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# Immunohistochemical Profiling of Liver Metastases and Matched-Pair Analysis in Patients with Metastatic Pancreatic Ductal Adenocarcinoma

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## RUNNING TITLE

Pattern of liver metastases in pancreatic cancer

## KEYWORDS

Pancreatic cancer, epithelial-mesenchymal-transition, metastases, CDX2, SMAD4

**Abstract**

Background: The purpose of the current study was to investigate the immunohistochemical (IHC) profile of liver metastases (LM) in patients with pancreatic ductal adenocarcinoma (PDAC).

Methods: Expression of 15 IHC markers in liver biopsies from 77 patients with PDAC, who were diagnosed between 2010 and 2014, were evaluated. In a separate subgroup analysis (n = 12), paired samples (LM and primary tumor) from the same patient were investigated for IHC profile differences.

Results: LM samples were classified as pancreatobiliary-type (PB-type) in 72 patients (93.5%), intestinal-type (INT-type) in four patients (5.2%), and squamous in one patient (1.3%). There was no significant difference in overall survival (OS) between LM of the PB-type or INT-type ($p = 0.097$). In a multivariate analysis, age <70 years ($p = 0.047$), absence of SMAD4 mutation ($p = 0.026$), absence of CDX2 expression ($p = 0.003$), and well to moderate differentiation were significant prognostic factors for better OS in patients with LM ($p = 0.031$). Analysis of paired tissue samples from LM and the primary tumor revealed a difference in CDX2 (50% increase, $p = 0.125$) and SMAD4 (33% loss of SMAD4, $p = 0.375$).

Conclusions: CDX2 expression and SMAD4 mutation indicate a poor outcome in patients with LM of PDAC. Matched-pair analysis revealed differences in distinct IHC marker expression.

## 1. Introduction

The worldwide incidence of pancreatic cancer amounts to approximately 430,000 cases per year appears to be increasing [1]. Reflecting its poor prognosis, pancreatic ductal adenocarcinoma (PDAC) is currently the fourth leading cause of cancer-related death in Europe, and the 5-years overall survival (OS) rate of approximately 8% is among the lowest of all solid cancers [2]. Radical resection is the only potentially curative treatment, but the prognosis remains poor and relapse is frequent. Because of its late presentation, PDAC often results in a medical emergency requiring immediate intervention [3].

Metastatic spread occurs rather late in the genetic evolution of PDAC [4] suggesting that early detection of PDAC could improve the clinical outcome [5]. However, because there are few and unspecific symptoms, the disease is usually detected late, and around 80% of patients already have locally advanced tumor growth or distant metastasis at initial diagnosis, with the liver being the most frequent metastatic site [6]. Although previous studies on the feasibility of resecting pancreatic metastases in selected patients reported promising results [7, 8], the therapeutic options in stage IV pancreatic cancer remain limited. While most studies on the immunohistochemical profile of PDAC focus on the primary tumor, systematic histopathological investigation of LM is uncommon. The histopathological classification of PDAC in a pancreatobiliary (PB) and intestinal (INT) type has been reported previously [9-11]. Immunohistochemical (IHC) staining with a panel including cytokeratin (CK) 7, CK20, mucin (MUC) 1, MUC2, and CDX2 further aids in distinguishing between both histopathological types (PB-type: CK7+ MUC1+; INT-type: CK20+ MUC2+ CDX2+) [10]. PDAC with an INT-type in the primary tumor was reported as an independent predictor for better OS [12], but the impact of this type in LM has not been investigated.

Epithelial-mesenchymal transition (EMT), accompanied by loss of E-cadherin and expression of mesenchymal markers such as vimentin, has been associated with invasive tumor growth and metastatic spread in several solid cancers [13]. Furthermore, the EMT program is known to induce cancer stem cell formation and enhance chemoresistance in PDAC [14]. The presence and potential prognostic impact of EMT in LM of PDAC has not been investigated.

Somatic mutation of SMAD4 through deletion or intragenic mutation occurs in around 55% of PDAC patients [15]. The loss of SMAD4 expression is associated with distant metastasis and poor prognosis in patients with PDAC by altering cellular signaling in the transforming growth factor (TGF) β pathway [6]. CDX2 functions as a major transcriptional regulator of intestinal cell differentiation and is used as a marker to assign adenocarcinomas of unknown primary to colorectal lineage [16]. The reported prognostic implications of positive CDX2 staining in PDAC are unclear [16, 17].

As previously reported [18], different morphological patterns of LM are associated with different prognosis in patients with colorectal cancer. The infiltrative front at the LM tumor periphery has been classified as replacing, pushing, or desmoplastic [19]. However, the incidence and prognostic impact of different LM infiltration patterns in PDAC have yet to be investigated.

Previous studies suggested limited molecular divergence between primary tumors and distant metastases in an unpaired analysis [20]. To understand underlying factors of metastatic spread in patients with PDAC,

comparison of paired patient samples is essential but severely limited because sufficient tissue from both the primary tumor and LM is rarely available.

The purpose of the current study was to use immunostaining to characterize LM in patients with PDAC adenocarcinoma to identify prognostic factors. In a separate subgroup analysis, paired samples (primary tumor and LM) from the same patient were evaluated to identify differences in the IHC profile.

