (3%)	0 (0%)	1 (2%)	
Carcinosis	6 (21%)	1 (4%)	7 (13%)	
Other	5 (17%)	4 (17%)	9 (17%)	
ECOG PS				
0	19 (66%)	14 (61%)	33 (63%)	0.73[b]
1	10 (34%)	9 (39%)	19 (37%)	

umSFRP1, patients without SFRP1 promoter hypermethylation; phSFRP1, patients with SFRP1 promoter hypermethylation. [1]Missing 1 patient.
[a] Kruskal-Wallis one-way test of variance.
[b] Pearson chi-square test.

continuous variable slightly improved the p-value (Supplemental Table 3).

3.1.1. Prognostic impact of SFRP1 compared to CA19-9

To assess the prognostic accuracy of SFRP1 compared to CA19-9, ROC curves were computed at 12- and 24-months for a selection of CA19-9 cutoff values, Fig. 4.

Across both time points and all cutoff values for CA19-9, phSFRP1 was more accurate at discriminating short survival than CA19-9. Models were generally slightly improved by inclusion of both markers compared to only phSFRP1.

3.2. SFRP1 promoter hypermethylation as a potential predictor of treatment effect in stage IV PDAC

To assess a possible value of the biomarker in predicting effect of treatment, we evaluated interactions between treatment with chemotherapy and phSFRP1. FOLFIRINOX-treated patients were pooled with stage IV patients receiving gemcitabine (n = 83) and patients receiving BSC (n = 15) from our previous study establishing phSFRP1 as a prognostic biomarker in patients with gemcitabine-treated stage IV PDAC [16]. Baseline characteristics are presented in Supplemental Table 4.

A crude model including treatment (BSC, gemcitabine, or FOLFIRINOX) and SFRP1 methylation status yielded a statistically significant interaction at both 12- and 24-months, indicating that treatment effect varies according to SFRP1 methylation status. See Supplemental Figure 1. At 12-months, gemcitabine and FOLFIRINOX-treatment lowered risk of death by 33.3% (95% CI -47.2 -19.5) and 58.6%, respectively. However, when patients were both phSFRP1 and treated with either gemcitabine or FOLFIRINOX the risk increased by 33.3% and 36.9% respectively.

3.3. SFRP1 promoter hypermethylation in patients with stage III PDAC treated with best supportive care or palliative chemotherapy

Thirty-one serum samples (BIOPAC study) and 13 plasma samples (GIVTE study) were received from patients with stage III PDAC, treated with either gemcitabine (n = 26), FOLFIRINOX (n = 11), or BSC (n = 7). More patients in the phSFRP1 group received only best supportive care (p = 0.01). No significant differences were seen in either age, sex, BMI, weight, CA 19-9, PS, attempted curative surgery, location of tumor, or location of metastases according to promoter hypermethylation of SFRP1, see Table 2.

Patients with stage III PDAC and phSFRP1 had a mOS of 7.4 months compared to 10.2 months in patients with umSFRP1, Fig. 1B. The 2-year survival rate was 12% for umSFRP1 patients, and 0% for phSFRP1 patients.

In the crude model phSFRP1 was associated with a significantly increased risk of death at 24-months, but not at 12-months PhSFRP1 was not significantly associated with risk of death in the adjusted models. See Fig. 5.

