Clinical Cancer Research

Pancreatic Ductal Adenocarcinoma Subtyping Using the Biomarkers Hepatocyte Nuclear Factor-1A and Cytokeratin-81 Correlates with Outcome and Treatment Response

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Abstract

Purpose: Pancreatic ductal adenocarcinoma (PDAC) is associated with a dismal prognosis and poor therapeutic response to current chemotherapy regimens in unselected patient populations. Recently, it has been shown that PDAC may be stratified into functionally and therapeutically relevant molecular subgroups and that some of these subtypes can be recapitulated by IHC for KRT81 [quasi-mesenchymal (QM)/squamous/basal-like] and HNF1A (non-QM, overlap with exocrine/ADEX subtype).

Experimental Design: We validated the different outcome of the HNF1A/KRT81 PDAC subtypes in two independent cohorts of surgically treated patients and examined the treatment response to chemotherapy in a third cohort of unresectable patients. The first two cohorts included 262 and 130 patients, respectively, and the third independent cohort comprised advanced-stage PDAC patients who were treated with either FOLFIRINOX (64 patients) or gemcitabine (61 patients).

Results: In both cohorts with resected PDAC, the HNF1A-positive subtype showed the best, the KRT81-positive subtype the worst, and the double-negative subtype an intermediate survival ($P < 0.013$ and $P < 0.009$, respectively). In the chemotherapy cohort, the survival difference between the double-negative and the HNF1A-positive subtype was lost, whereas the dismal prognosis of KRT81-positive PDAC patients was retained ($P < 0.021$). Patients with a KRT81-positive subtype did not benefit from FOLFIRINOX therapy, whereas those with HNF1A-positive tumors responded better compared with gemcitabine-based treatment ($P < 0.038$).

Conclusions: IHC stratification recapitulating molecular subtypes of PDAC using HNF1A and KRT81 is associated with significantly different outcomes and responses to chemotherapy. These results may pave the way toward future pretherapeutic biomarker-based stratification of PDAC patients. *Clin Cancer Res; 24(2); 351–9. ©2017 AACR.*

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a highly aggressive neoplasm with one of the poorest survival rates of all major malignancies (1) and the third most common cancer-related cause of death in the Western world (2). For patients with resected PDAC and optimal adjuvant chemotherapy, the median overall survival (OS) is 28 months (3), but most patients are either primarily diagnosed with advanced PDAC or relapse after surgery. Thus, initial systemic palliative treatment is applied in the majority of patients. Even with modern antineoplastic regimens, however, the median OS for advanced PDAC does not exceed 12 months, and some of the most effective regimens are associated

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Translational Relevance

On the basis of recent high-impact studies of RNA transcriptome data, we have developed a three-tier subtyping system for pancreatic ductal adenocarcinoma (PDAC) based on the IHC detection of two protein markers (namely, KRT81 and HNF1A), which was of prognostic value in a primary resected cohort and of predictive value concerning drug response *in vitro* (Noll and colleagues, Nature Medicine, 2016). In this work, we have further refined our IHC-based subtyping algorithm to allow its application in a routine diagnostics setting. We have also validated the prognostic value in an independent cohort of primary resected and another independent cohort of advanced-stage PDAC patients treated by primary chemotherapy. We could also show a predictive value of our subtyping system in the primary chemotherapy setting. The proposed subtyping system could provide clinicians with relevant additional information for communication of expectable prognosis and planning of systemic therapies.

with significant clinical side effects (4, 5). Thus, prognostic and predictive molecular markers identifying those patients with the highest benefit of surgery or the different chemotherapeutic regimens would be highly welcome.

One of the reasons why almost all novel therapeutic approaches have failed to markedly improve survival in this disease is probably the fact that PDAC is not a homogeneous disease with a well-known set of sequentially acquired driver mutations as previously believed (6–8) but likely comprises molecularly diverse sets of subtypes identified by RNA expression or DNA translocation profiling that cannot be deciphered by morphology or mutational profiling (9–12). This difficulty might severely hamper the selection of the right treatment modality for individual patient both in the trial and in the clinical routine setting.

In a landmark study using transcriptional profiling of microdissected tumors, Collisson and colleagues proposed three subtypes of PDAC defined by (i) high expression levels of adhesion-associated and epithelial genes termed "classical," (ii) high expression levels of mesenchyme-associated genes termed "quasi-mesenchymal," and (iii) high expression levels of digestive enzyme genes termed "exocrine-like" (10). In 27 patients for whom clinical data were available, the "quasi-mesenchymal" subtype showed a significantly poorer survival compared with the "classical" and "exocrine-like" subtype (10). In a subsequent study by Bailey and colleagues based on unsupervised clustering of RNA sequencing (RNA-seq) data, four subtypes were distinguished (9). The first was termed "squamous" as it showed RNA expression patterns similar to the C2-squamous–like class of tumors defined in The Cancer Genome Atlas pan-cancer studies. A significant overlap between this subtype and the quasi-mesenchymal subtype proposed by Collisson and colleagues can be assumed as the authors proposed a "complete loss of endodermal identity" in this subgroup and showed that it was also associated with significantly poorer survival compared with other subtypes. The second subtype termed "pancreatic progenitor" was defined by transcriptional upregulation of genes involved in pancreatic development and associated with IPMN-derived and colloidal carcinomas. The "aberrantly differentiated endocrine exocrine (ADEX)" subtype was characterized by an upregulation of genes involved in later stages of pancreatic maturation. The "immunogenic" subtype showed an RNA expression profile similar to the pancreatic progenitor class subtype but in addition had evidence of a significant immune infiltrate. A further study by Moffitt and colleagues proposed two "tumor-specific subtypes" after virtual microdissection of 4 × 44K DNA microarray and RNA-seq data termed "classical" and "basal-like," the latter showing significant overlap with the "quasi-mesenchymal" and "squamous" subtypes and again associated with poorer survival (11).