## 2. Methods

### *2.1. Patients and tissues*

Following approval by the regional ethics committee in Stockholm, data were screened from 427 patients who had histologically confirmed LM of metastatic adenocarcinoma and who were included in the cancer register at the Karolinska Institute, Stockholm, Sweden. All patients who met the following criteria were included in this retrospective study: (i) diagnosis of metastatic PDAC on imaging (CT, PET-CT, MRI or EUS); (ii) histomorphology of the LM that is consistent with metastatic PDAC; and (iii) formalin-fixed paraffin-embedded (FFPE) liver biopsy tissue that was available for IHC investigation. Patients were excluded if they had other tumor entities, in particular colorectal cancer (CRC), biliary tract cancer, and primary liver neoplasms, as well as neuroendocrine tumors. Seventy-seven (n = 77) patients diagnosed with metastatic PDAC (mPDAC) between 2010 and 2014 at the Division of Upper Gastroenterology, Karolinska University Hospital, Stockholm, Sweden were included in the study. Tumor staging was conducted according to the 7th edition of the Union for International Cancer Control (UICC) tumor node metastasis (TNM) classification [21].

Diagnostic workup included acquisition of tumor samples from mPDAC by image-guided punch biopsies of the liver. FFPE blocks were serially sectioned at a thickness of 4 µm. Tissue sections were deparaffinized, pretreated with epitope retrieval solutions and stained on a BOND-MAX automated stainer (Leica Biosystems, Newcastle upon Tyne, U.K.) with 15 different antibodies (Table 1).

### *3.2. Immunohistochemical scoring*

Only nuclear staining was considered to be a positive result for CDX2, P53, EZH-2, PDX1, TTF-1, and SMAD4. For all remaining markers, cytoplasmic or membranous staining was considered to represent a positive result. The extent of staining was scored as 0 (no staining), 1 (staining in 1–9% of tumor cells), 2 (staining in 10–49% of tumor cells), or 3 (staining in 50–100% of tumor cells). To account for intratumor heterogeneity, five representative high-power fields (200× magnification) were scored separately for each IHC marker. The expression level that was considered to be a positive result was defined before statistical analysis for all markers as staining in 50–100% of tumor cells [22]. As an exception, SMAD4 mutation was defined as positive if staining was lost in 50–100% of tumor cells. Staining intensity was recorded as 0 (none), 1 (weak), 2 (intermediate), or 3 (strong), but it was not used for scoring [16]. Semiquantitative histopathological evaluation of IHC slides was performed by two independent observers (TH, CSV), including one expert in pancreatic pathology. Interobserver agreement was almost perfect, with a median kappa-value of 0.87 (range, 0.56–0.94).

The histological type of PDAC was classified as PB or INT for each LM, according to the criteria that were established and used in previous studies [9, 11, 23]. Cases showing a combination of features were categorized according to the dominant pattern.

The EMT status was evaluated as previously described [24], based on staining for epithelial membrane antigen (EMA) as an epithelial marker and vimentin as a mesenchymal marker. EMT status was determined as epithelial (vimentin score / EMA score <1), intermediate (vimentin score / EMA score = 1), or mesenchymal (vimentin score / EMA score >1), as previously described [13].

Further morphological features, i.e. the grade of differentiation and pattern of tumor infiltration into adjacent liver tissue, were evaluated by one pathologist only (CSV). The grade of differentiation was categorized as 1 (well), 2 (moderate), or 3 (poor), and the histopathological growth pattern (HGP) of LM was classified as pushing, replacing, or desmoplastic, according to international consensus guidelines [25].

### *3.3. Matched Pairs*

Following approval by the ethics committee at Heidelberg University, 12 patients for whom paired samples from the primary PDAC and LM were available were identified at the European Pancreas Center, Heidelberg University Hospital, Heidelberg, Germany. Tissues from resection specimens of the primary tumor were compared with tissue from surgical biopsies from the LM for each individual patient. Patients with insufficient tissue for investigation were not included in the evaluation. To ensure consistency in staining, IHC was performed at the Department of Pathology at Karolinska University Hospital, Stockholm, Sweden, according to the above-mentioned procedures. IHC for all 15 markers was compared between primary tumors and the corresponding LM. Histological parameters, such as tumor grade in primary and LM and the HGP of the LM, were also evaluated. The IHC slides were assessed by two independent observers (TH, CSV) who were both blinded to the clinicopathologic parameters. The interobserver agreement for the paired patient samples was almost perfect, with a median kappa-value of 0.80 (range, 0.40–1.00).

### *3.4. Statistics*

Statistical analysis was performed using IBM® SPSS Statistics 25 (IBM™, Armonk, New York, USA). Relationships between different variables were tested using Spearman's rank correlation. Associations between

histological differentiation (i.e. PB and INT) and IHC staining panels were assessed using Fisher's exact test and the Chi-square test. OS, which was determined from the histological confirmation of LM until death or last follow-up, was calculated using the Kaplan–Meier method. Prognostic factors, such as sex, age, tumor grade and IHC marker expression, were evaluated for significance using the log-rank test.

A univariate analysis was conducted using the Cox proportional hazards regression modal and a p-value <0.05 was considered to be statistically significant. All variables that were tested significant in the univariate analysis were included in the multivariable analysis. The interobserver agreement was calculated using Cohen's kappa and classified as moderate ($0.41 < \kappa < 0.60$), substantial ($0.61 < \kappa < 0.80$), or almost perfect ($\kappa > 0.80$). McNemar's test was used to compare paired primary PDAC and LM patient samples.