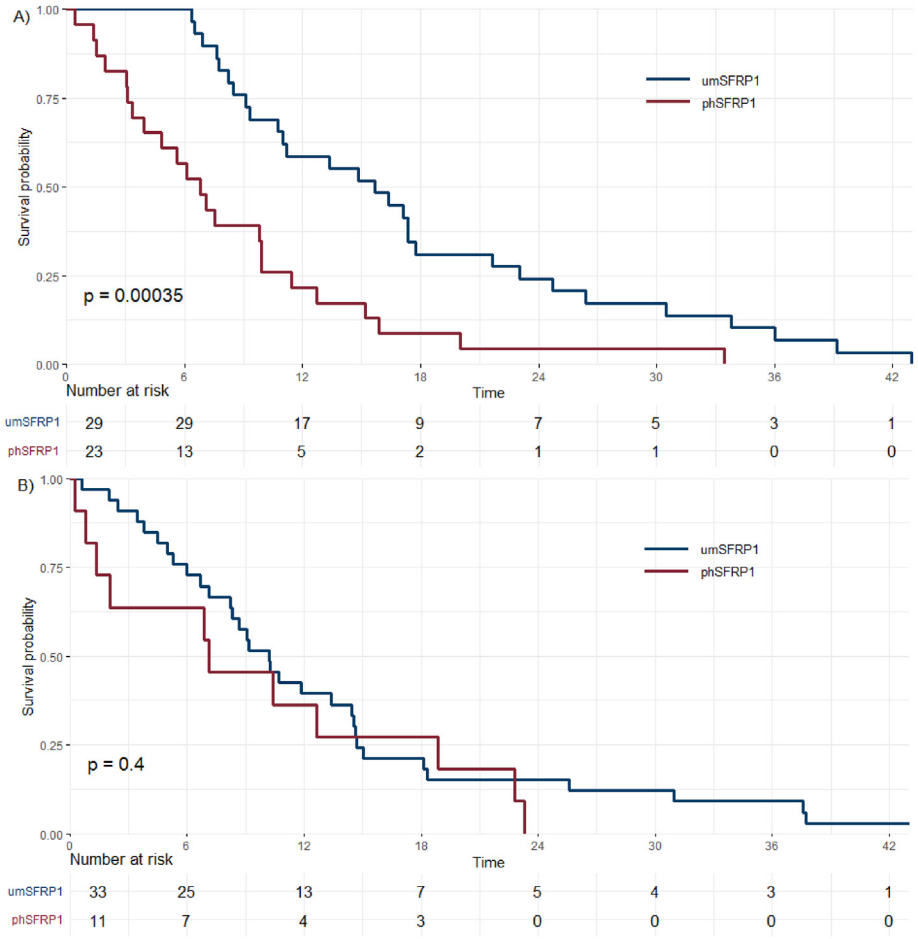

Fig. 1. Kaplan-Meier survival curves with log-rank tests for patients with PDAC. A) FOLFIRINOX-treated stage IV patients by SFRP1 promoter hypermethylation status. B) patients with stage III PDAC by SFRP1 promoter hypermethylation status. PhSFRP1, patients with a promoter hypermethylation of the SFRP1 gene; umSFRP1, patients without a promoter hypermethylation of the SFRP1 gene. Risk table shows the number of patients at risk in each group in 6-month intervals.

