SPANDIDOS PUBLICATIONS

Significant overexpression of SPARC/osteonectin mRNA in pancreatic cancer compared to cancer of the papilla of Vater

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Abstract. Cancer of the papilla of Vater (CPV) has a significantly better outcome compared to pancreatic cancer (PC) after curative resection. Increasing evidence suggests that prognostic differences are influenced by a different tumor biology. Secreted protein acidic and rich in cystein (SPARC)/osteonectin is a multifunctional matricellular protein involved in cell-matrix interactions and might be involved in tumor pathogenesis and progression. We examined quantitative SPARC mRNA expression in CPV and PC to evaluate if varying expression might contribute to the different biologic behaviour of these entities. Quantitative real-time reverse transcription-PCR was performed to analyze expression of SPARC mRNA in a series of 31 PC and 8 CPV specimens and corresponding uninvolved pancreatic tissues. Relative mRNA levels (ratio tumor/normal) were calculated as (SPARC/ß-actin in tumor)/(SPARC/ß-actin in paired normal tissue). SPARC expression levels were associated with clinical and histopathological parameters. SPARC mRNA expression was detected in all tumor and normal tissues of the pancreas and papilla of Vater. In pancreatic cancer, 15/31 (48.4%) patients showed overexpression of SPARC (ratio tumor/normal >1) whereas in CPV only 1/8 (12.5%) exhibited SPARC overexpression and this difference was statistically significant ($p<0.05$, Mann-Whitney test). No associations were detected with T- and N-categories, grading or prognosis. In conclusion, SPARC mRNA overexpression is significantly more frequent in CP than CPV and adds further evidence that CP and CPV are biologically different tumor entities.

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Introduction

Adenocarcinoma of the pancreas (PC) is currently the fifth leading cause of cancer-related deaths in North America and Europe with rising incidence and a five-year survival rate <5% (1-3). Although various molecular alterations were identified, which have improved our understanding for the carcinogenesis of this disease, the molecular basis for the dismal outcome of pancreatic cancer is still unknown. New treatment regimens based on molecular classifications of the individual tumor may provide improvements in outcome for patients with pancreatic carcinoma.

Cancer of the papilla of Vater (CPV) is a rare disease representing 6-12% of all periampullary malignancies (4) with an estimated incidence of 2.9 per million (5). In contrast to pancreatic cancer, it has a good prognosis with a 5-year survival rate of >40% after curative resection (6,7). Because of its anatomical localization, CPV becomes symptomatic at an earlier stage than pancreatic cancer. Therefore, most of the patients are candidates for surgical therapy. Nevertheless, there is a raising body of evidence suggesting that biological differences might also contribute to the different prognosis (8).

Secreted protein acidic and rich in cystein (SPARC) also called osteonectin belongs to a group of bone matrix-associated factors that mediate cell-matrix interactions but do not primarily serve structural roles (9). The role of SPARC in carcinogenesis has not been elucidated in detail. In human melanoma cell lines, SPARC protein overexpression is associated with invasive behavior (10), and suppression of SPARC expression by antisense RNA abrogates the tumorgenicity of these cells (11). On the other hand, SPARC protein expression in ovarian cancer cells is inversely correlated with the degree of malignancy, and overexpression induces apoptosis in cancer cells (12). Transfection of SPARC cDNA into an ovarian carcinoma cell line reduced its growth rate in culture and its ability to induce tumors in rodents (13). Transfection of SPARC cDNA into breast cancer cell lines inhibited cancer cell proliferation by slowing progression into S phase (14). Overexpression of SPARC protein or RNA has be reported in esophageal cancer (15), breast cancer (16),

