Ductal adenocarcinoma of the pancreas usually retained SMAD4 and p53 protein status as well as expression of epithelial-to-mesenchymal transition markers and cell cycle regulators at the stage of liver metastasis

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There are limited data on the biology of metastatic pancreatic ductal adenocarcinoma (PDAC). The aim of the present study was to compare the expression of immunohistochemical markers that may be involved in the development of metastatic disease in primary PDAC and in synchronous liver metastatic tissues. Thirty-two stains (corresponding to proteins encoded by 31 genes: *SMAD4*, *TP53*, *ACTA2*, *CDH1*, *CDKN1A*, *CLDN1*, *CLDN4*, *CLDN7*, *CTNNB1*, *EGFR*, *ERBB2*, *FN1*, *KRT19*, *MAPK1/MAPK3*, *MAPK14*, *MKI67*, *MMP2*, *MMP9*, *MUC1* (3 antibodies), *MUC5AC*, *MUC6*, *MTOR*, *MYC*, *NES*, *PTGS2*, *RPS6*, *RPS6KB1*, *TGFB1*, *TGFBR1*, *VIM*) were evaluated using tissue microarray of 26 pairs of primary PDACs and their liver metastases. There were no significant differences in expression levels of examined proteins between primary and secondary lesions. In particular, metastatic PDAC retained the primary tumour's SMAD4 protein status in all and p53 protein status in all but one case. This surprising homogeneity also involved expression levels of markers of epithelial-to-mesenchymal transition as well as cell cycle regulators studied. In conclusion, the biological profiles of primary PDACs and their liver metastases seemed to be similar. Molecular alterations of PDAC related to a set of immunohistochemical markers examined in the present study were already present at the stage of localized disease.

Key words: pancreatic neoplasms, ductal adenocarcinoma, metastasis, immunohistochemistry.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive human cancers. Most patients with PDAC are diagnosed at the stage of locally advanced and/or metastatic disease, and therefore they are not candidates for tumour resection. Although relative five-year survival in patients with PDAC increased from 1975 to 2008, it still does not exceed 10% [1]. Median overall survival of patients with metastatic PDAC has increased from 2 to 3 months during the past 20 years [2].

Significant effort has recently been made to examine PDAC at genomic, transcriptomic, and proteomic levels, aiming to identify screening, diagnostic, prognostic, and predictive biomarkers for PDAC [3-10]. Despite this, many questions concerning the molecular pathology of PDAC still remain unanswered [4, 5, 11-13].

One important factor limiting the progress in understanding the biology of PDAC is lack of availability of neoplastic tissues for research [13-16]. In the majority of studies on PDAC, neoplastic tissues were obtained from patients with non-advanced, i.e. potentially resectable

disease. That is why there are a lot of data on pancreatic carcinogenesis at the stage of localized disease but less is known about the biology of metastatic PDAC. Metastases of PDAC are usually examined using xenografted tissues, engineered mouse models or in rapid autopsy donation programs [9, 10, 17-19].

The transforming growth factor-β (TGF-β) signaling pathway is an essential component of human carcinogenesis, as it is involved in the control of cell growth, differentiation, and apoptosis [20-22]. TGF-β may serve as tumour suppressor, but in the advanced stage of cancer it may paradoxically hasten progression of the disease, for example by induction of epithelial-to-mesenchymal transition (EMT) [23-25]. The tumour suppressor gene *SMAD4/DPC4/MADH4* is a core component of the TGF-β signaling pathway [17, 21, 23, 26, 27]. According to recent studies, *SMAD4* plays a pivotal role in the development of metastatic PDAC [17, 19, 20, 28]. Alterations of *SMAD4* may potentially serve as a risk factor and a marker of distant tumour dissemination vs. a locally destructive growth [17, 19, 28]. Additionally, TGF-β may still operate via non-canonical, *SMAD4*-independent mechanisms, in particular through EGFR/Ras/Raf/ERK/MAPK, PTEN/PI3K/AKT/mTOR, or MEKK1/MKK4/p38 MAPK pathways [20, 23-27, 29].

TP53 is another gene involved in the pathogenesis of PDAC [3, 17-19]. Mutations of *TP53* are among the 3 most frequent genetic alterations in PDAC (following mutations in *KRAS* and *CDKN2A*) [3, 19, 30]. Together with *SMAD4, TP53* may be involved in determination of the PDAC progression pattern [17, 19], and it may serve as a marker of high metastatic potential of the tumour [19].

The aim of the present study was to compare primary PDAC and their synchronous liver metastases in the context of expression levels of immunohistochemical (IHC) markers which may be involved in the development of metastatic disease. In particular, proteins coded by *SMAD4* and *TP53* were studied. Some markers of EMT and cell cycle regulators were also examined. In contrast to the majority of previous reports on metastatic PDAC, material used here was obtained from patients submitted to surgical treatment rather than from autopsy.

Material and methods

Literature search and TGF-β, p53 and 'Pancreatic cancer' signaling pathways

The PubMed database was searched (last search in December 2013) for proteins which may be potentially useful as biomarkers of metastatic PDAC. The search was focused on: 1) proteins examined simultaneously in both primary and distant metastatic human PDAC tissues using IHC, 2) SMAD4 and p53 protein expression patterns in human PDAC. Among the key words used in the search were: 'pancreas', 'pancreat-

ic', 'cancer', 'carcinoma', 'adenocarcinoma', immuno-histochemistry', 'immunohistochemical', 'immuno-histochemically', 'primary', 'metastatic', 'metastasis', 'SMAD4', 'DPC4', 'MADH4', 'p53', and 'TP53'. Further papers were identified by cross-referencing. No chronological criteria were applied.

Data on 'TGF-β signaling pathway', 'p53 signaling pathway' and 'Pancreatic cancer' signaling pathway were retrieved from the KEGG database [31]. Proteins interacting with SMAD4 and p53 proteins were identified using STRING 9.05 [32].