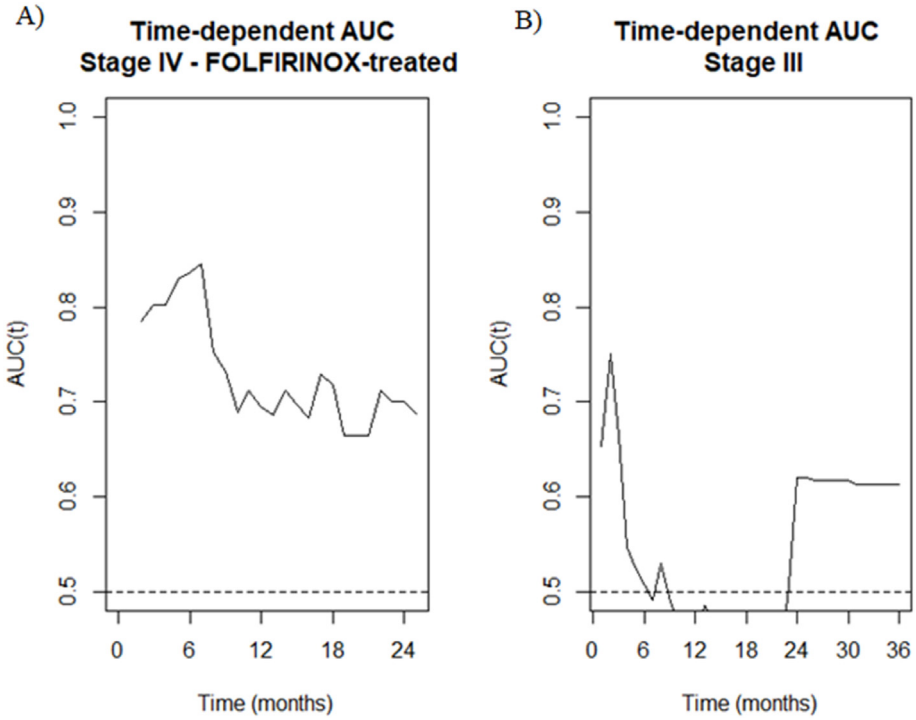

Fig. 2. Inverse probability of censoring weighting (IPCW) estimation of cumulative/dynamic time-dependent AUC plots. Fig. 2A: Stage IV FOLFIRINOX-treated patients. Fig. 2B: Stage III patients.

SFRP1 methylation status showed modest discriminatory capacity for differentiating between patients who died at time *t* and those who lived beyond (Fig. 2B). The biomarker was most accurate between 0 and 4 months follow-up with AUC(t) ranging from 0.77 to 0.65 at 4 months. From 4 months to 15-month follow-up, the AUC(t) was approximately 0.55. From 15 to 23 months, AUC(t) indicates a protective effect of phSFRP1 in this subgroup. The 1-year concordance was estimated to be C = 0.54.

4. Discussion

Epigenetic dysregulation is vital in carcinogen esis and could be a cornerstone in understanding the uncontrollable progression of pancreatic cancer [29]. Here we show that promoter hypermethylation of SFRP1 measured by cfDNA in plasma is significantly associated with shorter survival in patients with stage IV FOLFIRINOX-treated PDAC. Previously, we found phSFRP1 to be a prognostic biomarker for survival in patients with gemcitabine-treated stage IV PDAC [16]. Combined with results of the current study, this shows that phSFRP1 has potential as a prognostic biomarker for stage IV PDAC, regardless of the chosen 1. line palliative chemotherapy. The mOS of stage IV FOLFIRINOX-treated patients was similar to that reported in a large real-world Danish cohort of patients with PDAC [4]. This indicates our study population is representative of at least the Danish population.

SFRP1 methylation status was significantly associated with shorter survival of stage IV FOLFIRINOX-treated PDAC patients in both the crude and adjusted models. The estimated effect of the associations was approximately equal in the crude and adjusted model, indicating no confounding. Neither age above 65 years, sex, nor PS were significantly associated with survival.

In ROC analyses, models with phSFRP1 were more accurate in predicting mortality than CA 19-9 at all cutoffs. The accuracy was slightly improved when including both phSFRP1 and CA 19-9. This provides further evidence of the utility of phSFRP1 and indicates that phSFRP1 could supplement CA 19-9 measurements as a prognostic biomarker. The most accurate model at 12 months was using the cutoff of 860 - the median of the current study. These estimates are likely slightly optimistic; however, the same tendency was seen in all other examined CA 19-9 cutoffs. Time-dependent analysis of discriminatory power indicates that phSFRP1 is a promising both short-term and long-term prognostic biomarker for the survival of FOLFIRINOX-treated stage IV patients with PDAC. The accuracy of the biomarker was the highest within 0–6 months of diagnosis.