The molecular determination of the different subtypes proposed so far is technically demanding and therefore difficult to use in clinical practice, especially in patients for whom only small biopsies are available. Our group has therefore recently sought to identify IHC markers that are able to distinguish clinically relevant subtypes. We found that IHC screening for cytokeratin 81 (KRT81) and hepatocyte nuclear factor 1A (HNF1A) expression identifies PDAC subtypes with biological and prognostic relevance in an easy-to-use, reliable, and time- and cost-effective way (13). KRT81 was originally described in hair follicle formation and growth (14) but was previously associated with human cancer such as non–small cell lung cancer or non-Hodgkin lymphomas (15–17). The transcription factor HNF1A was originally delineated as a regulator of glucose metabolism with possible importance in diabetes (18–20) but recently also described as a mediator of B-cell differentiation (21). The KRT81- and HNF1A-derived PDAC subtypes correspond to some extent to those proposed by Collisson, Bailey, and Moffitt and colleagues (13). In this context, KRT81 positivity mainly delineates tumors falling into the QM/squamous/basal-like groups, while HNF1A selects a distinct biologically different set of non-QM/squamous/basal-like tumors with a specific clinical behavior possibly due to an inherent ability of intracellular drug metabolism (13) potentially enriched in the exocrine-like/ADEX group of tumors.

In this study, we set out to test for the translational validity of these recent high-impact functional studies on PDAC, introducing novel subtyping approaches. We evaluate the prognostic and predictive value of previously introduced markers, which to some extent recapitulate these subtypes and test for the clinical impact of the novel concept of molecular PDAC subtyping.

Materials and Methods

Patient cohorts

Three retrospective cohorts were used in this study. First, the primary resected 1 (PR1) cohort, a cohort investigated previously (13) consisting of 262 individuals that received partial pancreatoduodenectomy for PDAC between 1991 and 2006 at the Charité University Hospital (Berlin, Germany). Grading and staging followed the World Health Organization (WHO) recommendations at the time of cohort generation [tumor–node–metastasis (TNM) classification of the 7th edition]. The use of this tumor cohort for biomarker analysis has been approved by the Institutional Review Board (IRB; ethics committee) of the Charité University (EA1/06/2004). Data concerning the application of adjuvant chemotherapy could be obtained for 204 patients, 146 of which having received chemotherapy.

Second, an independent primary resected 2 (PR2) patient cohort consisting of 130 primary resected PDAC patients that underwent an elective pancreatic resection at the Department of Surgery, Klinikum rechts der Isar, TU München, Germany,

between July 2007 and July 2011 with a final histopathologic diagnosis of PDAC. Grading and staging followed the WHO recommendations at the time of cohort generation (TNM classification of the 7th edition). Clinical data and follow-up were obtained from a patient database, by reviewing the medical charts and directly contacting the patients and/or their physicians. The observation period for each patient started with the surgical resection. The study was approved by the Institutional Review Board (ethics committee) of the TU Munich, Germany (documents no. 1926/2007 and 126/2016 S). Data concerning the application of adjuvant chemotherapy could be obtained for 101 patients, 76 of which having received chemotherapy.

Third, the primary chemotherapy (PC) cohort consisting of 125 patients with histologically proven diagnosis of ductal pancreatic adenocarcinoma, unresectable (metastasized or locally advanced) disease, and start of first-line treatment with either FOLFIRINOX (64 patients) between January 2010 and June 2014 or gemcitabine-based therapy (21 patients gemcitabine monotherapy, 40 patients gemcitabine + erlotinib) between January 2007 and December 2011 at the Department of Medical Oncology, National Center for Tumor Disease (NCT), Heidelberg, Germany. The data were maintained via a prospective database, the NCT clinical cancer registry. Use of patient data was approved by the ethics commission of the faculty of medicine of the University Heidelberg (Vote Nr. S-267/2013). The observation period for each patient started with initiation of first-line treatment. The follow-up period for this analysis ended on November 15, 2015. Patient material was accessed according to the regulations of the Tissue Bank of the National Center for Tumor Diseases (NCT) Heidelberg and Institute of Pathology, University Hospital Heidelberg and ethical vote by the IRB (ethics committee) of the University of Heidelberg (nr. 160277).

This study was performed in accordance with the Declaration of Helsinki. Written consent of subjects was obtained.

For an overview of the clinical characteristics of investigated patient cohorts, see Supplementary Table S1.

