Cellular Histone Modification Patterns Predict Prognosis and Treatment Response in Resectable Pancreatic Adenocarcinoma: Results From RTOG 9704

Ananya Manuyakorn, Rebecca Paulus, James Farrell, Nicole A. Dawson, Sheila Tze, Gardenia Cheung-Lau, Oscar Joe Hines, Howard Reber, David B. Seligson, Steve Horvath, Siavash K. Kurdastani, Chandhan Guha, and David W. Dawson

From the Department of Pathology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok Thailand; Radiation Therapy Oncology Group, Philadelphia, PA; Department of Medicine, Department of Pathology and Laboratory Medicine, and Department of Surgery, Jonsson Comprehensive Cancer Center; Departments of Human Genetics and Biostatistics in the School of Public Health; Department of Biological Chemistry; and Eli and Edythe Broad Center of Regenerative Medicine and Stem Cell Research, David Geffen School of Medicine, University of California, Los Angeles, CA; and Department of Radiation Oncology, Montefiore Medical Center, Albert Einstein College of Medicine, Bronx, NY.

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Corresponding author: David W. Dawson, MD, PhD, Dept of Pathology and Laboratory Medicine, David Geffen School of Medicine at UCLA Medical Center, 10833 Le Conte Ave, Los Angeles, CA 90095-1732; e-mail: ddawson@mednet.ucla.edu.

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ABSTRACT

Purpose

Differences in cellular levels of histone modifications have predicted clinical outcome in certain cancers. Here, we studied the prognostic and predictive value of three histone modifications in pancreatic adenocarcinoma.

Methods

Tissue microarrays (TMAs) from two pancreatic adenocarcinoma cohorts were examined, including those from a 195-patient cohort from Radiation Therapy Oncology Group trial RTOG 9704, a multicenter, phase III, randomized treatment trial comparing adjuvant gemcitabine with fluorouracil and a 140-patient cohort of patients with stage I or II cancer from University of California, Los Angeles Medical Center. Immunohistochemistry was performed for histone H3 lysine 4 dimethylation (H3K4me2), histone H3 lysine 9 dimethylation (H3K9me2), and histone H3 lysine 18 acetylation (H3K18ac). Positive tumor cell staining for each histone modification was used to classify patients into low- and high-staining groups, which were related to clinicopathologic parameters and clinical outcome measures.

Results

Low cellular levels of H3K4me2, H3K9me2, or H3K18ac were each significant and independent predictors of poor survival in univariate and multivariate models, and combined low levels of H3K4me2 and/or H3K18ac were the most significant predictor of overall survival (hazard ratio, 2.93; 95% CI, 1.78 to 4.82) in the University of California, Los Angeles cohort. In subgroup analyses, histone levels were predictive of survival specifically for those patients with node-negative cancer or for those patients receiving adjuvant fluorouracil, but not gemcitabine, in RTOG 9704.

Conclusion

Cellular levels of histone modifications define previously unrecognized subsets of patients with pancreatic adenocarcinoma with distinct epigenetic phenotypes and clinical outcomes and represent prognostic and predictive biomarkers that could inform clinical decisions, including the use of fluorouracil chemotherapy.

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INTRODUCTION

Pancreatic adenocarcinoma is a highly aggressive and lethal cancer, for which there are limited therapeutic options. Along with genetic events, tumor-associated epigenetic alterations are important determinants in the initiation and progression of pancreatic cancer^{1,2} and represent promising biomarkers and therapeutic targets. Epigenetic alterations in cancer include genome-wide and locus-specific changes in DNA methylation and post-translational histone modifications, which influence chromatin accessibility and gene activity.³⁻⁵

Locus-specific changes in histone acetylation or methylation have been linked to the altered expression of several critical genes in pancreatic cancer,⁶⁻⁹ whereas widespread changes in gene expression seen on microarrays after treatment of cell lines with histone deacetylase inhibitors suggest that histone modifications may play a much broader role in regulating gene expression in pancreatic cancer.^{9,10}

Cancer-associated genome-wide alterations in histone modifications include changes in their levels and distribution across the genome, such as at gene promoters, repetitive DNA sequences, and other

heterochromatin regions.⁵ Finally, heterogeneity in cellular levels of histone modifications across a given tumor, as demonstrated by cell-to-cell differences in immunohistochemical staining of tumor cell nuclei,¹¹ adds an additional layer of complexity to the spectrum of changes that typify the cancer epigenome.