Study cases

The institutional pathology files were searched for patients who underwent surgical (open) incisional biopsy of both primary PDAC (primary tumour mass) and synchronous hepatic metastasis between 2006 and 2012. Biopsy material was fixed in 10% buffered formalin and then routinely embedded in paraffin. The primary diagnosis of PDAC was established histopathologically using a reference source [33]. Instead of the standard WHO grading system [33], the percentage of 'non-gland forming' tumour tissue [34] was assessed in each sample of primary and secondary tumour. This approach seemed to be suitable for examination of small volumes of tumoral tissue present in incisional biopsies, as it does not include assessment of mitotic activity, in contrast to the WHO system [33].

Tissue microarray

Tissue microarray (TMA) was prepared using a manual instrument (MTA-1, Beecher Instruments, Sun Prairie, USA). A single core (diameter 1.5 mm) was taken from each paraffin block aiming to sample the tumour and simultaneously not to diminish the diagnostic value of the tissue embedded in the paraffin block. The cores were incorporated in a new recipient TMA block.

Immunohistochemistry

Thirty-two IHC stains were available for the present study (gene symbols in parentheses): SMAD4 (*SMAD4*), p53 (*TP53*), cytokeratin 19 – CK19 (*KRT19*), epidermal growth factor receptor – EGFR (*EGFR*), phospho-mTOR (*MTOR*), HER2/neu (*ERBB2*), MUC1 (*MUC1*), MUC1 core (*MUC1*), epithelial membrane antigen – EMA (*MUC1*), MUC5AC (*MUC5AC*), MUC6 (*MUC6*), vimentin (VIM), smooth muscle actin – SMA (*ACTA2*), fibronectin (*FN1*), metalloproteinase 2 – MMP2 (*MMP2*), metalloproteinase 9 – MMP9 (*MMP9*), E-cadherin (*CDH1*), β-catenin (*CTNNB1*), claudin 1 (*CLDN1*), claudin 4 (*CLDN4*), claudin 7 (*CLDN7*), nestin (*NES*), p21WAF1/CIP1 (*CDKN1A*), Ki-67 (*MKI67*), c-myc (*MYC*), cyclooxigenase 2 – COX2 (*PTGS2*), phospho-p70 S6 kinase (*RPS6KB1*),

phospho-p44/p42 MAPK (*MAPK1/MAPK3*), phospho-S6 ribosomal protein (*RPS6*), phospho-p38 MAPK (*MAPK14*), TGF-β1 (*TGFB1*), TGFβ receptor type 1 – TGFβ-R1 (*TGFBR1*). Details on antibodies used, corresponding gene symbols and their functional categories [35, 36], clonality, dilutions, antigen retrieval procedures, detections systems, and cellular stain distribution patterns are presented in the Supplementary Table (available online). Importantly, 32 IHC stains corresponded to 31 proteins, as different glycoforms of MUC1 protein were examined with 3 antibodies (MUC1, MUC1 (core), and EMA), and a single antibody [phospho-p44/p42 MAPK (Erk1/2) (Thr202/Tyr204)] detected 2 related proteins (gene symbols: *MAPK1/MAPK3*).

Four-micrometer thick sections were cut from a TMA block and mounted on Superfrost Plus slides (Menzel-Glaser, Braunschweig, Germany). Slides were deparaffinized and rehydrated in a routine manner, submitted to appropriate antigen retrieval and peroxidase block, and incubated with primary antibodies. Diaminobenzidine was used for visualization. Slides were counterstained with hematoxylin. IHC stains were performed in automated machines (Dako Autostainer or Ventana Benchmark), with the exception of c-myc staining, which was performed manually. In negative controls, primary antibodies were omitted.

Interpretation of stains

The TMA core was considered adequate if at least 20 unequivocal neoplastic cells were recognized. The PDAC case was considered suitable for examination if both the primary tumour core and the metastasis core were adequate – otherwise the entire case (primary tumour and metastasis) was excluded.

For each core, stain intensity score (0 – none, 1+ weak, 2+ moderate, 3+ strong) and stain extent (in percentages) were recorded. Histoscores were obtained by multiplying the particular intensity scores and values of stain extent and adding the products obtained for each stain intensity score (histoscore range: 0-300) [37]. Results of assessment of phospho-p70 S6 kinase stain were recorded in 2 ways: taking into account the nuclear stain only, and additionally as a separate variable irrespective of cellular stain localization pattern (nuclear and/or cytoplasmic).

Three stains (SMAD4, p53, Ki67) were scored differently: the SMAD4 stain was considered 'abnormal' in case of complete loss of the stain in tumoral cells. Non-neoplastic tissues (stromal cells, vessels, pancreatic parenchyma) showed nuclear and/or cytoplasmic staining and served as an internal control [38]. The p53 stain was considered 'abnormal' if there was a complete lack of nuclear stain in tumoral cells (which is usually compatible with intragenic deletion, or frameshift mutation, or nonsense mutations of *TP53* [18, 39, 40]) or if more than 30% of tumoral

cells showed strong nuclear staining (which is usually compatible with missense mutation of *TP53* [40]). The result of the p53 stain was considered 'normal' if examined tissues showed only a scattered (less than 5% of cells), usually weak nuclear reaction. The latter pattern in non-neoplastic cells served as an internal control [40]. For assessment of the Ki67 proliferative index, the stain intensity was not taken into account – the results are shown as percentages of positive nuclei (histoscore range 0-100).

In silico analysis

Interactions between proteins studied were examined using STRING 9.05 [32]. Proteins which directly interact between SMAD4 and p53 proteins were identified with VisANT 4.0 ('Shortest Path' topology option) [41]. Enrichment of sets of proteins/genes in KEGG pathways was performed using DAVID [42]. A compendium of potential PDAC biomarkers [43] was searched for proteins identified in enrichment analysis.