IHC

IHC staining was done on tissue microarrays (TMA) of primary tumors in the primary resected cohorts. In the PR1 cohort TMAs were generated as described previously (13, 22). In short, three tumor cores (diameter 1.5 mm) of representative tumor areas selected by a board-certified pathologist on H&E-stained slides were punched out of formalin-fixed paraffin-embedded tissue blocks and arranged in a newly generated paraffin block. In the PR2 cohort, a minimum of 2 and (where feasible) up to 3 tumor cores (diameter 1 mm) of tumor areas previously marked by a board-certified pathologist were included. In both cohorts, TMAs were made using a tissue microarrayer (Beecher Instruments). In addition, 40 corresponding whole-tissue slides of the PR2 cohort were investigated to prove feasibility of TMA-based molecular subtyping. In the PC cohort, whole-tissue slides of 62 surgical and 63 needle biopsies were used. A TMA was not constructed due to the small sample size of some pretherapeutic biopsies.

All IHC stainings were done by an experienced technical assistant. For two of the cohorts (PR2 and PC), IHC staining was done by hand after epitopes were unmasked by boiling slides in citrate buffered distilled water (pH 6) for 15 minutes in a pressure cooker and allowing a 30-minute cooldown period. The Dako REAL Peroxidase Detection System Kit was used according to the manufacturers' specifications, including the ready-to-use anti-rabbit/mouse secondary antibody (catalog no. K5003). Primary antibodies used were rabbit polyclonal anti–HNF-1A antibody (catalog rr. sc-8986) at a dilution of 1:100, mouse monoclonal anti-Keratin 81 antibody (catalog nr. sc-100929) at a dilution of 1:500, both from Santa Cruz Biotechnology Inc. Primary antibodies were incubated for 2 hours at room temperature.

The PR1 cohort was processed following a slightly different protocol as IHC was performed on a BenchMark XT automated stainer (Ventana) with identical primary antibodies using the ultraVIEW DAB Detection Kit (all reagents from Ventana). Briefly, the tissue sections were deparaffinized with EZ Prep at 75°C and 76°C, heat pretreated in cell conditioning 1 (CC1) for antigen retrieval at 76°C to 100°C and then incubated with both primary antibodies diluted in antibody diluent 1:200 for 32 minutes at 37°C after inactivation of the endogenous peroxidase using UV inhibitor for 4 minutes at 37°C. The slides were incubated with a secondary antibody followed by the application of HRP Universal Multimer for 8 minutes. Antibodies were detected using chromogen (for 38 minutes). Before mounting, slides were counterstained manually with hematoxylin for 10 minutes and blued in water for 10 minutes. A positive control was included in each run giving comparable results as in the previously established manual protocol.

All evaluations of IHC stained slides were done by an experienced, board-certified pathologist. A barely discernable light brown nuclear staining of HNF1A only visible at high magnifications (at least ×100) was considered "weak," a heterogeneous nuclear staining of varying shades of medium to dark brown "medium," and a homogeneous dark brown staining "strong". If tumor cells showed medium to strong nuclear staining of HNF1A, tumors were classified as "HNF1A-positive." HNF1A is moderately to strongly expressed in epithelial cells of small intestine, which was used as a positive control. To delineate "KRT81-positive" cases as a distinct subgroup, a cutoff of >30% KRT81-expressing tumor cells was introduced for KRT81 positivity. The fraction of KRT81-positive tumor cells was determined by eyeball estimation, as an exact "counting" of single tumor cells in this context was difficult due to the strong cytoplasmic staining of KRT81 interfering with the exact recognition of cellular borders and less likely to be (adequately) performed in a diagnostic routine setting. This cutoff was chosen for two reasons: First, we wanted a cutoff that is high enough to definitely avoid overinterpretation of single, often KRT81-positive, budding tumor cells (even in small samples) without sacrificing too much sensitivity in KRT81-positive tumor detection, and 30% seemed ideal in that regard as most samples of our cohorts were clearly above or below this threshold, resulting in a low number of "borderline" cases. Second, we aimed at a scoring system easily applicable in pathologic routine diagnostics also making 30% an ideal cutoff as a tumor fraction of approximately 1/3 (which is the lowest expression considered KRT81-posistive) is comparably easy to discern by eyeball estimation. CK81 is expressed in hair follicles; therefore, normal skin was used as positive control. We included control tissues in our staining runs as is standard procedure in our laboratory. If tumors were negative for both markers, they were classified as "double negative."

To assess the level of interobserver reliability, the largest cohort (PR1) was reevaluated independently by a second rater.

Statistical analysis

All statistical analysis was done using SPSS statistics software by IBM Corp. version 23.0.0.0. All differences in survival were assessed by Kaplan–Meier analysis and subsequent log-rank test. Associations of markers with each other or clinicopathologic characteristics were investigated by χ^2 tests or Fisher exact test (if one or more cells showed counts below the expected minimum count) for nominal or ordinal variables, Spearman correlation coefficient for two-scaled variables (such as patient age and survival time), and one-way ANOVA for scaled variables with nominal/ordinal variables (such as patient age and subtype). Multivariate analysis was done by Cox regression modeling. $P < 0.05$ was considered statistically significant.

