ANTICANCER RESEARCH *40*: 1267-1275 (2020)
doi:10.21873/anticanres.14068

Characterization of Pancreatic and Biliary Cancer Stem Cells in Patient-derived Tissue

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Abstract. *Background/Aim: Pancreatic ductal adenocarcinoma (PDAC) and extrahepatic cholangio-carcinoma (eCC) represent two cancer entities with devastating prognoses. Despite recent progress in research and treatment, therapy remains challenging. Cancer stem cells (CSCs) have been shown to play an important role in metastasis and chemoresistance. Therefore, CSCs may play a promising role as a potential therapeutic target. Materials and Methods: A total of 31 patients (23 PDAC, 8 eCC) were included in the study. CSCs were analyzed in a single-cell suspension of tumor samples via fluorescence-activated cell scanning (FACS) with a functional Hoechst 33342 staining as well as a cell surface marker staining of the CSC-panel (CD24, CD44 and EpCAM) and markers to identify fibroblasts, leukocytes and components of the notch signaling pathway. Furthermore, the potential presence of CSCs among primary cancer-associated fibroblasts (CAFs) was assessed using the same FACS- panel. Results: We showed that CSCs are present in patient-derived dissociated tumor tissue. The functional and surface marker profile of CSC-detection did in fact correlate. The amount of CSCs was significantly correlated with tumor characteristics such as a higher UICC stadium and nodal invasion. CSCs were not restricted to the epithelial cell fraction in tumor tissues, which has been verified in independent analysis of primary cell cultures of CAFs. Conclusion: Our study confirms the in vivo presence of CSCs in PDAC and eCC, stating a clinical significance thereof and thus their plausibility as therapeutic targets. In addition, stem-like cells also seem to constitute a part of the CAFs.*

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Key Words: Pancreatic cancer, cancer stem cells, cancer-associated fibroblasts, tumor dissociation, FACS.

Pancreatic cancer consists of pancreatic ductal adenocarcinoma (PDAC) in 95% of patients and represents one of the deadliest cancer types, with a 5-year relative survival rate of 8% (1, 2). The survival rate has barely improved over the last decades, with the incidence and mortality projected to increase (3). Surgical resection remains the only curative option (4). Extrahepatic cholangiocarcinoma (eCC), comprising perihilar (Klatskin) and distal cholangiocarcinoma (dCC), shares several characteristics with PDAC, such as embryological development and many patterns of tumorigenesis, while having a slightly better prognosis (5-7). In the past, chemotherapy consisting mainly of gemcitabine in combination with nab-paclitaxel or FOLFIRINOX in PDAC as well as gemcitabine and cisplatin in eCC has been established as the standard of care in palliative patients (8, 9). New adjuvant treatment regimens have also improved survival in patients with PDAC as well as with eCC (10, 11). However, even in patients who have undergone R0 resection followed by adjuvant treatment, the long-term survival remains poor, with a 5-year survival rate of 15-20% for PDAC and 27-30% for eCC (5, 12). Thus, new therapies are urgently awaited (13).

Cancer stem cells (CSCs) seem to be a promising target for future therapeutic approaches. CSCs are characterized by the potential of self-renewal and multilineage differentiation and play a significant role in tumor initiation, tumor progression, metastasis, tumor recurrence and resistance to different chemotherapy protocols such as gemcitabine (14-16). Having first been found in hematological malignancies (17), CSCs have also been described in most solid tumors, including PDAC and eCC. In biliopancreatic carcinomas, CSCs have been characterized as a small CD24+ CD44+

EpCAM+ triple-positive subpopulation (15, 18). However, while CSCs are known to play a central role during tumorigenesis in xenograft mouse models (19, 20), the clinical relevance for patients remains unclear.

In contrast to differentiated cancer cells, CSCs express more efflux transporters such as ABCG2. In detail, CSCs can be found as a weakly stained so-called side population (SP) after staining with the DNA binding, actively effused fluorescent Hoechst 33342 (plotted Hoechst red vs Hoechst blue) (21). This side population (SP) has not yet been completely characterized, as it remains unclear whether the stromal cells of the tumor also overexpress multidrug efflux transporters, contributing to the SP fraction.