Cellular patterns of histone modifications provide additional independent prognostic information for several tumor types, including prostate,^{12,13} kidney,¹² lung,^{12,14} gastric,¹⁵ ovarian,¹⁶ and breast cancers.¹⁷ Low cellular levels of histone H3 lysine 27 trimethylation (H3K27me3) also were recently associated with poor outcome in pancreatic cancer.¹⁶ However, cellular levels of histone modifications have not predicted response to a specific therapy. By using tissue microarrays (TMAs) from two large cohorts of patients with pancreatic adenocarcinoma, we examined the cellular levels of three histone modifications not previously studied in pancreatic cancer, including H3K4me2, histone H3 lysine 9 dimethylation (H3K9me2), and histone H3 lysine 18 acetylation (H3K18ac). These were chosen because they share a common cellular pattern that reproducibly predicts tumor aggressiveness across multiple epithelial malignancies, including prostate, lung, and kidney cancers.^{12,13} We found these modifications to be highly significant and independent prognostic factors in pancreatic cancer. In addition, we found that lower cellular levels of H3K4me2 and H3K9me2 predicted worse survival outcomes, specifically for patients receiving adjuvant fluorouracil chemotherapy. Our data indicate that cellular levels of histone modifications represent novel prognostic markers for pancreatic cancer and may be helpful in predicting response to fluorouracil.

METHODS

Patients With Pancreatic Adenocarcinoma and TMAs

The Radiation Therapy Oncology Group trial RTOG 9704 TMA consisted of 229 occurrences of pancreatic adenocarcinoma obtained from patients enrolled on RTOG 9704, a phase III, randomized, postoperative, adjuvant treatment trial comparing fluorouracil to gemcitabine before and

after chemoradiotherapy.¹⁸ In RTOG 9704, all patients received adjuvant chemotherapy (fluorouracil or gemcitabine) for durations of 1 month before and 3 months after chemoradiotherapy therapy, which included fluorouracil infusion as a radiation sensitizer. Clinicopathologic factors were collected as part of patient enrollment, as were treatment schedules and follow-up clinical information that included toxicity, overall survival, and disease-free survival. The University of California, Los Angeles stages I and II pancreatic cancer TMA consisted of 140 occurrences of AJCC stage I or II pancreatic adenocarcinoma from the University of California, Los Angeles Department of Pathology and Laboratory Medicine archives, which represented patients who underwent gross resection of tumor at University of California, Los Angeles Medical Center between 1987 and 2005. All work was performed with appropriate institutional review board approvals.

Immunohistochemistry

A standard, two-step, indirect immunohistochemical staining method was used, as previously described¹² (DAKO Envision System, Carpinteria, CA). Primary rabbit antihistone polyclonal antibodies were applied for 60 minutes at room temperature, and this included H3K9me2 (Upstate/Millipore, Billerica, MA) at 1:800, H3K18ac (Upstate) at 1:200, and H3K4me2 (Abcam, Cambridge, MA) at 1:800. Control staining was performed in identical fashion without primary antibody. Semi-quantitative assessment of the percentage of tumor cells with positive nuclear staining (range, 0% to 100%) was performed independently by two of three pathologists (D.D., N.D., or A.M.), who were blinded to all clinicopathologic and outcome variables. For each tumor, three representative cores of 0.6 mm in diameter (from RTOG 9704 TMA) or two representative cores of 1.0 mm in diameter (from University of California, Los Angeles TMA) were used. Median percent cell staining was calculated for each tumor by using all scores from both pathologists.

