

HMGA1 correlates with advanced tumor grade and decreased survival in pancreatic ductal adenocarcinoma

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Although pancreatic ductal adenocarcinoma is a common and almost uniformly fatal cancer, little is known about the molecular events that lead to tumor progression. The high-mobility group A1 (HMGA1) protein is an architectural transcription factor that has been implicated in the pathogenesis and progression of diverse human cancers, including pancreatic ductal adenocarcinoma. In this study, we investigated *HMGA1* expression in pancreatic ductal adenocarcinoma cell lines and surgically resected tumors to determine whether it could be a marker for more advanced disease. By real-time quantitative RT-PCR, we measured *HMGA1a* mRNA in cultured pancreatic ductal adenocarcinoma cell lines and found increased levels in all cancer cells compared with normal pancreatic tissue. To investigate HMGA1 in primary human tumors, we performed immunohistochemical analysis of 125 cases of pancreatic adenocarcinoma and 99 precursor lesions (PanIN 1–3). We found nuclear staining for HMGA1 in 98% of cases of pancreatic adenocarcinoma, but only 43% of cases of PanIN precursor lesions. Moreover, HMGA1 immunoreactivity correlates positively with decreased survival and advanced tumor and PanIN grade. These results suggest that HMGA1 promotes tumor progression in pancreatic ductal adenocarcinoma and could be a useful biomarker and rational therapeutic target in advanced disease.

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Although pancreatic ductal adenocarcinoma is common and highly lethal, little progress has been made in the treatment of this disease.¹ In the United States, more than 34 000 patients succumb to pancreatic ductal adenocarcinoma annually, making this malignancy the fourth leading cause of cancer death.¹ Most patients present with locally invasive or metastatic disease, for which there are no effective therapies. Even in patients with small primary lesions that are amenable to surgical resection, most will eventually die from metastatic progression despite adjuvant chemotherapy and radiation ther-

apy.^{1,2} These dismal epidemiological data suggest that pancreatic cancers metastasize before clinical detection in the majority of cases. Moreover, the abysmal outcome for most patients with pancreatic ductal adenocarcinoma underscores the urgent need to discover molecular pathways involved in tumor progression to facilitate the development of more effective therapies. Although progress has been made in elucidating early molecular lesions that lead to tumor initiation, little is known about those that underlie tumor progression and eventual metastases.

Pancreatic ductal adenocarcinomas are caused by noninvasive, precursor lesions after a series of well-characterized, stepwise molecular alterations that correspond to distinct histopathological entities.^{2–11} These precursor lesions, termed 'pancreatic intraepithelial neoplasia (PanIN),' are divided into three grades using an international classification system

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that is based on histopathological features, including the degree of architectural and cytological atypia.³⁻⁶ PanIN-1, a relatively common lesion, can be identified in approximately a third of all elderly persons, but epidemiological data suggests that it rarely becomes malignant.³⁻⁶ PanIN-1 lesions are further divided into PanIN-1A and PanIN-1B lesions on the basis of the presence of a papillary, micropapillary or a basally pseudostratified architecture identified only in PanIN-1B.⁴⁻⁶ Activation of the *K-RAS* oncogene and telomere shortening have been identified in PanIN-1 lesions, suggesting that these lesions could be early in tumor initiation.⁴⁻⁸ The intermediate stage, PanIN-2, is far more likely to be found in pancreata harboring invasive cancer.⁹ Loss of function of p16 (*INK4A*) by deletion, methylation, or mutation is a common molecular alteration present in PanIN-2 lesions.⁹ PanIN-3, formerly known as 'carcinoma-*in-situ*,' immediately precedes the development of invasive carcinoma, and is often found in association with adjacent invasive cancer.³⁻¹¹ Mutations that alter *TP53* and *MAD4/DPC4* are frequently found in PanIN-3 lesions that are present in association with sporadic pancreatic cancer.^{10,11} Studies to elucidate molecular lesions that drive the transformation of PanIN-3 to invasive carcinoma are lacking.