Expanding on the CSC hypothesis, it was stated that specific CSC characteristics can be reacquired by differentiated tumor cells in a cancer associated fibroblast (CAF)-driven environment (16). Such CAFs – formerly considered as bystanders in tumor formation – have meanwhile been shown to contribute to chemoresistance, metastasis and other malignant features (*e.g.* cell proliferation, ECM formation, promoting invasive velocity and cell motility, angiogenesis, immune suppression) (22-25). Additionally, the tumor immunological microenvironment is of great importance for the tumor as a whole and therefore has become a subject of interest in current therapeutic approaches such as the use of PD-L1-inhibitors (26-28). Furthermore, several embryological pathways have been found to be crucial for the development of stromal and cancer stem cells, among others, the Notch, Sonic Hedgehog, Wnt and Hippo pathways (25, 29-32).

In this analysis, we aimed to examine the population of CSCs in human PDAC and eCC samples. The characterization has been realized using functional Hoechst 33342-staining in combination with surface marker staining for epithelial tumor cells, CSCs, CAFs and leukocyte common antigen CD45. Since the Notch pathway has been described to play an important role in pancreatic CSCs (29-31, 33, 34), we used antibodies against different notch components (Notch1/Notch4), to assess their expression in PDAC and eCC tumor tissue. With a translational approach, the findings were correlated with histopathological tumor characteristics. We further analyzed whether fibroblasts contribute to the side population by staining patient-derived primary fibroblast cultures with Hoechst 33342.

Materials and Methods

Patients. Patients undergoing resection for histologically confirmed PDAC or eCC between April 2016 and July 2017, who gave informed consent before operation, were included in the analysis. Exclusion criteria were pregnancy, age under 18 years and insufficient tissue-samples. Procedures were conducted in accordance with the ethical standards of the responsible committee on human experimentation, following approval from the institutional review board (EA1/292/16) and in accordance with the Helsinki Declaration of 1975.

Dissociation of patient-derived tumor fragments. A sterile tumor tissue fragment was obtained with macro dissection by the pathologist immediately after resection. The tissue was first dissociated mechanically into pieces of approximately 1 mm^3, transferred into a Falcon® tube and been weighed. Consecutive dissociation steps were established to obtain a single cell suspension according to a protocol modified according to Li *et al*. (15) and Kim, *et al*. (19). Tissue fragments were incubated at 37˚C for 1 h in Collagenase D (Roche Diagnostics GmBH, Mannheim, Germany) 1 mg/ml, 25 ml/g tissue. Subsequently, the fragments were mechanically dissociated by pipetting, the suspension was filtered through a 40 μm cell strainer and centrifuged at 800 rpm at 4˚C for 5 min. Afterwards, the pellet was repeatedly washed, centrifuged and resuspended in 4˚C cold FACS-buffer consisting of PBS (Gibco by life technologies, Carlsbad, CA, USA) with 1 % BSA (Sigma Aldrich, St Louis, MO, USA) and 0.1 % NaN_3 (Merck, Darmstadt, Germany). Cell counting was conducted with a CASY TT® system (OLS OMNI Life Science, Bremen, Germany). The quality of single cell suspension was controlled on cytospin slides that were stained with hematoxylin and eosin.

Multicolor FACS-panel. A stem cell panel of conjugated antibodies, as described by Li *et al*. (15) was used for the following antigens: EpCAM, CD24, CD44; additionally FAP (fibroblast activation protein), pan-leukocytes marker CD45, and two components of the Notch pathway, Notch1 and Notch4 . This panel was accompanied by a Hoechst 33342-stain.