Statistical Analysis

To standardize and apply the same cutoffs for both TMAs, each tumor was assigned a percent rank value on the basis of median percent cell staining relative to its TMA data set by using the SAS system procedure RANK with TIES = LOW option, which assigns the smallest of the corresponding ranks for the ties data values. Each tumor then was assigned into a low- or high-level staining group on the basis of its percent rank, including H3K4me2 (< 60% v ≥ 60% rank), H3K9me2 (< 25% v ≥ 25% rank), and H3K18ac (< 35% v ≥ 35% rank). Interobserver variability was determined by unweighted κ scores on the dichotomized histone groups for each individual pathologist. Survival estimates were generated and visualized by using the

Fig 1. CONSORT diagram of patients in Radiation Therapy Oncology Group (RTOG) trial RTOG 9704 undergoing analysis of histone modifications. FU, fluorouracil; TMA, tissue microarray.

Kaplan-Meier method, and survival curves were compared by using the log-rank test. Multivariate Cox proportional hazards models were used to test statistical independence and significance of multiple predictors, and backward selection was performed by using the Akaike information criterion. Overall survival time was measured from the date of random assignment (onto RTOG 9704 TMA) or date of surgery (for University of California, Los Angeles TMA) to the date of death as a result of any cause or last follow-up. Disease-free survival time was only determined for RTOG 9704 and was measured from the date of random assignment to the date of first disease-free failure event, defined as local or regional disease relapse, distant disease, second primary, or death as a result of any cause.

RESULTS

Cellular Histone Modification Levels in Pancreatic Adenocarcinoma TMAs

Cellular levels of H3K4me2, H3K9me2, and H3K18ac were examined by immunohistochemistry in two different pancreatic adenocarcinoma TMAs. The first TMA consisted of patients enrolled on RTOG 9704, a phase III multicenter, randomized, controlled trial of 451 patients comparing gemcitabine with fluorouracil adjuvant chemotherapy in conjunction with fluorouracil chemoradiotherapy after complete gross resection of pancreatic adenocarcinoma.¹⁸ Of the original total of 229 treatment-naïve tumors in the RTOG 9704 TMA, 195

had diagnostic tumor present in the cores we examined, including 103 from patients in the fluorouracil treatment arm and 91 (or 92 for H3K9me2) from the gemcitabine treatment arm (Fig 1). As this represented 43% of the patients enrolled on RTOG 9704, we verified that missing patients did not alter baseline clinicopathologic parameters by χ^2 tests (data not shown). Providing an external validation set, the second TMA consisted of 140 patients with AJCC stage I or II pancreatic adenocarcinoma resected at a single institution (University of California, Los Angeles Medical Center). Representative staining for each of the three histone modifications is shown in Figure 2. Absence of nuclear staining indicates a bulk decrease of that histone modification in a given cell and, thus, assesses its cellular heterogeneity. Tumors ranged from 0% to 100% percent cell staining for each histone modification; H3K4me2 and H3K18ac were skewed toward overall higher percent cell staining, and H3K9me2 skewed toward overall lower percent cell staining (Fig 2).

Prior work in prostate, lung, and kidney cancers identified and validated the histone rule, a classifier that divides patients into high- and low-risk groups on the basis of the percent rank staining for each histone modification.^{12,13} We dichotomized tumors into low- or high-percent rank groups for each TMA. When varying the thresholds in our TMAs, we confirmed the optimal percent rank cutoffs for H3K4me2 (< 60 or $\geq$ 60) and H3K18ac (< 35 or $\geq$ 35) were identical

Fig 2. Cellular heterogeneity of histone modifications in pancreatic adenocarcinoma. (A) Representative immunohistochemistry for histone modifications at $\times 10$ or $\times 40$ (inset) objective from tumors of either low-grade (ie, patient 1) or high-grade (ie, patient 2) histology. The distribution of tumors according to indicated percentage of tumor cells with positive nuclear staining is shown for each histone modification in the (B) Radiation Therapy Oncology Group study 9704 or (C) University of California, Los Angeles stages I and II tissue microarray.