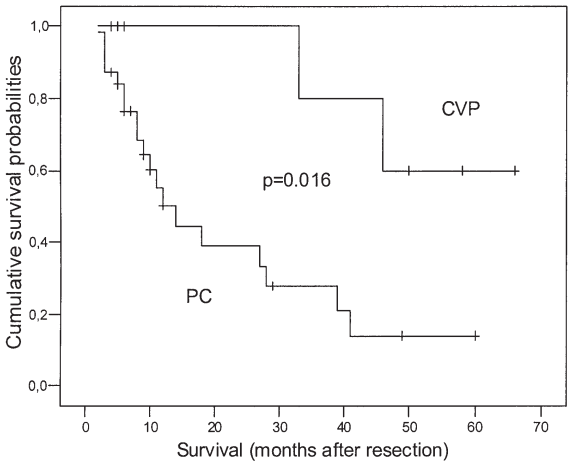

Figure 1. Kaplan-Meier survival curves of patients with pancreatic cancer (PC) (n=31) and cancer of the papilla of Vater (CVP) (n=8).

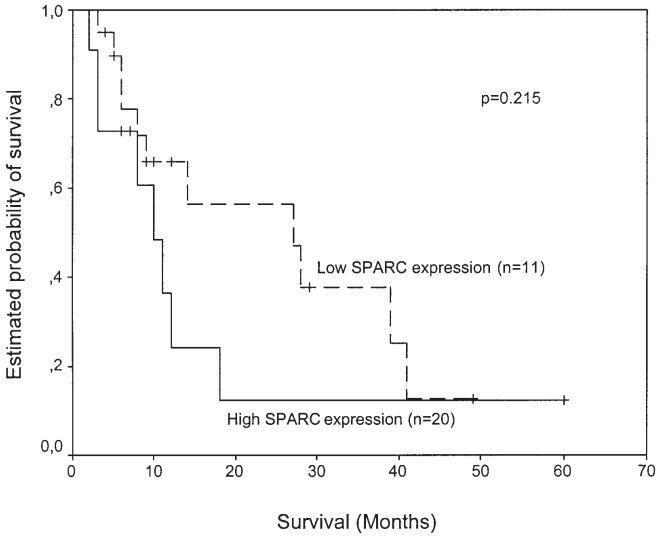

Figure 2. Estimated probability of survival of patients with pancreatic cancer showing low and high SPARC mRNA expression.

hepatocellular cancer (17), bladder cancer (18), and malignant melanomas (19). In pancreatic cancer, it was shown that SPARC expression is frequently lost in cell lines in conjunction with aberrant DNA methylation. Furthermore, SPARC was highly expressed by peritumoral fibroblasts and the expression of SPARC in fibroblasts was enhanced by coculture with pancreatic cancer cells. Exogenous SPARC inhibited the growth of pancreatic cancer cells *in vitro* (20).

We performed quantitative real-time RT-PCR assays to evaluate if SPARC is differentially expressed in CP versus CPV and if differences in SPARC expression were associated with clinico-pathological variables.

Materials and methods

Patients and specimens. Between July 1997 and December 2003 tumor and corresponding normal tissues from 63 patients, who underwent curative resection of pancreatic or ampullary tumors, were collected. Informed consent was obtained from all patients. Data and tissue collection were in accordance with the regulations of the local ethics committee.

For 31 patients with ductal adenocarcinoma of the pancreas and 8 patients with tumors of the papilla of Vater, matched tissue was available for gene analysis. The study population consisted of 24 (62%) men and 15 (38%) women, with a median age of 59.4 years (range, 33-81 years). All patients had adenocarcinomas. Tumor staging was performed according to the International Union Against Cancer (UICC) tumor node metastasis (TNM) classification. In patients with pancreatic cancer, one (3.2%) was in stage I, three (9.6%) in stage II, 24 (77.4%) patients were in stage III, and three (9,6%) were in stage IVa. In patients with CPV, five were in stage II, two in stage III and one patient was in stage IV.

Twenty-four patients underwent a Whipple's procedure, in 10 patients the pylorus was preserved. In four patients a left-sided pancreatic resection and in one patient a total pancreatectomy was performed. The mean follow-up for surviving patients was 9.5 months (range, 62 months) with no patient lost to follow-up.