The obtained primary tumor cells were resuspended to 1×10^6 cells/ml. The antibody-panel with and without Hoechst co-staining was applied, using 5 μl/ml Hoechst (Sigma Aldrich) from a 1 mg/ml stock solution and antibodies in a 1:100 dilution, each protected from light. Verapamil (Sigma Aldrich) at a concentration of 2 μl/ml from a 25 mM stock solution served as a negative control for the Hoechst staining. The staining was conducted as described by Goodell *et al*. (21, 35). During the establishment, DNA-intercalating fluorophores 7AAD and propidium iodide (PI) were added to exclude dead cells.

The stains were analyzed using BD LSR Fortessa® FACS with the additional UV filters 675/50 635 LP and 450/50 420 LP for Hoechst 33342 detection. Voltages and gates were set up in contrast with the unstained control and adjusted in the Hoechst co-staining where necessary. FACS data were analyzed using BD FACS Diva Software (Becton Dickinson, Franklin Lakes, USA) and FlowJo (Version vX.0.7, FlowJo LLC, OR, USA).

Patient-derived fibroblast culture and FACS analysis. To obtain a culture of cancer-associated fibroblasts, a modified outgrowth method (36) was applied. Resected tumor tissue was mechanically dissociated into tissue blocks of approximately 1 mm^3 and seeded in cell culture flasks with RPMI 1640 (Biochrom, Berlin, Germany) containing 10 % FCS (Biochrom) and 1% penicillin/streptomycin (Biochrom). The cell culture medium was changed after 24 h, and cultures were monitored daily.

Five different patients' confluent fibroblast cultures were harvested with trypsin/EDTA (0.02 %/0.05 % in PBS, Biochrom) and resuspended in FACS-buffer before staining, following the same staining protocol previously described for the dissociated tumor tissue.

Tumor characteristics. Histopathological assessment was carried out by a senior pathologist specialized in pancreato-biliary pathology.

All tumors were staged based on the AJCC Cancer Staging Manual, 7th Edition (2010) (37) using the TNM classification, which consists of the following assessments: T primary tumor, N regional lymph node metastasis and M distant metastasis. Further information regarding perineural, venous and lymphatic vessel invasion, as well as the resection margin status, was gathered.

Statistical analysis. Statistical analysis was conducted with Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA) and SPSS 22.0 (SPSS, Inc., Chicago, IL, USA). Associations between continuous variables were assessed by the Pearson correlation and between ordinal variables by the Spearman correlation. A Mann-Whitney *U*-test was executed to test for significant differences between two independent variables, not following a Gaussian distribution. When more than two groups were to be tested, Kruskal Wallis test was used, followed by a post hoc test (Tukey's and Dunn's) for confirmation. A Wilcoxon matched-pairs signed rank test was carried out on paired, not-normally distributed variables. In all tests, $p<0.05$ was considered to indicate a statistically significant difference.

Results

Characterization of the study population. Tumor samples from 31 patients were analyzed, comprising 14 women and 17 men suffering from the following entities: 23 PDAC (74.2%) and 8 eCC (25.8%), comprising 6 Klatskin tumors (19.4%) and 2 dCC (6.4%). The patients' median age was 67 years (range=49-83 years). The clinical characteristics of the cohort are displayed in Table I. An average number of 722,025 cells (median: 246,650 cells) were extracted from the dissected tumor tissue fragments, with a mean weight of 0.383 g (median: 0.2 g).

Table I. *Clinical characteristics of the study population (n=31).*

Clinical characteristics		
Entities		
PDAC	23	
Klatskin	6	
dCC	2	
Gender		
Female	14	
Male	17	
	PDAC	eCC
TNM classification		
T1	3	0
T2	12	5
T3	7	3
T4	1	0
N0	10	7
N1	13	1
M0	21	8
M1	1	0
MX	1	0
Perineural invasion		
Pn0	3	1
Pn1	20	7
Venous invasion		
V0	20	7
V1	3	1
Lymphatic vessel invasion		
L0	16	7
L1	7	1
Neoadjuvant chemotherapy		
Yes	4	0
No	19	8