The mOS of the umSFRP1 patients was approximately 5 months longer than what has previously been achievable with any palliative chemotherapy in this patient group [4]. In contrast, patients with phSFRP1 had a mOS comparable to the less effective treatment gemcitabine, where the expected mOS is roughly 6 months [4]. This difference is substantial, as patients receiving gemcitabine are generally in a too poor physical condition to receive the more effective chemotherapy regimens. Unfortunately, no tissue samples were available for cross-validation of results. However, phSFRP1 has previously been demonstrated in PDAC tissue [13–15]. Additionally, a recent study has linked low DNA methylation of SFRP1 in PDAC tissue to better prognosis [30]. At least in Denmark, the overall resection rate is approximately 20%, as patients with locally advanced or metastatic PDAC are generally not offered surgery [4]. This emphasizes the need for more minimally invasive approaches, such as liquid biopsies, for prognostication in the majority of cases where retrieval of additional tissue is not feasible.

Previously, SFRP1 overexpression has been linked to longer progression-free survival and overall survival in several cancers including lung carcinoma, nasopharyngeal carcinoma, and PDAC [14,31,32]. Knocking out SFRP1 has shown to increase resistance to paclitaxel, doxorubicin, and cisplatin in breast cancer [33]. Likewise, SFRP1 restoration has been linked to taxane resensitization in

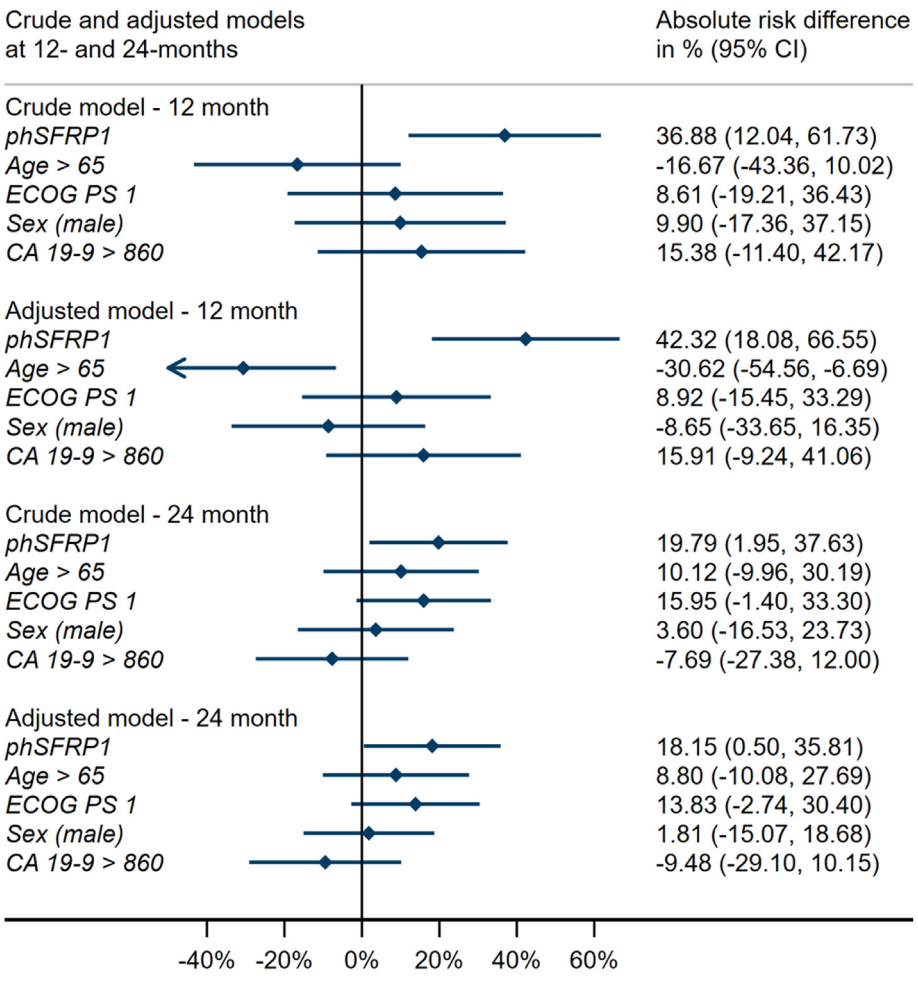

Fig. 3. Crude and adjusted risks of death in patients with FOLFIRINOX-treated stage IV PDAC at 12- and 24 months. umSFRP1, patients without SFRP1 promoter hypermethylation; phSFRP1, patients with SFRP1 promoter hypermethylation.