The *high mobility group A (HMGA1)* oncogene is widely expressed during embryonic development,¹² and is overexpressed in diverse, high-grade human cancers, including pancreatic ductal adenocarcinoma.¹³⁻²⁶ This gene encodes the HMGA1a and HMGA1b protein isoforms, which function in modulating gene expression.¹³⁻²⁵ Increasing evidence indicates an important role for these proteins in promoting tumor progression.¹³⁻²⁴ HMGA1 proteins induce a transformed phenotype with anchorage-independent cell growth in immortalized, cultured cells derived from diverse normal tissue.^{14,15,17,18,27} Furthermore, forced overexpression of *HMGA1* is associated with resistance to anoikis in pancreatic ductal adenocarcinoma cell lines.²² Interfering with its expression inhibits anchorage-independent cell growth, cellular motility, migration, and invasion in some cancer cell lines.^{14,15,17-22,26} In addition, transgenic mice overexpressing HMGA1 develop aggressive malignancies.^{19,21,25} More recently, studies with an orthotopic tumor mouse model showed that knockdown of *HMGA1* expression in human pancreatic cancer cells blocked tumor growth and metastatic progression.^{20,26} Taken together, these findings suggest that *HMGA1* could participate in transformation in pancreatic ductal adenocarcinoma and promote tumor progression.

In this study, we investigated *HMGA1* expression at the mRNA and protein levels in pancreatic ductal adenocarcinoma. We found that *HMGA1* is overexpressed in cultured pancreatic ductal adenocarcinoma cell lines compared with normal pancreatic tissue. Moreover, increasing protein levels correlate with decreased survival and more advanced tumor and PanIN grade. Although further studies are

required, our findings suggest that *HMGA1* may be an important driver of tumor progression and potential therapeutic target in this highly lethal malignancy.

Materials and methods

Cultured Human Pancreatic Ductal Adenocarcinoma Cells and Normal Human Pancreas

The pancreatic ductal adenocarcinoma cell lines have been described previously.²⁸ Briefly, MIA PaCa-2 (CRL-1420), and PL-1 (Panc 02.13, CRL-2554) were obtained from the American Type Culture Collection and were cultured as recommended. MIA PaCa-2 cells were generated from a primary undifferentiated pancreatic carcinoma in a 65-year-old Caucasian man that involved the pancreatic body and tail and extended to involve the periaortic soft tissues.²⁹ PL-1 cells were derived from a primary pancreatic ductal adenocarcinoma in a 64-year-old Caucasian woman that involved the duodenum, but did not metastasize to lymph nodes.³⁰ XPA-3 (PX154) cells are xenografted tumor cells and were kindly provided by Dr Anirban Maitra.³¹ They originate from a primary pancreatic ductal adenocarcinoma in a 77-year-old man that extended to involve the duodenum and peripancreatic soft tissues and metastasized to lymph nodes. Normal human pancreas was obtained from a 35-year-old man (Becton Dickinson, San Jose, CA, USA).

Quantitative RT-PCR of Cultured Human Pancreatic Ductal Adenocarcinoma Cells and Normal Human Pancreas

Quantitative RT-PCR was performed by Taqman chemistry according to our previous report.¹⁹ *HMGA1a* mRNA levels were normalized to human phosphoprotein as an internal control. Repeat experiments with normalization to the additional control genes, β -glucuronidase and β -actin, were also conducted.

Tissue Microarrays

Tissue samples were obtained from the pathology archives of The Johns Hopkins Medical Institutions (JHMI, Baltimore, MD, USA) as described previously.²⁸ Briefly, four 2-mm cores of pancreatic ductal adenocarcinoma were obtained from 125 patients, including two cores obtained from tumor in the pancreatic parenchyma and two cores obtained from tumor in the peripancreatic soft tissue and lymph nodes when infiltrated. Pathological diagnosis, grade and stage, as well as patient survival information were retrieved from the medical record. Staging was determined according to the American Joint Committee on Cancer guidelines.³² PanIN tissue microarray blocks were also created as described previously.³³ In short, 90 PanIN lesions were selected from 33 patients with pancreatic

ductal adenocarcinoma. Nine additional PanIN lesions from eight separate cases were identified on the pancreatic ductal adenocarcinoma tissue microarrays. PanIN lesions were separated into PanIN-1, PanIN-2 or PanIN-3 grades either at the time of microarray creation or after a review by AH.