Correlation of the CSC fraction with tumor entities and cell subtypes. A side population (SP) was detected in all 31 patients' tumor samples stained with Hoechst 33342 (Figure 1). The median SP fraction was 2%, ranging between 0.1% and 6.8%. The proportion of the SP fraction correlated with the expression of tumor stem cell markers EpCAM (r=0.7679, $p=0.0019$) and CD24 (r=0.6970, $p=0.0205$) in PDAC and eCC (Figure 2). The size of the SP fraction further showed a trend to correlate with the number of cancer-associated fibroblasts (CAFs) described by FAP-positive cells (r=0.5188, $p=0.1557$). The side population varied significantly in relation to the tumor entity ($p=0.047$, Kruskal-Wallis test) and thus varied among PDAC, Klatskin-tumor and dCC. The post hoc Dunn's multiple comparisons test did not, however, show any significant differences. When comparing eCC and PDAC, no differences in regard to the expression of cell surface markers (CD24, CD44, EpCAM, CD45, FAP, Notch1, Notch4) could be shown.

Correlation between the side population and cell surface markers. The expression of CD24, EpCAM and Notch4, as well as of the double (CD24+EpCAM+) and triple (CD24+ CD44+ EpCAM+) stem cell marker panel was significantly higher in the SP fraction compared to the nonSP fraction (CD24 $p<0.0001$, EpCAM $p=0.0007$, Notch4 $p<0.0001$, double $p=0.0012$, triple $p=0.0171$; Figure 3). Additionally, FAP-positive cells showed a trend of enrichment in the SP fraction compared to the non-SP fraction (mean 10.60% vs. 5.22%), indicating that there might be fibroblasts present in the stem cell-like fraction. However, the results did not reach significance.

Stem cell-like fraction in patient-derived cancer-associated fibroblasts. To further examine this finding, we analyzed CAF cultures acquired from five different patients with our FACS panel. These fibroblasts showed a stem cell-like population in the functional Hoechst 33342 stain, ranging between 0.30% and 3.89% (Figure 4). All cultures were negative for the epithelial marker EpCAM and the pan-leukocytes marker CD45, whilst being positive for CD44 (range=71.0%-98.9%).

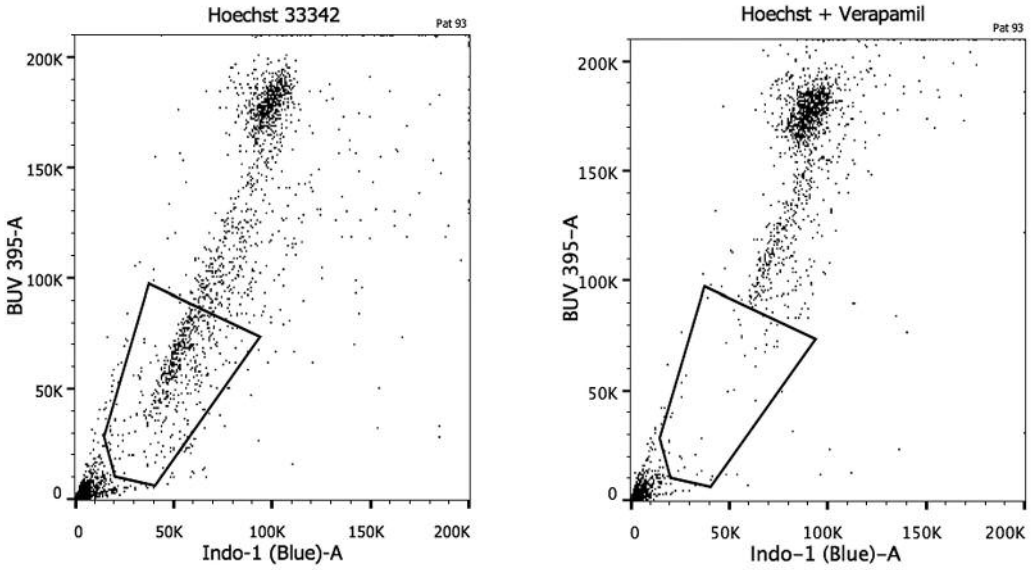

Figure 1. *Representative analysis of dissociated PDAC tissue stained with Hoechst 33342 (a) and Hoechst 33342 plus verapamil as a negative control (b). The side population (SP) is blocked when adding verapamil (SPP= 6.8% vs. 0.6%).*