Table 1. Multivariate Proportional Hazards Analyses: Overall and Disease-Free Survival for RTOG 9704 Pancreatic Cancer TMA

Histone Modification Group	Overall Survival				Disease-Free Survival			
	Adjusted HR*	95% CI	P†	Adjusted for‡	Adjusted HR*	95% CI	P†	Adjusted Analysis‡
Low H3K4me2	1.48	1.05 to 2.08	.02	1, 2, 4, 6	1.34	0.97 to 1.84	.08	6
Low H3K9me2	1.44	1.04 to 2.00	.03	2, 4, 6	1.69	1.23 to 2.31	.001	3, 4
Low H3K18ac	1.27	0.91 to 1.76	.16	2, 4, 6	1.33	0.97 to 1.82	.08	3
H3K4me2 and H3K18ac§	1.52	1.06 to 2.27	.02	1, 2, 4, 6	1.39	0.99 to 1.95	.053	3, 6
H3K4me2, H3K9me2, or H3K18ac	1.64	1.12 to 2.40	.01	1, 2, 4, 6	1.68	1.17 to 2.41	.005	4, 6
H3K4me2, H3K9me2, or H3K18ac¶	0.63	0.41 to 0.96	.03	1, 2, 4, 6	0.71	0.37 to 0.87	.02	3

Abbreviations: RTOG, Radiation Therapy Oncology Group; TMA, tissue microarray; HR, hazard ratio; H3K4me2, histone H3 lysine 4 dimethylation; H3K9me2, histone H3 lysine 9 dimethylation; H3K18ac, histone H3 lysine 18 acetylation.

*HR of 1 indicates no difference between the two groups of patients for the listed histone variable, whereas an HR > 1 indicates an increased risk of death/failure for histone group listed. With the exception of histone variable, which was fixed, only variables that were kept in the model after backward selection are listed in the adjusted for column.

†P value from χ^2 test that used the Cox proportional hazards model.

‡The following covariates were used: (1) nodal involvement (no v yes), (2) tumor differentiation (not poor v poor), (3) T stage (T1-T2 v T3-T4), (4) age (continuous), (5) sex (female v male), (6) Karnofsky performance status (90-100 v 60-80), (7) treatment arm (gemcitabine v fluorouracil), and (8) surgical margins (negative/unknown v positive).

§One or both low level.

||One or more low level.

¶One or more high level.

and that for H3K9me2 (< 25 or $\geq$ 25) was close to the histone rule established (< 30 or $\geq$ 30) in other tumor types.^{12,13} Good interobserver agreement for dichotomized histone groups was seen on the basis of these cut-offs, including unweighted κ scores of 0.568 (95% CI, 0.427 to 0.755) for H3K4me2, 0.751 (95% CI, 0.624 to 0.877) for H3K9me2, and 0.616 (95% CI, 0.477 to 0.755) for H3K18ac. No significant associations were found between baseline clinicopathologic parameters and dichotomized histone groups, with the exceptions of low H3K4me2 and node-negative status (N0) approaching statistical significance in RTOG 9704 ($P = .051$, χ^2 test; Appendix Table A1, online only) and a significant association between low H3K4me2 and low pathologic T stage in the University of California, Los Angeles TMA ($P = .007$, χ^2 test; Appendix Table A2, online only). Although low H3K4me2 was associated with worse prognosis in both TMAs (Tables 1 and 2), N0 status (RTOG TMA), and low pathologic T stage (University of California, Los Angeles TMA) were inversely associated with better prognosis. These data indicate that patient histone groups are decoupled from other pathologic staging parameters known to predict clinical outcome.

Histone Modification Levels Predict Survival in Pancreatic Cancer

In the RTOG 9704 TMA, low H3K4me2 or low H3K9me2 were significant and independent predictors of worse overall and disease-free survival by multivariate proportional hazards analyses, whereas low H3K18ac trended toward worse overall and disease-free survivals (Table 1). Kaplan-Meier survival curves visualized significant associations between low levels of H3K4me2 or H3K9me2 and worse overall survival (Appendix Fig A1, online only). Combinations of two or more histone modifications could also be used to group patients where combined low level histone groups were again significant and independent predictors of worse overall and disease-free survival (Table 1).