Results

IHC PDAC subtyping

We have previously shown that PDAC tumors can be stratified into three subtypes using IHC for HNF1A and KRT81 (13). We have further improved the stratification algorithm and now consider tumors only HNF1A-positive if moderate or strong nuclear staining of HNF1A is visible. Weak inconsistent focal staining is considered negative. For KRT81, we noted that KRT81 positivity can be frequently observed in single tumor cells budding off tumor glands in otherwise clearly negative tumors, which from a purely morphologic viewpoint seemed to reflect an epithelial-to-mesenchymal transition of single cells at the invasive front in all subtypes of PDAC rather than a distinct phenotype of tumor. We therefore defined a cut-off value of >30% KRT81-expressing tumors cells to define KRT81 positivity.

A small portion of tumors (3/262 cases of the PR1, 18/130 cases of the PR2, and 5/125 cases of the PC cohort) were considered unclassifiable by our method as they showed coexpression of both markers. These cases were excluded from subsequent analysis. For exemplary pictures of IHC stainings and overview of subtyping algorithm, see Fig. 1. When the largest cohort was independently evaluated by a second observer, a moderate level of agreement was observed (Cohen κ 0.543).

Frequency distribution and clinicopathologic patient characteristics of PDAC subtypes

Our algorithm allowed for subtyping of 259 cases (99%) of the PR1 cohort, 112 cases (86%) of the PR2 cohort, and 120 cases (96%) of the PC cohort (Supplementary Fig. S1A–S1C; Supplementary Table S1). In the PR1 cohort, the double-negative subtype was most common with 165 cases (63%), followed by the KRT81-positive subtype with 59 cases (23 %) and the HNF1A-positive subtype with 35 cases (13%). The second cohort of primary resected PDAC patients (PR2) showed a different frequency distribution with the HNF1A-positive subtype being most prevalent (50 cases, 39%) followed by the double-negative subtype (41 cases, 32%) and the KRT81-positive subtype (21 cases, 16%). In the nonresectable (PC) cohort, the double-negative subtype was most commonly found (62 cases, 50%), followed by the HNF1A-positive subtype (47 cases, 38%) and the KRT81-positive subtype (11 cases, 9%). Comparison of TMA-derived molecular subtypes with subtyping of corresponding whole-tissue slides in 40 cases of the PR2 cohort showed a good correlation ($P = 0.038$; Supplementary Table S2).

For the PC cohort, there was no apparent association between the variables sex, age, localization of primary tumor, and site of metastasis or Eastern Cooperative Oncology Group (ECOG) performance status with biological subtype (Supplementary Table S3A). In the PR1 cohort, an association of subtype with patient sex was found ($P = 0.04$); however, this observation could

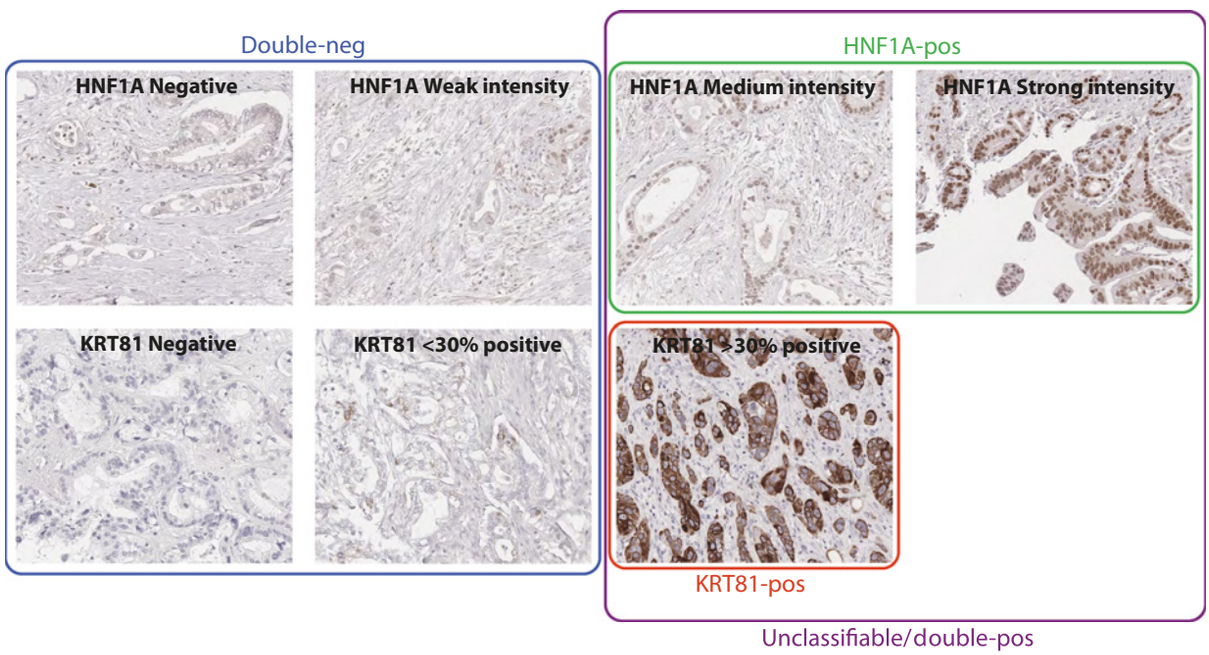

Figure 1.
Exemplary images of IHC stainings and overview of subtyping algorithm.