Immunohistochemistry

Immunohistochemistry was performed as we described previously.²⁸ Each pancreatic ductal adenocarcinoma tissue microarray was immunohistochemically stained for HMGA1 protein (HMGA1, dilution 1:25 from a stock solution of 200 µg/ml, Santa Cruz Biotechnologies, no. sc-8982, Santa Cruz, CA, USA) following the manufacturer directions. Specific nuclear immunoreactivity of more than 5% of neoplastic cells was interpreted as positive HMGA1 staining and was scored using a 9-point scale on the basis of the product of staining intensity (no staining = 0, weak = 1, moderate = 2, strong = 3) and staining extent (% of positive cells; <5% = 0, 5–30% = 1, 30–60% = 2, >60% = 3). The highest score per case was used for subsequent analyses. PanIN tissue microarrays were also stained for HMGA1 and interpreted as described above. All scores for PanIN lesions were used for subsequent analysis. At the dilutions used in this study, there was no significant cross-reactivity with HMGA2 using an antibody shown to be specific for HMGA2.²⁸ The tissue microarrays were evaluated microscopically in a blinded manner by a single investigator (AH).

Statistical Analysis

Results are expressed as mean ± s.e. Student's *t*-test was used to compare *HMGA1* mRNA levels from pancreatic ductal adenocarcinoma cells and normal tissues. To determine whether HMGA1 immunoreactivity correlated with more advanced disease, clinical and pathological parameters were investigated, including overall survival, tumor grade, lymph node metastases and stage. A nonparametric rank-sum test was used to evaluate the relationship between HMGA1 immunoreactivity and survival. HMGA1 protein levels were compared with tumor grade, lymph node metastases, and stage using the χ^2 test. Furthermore, HMGA1 protein expression was compared with PanIN grade using the two-sided Fisher's exact test.

Results

HMGA1a Overexpression in Cultured Pancreatic Ductal Adenocarcinoma Cells

To determine whether *HMGA1a* is overexpressed in pancreatic ductal adenocarcinoma, we measured *HMGA1a* mRNA levels by quantitative RT-PCR in cultured pancreatic ductal adenocarcinoma cells. We observed increased levels in all cultured cells

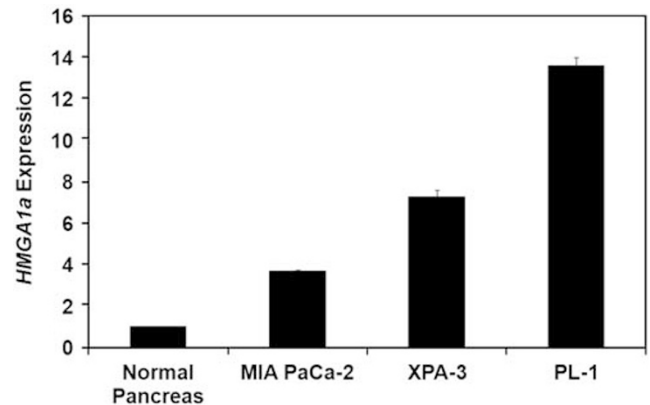


Figure 1 *HMGA1a* mRNA expression in human pancreatic ductal adenocarcinoma cell lines. *HMGA1a* mRNA levels were measured by quantitative RT-PCR and normalized to human phosphoprotein. Normal human pancreatic tissue from a healthy 35-year-old donor was used as a control and assigned a value of 1. *HMGA1a* mRNA levels are significantly above control in all cell lines (4-fold ± 0.08, $P < 0.0001$ for MIA PaCa-2 cells; 7-fold ± 0.3, $P < 0.0001$ for XPA-3 cells and 14-fold ± 0.4, $P < 0.0001$ for PL-1 cells vs 1 ± 0.05 for normal human pancreas). Three independent experiments were performed in triplicate; results represent the mean ± s.e. from independent experiments. Normalization to β -glucuronidase and β -actin as control genes produced similar results in separate experiments (not shown).