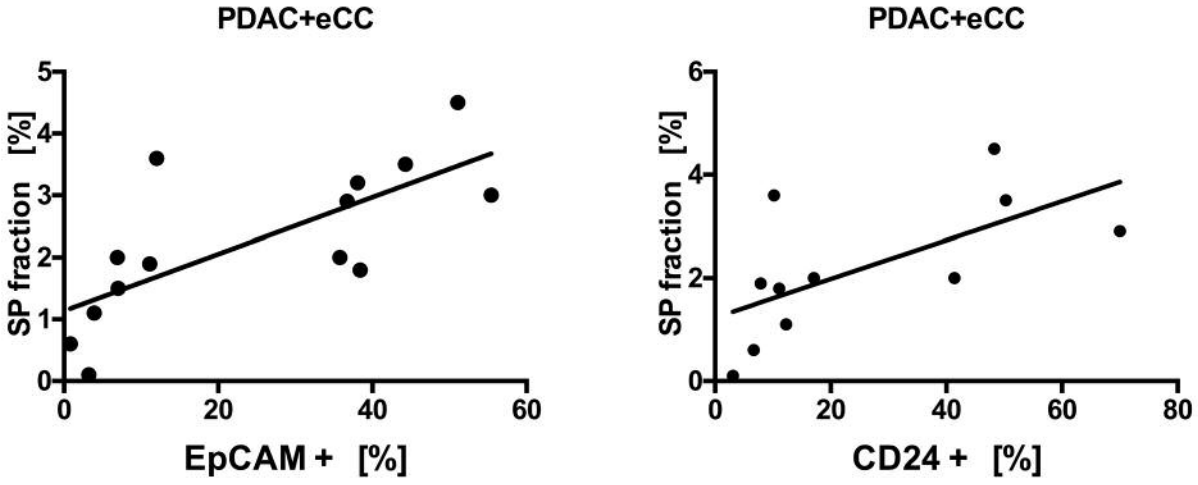

Figure 2. *EpCAM+ (a) and CD24+ (b) cells in relation to the size of the side population (SP).*

Clinical correlations. When comparing the clinical characteristics such as the UICC classification, TNM stage and grading, the SP fraction was significantly larger in tumors with a higher UICC staging (p=0.0106) and those with nodal metastasis (p=0.0332; Figure 5). This finding was also observed in comparison to the T status of the primary tumor but was not statistically significant (higher T status in patients with higher proportion of SP). Other parameters, such as grading, perineural invasion, vascular tumor invasion and lymphatic vessel invasion were not significantly correlated with the size of the SP fraction. We also observed a significant correlation between the percentage of CD24-positive cells and the UICC stage (r=0.5405, p=0.0460).

Discussion

For the first time, we demonstrated that the dissociation of patient-derived tumor tissue followed by Hoechst 33342 and cell surface staining was feasible in PDAC and eCC. We provided the largest cohort of patients with PDAC to be analyzed with this method.