Tissues were frozen immediately in liquid nitrogen and stored at - 80˚C until further processing. The definitive histology of the tissues used for RNA isolation was confirmed in serial sections analyzed by a staff pathologist (S.E.B.).

Quantitative RT-PCR. Total RNA was isolated using Trizol reagent (Life Technologies/Gibco, Grand Island, NY) and quantified at $A_{260/280mm}$ (Smart Spec; Biorad, Hercules, CA). Total cellular RNA (0.5 μg) was reverse-transcribed using an oligo (dT) 18 primer and MMLV (Moloney murine leukemia virus) reverse transcriptase (Clontech Lab.; Palo Alto, USA) according to the manufacturer's recommendations. Placenta RNA from this kit was used to prepare standard curves. An amount of 25 ng of cDNA was taken for real-time PCR using the TaqMan ABI PRISM-7900HT sequence detection system (Applied Biosystems, Darmstadt). By means of fluorescence emission this technique allows to find the cycling point when the PCR product is detectable (threshold cycle). This Ct value correlates to the starting quantity of the target (21,22). To normalize the amount of total RNA present in each reaction, the housekeeping gene ß-actin was amplified.

Primers used for PCR amplification were chosen to encompass intron between exon sequences. Primers and probes were designed for full length cDNA sequences blasted against human genomic sequences to identify exon-exon junctions (NCBI), using Primer Express software (Applied Biosystems). The ß-actin probe was labeled with 5'-VIC and 3'-minor groove binder/non-fluorescent quencher (Applied Biosystems). The SPARC probes were labeled at the 5' end with FAM and at the 3' end with the quencher TAMRA (Eurogentec, Seraing, Belgium). The sequences for the primers and probes were: SPARC sense 5' TCT TCC CTG TAC ACT GGC AGT TC 3', anti-sense 5' AGC TCG GTG TGG GAG AGG TA 3', probe 5' CAG CTG GAC CAG CAC CCC ATT GAC 3'.

Each probe was analyzed using MALDI-TOF and reliability of PCR amplification and detection was verified on serial dilutions of standard cDNAs prior to analyses of patient samples. To ensure that the genomic DNA was amplified, the assays were checked with RNA samples minus reverse transcription control as well as with genomic DNA as template.

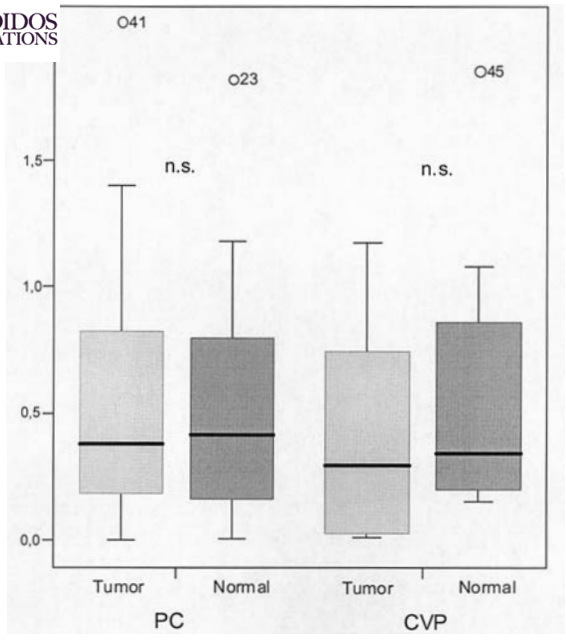

Figure 3. Median expression of SPARC in pancreatic cancer (PC) (n=31) and cancer of the papilla of Vater (CVP) (n=8).