Table 1 Distribution of cases represented on the pancreatic ductal adenocarcinoma tissue microarrays by stage, lymph node involvement, grade and mean survival

Stage	n	N1 ^a	Grade				Survival (months)
			1	2	3	Unknown	
Ia/b	3	0	0	2	1		31.0
IIa	18	0	2	10	6		18.4
IIb	101	101	2	53	43	3	17.4
III	3	2	0	1	2		13.3

^aN1 indicates lymph node involvement.

compared with *HMGA1a* mRNA levels in normal pancreatic tissue (Figure 1). Notably, *HMGA1* mRNA was increased by fourfold in MIA PaCa-2 cells ($P < 0.001$ by Student's *t*-test), sevenfold in the XPA-3 ($P < 0.001$ by Student's *t*-test), and 14-fold in PL-1 cells ($P < 0.001$ by Student's *t*-test). All of these cell lines were derived from tumors from patients with advanced disease. MIA PaCa-2 cells originate from an undifferentiated pancreatic carcinoma that extended to periaortic tissues.²⁹ XPA-3 cells were generated from a primary tumor that extensively involved regional tissues and metastasized to lymph nodes³¹ and PL-1 cells are from a primary tumor that extended to the duodenum.³⁰

Patient Characteristics

The mean age for patients was 67.5 years (range: 32–90 years). 64 patients were men and 61 patients were women. Table 1 displays the available stage, grade