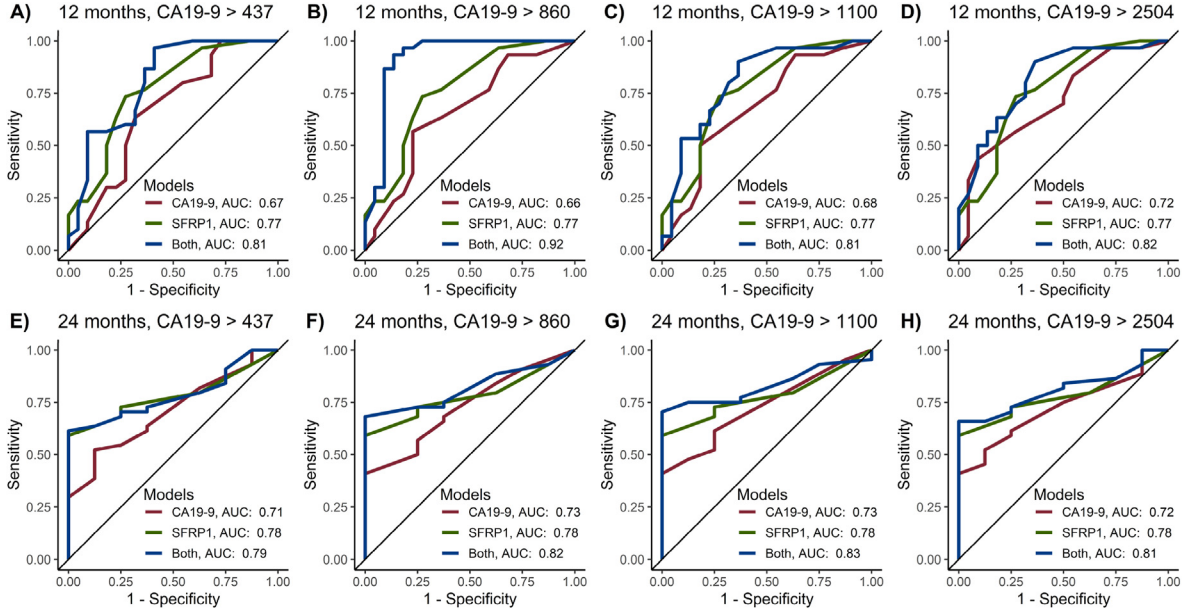

Fig. 4. Prognostic accuracy of models at the 12- and 24 months point with different cutoffs for CA19-9. Models include either CA19-9, phSFRP1 or both as well as the variables ECOG PS, age >65 years, and sex. A) 12 months, CA19-9 cutoff 437. B) 12 months, CA19-9 cutoff: 860C) 12 months, CA19-9 cutoff: 1100 D) 12 months, CA19-9 cutoff 2504 E) 24 months, CA19-9 cutoff: 437. F) 24 months, CA19-9 cutoff: 860. G) 24 months, CA19-9 cutoff: 1100. H) 24 months, CA19-9 cutoff: 2504.

Table 2
Characteristics of patients with stage III PDAC treated with best supportive care or palliative chemotherapy.