neither be confirmed in the PR2 cohort ($P = 0.25$) nor the PC cohort ($P = 0.51$). No statistically significant association of subtypes with age, tumor stage at resection, resection status, or nodal status was observed in the PR1 or PR2 cohort (Supplementary Table S3B). Tumor grade of both primary resected cohorts (Supplementary Fig. S2A and S2B) as well as in-depth analysis of histomorphologic growth patterns available for the PR2 cohort revealed no statistically significant association with molecular subtype ($P = 0.161$; Supplementary Fig. S2C), despite the fact that some rare growth pattern variants (specifically the squamoid and micropapillary) were consistently associated with KRT81 expression. The frequency of application of adjuvant chemotherapy did not differ significantly between primary resected cohorts or IHC-derived subtypes (Supplementary Fig. S3A and S3B), and similar survival effects of IHC-derived subgroups could be seen in Kaplan–Meier analysis, while a statistical level of significance was lost in most groups due to decreased group sizes (Supplementary Fig. S3C–S3F).

Prognostic value of PDAC subtypes

The HNF1A-positive subtype showed the best [median 1,048 days (PR1) and 745 days (PR2)], the double-negative subtype an intermediate (median 395 days in PR1 and 491 days in PR2), and the KRT81-positive subtype the worst survival in both primary resected cohorts [median 287 days (PR1) and 321 days (PR2); $P = 0.035$ (PR1) and 0.006 (PR2); Fig. 2A and B). In contrast to the two cohorts of primary resected tumors, there was no statistically significant survival difference between the HNF1A-positive and the double-negative subtype in the PC cohort of nonresectable patients receiving first-line chemotherapy (median survival of 347 compared with 377 days), while the poorer survival of patients with tumors of the KRT81-positive subtype was retained (median survival of 175 days; $P = 0.004$; Fig. 2C).

Multivariate analysis revealed the investigated PDAC subtypes as independent prognostic factors in the PR cohorts ($P = 0.013$ in PR1; 0.009 in PR2) just as higher initial tumor stage (P = 0.037 in PR1; 0.001 in PR2) and incomplete resection status in the PR1 cohort (P = 0.004). In multivariate analysis of the PC cohort, the KRT81-positive subtype was independently associated with a significant survival disadvantage ($P = 0.021$). For an overview of multivariate analysis, see Tables 1A and 1B.

Predictive value of PDAC subtypes

When subtype-dependent response to chemotherapy was analyzed, the KRT81 subtype was associated with a worse tumor control compared with the other two subtypes, with 70% of patients having progressive disease as best response (4/6 patients treated with FOLFIRINOX, 3 of 4 patients treated with gemcitabine; $P = 0.033$; Supplementary Fig. S4). Unstratified comparison of treatment groups showed no statistically significant survival advantage of FOLFIRINOX-treated compared with gemcitabine-based treated patients (P = 0.565; Fig. 3A). After stratification for molecular subtype, there was a significant difference between gemcitabine-based and FOLFIRINOX-treated patients regarding initial tumor control only in HNF1A-positive tumor patients ($P = 0.038$; Figs. 3B–D) as 53% of patients in the gemcitabine group had progressive disease at the first staging, whereas this was the case only in 19% in the FOLFIRINOX group (Table 2). When subgroup-specific survival was analyzed separately for patients treated with FOLFIRINOX and gemcitabine, no statistically significant survival difference could be seen, yet there was a trend toward a difference in survival between the HNF1A-positive subtype and double-negative subtype in the gemcitabine group compared with a very similar survival of these two subtypes in the FOLFIRINOX group (Figs. 3E and F; Supplementary Figs. S5A and S5B). There was no indication that patients with the KRT81-positive subtype benefitted from more intensive chemotherapy with FOLFIRINOX, as survival under FOLFIRINOX for these patients was even worse when compared with gemcitabine-treated patients (108 days compared with 175 days; Supplementary Fig. S5C).

Discussion

In a variety of solid tumors, molecular subtyping of morphologically indistinguishable tumors has heavily influenced oncologic treatment strategies and led to significant improvement of survival (23–27). Unfortunately, no such approach could be implemented in PDAC, so far. It has been argued, that PDAC is a genomically homogenous disease with almost universally present driver mutations in *KRAS, CDKN2A, SMAD4*, and *TP53*, and therefore, molecular subtyping might not be useful in this tumor entity (6–8).