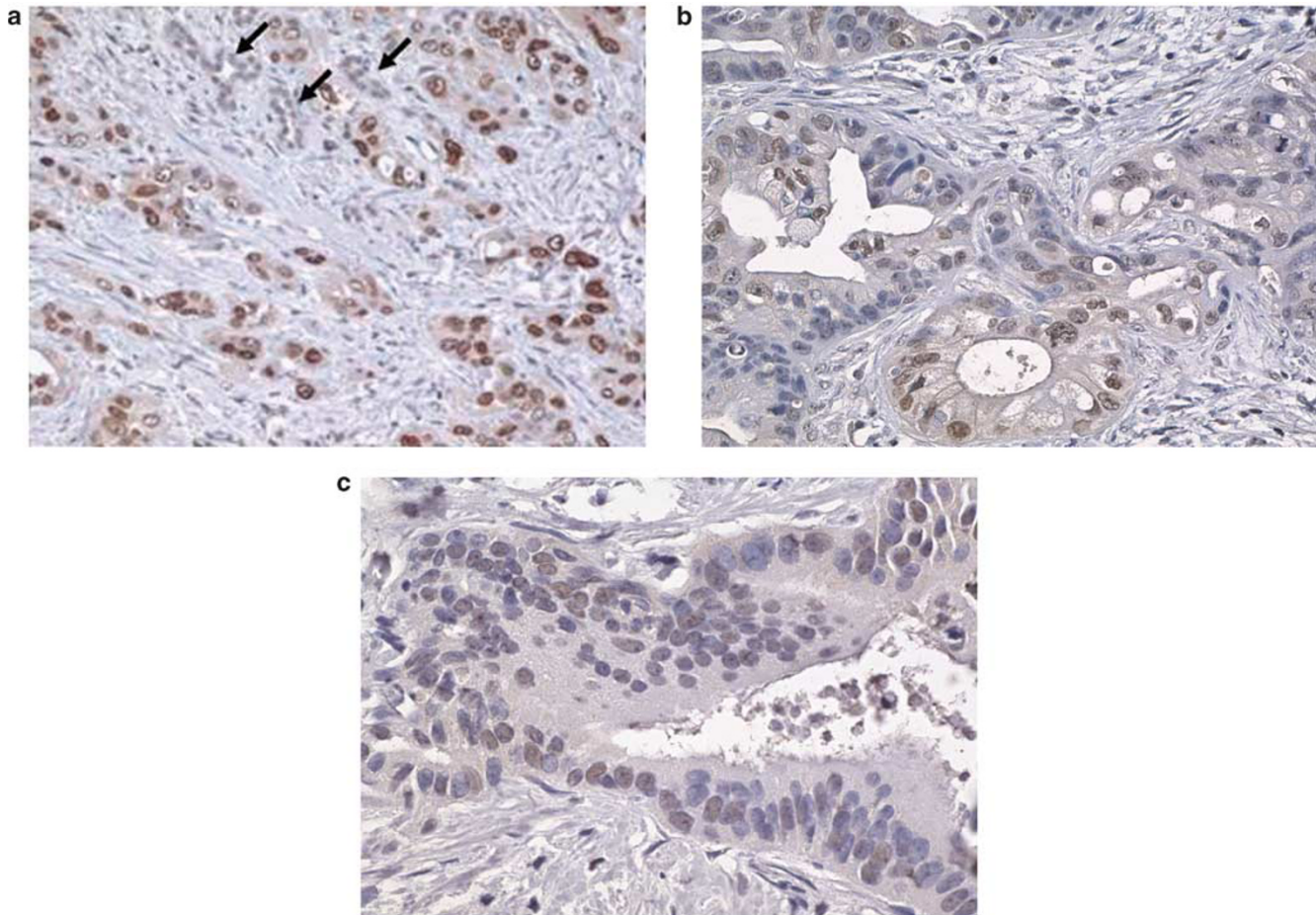


Figure 2 HMGA1 protein was detected in the majority of PDA by immunohistochemistry and increased levels of HMGA1 immunoreactivity correlated inversely with tumor differentiation (HMGA1 antibody, Gill hematoxylin counterstain). (a) Strong immunoreactivity in poorly-differentiated pancreatic ductal adenocarcinoma (magnification X 64, score 9). There is limited HMGA1 reactivity in benign pancreatic ducts (arrows). (b) Moderate immunoreactivity in moderately-differentiated pancreatic ductal adenocarcinoma (magnification X 100, score 6). (c) Weak immunoreactivity in well-differentiated pancreatic ductal adenocarcinoma (magnification X 160, score 2).

and survival information for these patients. Tumor grade was available for 122 of 125 patients. Survival information was available for 120 of 125 patients. Clinical observation ranged from 2 to 47 months (23.4 ± 9.8 , mean \pm s.d.), and 67 patients expired during this study period.

HMGA1 Immunoreactivity in Pancreatic Ductal Adenocarcinoma

As *HMGA1a* was overexpressed in all pancreatic ductal adenocarcinoma cell lines studied, we next sought to determine whether HMGA1 protein is overexpressed in more advanced disease in pancreatic ductal adenocarcinoma. To this end, we assessed HMGA1 protein levels by immunohistochemical analysis in tissue microarrays that included normal pancreatic tissue, PanIN 1–3 lesions, and primary pancreatic ductal adenocarcinomas from tumors with varied grades of differentiation (well-differentiated ($n=4$), moderately differen-

tiated ($n=66$), poorly differentiated ($n=52$)). HMGA1 immunoreactivity was identified in 123 of 125 (98%) cases of pancreatic ductal adenocarcinoma, with scores ranging from 1 to 9 (Figure 2). Of positive cases, 22 (18%) displayed limited reactivity (score <3), 53 (42%) displayed moderate reactivity (score 3–6) and 48 (39%) displayed strong reactivity (score >6). Normal tissues represented on the tissue microarrays, including benign pancreatic ducts, displayed limited to no HMGA1 immunoreactivity. Benign biliary epithelium was a notable exception and occasionally displayed moderate-to-strong HMGA1 nuclear immunoreactivity in the majority of cells.

HMGA1 Protein Expression Correlates with Patient Survival and Tumor Grade

As HMGA1 immunoreactivity was increased in the pancreatic adenocarcinoma samples compared with normal pancreatic tissue, we hypothesized that it

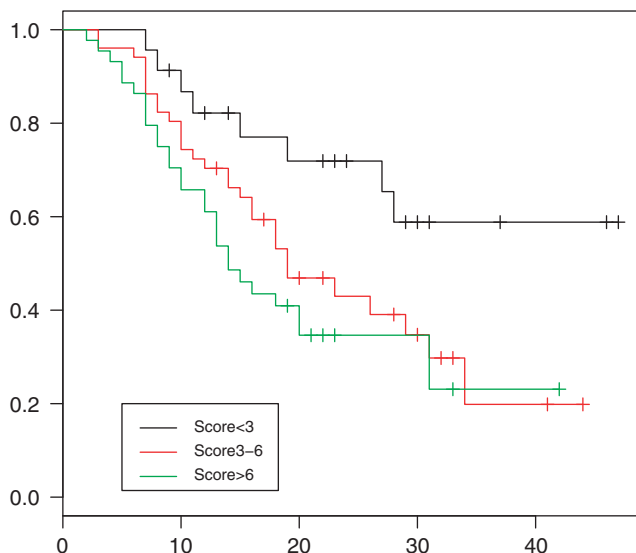


Figure 3 A Kaplan-Meier curve demonstrates that HMGA1 reactivity inversely related to mean survival. Patients with limited to no HMGA1 reactivity (score <3) had longer survival than those with moderate or strong HMGA1 reactivity (score 3–6 and score >6, respectively). Further, patients with the strongest HMGA1 reactivity (score >6) had the shortest survival ($P=0.0175$ by nonparametric rank sum test).