Statistical analysis

Non-parametric statistical tests were applied to analyze the study data because of the relatively small number of cases and non-normal distributions of many variables. Paired histoscores of IHC stains in primary and secondary neoplastic tissues were compared using the Wilcoxon signed-rank test. For non-matched variables, the Mann-Whitney U test was used. For correlations, Spearman rank correlations coefficients were calculated. For categorical variables, Fisher's exact test or McNemar's test was used as appropriate. Heatmaps were prepared using Gene-E software [44], which was also used for unsupervised hierarchical clustering (HC) analyses based on Euclidean distances and average linkage method. The false discovery rate (FDR) was controlled using the Benjamini-Hochberg approach [45]. Statistical significance was set at an FDR-adjusted p-value less than or equal to 0.05. Statistical analyses other than HC and FDR calculations were performed using Statistica 10 software (StatSoft, Tulsa, USA).

Ethics

The Institutional Review Board granted permission to perform the present study without a detailed review necessary for prospective interventional studies.

Results

Literature search and TGF-β, p53 and 'pancreatic cancer' signaling pathways

The PubMed search revealed 44 proteins examined in matched primary and distant metastatic PDAC using IHC techniques (corresponding gene

symbols: *ACTN4, AGR2, ALDH1A1, ALDOB, ANXA10, CD82, CDH1, CLDN4, CLDN18, CTNNA1, CXCR4, ECD, EGF, EGFR, ENO3, EPHA2, HK2, IDH3A, L1CAM, LCN2, LDHA, LGALS1, LGALS3, MAP2K4, MDM2, MKI67, MUC1, MUC2, MUC4, MUC5AC, MUC5B, MUC6, MUC7, MUC16, MUC17, PKM2, PROM1, RUNX3, S100P, SFN, SLC2A1, SMAD4, TP53, VCAN*). In addition to mentioned mucin core proteins (MUC), Remmers *et al.* [46] also examined some glycans in matched primary and metastatic PDAC tissues (CA19-9, SLeC, LeX, SLeX, Tn, STn, T, T on MUC1, Tn/STn on MUC1, Tn on MUC4).

IHC was also used to examine 49 proteins in distant metastatic PDAC (but not in primary lesions) or in non-matched sets of primary tumours and metastases (gene symbols: *AKT1, AGRN, AMACR, ANXA2, ARG1, BIRC2, BIRC3, CDC25B, CDH2, CDKN1A, CDX2, CEACAM5, CLDN1, CLDN2, CLDN3, CLDN7, CPS1, EIF4EBP1, ERBB2, ESR1, FASLG, FGF2, FSCN1, GPC3, HMGA1, HOXC8, HPSE, ITGB6, JUN, KRT7, KRT17, KRT19, KRT20, MMP1, MSLN, MTOR, PIP, PGR, PPARG, PSCA, PTEN, RPS6KB1, SBDS, SLC29A1, TGFB1, TP63, TTF1, TYMP, VIM*). Additional protein markers were examined in metastatic PDAC samples using antibodies of wider reactivity: polyclonal CEA (*CEACAM1/CEACAM5/CEACAM6)*, cytokeratin 8/18 (*KRT8/KRT18*), laminin (*LAMA*), p44/p42 MAPK (*MAPK1/MAPK3*).

Among proteins examined in metastatic PDAC and identified in the literature search, 9 were included in 'Pancreatic cancer' KEGG signaling pathway (gene symbols: *EGF, EGFR, ERBB2, MAPK1, MAPK3, TGFB1, TGFBR1, TP53, SMAD4*), 5 were included in the 'p53 signaling pathway' (gene symbols: *CD82, CDKN1A, MDM2, SFN, TP53*), and 3 were included in the 'TGF-β signaling pathway' (gene symbols: *RPS6KB1, SMAD4, TGFB1*).

The STRING database showed 61 proteins interacting with SMAD4 (confidence score set at 0.950, no 'white nodes' allowed) – 5 among them were previously examined in metastatic PDAC using IHC (gene symbols: *CDKN1A, ESR1, JUN, MAPK1, TGFB1*). The same database showed 184 proteins interacting with p53 (confidence score set at 0.950, no 'white nodes' allowed) – 12 of them were previously examined in metastatic PDAC using IHC (gene symbols: *CD82, CDKN1A, EGFR, EPHA2, ERBB2, ESR1, JUN, MAPK1, MDM2, PTEN, SFN, TGFB1*).

Study cases

Material from 33 patients was accepted as appropriate for the study. In a single case, original paraffin blocks were not available for the study and therefore that case was excluded.

TMA

Thirty-two cases were included in the TMA block. Due to the desmoplastic nature of tumoral tissues some cases showed only a few neoplastic cells in a core, or core cuts were lacking a significant number of TMA sections (defined here as more than 10 slides submitted for IHC). For that reason, 6 entire cases (both primary and secondary lesions) were excluded and the final study population consisted of 26 PDAC samples.

Demographic data and histopathological diagnoses

There were 16 males and 10 females with PDAC included in the study. Median age at diagnosis was 64 years (range 31-80 years). Histopathological examinations in 25 cases showed conventional PDAC, while a single patient suffered from sarcomatoid carcinoma. The median percentage on 'non-gland forming' tumour tissue in both primary and secondary lesions was 10% and ranged from 0% to 100% (the difference was not significant, Wilcoxon signed-rank test, p = 0.877).

Antibodies

The selection of antibodies for IHC assays was based on: 1) results of the literature search of biomarkers of metastatic PDAC, 2) a survey of 3 KEGG pathways: TGF-β, p53 and 'Pancreatic cancer' signaling pathway, 3) study on SMAD4 and p53 interacting proteins, and 4) the results of other studies that identified proteins which may be important for PDAC progression, in particular to the stage of distant metastases.