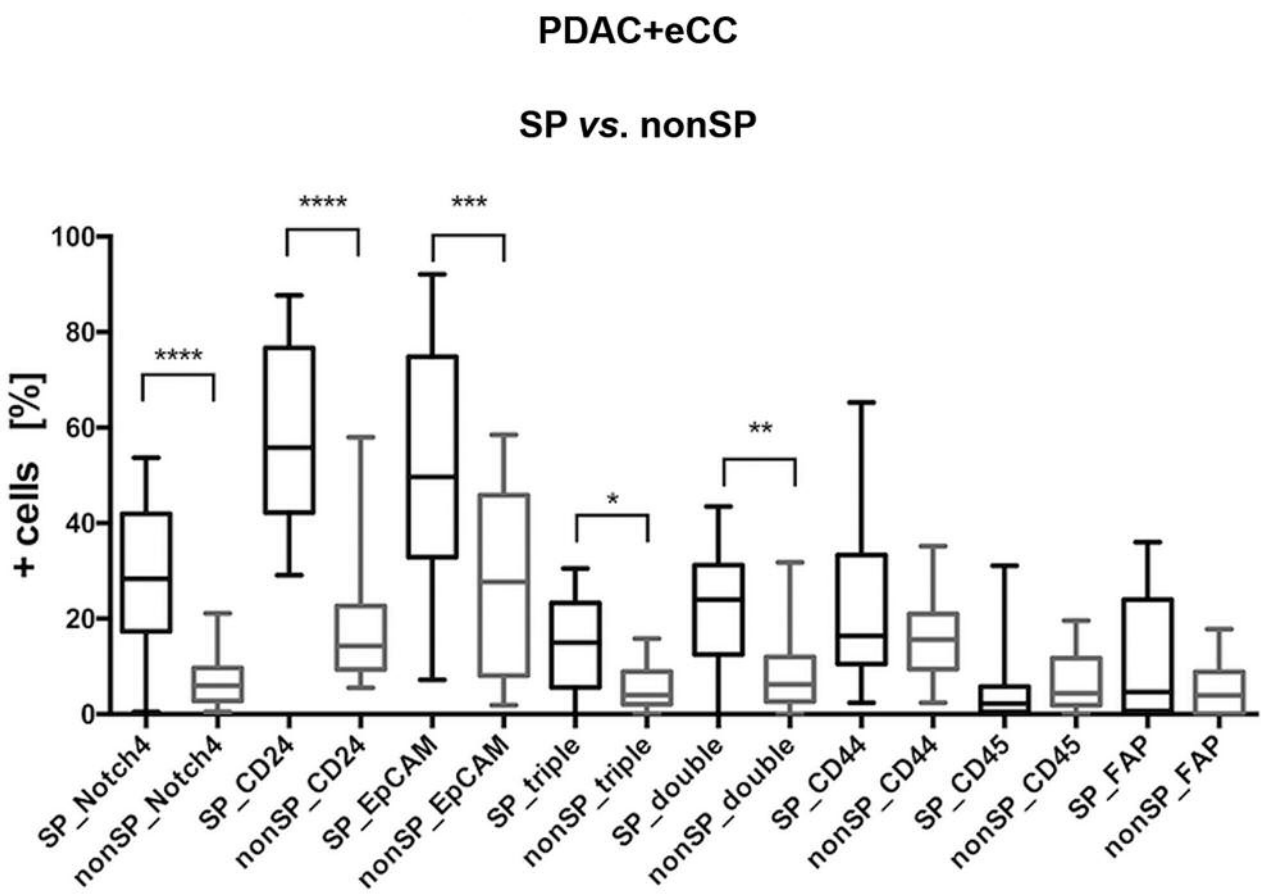

Figure 3. *Cell surface maker distribution in the side and non-side population (SP and nonSP) fraction in PDAC and dCC. Double staining indicating CD24 and EPCAM, and triple staining indicating CD24, EPCAM and CD44 staining.*