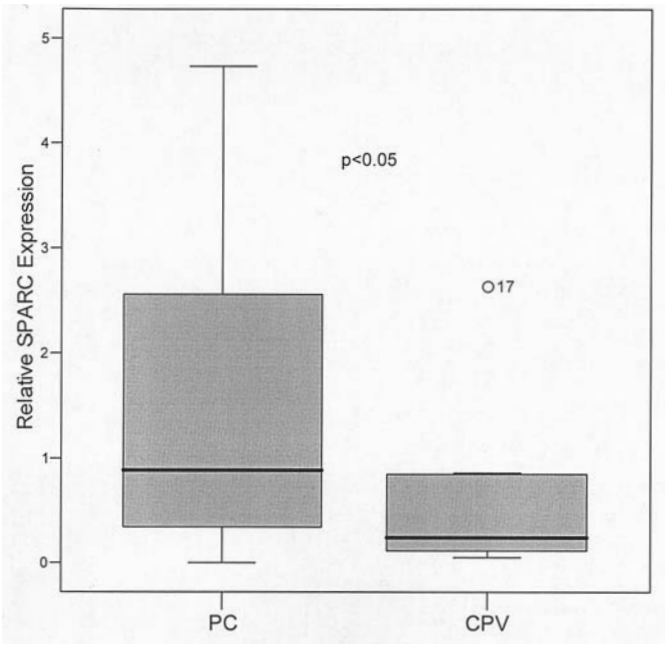

Figure 4. Relative expression (ratio tumor/normal) of SPARC mRNA in pancreatic cancer (PC) (n=31) and cancer of the papilla of Vater (CVP) (n=8).

The PCR reaction mixture contained 300 nM of each primer, 200 nM probe in a final volume of 20 μl. PCR conditions were 50˚C for 2 min, 95˚C for 10 min, followed by 40 cycles at 95˚C for 15 sec and 60˚C for 1 min. Initial template was calculated from the cycle number when the amount of PCR product passed a threshold set in the exponential phase of the PCR reaction (CT-value). Relative gene expression levels were calculated using standard curves generated by serial dilutions of placenta cDNA (Clontech Lab.). All analyses were done in triplicate.

Absolute expression levels were calculated as SPARC/ß-actin in tumor and normal tissues, respectively. Relative mRNA expression levels (ratio tumor/normal) were calculated as (survivin/ß-actin in tumor)/(SPARC/ß-actin in paired normal tissue).

Statistical analysis. The gene expression analyses yield values which are expressed as ratios between two absolute measurements: the gene of interest and the internal reference gene ß-actin. Gene expression levels were described using the median as a point estimator and the range of values. Cut-off values for discrimination of dichotomized mRNA expression levels and clinico-pathologic parameters were derived from receiver operating curve (ROC) data (area under the curve and the 95% confidence interval) according to Metz *et al* (23). Associations between gene expression levels and clinico-pathological parameters were evaluated using the χ^2 test for dichotomized variables, Wilcoxon rank test for paired variables and the Mann-Whitney test for independent variables applying Fisher's exact testing for significance.

Partitioning of gene expression levels to construct prognostic groups was performed according to LeBlanc and colleagues (24). Briefly, the best cut-off value for a supposed prognostic variable is determined by simulating the log-rank test for all observed covariate values within the entire data set. The minimal log-rank p-value determines the best cut-off value for dichotomization of the covariate. Kaplan-Meier (25) plots were used to describe the survival distribution and the log-rank test was used to evaluate for survival differences (26).

The level of significance was set to $p<0.05$ in all statistical testing. Unless otherwise specified, p-values were given for two-sided testing.

All statistical tests were performed using the software package SPSS for Windows, version 12.0, Chicago, IL, USA.

Results

SPARC mRNA expression. Expression of SPARC mRNA was detected in all pancreatic cancer specimens, in all cancers of the papilla, and in all normal pancreatic specimens. Median SPARC gene expression was similar in tumor (0.38) and normal (0.41, p=0.35) pancreatic tissue. Also, in cancer of the papilla the median SPARC was similar in tumor (0.29) and normal (0.33) specimens (p=0.12) (Fig. 3).