As mentioned above, 32 antibodies (corresponding to 31 proteins) were used in the study. Twenty of these proteins were previously studied in metastatic PDAC, but only 9 of them were examined in matched pairs of primary and secondary lesions. Thirteen of the examined proteins were identified in 3 KEGG pathways, including two (TGFBR1 and MYC) which were not previously examined in metastatic PDAC using IHC. Four and 8 of the examined proteins were identified in SMAD4 and p53 STRING interaction networks, respectively, including two proteins (MAPK14, PTGS2) which were not examined in metastatic PDAC using IHC and were not identified in 3 KEGG pathways. The study also included IHC stains for: 1) phospho-S6 ribosomal protein (Ser240/244) (*RPS6*), an effector of the PTEN/PI3K/AKT/mTOR pathway involved in PDAC development [47, 48], 2) nestin (*NES*) – a stem cell marker involved in EMT in PDAC [49], 3) β-catenin (*CTNNB1*) – a marker of canonical Wnt signaling pathway, previously shown to interact with SMAD4 in PDAC [50], 4) smooth muscle actin (*ACTA2*) – an EMT marker [51], 5) metalloproteinase 2 (*MMP2*) and 6) metalloproteinase 9 (*MMP9*) – EMT mark-

ers of prognostic significance in PDAC [25, 52], and 7) fibronectin (*FN1*) – another EMT marker, whose expression in PDAC was previously correlated with worse survival and MAPK pathway activation [37].

SMAD4 IHC stain

SMAD4 protein status was examined using mouse monoclonal B-8 antibody. This antibody was previously validated and recognized as an excellent IHC surrogate marker of *SMAD4* status [38]. Loss of SMAD4 stain in almost all cases of PDAC correlates with homozygotous deletion or truncating mutation combined with loss of heterozygosity of *SMAD4*. Half (13/26) of the study cases showed SMAD4 protein loss in both primary and metastatic lesions (Fig. 1A). Patients with retained SMAD4 expression and those with SMAD4 loss did not differ in the context of their age at diagnosis as well as the percentage of non-gland forming tumoral tissue (data not shown). In concordance with previous reports based on autopsy data [17, 19], there was agreement of SMAD4 protein status between primary tumours and liver metastases.

Comparison of protein expression levels in primary and secondary tumours in subgroups defined based on SMAD IHC stain

Primary tumours with retained SMAD4 expression showed a significantly higher median histoscore for HER2/neu stain than primary tumours with SMAD4 loss (median values: 15 vs. 0, respectively, Mann-Whitney U test, FDR-adjusted p = 0.028). No other differences regarding protein expression levels were seen in primary or secondary lesions in subgroups defined based on SMAD4 protein status (data not shown).

p53 IHC stain

Twenty-three (88.5%) cases of primary PDAC showed 'abnormal' p53 staining (Fig. 1B). This value

was similar to the result in a previous report based on resection specimens (81.1%) [39]. Seven samples of 23 cases with an 'abnormal' p53 IHC result showed total loss of stain compatible with *TP53* truncating mutations. 'Abnormal' p53 staining was seen in 24 (92.3%) samples of liver metastases – a single case with 'normal' staining in the primary tumour showed p53 protein accumulation in a metastatic focus. High concordance of p53 stain results in primary and metastatic PDAC was in agreement with previous reports [17-19]. The small number of cases with 'normal' p53 stain result (n = 3) did not allow for comparisons of clinicopathological variables and IHC results between groups defined based on p53 protein status.

Comparison of protein expression levels between primary tumours and liver metastases

The heat map illustrating the results of IHC stains is presented in Fig. 2. As shown in the Table I, there were no significant differences in histoscores of 30 IHC stainings (SMAD4 and p53 excluded) between primary tumours and liver metastases were lacking (FDR-adjusted p values were larger than 0.05). Similar results were obtained in a separate analysis of subgroups of tumours with 'normal' and 'abnormal' SMAD4 stain results (data not shown).

Correlations between corresponding protein expression levels in primary and metastatic PDAC

Not surprisingly, there were significant correlations between expression levels of almost all proteins in pairs of primary and metastatic PDAC (Table I).

Hierarchical clustering

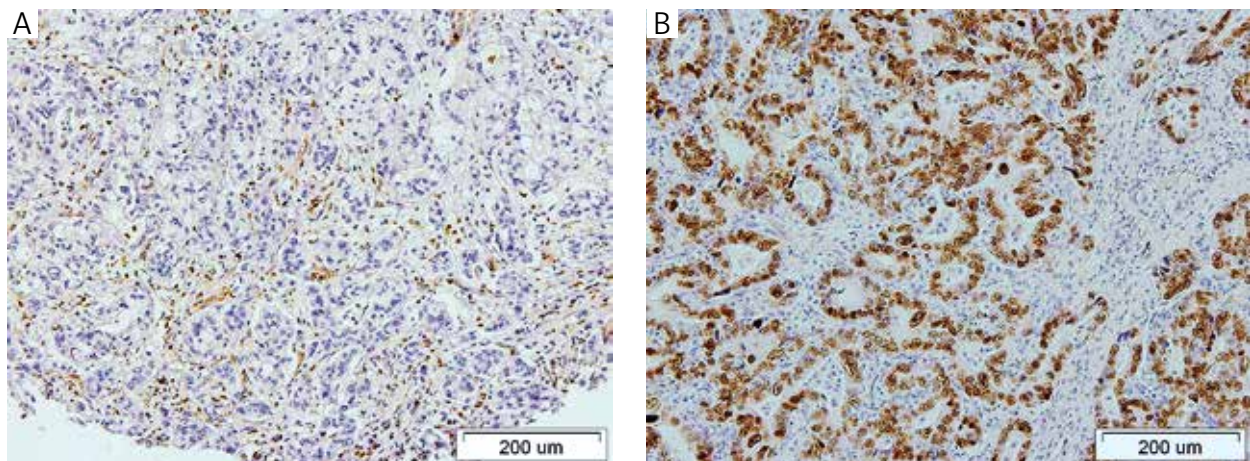Three clusters (Fig. 2) among study cases were distinguished:

Fig. 1. SMAD4 and p53 in PDAC: **A)** SMAD4 loss in PDAC; **B)** p53 overexpression in PDAC
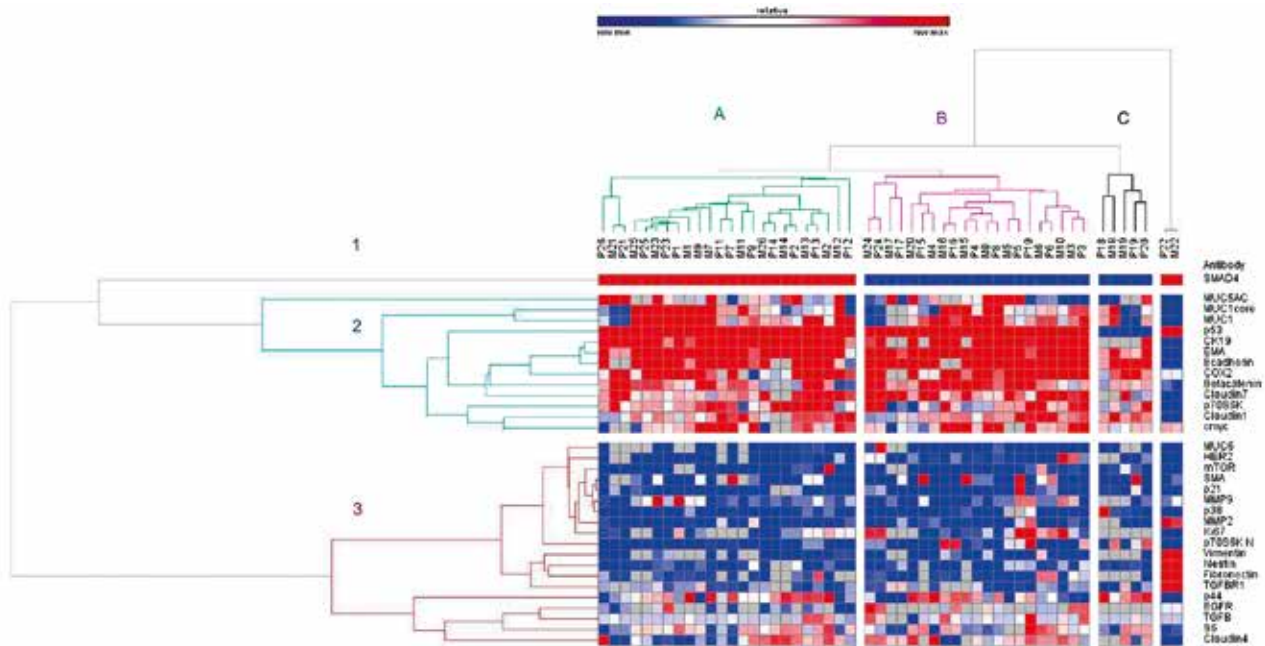
Fig. 2. Heatmap of results of immunohistochemical stains and unsupervised hierarchical clustering of protein expression patterns in studied samples of ductal adenocarcinoma of the pancreas. Study cases with no data indicated in grey. SMAD4 and p53 'abnormal' stains results indicated in red

P – primary tumour, M – liver metastasis, N – nuclear

- cases with 'abnormal' SMAD4 and p53 stains (cluster A),
- cases with 'normal' SMAD4 stain and 'abnormal' p53 stain (cluster B),
- cases with 'normal' SMAD4 and p53 stains (cluster C).

A case of sarcomatoid carcinoma (no. 22) did not cluster with the rest of the study cases. The HC approach was also applied to recognize subgroups of examined IHC biomarkers. This resulted in identification of 3 clusters:

- SMAD4 cluster (cluster 1),
- cluster enriched in many, but not all epithelial markers and p53 (cluster 2),
- cluster enriched (albeit not specifically) with mesenchymal markers (cluster 3).

Importantly, HC also showed relatively large homogeneity of protein expression patterns of primary PDAC and corresponding liver metastases. Some pairs of study cases (in particular in cluster B) clustered at the first node of the dendrogram. The results of HC did not differ much irrespective of normalization of data (log$_2$ transformation, quantile normalization) and calculation approach (Kendall's tau as an alternative to Euclidean distances metric).

In silico analysis

Interactions between proteins examined in the study and visualized using the STRING tool [32] are presented in Supplementary Fig. 1 (available online). Obviously, the set of 30 examined proteins (MUC5AC was not available for the analysis and therefore excluded) was significantly enriched in interactions (159 observed instead of 9.34 expected, p = 0). STRING revealed a further 118 proteins interacting with the set of 30 examined proteins with the confidence score of 0.999 (Supplementary Fig. 2; available online). According to DAVID, the set of 118 proteins was enriched in 32 KEGG signaling pathways other than the 'Pancreatic cancer' pathway, 'p53 signaling pathway' and 'TGF-β signaling pathway' (FDR-adjusted p < 0.05). These included: 'Pathways in cancer', 'Cell cycle', 'erbB signaling pathway', 'Wnt signaling pathway', 'Adherens junctions', 'Focal adhesions', 'Ubiquitin mediated proteolysis', 'Jak-STAT signaling pathway', 'MAPK signaling pathway', and 'mTOR signaling pathway' among others. Fifty-five among these 118 proteins/genes were previously identified as potential biomarkers of PDAC based on literature data (*AKT1*, *ATM*, *BLC2*, *CAV1*, *CBLB*, *CCNA2*, *CCND1*, *CCND2*, *CCND3*, *CCNE1*, *CDH2*, *CDH5*, *CDK2*, *CDK4*, *CDKN2A*, *CHEK1*, *CTNNA1*, *CTN-ND1*, *DCN*, *DUSP1*, *EGF*, *ERBB3*, *FKBP1A*, *FOS*, *HIF1A*, *ITGA5*, *JUN*, *JUP*, *LEF1*, *MAPK8*, *MDM2*, *MDM4*, *MEF2A*, *MSH2*, *NEDD8*, *PCNA*, *PML*, *RAD51*, *RRM2B*, *SKI*, *SMAD2*, *SMAD3*, *SMAD7*, *SP1*, *SRC*, *STAT1*, *STAT3*, *SUMO1*, *TCF4*, *TGFA*, *TGFB3*, *TGFBR2*, *TIMP2*, *UBC*, *UBE2I*) [43].