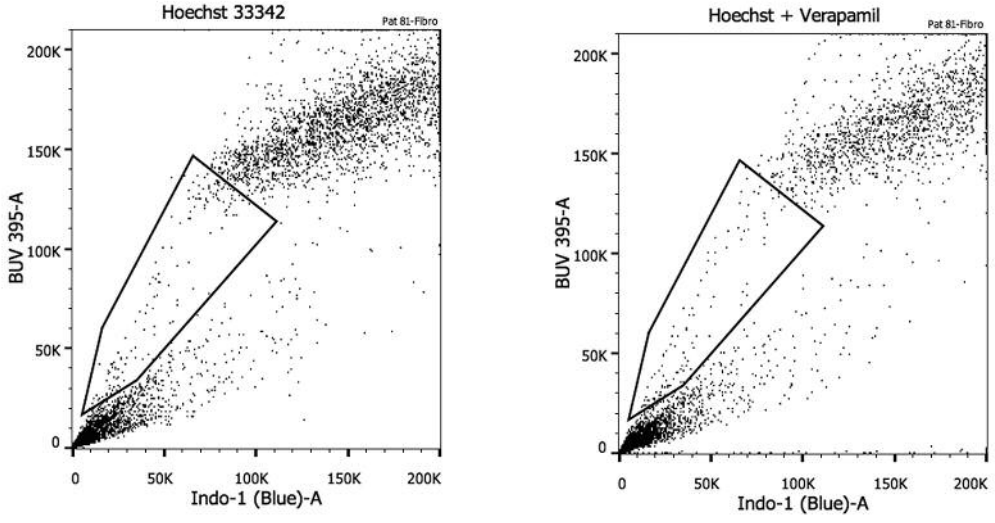

Figure 4. *Cancer-associated fibroblasts isolated from a PDAC, showing a distinct side population (SP) in the Hoechst 33342 staining (a) and the Hoechst 33342 staining with verapamil control (b); (SP=1.7% vs. 1.5%).*

The size of the side population detected in our cohort is comparable with those of cell lines and human tumor samples found in the literature (15, 38-42). Additionally, we assessed a correlation between the two detection methods of CSCs, namely the SP and the CD24+ EpCAM+ population. As discussed to what extent functional Hoechst staining is applicable to detect CSCs, the good correlation with the cell surface marker panel can be seen as a validation.

The overexpression of Notch4 in SP cells indicates the importance of the Notch pathway, as previously described (29-31, 34), potentially providing further evidence for the role of embryological factors in the tumorigenesis of these entities. However, Notch4 is only one of the many components of the Notch pathway, and further analysis needs to be conducted.

We detected FAP-positive cells in the SP fraction. These cells were – however not significantly – enriched in the SP fraction compared to the non-SP fraction. This finding indicates that there may be fibroblasts present among stem-like cells in tumors, visualized by patient-derived primary CAF cultures displaying a SP in the Hoechst 33342 staining. To our knowledge, CAFs have not yet been previously characterized to contain a SP fraction in PDAC or cholangiocarcinoma. This characterization could be another step towards the recognition of CAFs as truly malignant cells. Potential stem cell-like features have indeed been described for CAFs of other tumor entities, such as breast, colon and hepatocellular carcinomas (43-45). As our findings were only represented by a pilot investigation, larger numbers of patient-derived CAFs certainly need to be examined, possibly in correlation with additional CAF and CSC markers.

Furthermore, CAFs have been described to form a stem cell niche for CSCs. In such a microenvironment, CAFs seem to play an important role in the mechanisms of chemotherapeutic drug resistance (24, 46, 47). This hypothesis is supported by our findings, as tumors with a larger fibroblast population might have a larger SP fraction (results did not reach significance).

To date, despite strong evidence from animal studies, few correlations between CSCs and clinical parameters in patients have been examined (39-41, 48). Our data showed that a higher UICC stage, as well as the presence of a nodal metastasis, was significantly correlated with more CSCs in tumor samples. This finding was consistent using functional Hoechst-staining and CD24 cell surface staining. Thus, we provided evidence for a clinical impact of CSCs *in vivo*.

Several limitations of this study should be addressed. We encountered challenges on the way to multicolored FACS. The number of stains per tissue was vastly limited by the cell count and thus by the size of the mostly small obtained tumor fragment. Hence, it was not always possible to obtain all stains. Debris and dead cells could not be entirely outgated due to the lack of detection of ECM-debris by dead cell staining, such as 7AAD and PI (35, 49).