Characteristics	umSFRP1	phSFRP1	All	p-value
Number	33	11	44	
Age, years (mean, range)	66 (42–86)	67 (52–79)	66 (42–86)	0.47[d]
Sex				
Male	17 (52%)	3 (27%)	20 (45%)	0.16[e]
Female	16 (48%)	8 (73%)	24 (55%)	
Weight, (mean, range)[a]	71 (44–133)	67 (56–90)	70 (44–133)	0.50[d]
BMI[a]				
<18.5	5 (17%)	2 (22%)	7 (17%)	0.72[e]
18.5–25	14 (47%)	5 (56%)	19 (46%)	
>25	11 (37%)	2 (22%)	13 (32%)	
CA 19–9, (mean, range)[b]	1249 (3–27900)	373 (3–1000)	1025 (3–27900)	0.68[d]
Curative surgery attempted				
No surgery	24 (73%)	7 (64%)	31 (70%)	0.57[e]
Surgery	9 (27%)	4 (36%)	13 (30%)	
Type of treatment				
Best supportive care	2 (6%)	5 (45%)	7 (16%)	0.01[e]
Gemcitabine	21 (64%)	5 (45%)	26 (59%)	
FOLFIRINOX	10 (30%)	1 (9%)	11 (25%)	
Series of chemotherapy (mean, range)[c]	6 (0–20)	6 (0–20)	6 (0–20)	0.24[d]
Location of the primary tumor				
Caput	25 (76%)	10 (91%)	35 (80%)	0.76[e]
Corpus	4 (12%)	1 (9%)	5 (11%)	
Unknown	3 (9%)	0 (0%)	3 (7%)	
Diffuse	1 (3%)	0 (0%)	1 (2%)	
ECOG PS				
0	15 (45%)	7 (64%)	22 (50%)	0.58[e]
1	13 (39%)	3 (27%)	16 (36%)	
2	5 (15%)	1 (9%)	6 (14%)	

[a] Missing 5 patients.
[b] Missing 1 patient.
[c] Missing 1 patient. umSFRP1, patients without a promoter hypermethylation of the SFRP1 gene; phSFRP1, patients with a promoter hypermethylation of the SFRP1 gene.
[d] Kruskal-Wallis one-way test of variance.
[e] Pearson chi-square test.

taxane-resistant lung adenocarcinoma cell lines and nude mice [32].

This suggests the observed short survival in stage IV PDAC patients with phSFRP1 could be caused by phSFRP1-mediated resistance to chemotherapy. This might indicate that chemotherapy may not be beneficial for patients with phSFRP1, regardless of their PS. A biomarker able to predict the effect of chemotherapy would be ideal to guide clinicians in the choice of treatment, with the purpose of finding the optimal balance of quantity of life while retaining the quality.

To evaluate this, we supplemented our analysis with interaction terms. Interaction terms between phSFRP1 and treatment with chemotherapy were significant in both crude and adjusted analysis, indicating that treatment effect varies by methylation status [22]. PhSFRP1 was significantly associated with an increased risk of death when treated with either gemcitabine or FOLFIRINOX, compared to umSFRP1 patients. This indicates a predictive value of the biomarker to predict response to chemotherapy. The effects of this may be wide-reaching as there are currently no such predictive biomarkers available in patients with PDAC. However, the analysis is somewhat limited by the small control group, and results must be reproduced in larger, preferably prospective cohorts. Further, a question is the comparability of patients, as those fit for treatment with FOLFIRINOX are in a substantially better condition than patients who receive only BSC. However, a true control group may not be feasible as it would require randomization of patients with PS 0–1 to BSC. Thus, phSFRP1 becomes an attractive target for at least two forms of targeted treatment. One is hypomethylating agents (HMA) such as the DNA methyltransferase inhibitors decitabine and azacitidine. These drugs are used in patients with acute myeloid leukemia ineligible for allogeneic hematopoietic stem cell transplantation or intensive chemotherapy [34]. Treatment with HMA is superior to conventional care regimens in these patients and can induce complete remission [34].

Treatment with HMA has been linked to re-expression of SFRP1 in cell lines from both triple-negative breast cancer, clear cell renal cell carcinoma, laryngeal carcinoma, nasopharyngeal carcinoma, and PDAC [31,35–37].

This suggests that administration of demethylating agents for these patients could potentially reverse the phSFRP1-mediated chemotherapy resistance and sensitize the tumor to chemotherapeutics. Additionally, in patients with umSFRP1, this could potentially prolong the time the patients are sensitive to chemotherapy. There are currently several clinical trials examining the role of various epigenetic therapies in PDAC [38].