To independently validate the results of the RTOG 9704 TMA, histone groups were separately examined in the University of California, Los Angeles stages I and II pancreatic cancer TMA. Low-level groups for H3K4me2, H3K9me2, and H3K18ac were each significant

and independent predictors of worse overall survival in the University of California, Los Angeles stages I and II TMA by multivariate Cox regression analyses (Table 2). Kaplan-Meier survival curves confirmed significantly reduced median survival times for each low histone grouping (Fig 3), including low versus high H3K4me2 (1.68 years; 95% CI, 1.02 to 2.33; v 3.66 years; 95% CI, 1.84 to 5.49; $P < .001$), low versus high H3K9me2 (1.68 years; 95% CI, 0.74 to 2.61; v 2.39 years; 95% CI, 1.76 to 3.03; $P = .039$), and low versus high H3K18ac (1.56 years; 95% CI, 1.25 to 1.86 v 2.74 years; 95% CI, 2.04 to 3.43; $P = .006$). Combined low H3K4me2 and/or low H3K18ac versus high H3K4me2 and high H3K18ac was the most highly significant and

Table 2. Multivariate Proportional Hazards Analyses: Overall Survival for UCLA Stages I and II Pancreatic Cancer TMA

Histone Modification Group	Overall Survival			
	Adjusted HR*	95% CI	P†	Adjusted Analysis‡
Low H3K4me2	2.39	1.53 to 3.73	< .001	2
Low H3K9me2	1.62	1.04 to 2.51	.032	2, 3
Low H3K18ac	1.66	1.09 to 2.52	.018	1, 2, 3, 4
H3K4me2 and H3K18ac (one or both low)	2.93	1.78 to 4.82	< .001	2, 3, 4
H3K4me2, H3K9me2 or H3K18ac (one or more low)	3.13	1.82 to 5.37	< .001	2
H3K4me2, H3K9me2 or H3K18ac (one or more high)	0.67	0.38 to 1.18	.17	1, 2, 3, 4

Abbreviations: UCLA, University of California, Los Angeles; TMA, tissue microarray; HR, hazard ratio; H3K4me2, histone H3 lysine 4 dimethylation; H3K9me2, histone H3 lysine 9 dimethylation; H3K18ac, histone H3 lysine 18 acetylation.

*HR of 1 indicates no difference between the two groups of patients for the listed histone variable, whereas an HR > 1 indicates an increased risk of death/failure for histone group listed. With the exception of histone variable, which was fixed, only variables that were kept in the model after backward selection are listed in the adjusted for column.

†P value from χ^2 test that used the Cox proportional hazards model.

‡The following covariates were used: (1) nodal involvement (no v yes), (2) tumor differentiation (not poor v poor), (3) T stage (T1-T2 v T3-T4), (4) age (continuous), (5) sex (female v male), (6) Karnofsky performance status (90-100 v 60-80), and (7) surgical margins (negative/unknown v positive).

Fig 3. Overall patient survival in the University of California, Los Angeles stages I and II pancreatic cancer tissue microarray (TMA) on the basis of indicated histone modification group. Kaplan-Meier plots visualize survival probabilities for the high-level (blue line) versus low-level (gold line) histone group for (A) H3K4me2, (B) H3K18ac, (C) H3K9me2, and (D) low H3K4me2 and/or H3K18ac versus high H3K4me2 and H3K18ac. P values are for log-rank tests.

independent predictor of survival by multivariate proportional hazards analysis (Table 2), with respective median survival times of 1.70 years (95% CI, 1.20 to 2.20) versus greater than 5 years (CI cannot be determined), as determined by Kaplan-Meier survival analysis (log-rank test $P < .001$; Fig 3). Therefore, the University of California, Los Angeles cohort validates cellular histone modifications are significant and independent prognostic markers for grossly resected pancreatic adenocarcinoma.