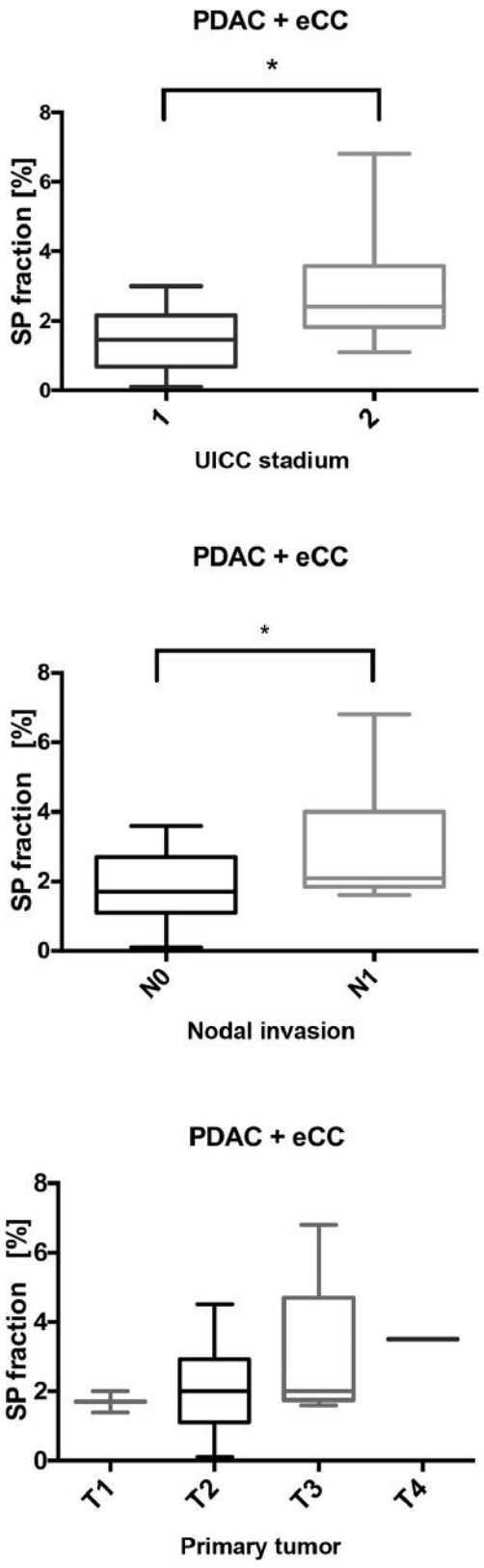

Figure 5. *Size of the side population (SP) in relation to the UICC stadium (a), the N status (b) and the T status (c).*

As Hoechst 33342 can also be detected with the filters used for brilliant violet stains, especially BV421, complete compensation was often not possible and impeded our Notch1 and partly our CD44 staining. This could be a possible cause for the fact that our triple stain (EpCAM+CD24+CD44+) did not correlate with the functional Hoechst stain, while the double stain (EpCAM+CD24+) did correlate.

Conclusion

In this study, we were able to confirm the *in vivo* presence of CSCs in PDAC and eCC and provide evidence for their clinical significance. We showed that the detection of CSCs in pancreatic and biliary dissociated primary material is feasible and that both methods – functional SP and surface marker staining – correlate not only with each other but also with histopathological parameters. Thus, we support the evidence of CSCs as a promising therapeutic target *in vivo* and discuss a subset of CAFs as potential stem-like cells. Hence, we attribute further malignant features to CAFs and underline their meaning in tumorigenesis and further therapeutic options. In addition, we presented the immediate FACS analysis of patient-derived tissue directly after resection as a practicable method for a broad field of potential applications.

Conflicts of Interest

The Authors declare no conflicts of interest.

Authors' Contributions

JP, MB, IMS, RBS and JG developed the project. JG performed the experiments. JG and RBS analyzed the results, ARS, ES and AS contributed to the interpretation of the results. JG and RBS wrote the paper. MB revised the paper.

Acknowledgements

Dr. Rosa Schmuck is participant in the BIH-Charité Clinician Scientist Program funded by the Charité - Universitätsmedizin Berlin and the Berlin Institute of Health.

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Received January 23, 2020
Revised February 10, 2020
Accepted February 14, 2020