## 3. Results

### *3.1. Clinicopathologic Assessment*

Most tumors were located in the pancreatic head (n = 28, 40.6%), followed by the pancreatic body (n = 21, 30.4%) and tail (n = 20, 28.9%). Most tumors were in advanced stages (T3/4, n = 53, 76.8%) and had spread to regional lymph nodes. The median level of carbohydrate-antigen 19-9 (CA19-9) at initial diagnosis was 2223 kU/L (range, 6–1044000 kU/L). Surgical resection was performed in only seven patients (9.1%) without distant metastasis at the initial diagnosis. However, all seven patients rapidly developed LM before receiving other treatments, such as chemotherapy. At the time of liver biopsy acquisition, additional metastases were manifest in the lungs (n = 14, 18%) and the peritoneum (n = 7, 9.1%). None of the patients received chemotherapy before acquisition of LM tissue specimens. Most LM were moderately (n = 37, 48.7%) or poorly differentiated (n = 32, 42.1%). Only seven liver biopsy specimens contained well-differentiated tumor (9.2%). The histopathological infiltration pattern was replacing in 47 patients (79.6%) and pushing or desmoplastic in six patients (10.2%). In 18 liver biopsies, the tumor–liver interface was insufficiently represented such that the infiltration pattern could not be assessed. Detailed clinicopathologic features are listed in Table 2.

### *4.2. Immunohistochemistry*

Representative tissue sections for IHC were available for all IHC markers in all patients, except for PDX1, which could not be stained in 15 of 78 patients (19.2%). According to previously published results [9, 23], LMs were classified based on their morphological features as PB in 72 patients (93.5%), INT in four patients (5.2%), and squamous in one patients (1.3%). The prevalence of INT-type LM in the current analysis was low (5.2%). Based on previous studies on primary PDAC [11, 12], CK7 and MUC1 expression was attributed to PB-type

LM, while positive staining for CK20, MUC2, and CDX2 was considered to be diagnostic for INT-type LM. IHC staining in PB-LM was positive for CK7 in 72 patients (93.5%) and for MUC1 in 52 patients (67.5%). INT-type LMs were positive for CK20 in three patients (75%), MUC2 in one patient (25%), and CDX2 in four patients (100%). A representative case of INT-type differentiation in a LM is shown in Figure 1 A–D.

Immunostaining for EMA as a surrogate marker for E-cadherin expression was positive in all patients (100%). However, vimentin as a typical mesenchymal marker was positive in only ten patients (13.0%). Thus, the EMT status was categorized as epithelial in 67 LMs (87.0%), as intermediate in ten patients (13.0%), and as mesenchymal in zero patients (Figure 2).

IHC labeling for p53 and SMAD4 is known to reliably reflect the respective gene status; gene mutation is associated with a loss of IHC staining for SMAD4 and gain of staining for p53. Nuclear staining for p53, a marker of chromosomal instability in mPDAC [26], was positive in 44 patients (57.1%). Among the 77 patients, 42 patients (54.5%) showed a loss of SMAD4 expression indicating tumor suppressor gene inactivation.

In clinical practice, the markers TTF-1, PDX1, and mesothelin are often used to confirm the pancreatic origin of the LM (PDX1, mesothelin) and exclude any spread from a primary lung cancer (TTF-1). In addition, mesothelin has been reported previously as a putative biomarker that was found to be superior to pathologic features in predicting OS [27]. TTF-1 was negative in most LMs (n = 76, 98.7%). The evaluation of PDX1 was incomplete: among the 62 patient samples that could be stained for PDX1, were 33 were positive (53.2%), while staining for mesothelin was positive in 57 samples (74.0%). Detailed IHC findings are shown in Table 3.

*4.3. Survival Analysis*

The median OS in all patients was 3.1 months (95% confidence interval [CI] 1.8–4.3 months). At the end of follow-up, 76 of 77 patients (98.7%) had died and one patient (1.3%) was lost to follow-up. Previously published studies suggested a better outcome for patients with INT-type PDAC [12]. There was no significant difference in OS between PB-type and INT-type LM in the current analysis ($p = 0.097$).

Because all LM specimens were positive for the epithelial marker EMA, the EMT status was either epithelial or intermediate, depending on the expression of the mesenchymal marker vimentin. Patients with positive vimentin expression (intermediate phenotype) had a worse OS at 1.5 months (95% CI 1.1–1.8 months) compared to 3.3 months (95% CI 2.3–4.2 months) for patients with negative staining (epithelial phenotype). However, this trend did not reach significance ($p = 0.100$).

There was no significant difference in outcome in patients with LM that stained positive for p53 compared to those with absence of nuclear staining ($p = 0.854$). Determination of SMAD4 status may be of value in distinguishing between PDAC with a tendency for local invasive growth or metastatic spread [6, 28]. In the current study, deletion of SMAD4 (negative staining) in LM was associated with a worse OS of 1.7 months (95% CI 0.04–3.4 months) compared to 3.4 months (95% CI 1.5–5.3 months) for positive staining ($p = 0.044$). The impact of CDX2 expression on the outcome of patients with PDAC remains unclear [16, 17]. In the current analysis on CDX2, immunostaining of LM in patients with mPDAC showed a significant correlation with OS.

Patients with LM that stained positive for CDX2 had a significantly worse OS than those with CDX2-negative LM (1.4 months versus 3.3 months; p = 0.008).

Furthermore, LMs with histological grade 1 and 2 were associated with a significantly better outcome (3.8 months; 95% CI 2.3–5.4 months) than those with grade 3 LM (1.5 months; 95% CI 1.1–1.9 months; p = 0.037).

Clinicopathologic features and IHC markers that significantly correlated with outcome in the univariate analysis (age, gender, grade of differentiation, and SMAD4, CDX2, and vimentin expression) were included in the multivariate Cox regression model. Significant prognostic factors for longer OS in the multivariate analysis were age <70 years (p = 0.047), absence of SMAD4 mutation (p = 0.026), absence of CDX2 expression (p = 0.003), and moderately or well differentiated LM (p = 0.031). Long-term survival (>2 years) occurred in only one patient with PB-type LM and replacing HGP.