Histone Modifications Predict Prognosis in Node-Negative Pancreatic Cancer

Tumor stage, lymph node involvement, or histologic grade are important predictors of clinical outcome in pancreatic cancer.¹⁹ However, even within these clinicopathologic subgroups, there remains a wide range of survival outcomes. Because low cellular histone levels previously predicted aggressive tumor behavior in subsets of patients with low Gleason score prostate cancer,¹² low-stage lung cancer, or localized kidney cancer,¹³ we performed similar subgroup analysis on patients with pancreatic cancer stratified by T stage, N stage, or histo-

logic grade. The prognostic significance of low H3K4me2, low H3K18ac, or both combined was strengthened for the RTOG 9704 TMA when analysis was limited to patients with node-negative disease (Table 3). This was separately validated for patients with node-negative pancreatic cancer in the University of California, Los Angeles stages I and II TMA, and most significantly (hazard ratio, 5.00; 95% CI, 2.25 to 11.1; $P < .001$) for the patient group defined by low levels of H3K4me2 and/or H3K18ac (Table 3). By contrast, histone groups did not discriminate differences in survival for the subset of patients with node-positive pancreatic cancer in either TMA (data not shown). These findings indicate cellular histone levels may be best utilized as prognostic markers for node-negative pancreatic cancer.

Histone Modification Levels Predict Response to Adjuvant fluorouracil Chemotherapy

We next examined whether histone levels were able to predict response to fluorouracil or gemcitabine adjuvant chemotherapy in the RTOG 9704 TMA. First, we stratified patients on the basis of histone groups and performed Kaplan-Meier survival analysis to compare

Table 3. Multivariate Proportional Hazards Analyses: Overall Survival for Patients With Node-Negative Disease in either the RTOG 9704 or UCLA Stages I and II Pancreatic Cancer TMAs

Histone Modification Group	RTOG 9704 TMA				UCLA Stages I and II TMA			
	Hazard Ratio	95% CI	P*	No. of Patients	Hazard Ratio	95% CI	P*	No. of Patients
Low H3K4me2	2.70	1.27 to 5.74	.01	69	4.46	2.07 to 9.59	< .001	65
Low H3K9me2	1.35	0.75 to 2.43	.32	70	2.26	1.04 to 4.94	.040	63
Low H3K18ac	1.89	1.05 to 3.43	.04	69	2.70	1.37 to 5.31	.004	65
H3K4me2 and H3K18ac†	3.61	1.57 to 8.30	.003	68	5.00	2.25 to 11.1	< .001	65

NOTE. Separate analyses were performed on either the RTOG 9704 TMA or the UCLA stage I and II TMA for only the subset of patients with node-negative cancer. HR of 1 indicates no difference between the two groups of patients for the listed histone variable, whereas an HR > 1 indicates an increased risk of death/failure for histone group listed. In addition to histone status, the following covariates were used in the analysis of each TMA: tumor differentiation (not poor v poor), T stage (T1-T2 v T3-T4), age (continuous), sex, and surgical margins (negative/unknown v positive). Karnofsky performance status (90-100 v 60-80) and treatment arm (gemcitabine v fluorouracil) were additional covariates used in the model for RTOG 9704.

Abbreviations: RTOG, Radiation Therapy Oncology Group; UCLA, University of California, Los Angeles; H3K4me2, histone H3 lysine 4 dimethylation; H3K9me2, histone H3 lysine 9 dimethylation; H3K18ac, histone H3 lysine 18 acetylation.

*P value from χ^2 test that used the Cox proportional hazards model.

†One or both low level.