could be a marker for more advanced disease. We therefore determined whether HMGA1 protein levels correlate with clinicopathologic parameters indicative of disease progression, including decreased survival, poor tumor differentiation (high grade), lymph node metastases, and stage. We found that HMGA1 immunoreactivity was inversely associated with mean survival. Patients with limited to no reactivity had longer survival than those with strong reactivity (<3: 23.9 months, 3–6: 17.6 months, >6: 15.0 months, $P=0.0175$ by nonparametric rank-sum test, Figure 3). Furthermore, increased immunoreactivity for HMGA1 was positively correlated with tumor grade. Tumors with an HMGA1 score of ≥ 4 were less differentiated when compared with tumors with an HMGA score <4 ($P=0.03$, χ^2 test). We did not find a correlation between HMGA1 immunoreactivity and lymph node metastases or overall stage.

HMGA1 Protein Levels are Increased in High-Grade PanINs

Only 43% (43/99) of PanINs demonstrated nuclear HMGA1 immunoreactivity. Of these HMGA1-positive lesions, 86% (37/43) displayed low HMGA1 protein expression (score <3). Notably, PanIN-3 lesions were the most likely to have demonstrable HMGA1 staining. In fact, HMGA1 staining was positively correlated with increasing PanIN grade. Specifically, 78% of PanIN-3 lesions and 55% of PanIN-2 lesions exhibited HMGA1 staining, whereas only 36% of PanIN-1 lesions exhibited HMGA1

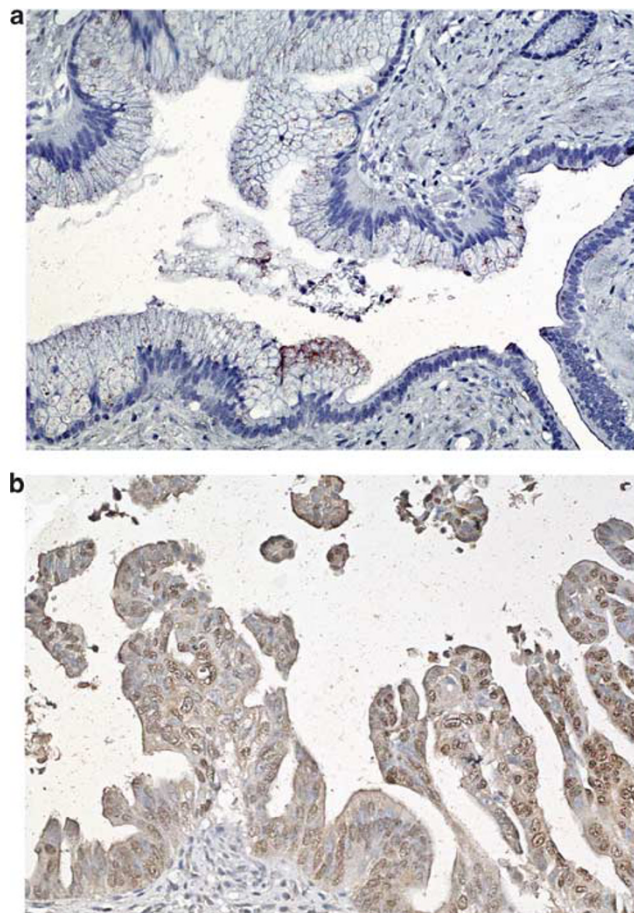


Figure 4 HMGA1 protein expression in PanIN determined by immunohistochemistry correlates with grade (HMGA1 antibody, Gill hematoxylin counterstain). (a) No HMGA1 immunoreactivity in a low grade PanIN lesion (PanIN-1B). (b) High HMGA1 immunoreactivity in a high grade PanIN lesion (PanIN-3).

immunoreactivity ($P=0.024$ by the two-sided Fisher's test, Figure 4).