*4.4. Paired Sample Analysis*

The median time interval between primary surgery and liver biopsy was 13.8 months (range, 8.7–19.6 months). Two-thirds of the paired patient samples (n = 8, 66.7%) were acquired synchronously and in one-third (n = 4, 33.3%) of sampling was metachronous. All primary tumors (n = 12, 100%) were PDAC. The majority of neoplasms were located in the pancreatic head (n = 8, 66.7%), followed by the pancreatic body (n = 2, 16.7%) and tail (n = 2, 16.7%).

All primary tumors were at an advanced stage (T3, n = 11, 91.7%; T4, n = 1, 8.3%) and 66.7% (n = 8) of patients showed spread to the regional lymph nodes. Distant metastasis was apparent in nine patients (75.0%) at the time of initial diagnosis. None of the patients had additional distant metastases (e. g. peritoneal) at the time of liver biopsy acquisition. The median level of CA19-9 at initial diagnosis was 1410 kU/L (range, 9–20,545 kU/L). Four patients (33.3%) received neoadjuvant chemotherapy before acquisition of surgical biopsy specimens. Most LMs had moderate (n = 7/12, 58.3%) or poor differentiation (n = 4/12, 33.3%), and reflected faithfully the differentiation of the corresponding primary tumors with seven moderately (n = 7/12, 58%) and three poorly (n = 3/12, 25.0%) differentiated carcinomas, respectively. The HGP of LM was categorized as replacing (n = 4/12, 33.3%), desmoplastic (n = 3/12, 25.0%), pushing (n = 2/12, 16.7%), mixed (n = 1/12, 8%), or undetermined (n = 2; 16.7%).

While the IHC profile of LM was highly similar to that of their corresponding primary tumor, differences were observed for a distinct set of IHC markers. Expression of CDX2 differed in 58.3% of patients (50.0% gained and 8.3% lost in LM compared to the primary tumor; p = 0.125), and SMAD4 staining varied in 41.6% (33.3% lost and 8.3% gained in LM compared to the primary tumor; p = 0.375) in patient samples of LM compared to primary PDAC. In addition, a sensitivity analysis was performed including only cases with synchronous metastatic spread (n = 8, 66.7%). Heterogeneity persisted with a 50% increase in CDX2 expression and a 37.5% decrease in SMAD4 staining in LM compared to the primary tumor. A detailed overview of changes in IHC staining between LM and their corresponding primary tumor is shown in Figure 4.

## 4. Discussion

PDAC has a poor prognosis because of aggressive, locally invasive tumor growth and frequent metastatic spread. Metastases were previously shown to develop late in the lengthy genetic evolution of PDAC [4], but recent studies suggest that distant tumor spread may occur much earlier than anticipated [29]. Because of occult clinical symptoms and late presentation, only around 20% of patients with PDAC are eligible for surgical resection with curative intent [6]. To date, research, particularly histology- and IHC-based investigations, has mainly focused on resection specimens of primary PDAC, but there is a lack of data to support the growing demand for innovative clinical approaches in the metastatic setting. A better understanding of factors that are involved in metastatic tumor spread, development of prognostic biomarkers to guide clinical decision-making, and identification of new therapeutic targets should be considered in patients with metastatic PDAC. In this study we aimed at better characterizing LM of PDAC according to morphological features and IHC marker profiles that are known to be relevant for the prognosis or biology of primary PDAC. Furthermore, investigations were extended to a unique series of matched pairs, i.e. LM and their corresponding primary tumors, to identify potential changes in the IHC profile that might be of clinical or biological interest.

The liver is the most frequent metastatic organ site (up to 45% [30]), and an extensive diagnostic workup, including histomorphology, IHC, and molecular assays, often identifies the primary tumor site. The gene expression pattern of pancreatic metastases was reported to remain similar to the primary site [4]. A PB- and INT-type has been reported in primary PDAC based on morphological and IHC criteria. The distinction is believed to be of clinical relevance because the INT-type has been associated with a better outcome than the more common PB-type [10]. At some centers, this has been the rationale for considering surgical treatment of

isolated liver metastasis of an INT-type primary tumor [31]. However, no data are available on the clinical outcome of this patient cohort. In our study, LM was confidently assigned to a PB- or INT-subtype, based on morphological features and IHC staining. Thus, subtyping can also be applied to metastatic PDAC. However, the presumed difference in outcome between the PB- and INT-type does not apply to metastatic PDAC. The results should be interpreted with caution because there are few 5.1% INT-type LM patients, which influences the statistical analysis of the prognostic impact. The low rate of INT-type in PDAC is consistent with previous reports and differs from a much higher incidence in ampullary cancer [12].

Data on the significance of EMT in the development of PDAC metastases are unclear [13, 14, 24]. According to Zheng et al., EMT, which is an initiator of tumor plasticity [32] and cell migration, is not required for metastasis but it induces chemoresistance in pancreatic cancer [14]. In metastasis, vimentin may persist in tumor cells after returning to the epithelial state, resulting in an intermediate phenotype and representing a partial EMT without a complete switch to a mesenchymal differentiation [24]. Our results showed that 13.0% of LM were categorized as intermediate, suggesting some remaining mesenchymal activity. Consistent with previously published results, there was a trend towards a negative impact of intermediate EMT status on OS. Further evaluations, including novel EMT markers, such as Snail and Slug [14], and direct comparison of primary neoplasms with the metastatic site, are required.