adjuvant treatments. For each of the high-level histone subgroups, there were no significant differences in overall or disease-free survival for patients receiving either gemcitabine or fluorouracil (data not shown). In contrast, for the low H3K4me2 subgroup or low H3K18ac subgroup, there was worse disease-free survival for patients receiving fluorouracil versus gemcitabine (log-rank test $P = .014$ and $P = .015$, respectively), as well as a nonsignificant trend toward worse overall survival in the low H3K4me2 subgroup for fluorouracil versus gemcitabine (Appendix Fig A2, online only). Next, we stratified patients on the basis of adjuvant therapy and performed Kaplan-Meier survival analyses to compare low- versus high-histone groups. Low levels of H3K4me2 or H3K9me2 were significantly associated with worse overall survival in the subgroup of patients receiving fluorouracil but not in the subgroup of patients receiving gemcitabine (Fig 4). Univariate hazards models also indicated that low levels of H3K4me2, H3K9me2, or H3K18ac were associated with worse overall and disease-free survival in the subgroup of patients receiving fluorouracil but not in the subgroup receiving gemcitabine (Appendix Table A3, online only). Although exploratory in nature and requiring additional validation, these results suggest that low histone levels could indicate a subset of patients with pancreatic cancer may be less likely to derive benefit from fluorouracil adjuvant therapy.

DISCUSSION

We have found that cellular patterns of histone modifications provide significant and independent prognostic information in two large cohorts of patients with pancreatic adenocarcinoma. Similar to published findings with H3K27me3,¹⁶ we found a significant association between reduced cellular levels of H3K4me2, H3K9me2, or H3K18ac and worse prognosis in pancreatic adenocarcinoma. Our work here and other studies^{12,13} highlight the potential widespread applicability of cellular histone modification levels as cancer prognostic markers that could be incorporated into a standardized immunohistochemical assay. Such an assay will need to be additionally developed with carefully calibrated internal tissue and total histone antibody controls, as well as with absolute percent cell cutoffs for each tumor type, to be used reliably in the clinical laboratory setting.

Survival varies widely for patients with pancreatic adenocarcinoma, even within subsets of patients stratified by clinicopathologic

criteria, such as tumor grade, stage, or lymph node status. In both of our TMA data sets, histone levels were prognostic for the subset of patients with node-negative pancreatic cancer. This is consistent with previous reports, in which the prognostic value of histone modifications were largely confined to less-aggressive or early-stage cancers, including lower Gleason score prostate cancer,¹³ lower-stage lung cancer,¹² and localized kidney cancer.¹² Thus, cellular histone levels appear best suited as biomarkers when used in conjunction with routine clinicopathologic staging information and, perhaps specifically, in the context of early stage pancreatic cancer.

Genome-wide profiling studies that compare normal versus cancer cells indicate a dynamic interplay between active or repressive histone marks and altered gene expression.²⁰ Although changes in a histone modification at a particular genetic locus may predictably alter gene expression, the consequences of global changes in the levels of multiple histone modifications is more difficult to forecast, given their potential opposing functional effects on transcriptional activity and our incomplete understanding of their distribution across the cancer genome. Although decreased levels of nearly all histone modifications studied thus far have been linked to worse prognosis, these same histone modifications are variably associated with transcriptional activation (eg, H3K4me2 and H3K18ac) or transcriptional silencing (eg, H3K27me3 or H3K9me2).⁵ One possible explanation is that, similar to global DNA hypomethylation in cancer,²¹ bulk reductions in histone modifications may lead to genomic instability. In support of this hypothesis, prostate cancer cell lines with large differences in H3K9me2 levels have been shown to alter the distribution H3K9me2 almost exclusively at repetitive DNA elements and not at gene promoters.¹² Likewise, global losses of H4K16ac and H4K20me3 in cancer cells have been shown to occur primarily at repetitive elements in combination with DNA hypomethylation.²² Experimentally, reduction in H3K9me2 levels by knockdown of the histone methyltransferase G9a has been shown to induce chromosomal instability, whereas it has little impact on gene expression in cancer cell lines.²³ Additional studies are needed to determine the global distribution of histone modifications and the underlying reasons for their reduced levels in subsets of more clinically aggressive pancreatic cancer; studies that establish the direct effects of altered histone modification levels on genomic instability and gene transcription are needed as well.

Fig 4. Overall survival in Radiation Therapy Oncology Group trial RTOG 9704 tissue microarray for indicated histone modification after first stratifying on treatment arms. Patients were stratified on the basis of adjuvant chemotherapy: (A, B) fluorouracil; (C, D) gemcitabine. Kaplan-Meier plots then were used to visualize survival probabilities for patients with either high (blue line) or low (gold line) levels of (A, C) H3K4me2 or (B, D) H3K9me2. P values are for log-rank tests.