However, there is no doubt that individual patients respond very differently to the currently used chemotherapy

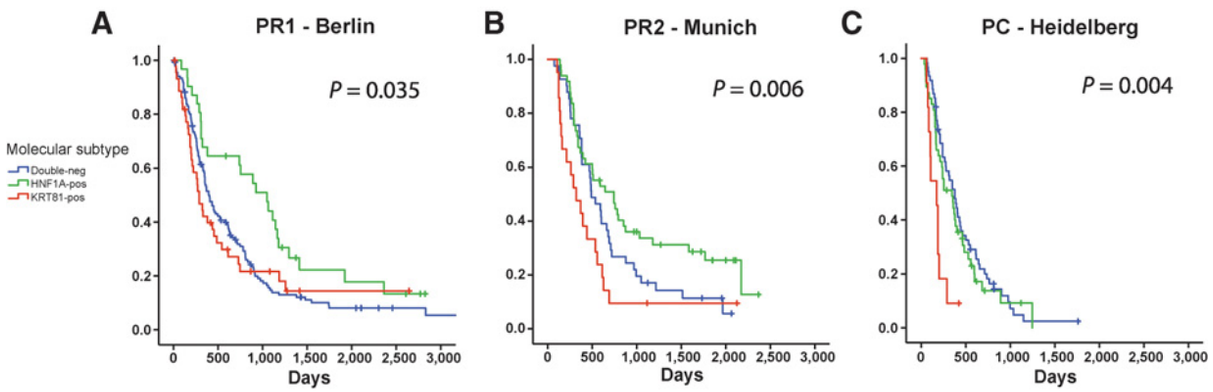

Figure 2.
Kaplan–Meier curves of survival differences between IHC-derived subtypes of PDAC. *P* values calculated by log-rank test. Ticks, censored cases.

Table 1A. Multivariate analysis of primary resected cohorts calculated by Cox regression modeling

PR1 (Berlin)		
	HR (OS; 95% CI)	***P***
Double-negative		0.013
HNF1A-positive	0.514 (0.325–0.811)	
KRT81-positive	1.036 (0.700–1.535)	
Stage I		0.037
Stage IIA	0.851 (0.406–1.785)	
Stage IIB	1.429 (0.735–2.780)	
Stage III	1.888 (0.774–4.606)	
Stage IV	2.345 (0.973–5.652)	
R1 status	1.624 (1.165–2.265)	0.004

PR2 (Munich)		
	HR (OS; 95% CI)	***P***
Double-negative		0.009
HNF1A-positive	0.646 (0.400–1.044)	
KRT81-positive	1.730 (0.937–3.195)	
Stage I		0.001
Stage IIA	0.520 (0.171–1.580)	
Stage IIB	1.458 (0.516–4.120)	
Stage III	1.709 (0.526–5.549)	
Stage IV	2.846 (0.740–10.943)	
R1 status	1.366 (0.870–2.146)	0.175

Abbreviation: CI, confidence interval.

Table 1B. Multivariate analysis of PC cohort calculated by Cox regression modeling

	HR (OS; 95% CI)	***P***
Double-negative		0.065
HNF1A-positive	1.221 (0.805–1.853)	
KRT81-positive	2.428 (1.144–5.155)	*0.021*
ECOG 0		0.003
ECOG 1	1.083 (0.696–1.687)	
ECOG 2	2.365 (1.312–4.262)	
ECOG 3	4.715 (1.496–14.858)	

protocols (28). As an example, 32% of patients treated with FOLFIRINOX show a complete or partial response, but 15% of patients have progressive disease at first staging, and these different responses are currently unpredictable (4). The molecular makeup of a given tumor is influenced by many parameters beyond mutational profiles alone. Consequently, recent work suggests that mutation-agnostic PDAC subclassifications that are mainly based on activated/suppressed pathways regulated by epigenetic modifications and posttranscriptional mechanisms might have therapeutic and prognostic impact (9–11). Although the proposed subtypes differed in some aspects, some convergent subgroups were identified as outlined in the introduction and reviewed recently (29).

As global expression-based signatures are hard to implement into a clinical routine diagnostic setting, we have developed a robust IHC classifier initially based on the RNA expression signatures for PDAC suggested by Collisson and colleagues (10) and refined by own data (13). Remarkably, IHC for HNF1A and KRT81 had enormous prognostic relevance in a cohort of 217 resected patients, with mean survival ranging from 43.5 months for HNF1A-positive patients to only 16.5 months for the KRT81-positive patients (13). However, the translational investigations in this article were only aiming to support the biological relevance of the subtypes in the human setting, but data were insufficient to argue for a real world implementation of such an algorithm.

In this study, we have further refined the IHC algorithm and thereby reduced the amount of double positive, unclassifiable patients. We have also validated the prognostic significance of the three subtypes in an independent cohort of 130 primary resected patients. Specifically, the HNF1A-positive subtype showed the best, the KRT81-positive subtype the worst, and the double negative subtype an intermediate survival in both investigated cohorts.

The cohort with the smallest investigated tumor area (PR2) yielded the highest number of unclassifiable cases, indicating a possible caveat when subtyping is performed on biopsy specimens of exceptionally small sizes. In our study, subtype frequencies varied considerably between cohorts, the reason for which was not clear. Additional studies are needed to investigate differences in subtype frequencies between cohorts, which might point to yet unknown risk factors for development of the specific PDAC subtype.

As protein expression levels have to be evaluated semiquantitatively to achieve subtyping, inherently introducing a certain level of subjectivity, and some borderline cases remain, we consider the interobserver variability acceptable despite the fact that only a moderate level of agreement was achieved (30).

Correlation of previously published data of in-depth histomorphologic analysis of the PR2 cohort (31) revealed no statistically significant association with IHC-derived molecular subtypes, indicating that our markers provide an additional layer of information that cannot be derived from thorough histopathologic examination.