VisANT revealed 49 proteins directly interacting between SMAD4 and p53. Two of them (p42 MAPK Erk2 (*MAPK1*) and p21WAF1/CIP1 (*CDKN1A*)) were examined in the present study using IHC. Accord-

Table I. Results of immunohistochemical stains in the study cases (SMAD4 and p53 stains not included)

Antibody	Number of evaluable cases (out of 26 cases in the study population)	Histoscores of primary tumour – median (IQR)	Histoscores of liver metastasis – median (IQR)	p*	p**
CK19	22	300 (300-300)	300 (300-300)	0.674	0.054
EGFR	13	80 (70-150)	80 (30-100)	0.420	0.003
Phospho-mTOR	24	0 (0-2.5)	0 (0-15)	0.420	0.003
HER2/neu	23	0 (0-20)	0 (0-20)	0.564	0.010
MUC1	25	200 (140-280)	250 (180-300)	0.420	0.003
MUC1core	25	150 (90-200)	180 (120-300)	0.420	0.003
EMA	26	300 (240-300)	300 (300-300)	0.420	0.003
MUC5AC	21	90 (20-260)	180 (80-300)	0.479	0.064
MUC6	20	0 (0-7.5)	0 (0-5)	0.420	0.008
Vimentin	19	0 (0-20)	0 (0-60)	0.460	0.003
SMA	24	0 (0-10)	0 (0-5)	0.564	0.269
Fibronectin	19	20 (10-70)	20 (10-30)	0.564	0.004
MMP2	25	5 (0-30)	5 (0-10)	0.420	0.064
MMP9	25	10 (5-15)	10 (0-15)	0.687	0.114
E-cadherin	23	300 (300-300)	300 (300-300)	0.564	0.003
β-catenin	25	210 (200-280)	250 (200-280)	0.866	0.003
Claudin 1	25	200 (150-250)	200 (150-225)	0.629	0.003
Claudin 4	24	155 (50-215)	180 (65-230)	0.420	0.003
Claudin 7	24	200 (175-290)	280 (180-300)	0.420	0.010
Nestin	24	15 (5-92.5)	20 (7.5-45)	0.420	0.003
p21WAF1/CIP1	25	1 (0-6)	1 (0-4)	0.488	0.053
Ki67	19	20 (5-60)	30 (10-50)	0.564	0.004
c-myc	23	200 (190-280)	200 (150-300)	0.915	0.048
COX2	24	300 (220-300)	300 (225-300)	0.885	0.028
Phopsho- p70 S6 kinase	26	200 (150-280)	195 (90-280)	0.687	0.064
Phopsho- p70 S6 kinase (nuclear stain only)	26	0 (0-60)	0 (0-0)	0.420	0.053
Phospho-p44/p42 MAPK	26	190 (100-240)	150 (60-260)	0.643	0.006
Phospho-S6 ribosomal protein	25	180 (90-200)	150 (60-180)	0.488	0.003
Phospho-p38 MAPK	25	0 (0-5)	0 (0-10)	0.687	0.048
TGF-β1	24	100 (100-120)	100 (100-130)	0.979	0.053
TGF-βR1	22	85 (40-100)	70 (20-110)	0.979	0.018

*Wilcoxon signed-rank tests, FDR-adjusted p-values
**Spearman rank correlation coefficients, FDR-adjusted p-values
IQR – interquartile range

ing to DAVID, the set of 49 proteins was enriched in 8 KEGG pathways (FDR-adjusted p < 0.05). These were: 'TGF-β signaling pathway', 'Pathways in cancer', 'Cell cycle', 'Ubiquitin mediated proteolysis', 'Adherens junctions', 'Notch signaling pathway', 'Chronic myeloid leukemia', and 'Prostate cancer'. Eighteen of 49 proteins found using VisANT were previously identified as potential biomarkers of PDAC (*CDKN1A, CEBPB, CHEK1, COPS5, ESR1, NOTCH4, PBK, PSMD11, RPS27A, SKI, SMAD2, SMAD3, SMAD7, SP1, SUMO1, UBC, UBE2I, UHRF2*) [43].

Results of *in silico* analysis were concordant with other studies describing the significant role of some identified proteins/genes in PDAC development, e.g. cyclin D1 (*CCND1*) protein over-expression and gene amplification [53], or mutations of *ATM, CDKN2A,* and *TGFBR2* [8]. This approach also suggested a role of the cell cycle regulator p21WAF1/CIP1 (*CDKN1A*) and kinase p42 MAPK Erk2 (*MAPK1*) in PDAC development and progression, in agreement with previous experimental reports [37, 54, 55].

Discussion

Concordance of protein expression patterns in primary and secondary PDAC

The present study showed that the IHC profile of PDAC liver metastases was similar to the profile of primary tumours. All liver metastases retained primary tumours' SMAD4 profile, and all but one retained the p53 protein expression pattern during metastatic progression. Moreover, none of the other examined markers showed a significant change in expression pattern when comparing primary and secondary PDAC tissues. Therefore, the biological profile of primary tumours and liver metastases seemed to be similar.