P53, one of several tumor suppressor genes that are frequently altered in PDAC, is mutated in around 75% of PDAC [15]. Positive staining for p53 in LM, which reflects a mutated TP53 gene [33], was observed considerably less frequent (57.1%) than reported for primary PDAC. The presence or absence of IHC p53

staining did not significantly associate with OS. According to previous studies, loss of SMAD4 staining may indicate widespread metastatic failure as opposed to localized invasive tumor growth [6]. Somatic SMAD4 mutational status was previously identified as an independent prognostic factor for both overall and disease-free survival in several studies [28, 34]. IHC staining for SMAD4 represents a reliable method to identify the mutational status. In the current study, 54.5% of LM showed loss of SMAD4 expression, which is consistent with published results for PDAC [15]. Furthermore, in the multivariate analysis, the status of SMAD4 had a significant impact on OS, which confirms the relevance of this IHC marker as a clinical prognosticator for PDAC with established metastasis.

CDX2 plays an important role in the proliferation and differentiation of intestinal cells and, therefore, it is used as part of a panel of IHC markers to distinguish between PB- and INT-type PDAC [12]. Previously published results on IHC staining for CDX2 in primary PDAC reported a significantly worse prognosis in patients with higher CDX2 expression [16]. In the current study, 17% of LM showed strong nuclear staining (reactivity in ≥50% of tumor cells), which is consistent with previous studies on primary PDAC with 10–40% positive staining of CDX2 [35-37]. Because CDX2 expression is rather heterogenous, various approaches for scoring IHC staining have been applied, e.g. classification as positive if any cancer cell showed nuclear staining [23]. This divergence in scoring is likely to have affected study results. In our analysis, strong CDX2 expression in LM was associated with a significantly shorter OS, suggesting that CDX2 staining may be a novel prognostic biomarker that indicates poor survival in mPDAC.

In patients with CRC, the pattern of infiltration of LM into the surrounding liver tissue has been described as pushing, replacing, or desmoplastic [38], with an unfavorable outcome reported for the replacing infiltration pattern [18]. For LM of PDAC, the pattern of infiltration into the surrounding liver parenchyma has not been investigated. Applying similar histological criteria as those defined for CRC liver metastasis, most patients (n = 47, 80.0%) in this study were classified as replacing, but correlation with other IHC markers or patient outcomes was not observed.

In this study, a unique series of paired tissue samples from LM and the corresponding primary PDAC was available for comparative analysis. In our evaluation, the IHC expression patterns of LM compared to PDAC were broadly similar, but they showed a marked difference from a distinct set of individual IHC markers, which is consistent with previous results from molecular analyses [20, 39]. In particular, IHC marker expression indicating prognosis (i.e. CDX2 and SMAD4) changed frequently between the primary tumor and the corresponding LM (58% and 42%). Even when cases with metachronous metastatic spread (n = 4, 33.3%) were excluded from analysis, heterogeneity in CDX2 and SMAD4 staining levels persisted in LM compared to the primary tumor. One-third (n = 4, 33%) of the patients gained SMAD4 mutations in liver metastases compared to the primary tumor. These findings raise the question of whether IHC profiling of LM may be of clinical relevance. The sample size was too small to confirm this discovery, but it allows hypothesis generation. A recent abstract published by Grewal et al. [40] included 26 patients with pancreatic cancer, and they reported that SMAD4 mutation was associated with metastatic transition. SMAD4 mutations, analyzed using next-generation sequencing, were acquired by each patient who presented with localized disease and then developed metastases, and this preceded CA19-9 elevations. These results are consistent with our findings that

the rate of SMAD4 mutations increases during metastatic spread. Additionally, more extensive evaluations of paired patient samples are required to identify patients who are at greater risk for developing metastatic disease and to expand our understanding of metastatic tumor progression biology.

A limitation of the current study is that tissue was available from only a single LM, even in patients with multifocal spread to the liver. The degree of heterogeneity between LM has been rarely investigated, but two seminal studies demonstrated that genetic heterogeneity of metastases reflects the pattern in the primary tumor [4, 41]. Furthermore, some of the patients had undergone neoadjuvant chemotherapy treatment, which may have had a profound impact on the LM pathology [42]. In this series of 77 LM samples, some of the LMs may have resulted from intrapancreatic bile duct cancer rather than PDAC because these cancers can be difficult to distinguish on imaging and it is not possible to distinguish between them using histological or IHC examination. Although the CDX2 and SMAD4 status had a significant impact on the clinical outcome, the OS was relatively short, even for patients with metastatic disease. Possible explanations might be the high metastatic load and the fact that almost all patients (n = 70, 90.1%) had LM at the initial diagnosis. The role of several novel IHC markers (e. g. EZH2 and PDX1) in metastatic pancreatic cancer remains unclear and should be further explored.

The aim of the current study was to characterize LM from PDAC using morphological and IHC examination, and to correlate these findings with survival. CDX2 expression and SMAD4 mutation indicated poor survival, while the INT-type LM was not associated with any difference in survival compared to the PB-type. In addition, paired patient sample analyses revealed a difference in immunoreactivity of LM and PDAC, but further studies are needed to confirm these findings.

**AUTHOR CONTRIBUTIONS:**

T. H.: Data curation, statistical analysis, investigation, validation, methodology, visualization, writing-original draft, writing-review, editing. C. S. V. (Caroline S. Verbeke): Data curation, pathology review, statistical analysis, investigation, validation, methodology, project administration, writing-review, editing. O. S., M. B. and R. H.: Data curation, validation, methodology, project administration, writing-review, funding, editing. W. R.: Data curation, investigation, visualization, writing-review, editing. C. V. (Christina Villard), C. M. and M. C.: Data curation, methodology, supervision, editing. M. L.: Data curation, statistical analysis, investigation, validation, methodology, visualization, writing-original draft, writing-review, project administration, supervision, funding, editing.

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**CONFLICTS OF INTEREST:**

The authors declare they have no conflict of interest.