In pancreatic cancer, 15 out of 31 (48.4%) patients showed overexpression (T/N ratio >1) of SPARC mRNA. In contrast, only in 1 out of 8 (12.5%) CPV demonstrated overexpression of SPARC mRNA. This difference was statistically significant ($p<0.05$, Mann-Whitney test, Fig. 4).

Association of SPARC mRNA expression with clinico-pathologic variables. No significant associations between gene expression levels and clinico-pathologic parameters such as UICC tumor stage, pT and pN-categories, or grading of the primary tumor were observed.

mRNA expression and survival of patients with PC and CPV. Median survival was 14 months (range 2-60 months) for patients with PC and significantly different from 39.5 months

(range, 4-66 months) for patients with CPV ($p<0.016$ log-rank test; Fig. 1).

Partitioning of gene expression levels to construct prognostic groups according to LeBlanc and colleagues (24) did not reveal any correlation between gene expression and survival of patients with CPV. For patients with pancreatic cancer, the largest segregation between survival probabilities was obtained with a relative SPARC expression level of 2.1 ($p=0.21$, log-rank test; Fig. 2). However, no statistical significance was reached by this segregation. Twenty patients displayed a low (<2.1) and 11 patients a high (≥2.1) SPARC gene expression. The median survival for patients with low gene expression levels was 27 months (95% CI, 7-47 months) in contrast to 10 months (95% CI, 6-14 months) for patients with high SPARC expression levels.

Discussion

The surgical therapy of carcinoma of the papilla of Vater and adenocarcinoma of the pancreas consists of a pylorus preserving or a classical pancreaticoduodenectomy (Whipple procedure). Despite this identical surgical approach, the long-term prognosis is different. Five-year survival rates of 0-25% for pancreatic cancer and 15-56% for CPV have been reported (1,6). It was shown that a different tumor biology, represented by higher expression levels of members of the EGF-R family (EGF-R, c-erbB2 and c-erbB3) and metastasis-associated genes, contributes to the different growth patterns of these tumors (8,27).

The role SPARC mRNA expression in pancreatic cancer and CPV has not been addressed so far. The overexpression of SPARC was correlated to progression, incidence of distant metastasis and survival in thin cutaneous malignant melanomas (19) and associated with worse prognosis in patients with urinary bladder cancer (18). This correlation was not found in studies examining esophageal (15) or breast cancer (16). In non-small cell lung cancer, coauthors of this study could not detect a difference in SPARC expression between malignant and normal lung tissues applying QRT-PCR (28). The results of these three studies are consistent with ours, showing no statistically significant difference in median SPARC mRNA expression between normal and malignant samples from patients with pancreatic cancer and CPV. However, SPARC overexpression (T/N ratio >1) was frequently detected (48.4%) in pancreatic cancer compared to CPV (12.5%) and this difference was statistically significant ($p<0.05$).

These data substantiate previous studies concerning different gene expressions between the two tumor entities (8,27) and add further evidence that they represent biologically different tumor entities.

There is a trend towards longer survival in pancreatic cancer patients with low SPARC gene expression compared to patients with a high intratumoral expression. This finding is also confirmed by two studies using immunohistochemistry for detection of SPARC expression in urinary bladder cancer (18) and non-small cell lung cancer (29). Sato *et al* (20) have shown that SPARC mRNA was predominantly expressed in stromal fibroblasts adjacent to the neoplastic epithelium in primary pancreatic cancers. Since laser-micro-dissection was not performed in this study, we cannot rule out that one source of SPARC mRNA would be from stromal fibroblast next to tumor cells. Even if this is the case, the difference between PC and CVP would remain although the source of SPARC mRNA is not the cancer cell itself.

In summary, we have shown that overexpression of SPARC mRNA is significantly more frequent in pancreatic cancer than cancer of the papilla of Vater and this finding adds further evidence that the two entities are biologically different.

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