Among IHC markers examined by other investigators in primary and metastatic PDAC and listed in the Results section, only 9 antigens (corresponding genes: *EPHA2, MAP2K4, MKI67, MUC2, MUC5B, MUC6, TYMP*, and 2 mucin antigens: T on MUC1, Tn/STn on MUC1) showed a statistically significant difference in extent and/or intensity of IHC staining in matched or non-matched samples of primary PDAC tissues and distant metastases [46, 56-59]. However, neither a decrease of MUC6 expression in secondary lesions in comparison to primary tumours [46], nor an increase of Ki67 proliferative index in metastases [58, 59] was confirmed in this study. In the present series MUC6 expression was rare in both primary and secondary lesions. The median Ki67 index value was non-significantly higher in metastases in comparison to primary tumours (30% vs. 20%, respectively). The discrepancy of results between studies might have resulted from tissue heterogeneity concerning expression of particular markers and/or differences in populations' characteristics.

SMAD4

The contribution of the TGF-β signaling pathway to pancreatic carcinogenesis was discovered early, but many aspects of that pathway are still under investigation [21, 23]. Alterations of genes involved in the TGF-β signaling pathway (e.g. *BMPR2, SMAD3, SMAD4, TGFBR2*) are present in virtually all PDAC cases, but among them *SMAD4* alterations are more prevalent [7]. Loss of SMAD4 is highly, but not fully specific for PDAC [3, 60] and it is detected in 24-85% (average 57%) of PDAC cases [26]. Both deletions and mutations of *SMAD4* contribute to SMAD4 loss [26, 38, 61]. Mechanisms which explain the role of SMAD4 loss in progression of cancer are only partially recognized [21]. SMAD4 is involved in changing the response of cells to TGF-β [27]. SMAD4 loss abolishes cell cycle arrest as well as cell migration following TGF-β administration [24]. SMAD4 loss may result in loss of TGF-β-inducible p21 expression [62]. SMAD4 protein status does not seem to be related to gender or age of the patient, tumor size, differentiation, presence of lymph node metastases or *KRAS* gene status [38, 63]. Some investigators have reported that loss of SMAD4 immunoexpression is more frequent in early-stage/resectable tumours [64]. It is not clear whether SMAD4 loss and/or *SMAD4* deletion/mutation are significant prognostic factor in PDAC [65], as results of studies on that issue are heterogeneous (reviewed in [26, 65]). Many [30, 61, 63, 64, 66, 67], but not all [68, 69] studies have shown that *SMAD4* loss/deletion/mutation is an unfavourable prognostic factor in PDAC.

Iacobuzio-Donahue *et al.* found that SMAD4 loss was more frequent in those autopsied patients with PDAC who died with widely metastatic disease (72%) than those who suffered from locally destructive disease (35%, p = 0.007) [17]. This discovery could be potentially of a great importance for PDAC patients, as it would allow a choice of the best available treatment options, targeted to minimize local or distant tumour growth. Specifically, SMAD4-deficient PDAC cell lines differ to some extent in their chemosensitivity in comparison to cell lines with retained SMAD4 [70]. SMAD4 loss is also associated with inferior recurrence-free survival in patients with PDAC who underwent resection and then treatment with erlotinib with adjuvant chemoradiation [71]. A randomized clinical trial correlating SMAD4 protein and gene status with survival in patients with unresectable PDAC without distant metastases treated with chemoradiotherapy is ongoing [16]. Results presented by Iacobuzio-Donahue *et al.* were confirmed by some [19, 28], but not all investigators [68, 69]. Bachet *et al.* [68] and Winter *et al.* [69] did not find a correlation of SMAD4 protein status and type of tumour recurrence (locoregional vs. distant).

SMAD4 status of metastatic disease usually correlates with SMAD4 status of primary tumours [17, 19], and the results of the present study were in agreement with these previous observations. Resected SMAD4-positive PDAC may rarely recur as SMAD4-negative disease [17], or SMAD4-negative liver metastasis may develop in a patient with SMAD4-positive primary lesion [19].

Detailed reviews on the role of SMAD4 and TGF-β in carcinogenesis of the pancreas and other organs are available [21, 23, 26].

TP53

The role of the tumour suppressor gene *TP53* in pancreatic carcinogenesis has been extensively studied, but its prognostic and/or predictive role is still controversial (reviewed in [3, 65]). Mutant p53 inhibits cell-cycle-related function of wild-type p53 [72]. Moreover, it induces chromosomal instability by inactivation of DNA repair pathways, inhibits p63 and p73 transcription factors, and increases tumour cell invasiveness [72]. A half to 75% of PDAC cases show *TP53* mutations [3, 19]. Mutations of *TP53* are slightly more prevalent in autopsied patients with metastatic PDAC (83%) than in autopsied patients with locally destructive tumours (71%, p = 0.037) [17]. There are differences in the proportion of patients with p53-positive IHC staining between studies (range 25-68%) [65]. In a recent study on p53 protein status in resectable PDAC cases using the same definition of 'abnormal' stain as applied in the present study, the altered IHC stain was seen in 81.1% of cases [39]. p53 immunoexpression and/or *TP53* mutations do not seem to have prognostic value in PDAC [30, 64, 65, 67], but in the mentioned recent study, an abnormal result of the p53 stain was associated with worse overall and disease-free survival at borderline statistical significance [39]. The *TP53* gene status of primary tumours is usually retained at the stage of metastases [17-19], and the series presented here confirmed these observations.

Molecular subtypes of PDAC

Hierarchical clustering showed that SMAD4 and p53 protein levels allowed identification of 3 separate clusters of PDAC. This result was in full concordance with the results of Yachida *et al*. [12, 19], which were based on autopsy samples. They showed that SMAD4 loss in PDAC is a very rare finding without coexistent *TP53* mutation, but *TP53* mutations are almost equally frequent in PDAC with altered and wild-type *SMAD4*. This fact suggested that *TP53* mutations occur earlier in the course of PDAC development than SMAD4 loss [19]. In cases of PDAC with SMAD4 loss, *TP53* usually undergoes missense mutations. Null mutations (nonsense, frameshift

mutation, of intragenic deletion) are more frequent in PDAC cases with retained SMAD4 protein (64%) than in cases with SMAD4 loss (38%, p = 0.046) [12, 19]. In the present study these observations on the relationship between p53 staining pattern and SMAD4 status could not be confirmed, possibly because of the relatively small number of examined cases (data not shown).