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**TABLES AND FIGURES**

**Table 1.** IHC antibody panel and pretreatment epitope retrieval solutions.

| Antibody | Clone | Dilution | Pretreatment* | Product code |
|---|---|---|---|---|
| CK7[1] | RN7 | 1 / 200 | H2 | NCL-L-CK7-560 |
| CK20[1] | PW31 | 1 / 25 | H2 | NCL-L-CK20-561 |
| MUC1[1] | Ma695 | 1 / 50 | H1 | NCL-MUC1 |
| MUC2[1] | Ccp58 | 1 / 100 | H2 | NCL-MUC2 |
| MUC5AC[1] | CLH2 | 1 / 50 | H2 | NCL-MUC-5Ac |
| MUC6[1] | CLH5 | 1 / 50 | H2 | NCL-MUC-6 |
| EMA[2] | E29 | 1 / 100 | H1 | Dako-M0613 |
| Vimentin[2] | V9 | 1 / 1500 | H1 | Dako-M0725 |
| CDX2[1] | AMT28 | 1 / 25 | H2 | NCL-CDX2 |
| SMAD4[3] | B-8 | 1 / 300 | H2 | Santa Cruz-sc-7966 |
| P53[1] | Do-7 | 1 / 300 | H1 | NCL-L-P53-D07 |
| Mesothelin[1] | 5B2 | 1 / 40 | V2 | NCL-L-MESO |
| EZH2[1] | 6A10 | 1 / 500 | V1 | NCL-L-EZH2 |
| TTF1[1] | SPT24 | 1 / 200 | H2 | NCL-TTF-1 |
| PDX-1[1] | EP139 | 1 / 100 | V1 | AC-0131RUOB |

*Pretreatments BOND-MAX automated IHC stainer: H1 = Bond epitope retrieval solution 1 citrate (20 minutes); H2 = Bond epitope retrieval solution 2 EDTA (20 minutes); [1]NovocastraTM (Leica Biosystems, Newcastle upon Tyne, U.K.); [2]DAKO™ (Agilent Pathology Solutions, Santa Clara, U.S.A.); 3Santa Cruz Biotechnology™ (Dallas, U.S.A.)

**Table 2.** Clinicopathologic features.

| | **Patients** | **%** |
|---|---|---|
| *Gender* | | |
| Female/ Male | 36 / 41 | 46.8 / 53.2 |
| *Age* | | |
| ≥ 70 years/ < 70 years | 28 / 49 | 36.4 / 63.6 |
| *Primary tumor site* | | |
| Head | 28 | 36.4 |
| Body | 21 | 27.3 |
| Tail | 20 | 26.0 |
| Not specified | 8 | 10.3 |
| *Initial CA19-9* | | |
| ≥ 1000 kU/l | 29 | 37.7 |
| < 1000 kU/l | 22 | 28.6 |
| Unknown | 26 | 33.7 |
| *Neoadjuvant treatment* | | |
| Yes/ No | 0/ 77 | 0/ 100.0 |
| *Surgical treatment primary tumor* | | |
| Yes/ No | 7/ 70 | 9.1/ 90.9 |
| *Initial clinical TNM-stage* | | |
| T1/2 | 16 | 20.8 |
| T3/4 | 53 | 68.8 |
| TX | 8 | 10.4 |
| N0 | 0 | 0 |
| N+ | 33 | 42.9 |
| NX | 44 | 57.1 |
| M1 | 77 | 100.0 |
| *Metastatic sites* | | |
| Liver | 77 | 100.0 |
| Lung | 14 | 18.2 |
| Peritoneum | 7 | 9.1 |
| Extraregional lymph nodes | 2 | 2.6 |
| *Differentiation (liver metastases)* | | |
| Well | 7 | 9.1 |
| Moderate | 37 | 48.0 |
| Poor | 32 | 41.6 |
| Unknown | 1 | 1.3 |
| *Infiltration pattern (liver metastases)* | | |
| Pushing | 6 | 7.8 |
| Replacing | 47 | 61.0 |
| Desmoplastic | 6 | 7.8 |
| Undetermined | 18 | 23.4 |

**Table 3.** Immunohistochemical profiling of liver metastases

| **IHC markers** | **Extent of staining** | | **Intensity of staining** | | | |
|---|---|---|---|---|---|---|
| | ≥ 50% | < 50% | negative | weak | intermed. | strong |
| *PB vs. INT* | | | | | | |
| CK7 | 72 (93.5%) | 5 (6.5%) | 0 (0%) | 0 (0%) | 5 (6.5%) | 72 (93.5%) |
| CK20 | 5 (6.5%) | 72 (93.5%) | 58 (75.3%) | 0 (0%) | 0 (0%) | 19 (24.7%) |
| MUC1 | 52 (67.5%) | 25 (32.5%) | 6 (7.8%) | 1 (1.3%) | 4 (5.2%) | 66 (85.7%) |
| MUC2 | 3 (3.9%) | 74 (96.1%) | 69 (88.3%) | 1 (1.3%) | 1 (1.3%) | 7 (9.1%) |
| MUC5AC | 52 (67.5% | 25 (32.5%) | 5 (6.5%) | 0 (0%) | 5 (6.5%) | 67 (87.0%) |
| MUC6 | 3 (3.9%) | 75 (96.1%) | 66 (85.7%) | 0 (0%) | 3 (3.9%) | 8 (10.4%) |
| CDX2 | 14 (18.2%) | 63 (81.8%) | 40 (51.9%) | 1 (1.3%) | 14 (18.2%) | 22 (28.6%) |
| *EMT* | | | | | | |
| Vimentin | 10 (13.0%) | 67 (87.0%) | 26 (33.8%) | 1 (1.3%) | 3 (3.9%) | 47 (61.0%) |
| EMA | 77 (100%) | 0 (0%) | 0 (0%) | 1 (1.3%) | 1 (1.3%) | 75 (97.4%) |
| *Gene mutations* | | | | | | |
| TP53 | 44 (57.1%) | 33 (42.9%) | 25 (32.5%) | 1 (1.3%) | 6 (7.8%) | 45 (58.4%) |
| SMAD4 | 35 (45.5%) | 42 (54.5%) | n. a. | n. a. | n. a. | n. a. |
| *Other markers* | | | | | | |
| PDX1 | 33 (53.2%) | 29 (46.8%) | 15 (24.2%) | 6 (9.7%) | 14 (22.6%) | 27 (43.5%) |
| Mesothelin | 57 (74.0%) | 20 (26.0%) | 13 (16.9%) | 46 (59.7%) | 16 (20.8%) | 2 (2.6%) |
| EZH2 | 43 (55.8%) | 34 (44.2%) | 8 (10.4%) | 5 (6.5%) | 22 (28.6%) | 42 (54.5%) |
| TTF1 | 1 (1.3%) | 76 (98.7%) | 76 (98.7%) | 0 (0%) | 0 (0%) | 1 (1.3%) |