However, a limitation of HMAs are their shotgun approaches – affecting the entire epigenome. They affect not only epigenetically silenced tumor suppressor genes, but may also affect epigenetically silenced oncogenes [39].

Another potentially interesting treatment is the recently proposed concept of mimetics [40]. The process involves identifying and validating drugs that phenotypically mimic proteins encoded by epigenetically silenced tumor suppressor genes. Treatment with mimetics may be able to restore the lost function of tumor suppressor genes. As a proof of concept of mimetic development, Dahl et al. identified a novel mimetic lead specifically inhibiting growth in SFRP1-inhibited cells by inhibiting the phosphorylated LRP6 receptor [40]. While still early in development, the methodology could open an entirely new paradigm of targeted therapies.

Either methodology could potentially contribute to individualized treatment for patients with stage IV PDAC. However, more research is required to determine the feasibility.

This study also examined the prognostic impact of phSFRP1 in patients with stage III PDAC. Dysregulation of the Wnt/B-catenin

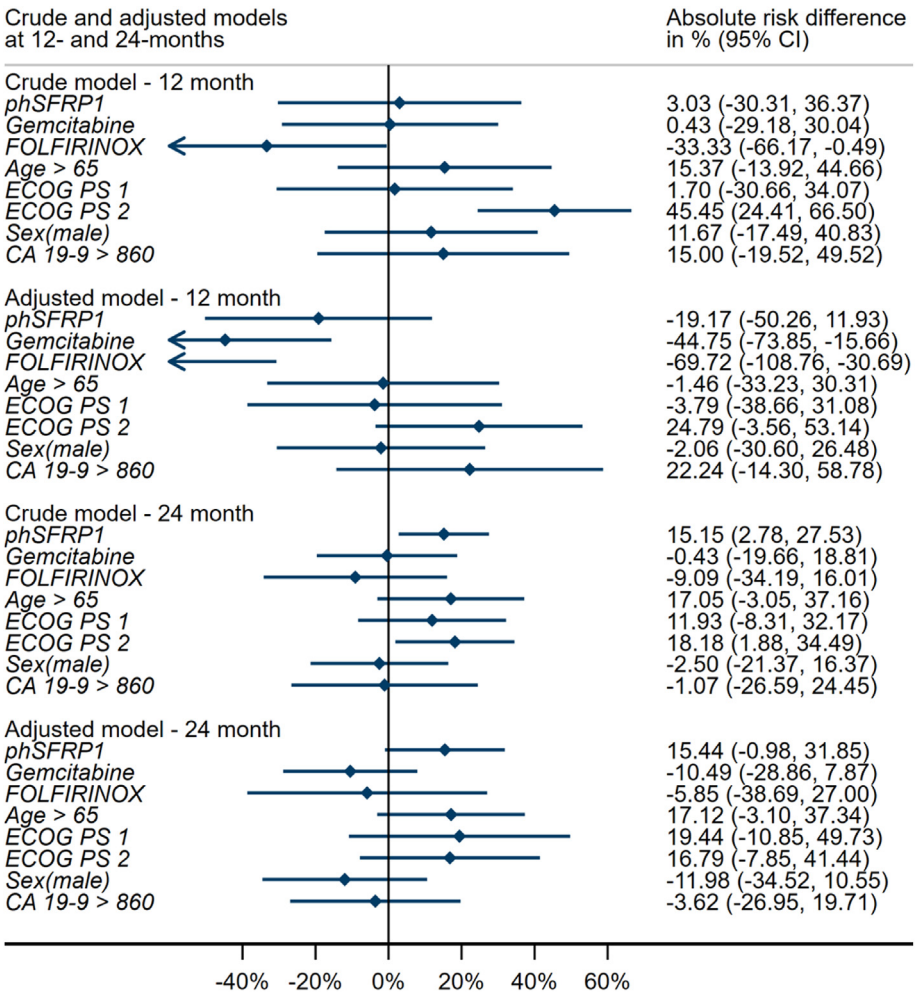

Fig. 5. Univariate and adjusted risks of death in patients with III PDAC at 12- and 24 months. umSFRP1, patients without SFRP1 promoter hypermethylation; phSFRP1, patients with SFRP1 promoter hypermethylation.

pathway would also be suspected to impact prognosis in these patients. Interestingly, the frequency of phSFRP1 among stage III patients was only approximately 20%, compared to 45–50% as seen in stage IV PDAC.