Discussion

Recent studies have identified molecular alterations that occur in PanIN precursor lesions as they progress to invasive ductal adenocarcinoma. Activating mutations in the *K-RAS2* gene and telomere shortening are believed to occur early because they are often observed in PanIN-1 lesions.^{4–6} Mutations that inactivate *p16(INK4A)* are frequently seen in PanIN-2 lesions,⁹ whereas inactivation of *TP53* and *MAD4/DPC4* are typically observed in PanIN-3 lesions.^{3,10,11} Unfortunately, elucidation of these early molecular events has not translated into improved patient survival. Moreover, the changes that contribute to tumor invasion, malignant progression, and eventual metastasis are poorly understood. As most patients with pancreatic cancer present with advanced disease or rapidly develop distant metastases, discoveries of later molecular

events that cause tumor progression are required to identify cellular pathways that could be targeted in therapy.

Previous studies have implicated *HMGA* genes and proteins in the pathogenesis of pancreatic ductal adenocarcinoma and other high-grade tumors. We recently showed that another *HMGA* family member, *HMGA2*, is overexpressed in pancreatic ductal adenocarcinoma cell lines.²⁸ Moreover, we found that *HMGA2* protein levels correlate with lymph node metastases and tumor grade by immunohistochemical analysis of primary human tumors. In another study using immunohistochemical analysis, *HMGA1* protein was found to be expressed in 15 ductal adenocarcinomas, including 6 metastatic lesions, but not in benign ductal epithelium. There was no correlation between protein levels and tumor differentiation in this relatively small, pilot study.¹⁶ *HMGA1* protein was also found to be increased in two intraductal papillary mucinous neoplasms (IPMNs).¹⁶ In a subsequent paper, this group also demonstrated increased *HMGA1* protein in pancreatic ductal adenocarcinoma cell lines using Western blot analysis, with no demonstrable levels in normal pancreas.³⁴ More recently, another group demonstrated *HMGA1* immunoreactivity in 93% of pancreatic ductal adenocarcinomas from 89 patients.²⁶ In this study, only the intensity of *HMGA1* protein expression was determined, and statistical analyses were based on the presence or absence of immunoreactivity. Unlike our study, no correlation between *HMGA1* staining and tumor differentiation was detected, although this could relate to the dichotomized analysis or the smaller sample size in the previous study.²⁶ However, similar to our study, there was a significant correlation between *HMGA1* immunoreactivity and survival. It is noteworthy that this previous study included a very limited number of samples without *HMGA1* immunoreactivity ($n = 6$, or 7%) as the basis for their survival analysis. In addition, it was not clear whether *HMGA1* expression was an early or late event in tumor progression because PanIN lesions were not examined. Functional studies in pancreatic ductal adenocarcinoma cell lines showed that knockdown of *HMGA1* interferes with cellular invasion,²⁰ anchorage-independent cell growth,²⁶ resistance to anoikis,²² and resistance to gemcitabine.^{35,36} Taken together, these findings indicate that *HMGA1* could drive tumor progression in pancreatic ductal adenocarcinoma.

In summary, we report that *HMGA1* is overexpressed in pancreatic ductal adenocarcinoma. We found high levels of *HMGA1* mRNA in pancreatic carcinoma cell lines compared with normal tissue. In addition, *HMGA1* proteins are present in high levels in pancreatic ductal adenocarcinoma, but not in benign pancreatic ductal epithelium. Furthermore, we show that *HMGA1* protein immunoreactivity correlates with measures of tumor

progression, including advanced tumor grade and decreased survival. Because *HMGA1* is not expressed in the majority of PanIN lesions and found predominantly in invasive carcinoma, our results suggest that it drives tumor progression. Together with functional studies demonstrating a role for *HMGA1* in tumor invasion, anchorage-independent cell growth, and metastatic progression, these findings indicate that *HMGA1* could also serve as a rational therapeutic target and potential biomarker in advanced disease. Future studies are required to identify critical downstream pathways induced by *HMGA1* that are amenable to therapeutic interventions.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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