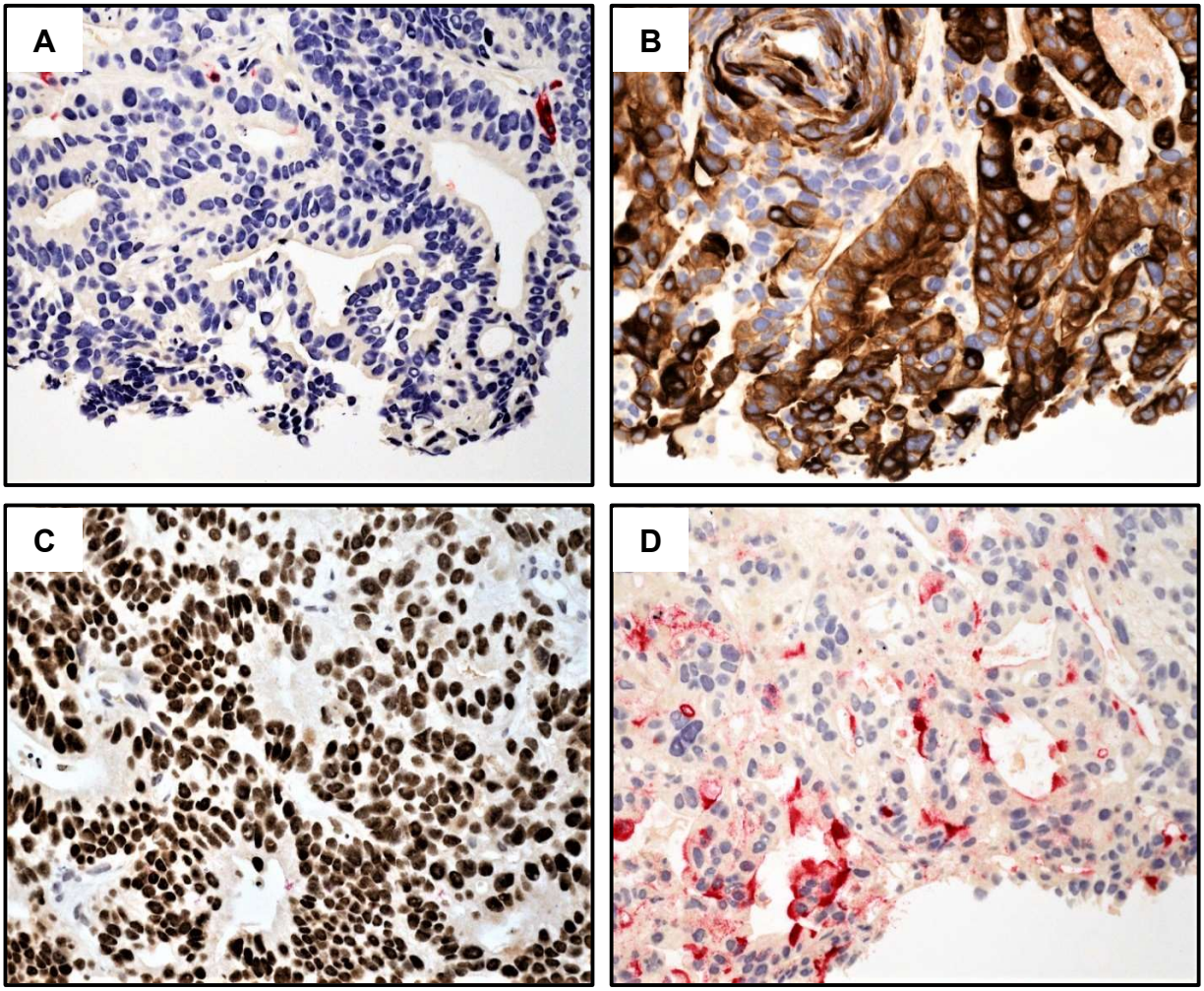


**Figure 1.** Depicted are four different IHC stainings (A-D, magnification 200X) of representative tumor samples from one LM with INT-type histological differentiation. Whereas expression of CK7 (A) was negative and MUC1 (D) less than 50%, immunoreactivity of CK20 (B) and nuclear reactivity of CDX2 (C) were pronounced.