We also examined the prognostic significance of the IHC subtypes in a cohort of patients undergoing palliative chemotherapy. In contrast to the resected patients that are homogenously left with none/only minimal residual disease after resection, this cohort represents a real-world mixture of patients with primary metastatic disease and unresectable locally advanced cancer treated with different types of chemotherapy. Still, even in this heterogeneous cohort, the KRT81-positive patients had a dismal prognosis when compared with the double-negative and HNF1A-positive patients.

In addition to the prognostic relevance of the IHC subtypes, it is even more clinically relevant to determine whether the subtypes can also be predictive for response to different chemotherapies. In patients treated with palliative chemotherapy, histology is often based on small core biopsies of liver metastases or even endosonography-guided fine-needle aspiration of the primary tumor, making simple subtyping based on IHC even more important than in resected patients. In our previous work (13), we have found that tumor cell lines of the HNF1A-positive subtype can be resistant to certain tyrosine kinase inhibitors and paclitaxel due to xenobiotic biotransformation by CYP3A5, but no data regarding the drugs used in our PC cohort (gemcitabine, irinotecan, oxaliplatin, 5-fluorouracil) have been published yet.

The results of our study suggest that KRT81-positive patients might not derive a relevant advantage from intensive chemotherapy with FOLFIRINOX, while HNF1A-positive patients might especially benefit from this protocol as it was the only subtype showing a significantly better initial tumor response compared with gemcitabine-based treated patients. A corresponding survival difference between gemcitabine-based and FOLFIRINOX-treated HNF1A-positive patients was observable in Kaplan–Meier analysis but did not reach a statistically significant level, a fact that might be attributed to confounders and loss of power as

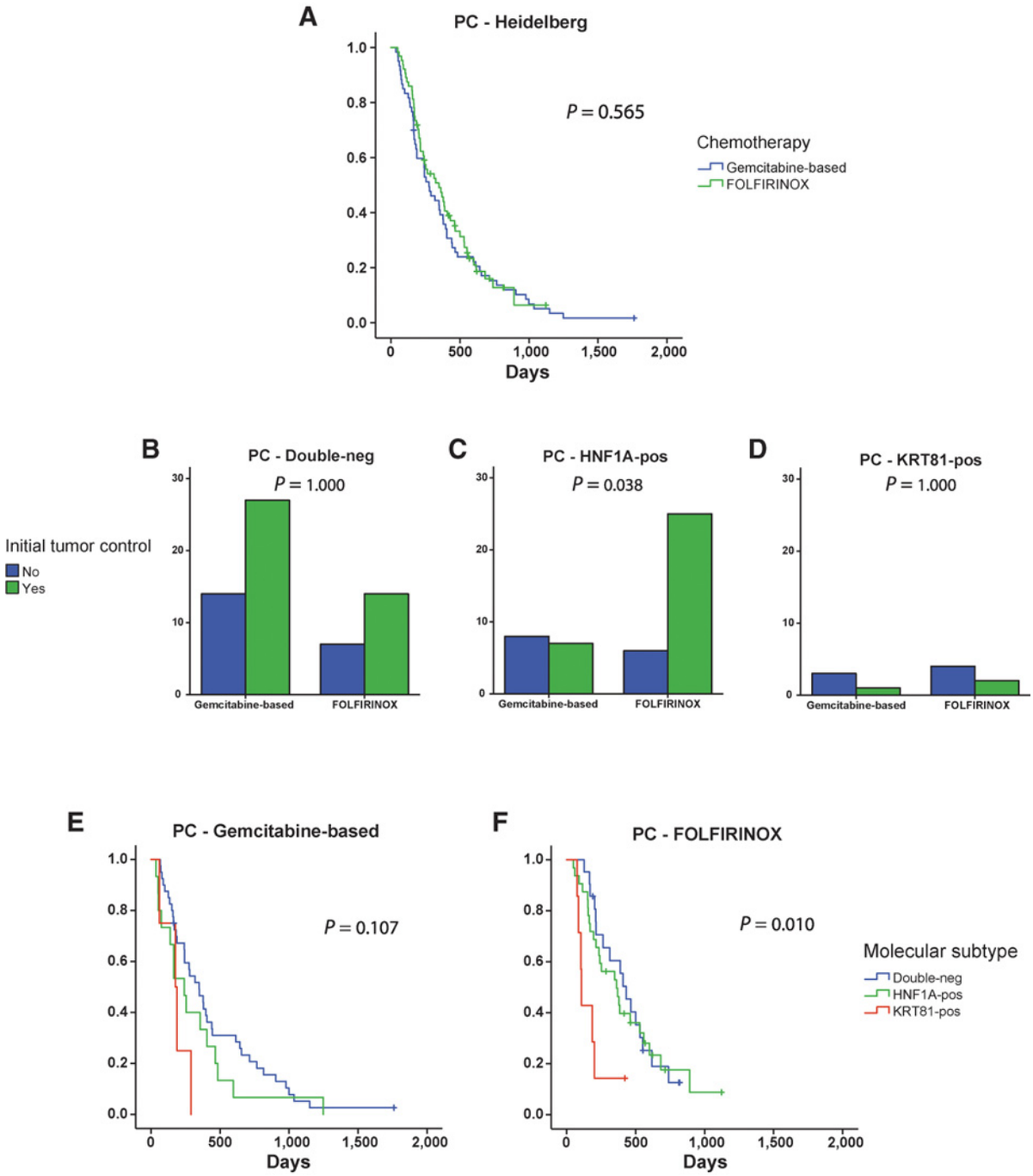

Figure 3.