This could indicate that metastatic tumors harbor a higher rate of phSFRP1 than non-metastatic tumors, but also by less shedding of DNA. The results indicate some prognostic impact of phSFRP1 in stage III patients. However, it is currently uncertain if it provides clinically actionable information. One possibility is that metastatic PDAC has other mechanisms which amplify the tumorigenesis of SFRP1 deactivation. The tumor microenvironment may also play a substantial role in the progression, drug resistance, shedding of DNA, and metastasis of PDAC [41].

Future, more extensive studies of SFRP1 methylation in cfDNA are planned to establish the relevance of this biomarker in patients with stage III PDAC.

The study is limited by the reliance of liquid biopsies on sufficient DNA leaking into the bloodstream to be technically detectable. The retrospective nature of the study could cause selection bias; however, this is partly offset by the prospective inclusion of the original studies and the blinded methylation analysis. Further, there is no censoring, as all patients were followed until death. A limitation of statistical modeling is poor registration of outcomes and covariates, but registration was completed prospectively. Toxicity data was not available, so it was not possible to examine a possible interaction between phSFRP1 and toxicity to chemotherapy. The dichotomization procedure has previously been demonstrated to not result in significant loss of information [19]. Classifying samples with low levels of methylation did not improve the accuracy of the biomarker. However, including the Ct value as a continuous variable slightly improved the p-value, at the expense of interpretability. This indicates that additional prognostic value could be gained from a fully quantitative method. An ongoing study will examine an updated digital droplet PCR-based approach in a larger cohort, which will allow complete quantification of gene methylation.

In conclusion, this study establishes SFRP1 as a prognostic biomarker in patients with stage IV PDAC receiving treatment with FOLFIRINOX, and we propose that SFRP1 has the potential to stratify patients with metastatic PDAC for sensitivity to 1. line palliative chemotherapy. After further validation, SFRP1 methylation status could be used up-front in a clinical setting to enable selection of patients most likely to benefit from chemotherapy. A routine assessment of phSFRP1, measured by cfDNA in plasma, is faster, safer, and more practical than conventional tissue biopsy. This would be a welcome addition to a patient group severely in need of prognostication and may allow for more individualized treatment options.

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Disclaimers

S.D.H., P.H.M., O.T.-U., and H.B.K. declare that they have a patent on a diagnostic panel including the promoter hypermethylation status of 8 genes. Application No. 16161073.8–1403 Pancreatic Cancer methylation Markers (18.03.2016).

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SDH, PHM, OTU, and HBK declare that they have a patent on a diagnostic panel including the promoter hypermethylation status of 8 genes. Remaining authors have nothing to declare. We thank the personnel at the research department of the Department of Surgery Aalborg University Hospital, especially biomedical laboratory scientist June L. Køjborg for her tireless assistance with the GIVTE biobank. We thank the biomedical laboratory scientists Vibeke H. Holm and Charlotte Falk for their great assistance with the BIOPAC biobank at Herlev Hospital. Many thanks to the nurses Marianne Melton, Betina Nielsen, Mette Tholstrup Bach, and Nina Spiegelhauer at Rigshospitalet for including patients in the BIOPAC study. Astrid Z. Johansen is thanked very much for the work on clinical data in the BIOPAC database. Medical doctors at Herlev Hospital and Rigshospitalet are thanked for their role in the inclusion of patients in the BIOPAC study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pan.2023.05.003.

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