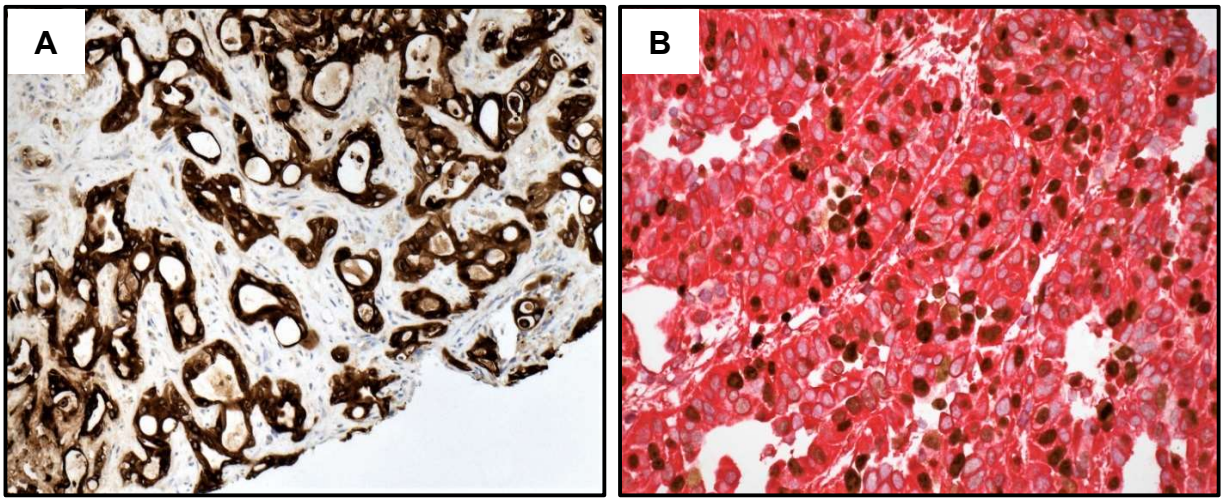


**Figure 2.** Depicted are representative tumor samples from LM with positive expression of EMA (A, magnification 100X) as well strong reactivity for vimentin (B, magnification 200X).

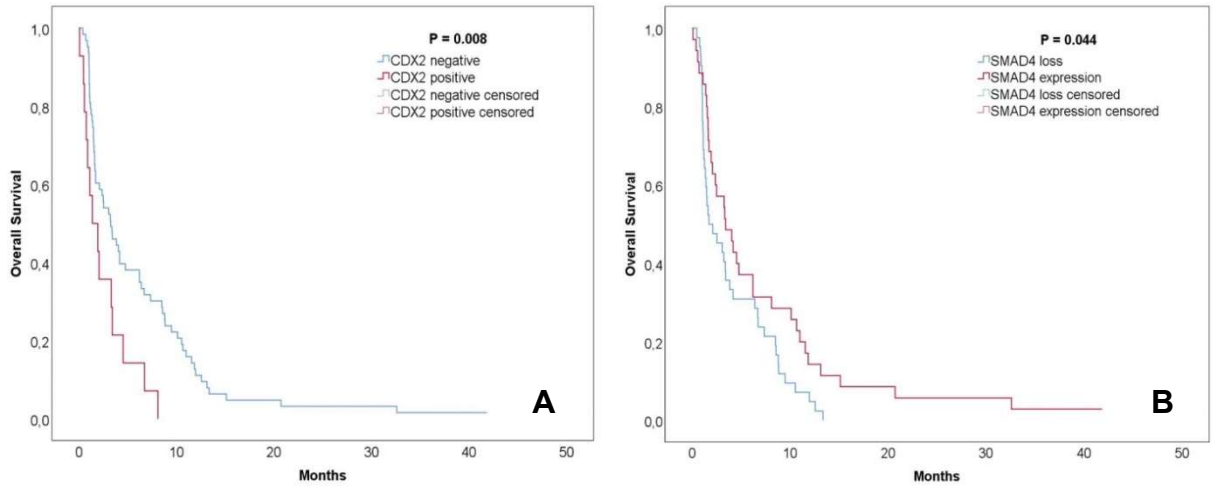


**Figure 3.** Median OS of patients with pronounced CDX2 expression (1.4 months, 95%-CI 0.1 – 2.9 months) vs. negative CDX2 staining (3.2 months, 95%-CI 1.8 – 4.7 months); Figure 3 A. Median OS of patients with loss of SMAD4 (1.7 months, 95%-CI 0.1 – 3.4 months) vs. SMAD4 expression (3.4 months, 95%-CI 1.5 – 5.4 months); Figure 3 B.

| IHC markers | Gained | Lost | Unchanged |
|---|---|---|---|
| *CDX2* | 50% | 8% | 42% |
| *SMAD4* | 8% | 33% | 58% |
| *Mesothelin* | 17% | 25% | 58% |
| *MUC1* | 17% | 25% | 58% |
| *PDX1* | 8% | 25% | 67% |
| *MUC5AC* | 25% | 0% | 75% |
| *TP53* | 8% | 8% | 83% |
| *EZH2* | 8% | 8% | 83% |
| *MUC6* | 0% | 8% | 92% |
| *TTF1* | 0% | 8% | 92% |
| *CK7* | 0% | 0% | 100% |
| *CK20* | 0% | 0% | 100% |
| *MUC2* | 0% | 0% | 100% |
| *Vimentin (FSP-1)* | 0% | 0% | 100% |
| *EMA* | 0% | 0% | 100% |

**Figure 4.** Overview of shifts in IHC marker staining between LM and corresponding primary PDAC. Gained expression was defined as IHC staining of 50-100% of tumor cells in LM and 0-49% in the primary tumor. Lost expression was defined as IHC staining of 50-100% of tumor cells in the primary tumor and 0-49% in LM. The expression of IHC markers CDX2 and SMAD4 changed in 58% and 42% of patients, respectively.