The present approach for adjuvant chemotherapy in resected pancreatic cancer primarily involves the choice between gemcitabine versus fluorouracil, and the evolving consensus is that gemcitabine provides improved survival benefit.²⁴ However, RTOG 9704 concluded that adjuvant gemcitabine provided only a nonstatistically significant survival benefit compared with adjuvant fluorouracil in the setting of fluorouracil-based chemoradiotherapy.¹⁸ The ESPAC-3 multicenter, randomized, controlled phase III trial of resected pancreatic ductal adenocarcinoma also recently reported no survival difference between adjuvant fluorouracil/folinic acid versus gemcitabine.²⁵ These studies highlight the need for predictive biomarkers better able to inform treatment decisions. Toward this end, accumulating data suggest the levels of one or more mediators of drug transport or metabolism may be useful in predicting response to gemcitabine²⁶⁻²⁹ or fluorouracil³⁰ chemotherapy in cancer. Our data here indicate cellular histone modification levels are an additional class of biomarkers that could help to predict response to fluorouracil. In keeping with our observation that low H3K18ac is associated with worse response to fluorouracil, certain histone deacetylase inhibitors (which

will act to increase global levels of H3K18ac) have been shown to act in synergy with fluorouracil to increase its cytotoxic and growth inhibitory effects in cancer cell lines.^{31,32} This appears to be caused at least in part by reduction in the levels of thymidylate synthase,^{31,33} which has been associated with resistance to fluorouracil chemotherapy. By extension, cellular levels of H3K18ac may help identify patients who are more likely to benefit from the addition of an histone deacetylase inhibitor to fluorouracil chemotherapy.

We have shown that histone modification levels indicate patients in RTOG 9704 who were more or less likely to derive survival benefit from adjuvant fluorouracil relative to gemcitabine. Although differences were modest and require validation, they raise the possibility that histone modification levels could serve as predictive biomarkers for adjuvant fluorouracil in pancreatic cancer, perhaps in combination with other fluorouracil predictive markers, such as thymidylate synthase or dihydropyrimidine dehydrogenase expression.³⁰ These results also merit the investigation of histone modification levels as predictive biomarkers in other cancers (ie, colorectal or breast) treated with fluorouracil. In conclusion, cellular histone modification levels

define previously unrecognized subsets of patients with pancreatic adenocarcinoma with distinct epigenetic phenotypes and clinical outcomes, with potential clinical value for prognosis or in predicting response to fluorouracil or histone-modifying agents.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Although all authors completed the disclosure declaration, the following author(s) indicated a financial or other interest that is relevant to the subject matter under consideration in this article. Certain relationships marked with a "U" are those for which no compensation was received; those relationships marked with a "C" were compensated. For a detailed description of the disclosure categories, or for more information about ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section in Information for Contributors.

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AUTHOR CONTRIBUTIONS

Conception and design: James Farrell, David Dawson

Financial support: David Dawson

Administrative support: David Dawson

Provision of study materials or patients: Ananya Manuyakorn, James Farrell, Sheila Tze, Oscar Joe Hines, Howard Reber, David B. Seligson, David Dawson

Collection and assembly of data: Ananya Manuyakorn, Nicole A. Dawson, Sheila Tze, Gardenia Cheung-Lau, Howard Reber, David B. Seligson, David Dawson

Data analysis and interpretation: Rebecca Paulus, Nicole A. Dawson, Howard Reber, David B. Seligson, Steve Horvath, Siavash K. Kurdistani, David Dawson

Manuscript writing: James Farrell, Oscar Joe Hines, David B. Seligson, Steve Horvath, Siavash K. Kurdistani, Chandhan Guha, David Dawson

Final approval of manuscript: Ananya Manuyakorn, Rebecca Paulus, James Farrell, Nicole A. Dawson, Oscar Joe Hines, Howard Reber, David B. Seligson, Steve Horvath, Siavash K. Kurdistani, Chandhan Guha, David Dawson