Kaplan–Meier curves of survival effects of chemotherapeutic regimens administered in the PC cohort (**A**) and survival differences between PDAC subtypes after stratification for chemotherapeutic regimens (**E** and **F**). Bar graphs of differences between PDAC subtypes with regard to initial tumor control under differing chemotherapeutic regimens (**B–D**). *y*-Axes of bar graphs represent total patient count. *P* value in bar graph **C** is calculated by two-sided Fisher exact test. *P* values in Kaplan-Meier curves (**A**, **E**, and **F**) are calculated by log-rank tests. Ticks, censored cases.

effective sample sizes are decreased by censoring. The survival difference between double-negative and HNF1A-positive types consistently observed in our primary resected tumor cohorts was lost in the patients treated with palliative chemotherapy. This might imply that in general, the double-negative subtype fares better under chemotherapy than the HNF1A-positive subtype (which generally has a better prognosis). Because of the heterogeneous nature of our PC cohort, the limited number of patients,

Table 2. Table of best response to chemotherapy at first staging of PC cohort (%, *n*)

	FOLFIRINOX			Gemcitabine based			All		
	PR	SD	PD	PR	SD	PD	PR	SD	PD
HNF1A-pos.	26 (8)	55 (17)	19 (6)	7 (1)	40 (6)	53 (8)	20 (9)	50 (23)	30 (14)
Double-neg.	29 (6)	38 (8)	33 (7)	0 (0)	66 (27)	34 (14)	10 (6)	56 (35)	34 (21)
KRT81-pos.	17 (1)	17 (1)	67 (4)	0 (0)	25 (1)	75 (3)	10 (1)	20 (2)	70 (7)
Combined	26 (15)	45 (26)	29 (17)	2 (1)	57 (34)	42 (25)	14 (16)	51 (60)	36 (42)

Abbreviations: PD, progressive disease; PR, partial regression; SD, stable disease.

and the retrospective nature of the study, our analysis regarding the predictive value of the suggested subtyping method can only be hypothesis generating and should be validated in prospective clinical trials. Clearly, it will also be interesting to see whether the *in vitro* studies showing paclitaxel resistance of the HNF1A-positive subtypes will be clinically relevant in patients treated with nab-paclitaxel (13, 32).

The subtypes identified by our two-marker IHC classifier do not fully recapitulate the expression-based subtypes defined by Collison and colleagues (10, 13) and may only partially overlap with some subtyping aspects defined previously (9, 11, 12). Indeed, the respective exact convergence has to be investigated in additional studies. However, the marker set likely captures a general biological principle, defining a "KRT81-positive" subtype related to the "quasi-mesenchymal" (10)/"squamous-like" (9)/"basal-like" (11) subtype and a second distinct subtype defined by HNF1A positivity that is related to the "exocrine-like" (10)/"ADEX" (9) subtype. This notion is also supported by biological differences that we have delineated for these subtypes (13). The double negative subtype, related to the "classical" subtype (10), might represent a mixture of different biologies, and it has to be noted that the "classical" subtype has already been divided into a "pancreatic progenitor" and an "immunogenic" subtype (9).

It should also be noted that Bailey and colleagues observed an increased frequency of mutations in chromatin remodeling pathways in PDAC of the squamous-like subtype (9), indicating that PARP or ATR (Ataxia telangiectasia and Rad3 related) inhibitors might provide a rescue therapy for patients with this exceptionally aggressive PDAC variant easily identified by our IHC-based subtyping method (33–36).

In conclusion, analysis of KRT81 and HNF1A expression by IHC is a reliable way to identify biologically relevant subtypes of PDAC that could easily be integrated into common pathologic practice. Our data confirm the prognostic value of the three subtypes in resected patients. Patients with KRT81-positive subtype also have a dismal prognosis in the palliative setting and might not benefit from gemcitabine and, especially, FOLFIRINOX chemotherapy. Specifically for this population, novel treatment approaches are urgently needed. Patients with double-negative subtype seem to benefit most from currently administered chemotherapeutic approaches in the palliative setting. Patients with HNF1A-positive tumors might respond better to intensive chemotherapy according to the FOLFIRINOX protocol. PDAC subtyping by our method opens up opportunities not only for future prospective observational and interventional trials but also for retrospective reanalysis of already existing study cohorts. Hopefully, these analyses will help to individualize treatment and finally improve the prognosis of patients with pancreatic cancer.

Disclosure of Potential Conflicts of Interest

A. Stenzinger reports receiving speakers bureau honoraria from AstraZeneca, Illumina, Novartis, and Thermo Fisher Scientific and is a consultant/advisory board member for AstraZeneca, Bristol-Myers Squibb, Illumina, Novartis, and Thermo Fisher Scientific. M.R. Sprick is listed as a co-inventor on a pending patent application on the use of KRT81 and HNF1A for stratification of PDAC patients that is owned by HI-STEM gGmbH. No potential conflicts of interest were disclosed by the other authors.

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