An attempt to perform cluster analysis of the study samples irrespective of SMAD4/p53 protein status was also undertaken. Two clusters were distinguished: a cluster of a small number of samples with low MUC1/MUC1core expression, and a large cluster of cases with higher MUC1/MUC1core expression (specific data not shown). This addressed the issue of glycosylation patterns in neoplastic tissues, which was recently examined and reviewed [46, 73-75]. MUC1 over-expression was previously recognized as a unfavorable prognostic factor in PDAC [73, 76].

EMT

EMT contributes to invasion, microenvironmental interactions and metastatic abilities of tumours [51]. EMT is possibly also involved in PDAC progression and chemoresistance [11, 77, 78]. TGF-β-induced EMT may be a SMAD4-independent process [24], but some reports have shown that SMAD4 loss may prevent EMT [27]. SMAD4-independent TGF-β-related signaling pathways may contribute to EMT in PDAC [29]. The EMT phenotype may be at least in part related to stem-cell features of PDAC cells and promotes their invasion [79]. Expression of mesenchymal markers (vimentin, fibronectin) is more pronounced in poorly differentiated PDAC [37, 80, 81] and, not surprisingly, it is an unfavorable prognostic factor [37, 81]. Loss of glandular differentiation is a surrogate marker of EMT in PDAC and it is an unfavorable prognostic factor in PDAC [80]. Importantly, not all aggressive PDAC show features of EMT, as some metastatic cancers may be extremely well differentiated. EMT does not seem to be necessary in all cases of PDAC for development of metastatic disease. Alternatively, a process opposite to EMT, i.e. mesenchymal-to-epithelial transition may cause restoration of epithelial characteristics of metastatic deposits of PDAC [11].

In the present study SMAD4-positive and SMAD4-negative tumours did not differ in terms of levels of expression of EMT markers. Similarly, liver metastases usually retained expression levels of these markers seen in primary tumours. A similar observation concerned tumour differentiation, which was usually retained at the stage of secondary lesions. This was in concordance with another study [17], according to which tumor dedifferentiation (defined as presence of 30% or more of undifferentiated carcinoma component in metastatic deposits but not in

primary tumours) is a relatively rare finding in autopsied patients with PDAC (4%).

Cell cycle

Cell cycle regulators are critical for tumour development and progression. Many of them serve or may potentially serve as diagnostic, prognostic, and predictive biomarkers for cancer patients [82]. In PDAC, deregulation of the G1/S checkpoint is of paramount importance for tumour development and it was recognized as one of core-signaling pathways altered in PDAC [7, 9]. Apart from the Ki67 index and gain of p53 alteration at the stage of metastasis in a single patient discussed above, the present study did not show clear-cut differences in expression levels of cell cycle regulators between primary and secondary PDAC lesions. This suggested that at least some cell cycle alterations are gained by cancer tissue at the stage of localized disease rather than during dissemination.

Biology of metastatic PDAC

The biology of metastases is still not fully understood [12, 13, 15, 83, 84]. Metastases are present in the majority of autopsied patients who were diagnosed with PDAC at the stage of potentially resectable or locally advanced disease [17]. Metastatic PDAC most commonly involves the liver [13, 17-19]. As shown in a study based on sequencing technology applied in autopsied patients with metastatic PDAC [9], genomic instability of cancer cells persists at the stage of disseminated disease and results in evolution of subclones which may further acquire new genetic alterations. The phylogenetic approach applied to genome data of primary tumour and corresponding metastases allowed recognition of patterns of progression of a particular cancer and reasonably explained similarities and/or differences of the genomic and proteomic profile of primary and secondary neoplastic lesions [9]. Surprisingly, in the present study there were no significant differences in protein profile examined using IHC between PDAC and synchronous liver metastases. This may be caused by the relatively low sensitivity of the IHC approach in detecting particular alterations of the molecular profile of tumours. Alternatively, IHC markers examined here concerned molecular alterations which were acquired at the stage of primary tumour. Importantly, many proteins examined here were previously identified as potential prognostic biomarkers in PDAC (reviewed in [36, 43, 65]). Similarly to the results of this study, Campagna *et al.* did not find differences in gene expression profiles between primary and metastatic PDAC using an oligonucleotide microarray platform [10].

The usefulness of molecularly targeted therapies in patients with metastatic PDAC has been extensively studied, with somewhat disappointing results [85]. For that reason personalized care is still not an available option for patients with PDAC [86]. Some of the IHC stains examined here may serve as biomarkers of signaling pathways recognized previously as therapeutic targets in PDAC (EGFR, Her2/neu, mTOR, COX2, TGF-β) [85, 87].

Study limitations

Due to the small amount of tissue available in paraffin blocks, only a single TMA core was taken from each lesion. This precluded an assessment of stain heterogeneity. However, at least SMAD4 and p53 stain results are usually homogeneous in PDAC tissues [39]. Another limitation of the study was visual stain scoring only, without the use of a computer-assisted scoring platform. However, this approach possibly did not diminish the results [88].

Conclusions

PDAC usually retained the primary tumour's SMAD4 and p53 protein status at the stage of synchronous liver metastasis. There was also surprising homogeneity of expression levels of selected epithelial-to-mesenchymal transition markers and cell cycle regulators between primary and distant secondary PDAC tissues. The biological profiles of primary PDACs and their liver metastases seemed to be similar. Molecular alterations of PDAC related to a set of immunohistochemical markers examined in the present study were already present at the stage of localized disease.

Acknowledgement

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