HMGA1 expression levels are elevated in pancreatic intraepithelial neoplasia cells in the Ptf1a-Cre; LSL-KrasG12D transgenic mouse model of pancreatic cancer

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Background: Pancreatic cancer is currently the third leading cause of cancer deaths in the United States and it is predicted to become the second by the year 2030. High-mobility group A1 protein (HMGA1) is an oncogenic transcription factor, localised and active in cell nuclei, that is linked to tumour progression in many human cancers, including pancreatic cancer. Overexpression of HMGA1 renders cancer cells resistant to chemotherapy. Although the Ptf1a-Cre; LSL-KrasG12D transgenic mouse is perhaps the most widely utilised animal model for human pancreatic cancer, expression levels of HMGA1 in pancreata from this mouse model have not been characterised.

Methods: Quantitative immunohistochemical analysis was used to determine nuclear HMGA1 levels in pancreatic tissue sections from Ptf1a-Cre; LSL-KrasG12D mice aged 5, 11, and 15 months. The H Score method was used for quantitative analysis.

Results: The HMGA1 levels were significantly elevated in pancreatic intraepithelial neoplasia (PanIN) epithelia compared with untransformed acinar tissues or fibroinflammatory stroma.

Conclusions: The PanINs have long been regarded as precancerous precursors to pancreatic adenocarcinoma. Significantly elevated HMGA1 levels observed in the nuclei of PanINs in Ptf1a-Cre; LSL-KrasG12D mice validate this animal model for investigating the role that HMGA1 plays in cancer progression and testing therapeutic approaches targeting HMGA1 in human cancers.

Pancreatic cancer (PC) is currently estimated to be the third leading cause of cancer related deaths in the United States (https://cancerstatisticscenter.cancer.org/#/) with a 5-year survival of <10% (Warshaw and del Castillo, 1992; Hingorani et al, 2003; Jemal et al, 2006; Ottenhof et al, 2009; Rahib et al, 2014; Siegel et al, 2016; American Cancer Society, 2017). Exocrine tumours in the pancreas account for over 95% of all pancreatic malignancies (Fesinmeyer et al, 2005). Specifically, pancreatic ductal adenocarcinoma (PDAC) comprises ~90% of these exocrine tumours and resists all conventional forms of chemotherapy and radiotherapy (Futura et al, 1992; Yadav and Lowenfels, 2013). Surgery is currently the most effective treatment for patients with PC. Unfortunately, most patients are not candidates for resection because of metastases or extensive local vascular involvement at diagnosis (National Cancer Institute, 2014; https://deainfo.nci.nih.gov/advisory/ctac/workgroup/pc/PDACframework.pdf). Despite surgery being the most effective method of treatment, only 3–4% of all patients who undergo resection receive a disease-free diagnosis with long-term survival. Precancerous lesions, referred to as pancreatic intraepithelial neoplasms (PanINs), are below the detection limit of current imaging capabilities (Chari et al, 2015). In 2014, there were 39 590 deaths as a result of PC (Stark and Eibl, 2016).
Animal model and breeding. The scientific name of the LSL Kras G12D mouse strain used was B6.129-Kras tm4Tyj that contains a G12D Kras mutant silenced by a Lox-stop-Lox (LSL) cassette. The scientific name of the P48-Cre mouse strain used was B6.Ptf1a(1tm1.1(cre)Cvw) that causes Cre-recombinase expression under control of the Cre knock-in at the Ptf1a-p48 locus that is primarily expressed in the pancreas after embryonic day 9.5. After cross-breeding Kras G12D mice with P48-Cre mice, offspring carrying both genetic elements express Cre-recombinase specifically in the pancreas, thus ‘unsilencing’ the oncogenic Kras mutant only in the pancreas, leading to PC progression (Hingorani et al., 2003). To monitor the progression from precancerous lesions to PC, cohorts of six female mice (double-negative controls and double-positive study) groups at ages 5, 11, and 15 months were generated. Although the IHC study was limited to female mice, standard H&E staining histological analyses of pancreata from male study mice were indistinguishable from female mice, and therefore considered representative of both genders. Mice were bred, sexed, tagged, genotyped, and killed according to procedures approved by the institutional animal care and use committee (IACUC) at Miami University.

Genotyping. Mouse DNA was obtained through an ear punch to determine the genotype of each mouse and to establish individual identities throughout the study. DNA extraction was conducted by submerging the ear punch in 25 μl of 25 mM NaOH/0.2 mM EDTA at pH 12 and then heating in a thermocycler, GeneAmp PCR System 9700 (Thermo Fisher Scientific, Florence, KY, USA), at 98 °C for 1 h. The samples were then neutralised with 25 μl of 40 mM Tris-HCl at pH 5. Samples were then stored in −20 °C until PCR amplification was conducted on the DNA with both the Kras and Cre primers. Dreamtaq Green Master Mix (Fisher Scientific, Singapore, KY, USA), MgCl2 (Fisher Scientific), and water were mixed together and either the Kras, Cre, or control primers (IDT, San Jose, CA, USA) were added and then combined with the DNA. For the Kras programme, the mixture was denatured for 30 s at 94 °C, then annealed for 1 min at 69 °C, and elongated for 1 min at 72 °C using the GeneAmp PCR System 9700 (Thermo Fisher Scientific) thermostapler for 35 cycles, then held at 4 °C when complete. For the Cre program, the mixture was denatured for 1 min at 94 °C, then annealed for 20 s at 69 °C, and elongated for 45 s at 72 °C using the thermostapler for 30 cycles, and then held at 4 °C when complete. Gel electrophoresis was then performed using an agarose gel stained with ethidium bromide to separate the amplified gene product. The presence or absence of amplified DNA was determined by inspection under a UV light on the UVP ChemiDoc-It 2 815 Imager (UVP, Upland, CA, USA). If present, the Kras mutation displayed a band at 550 bp and Cre displayed a band at 250 bp. Mice that carried both the Kras mutation and Cre-recombinase were categorised as study mice. Mice that lacked both the Kras mutation and Cre-recombinase were categorised as

MATERIALS AND METHODS

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control mice. In addition, internal controls were generated from each study mouse by analysing the normal tissue present within the pancreas image. This is further described in the Aperio analysis section.

Pancreata collection, processing, and sectioning. Based on predetermined age ranges, the dates for killing of mice were established. Female mice aged 5, 11, and 15 months were used in this study. These ages were selected based on reports that ~80% of the ducts were normal at 2.25 months, 68% were normal between 4 and 5 months, and 18% were normal between 7 and 10 months in this mouse model (Hingorani et al, 2003). Upon killing, pancreatic tissue samples were collected, stored overnight at room temperature in formalin, and changed to 70% ethanol and placed into 4 °C until further preparation. Pancreatic tissue samples were then processed using the Leica TP 1020 (Leica Biosystems, Buffalo Grove, IL, USA) and embedded using the Shandon Histocentre 3 (Thermo Fisher Scientific). Then, 5 μm sections of each pancreata were made by using Thermo-Shandon Finesse ME Microtome (Thermo Fisher Scientific) and placed on positively charged slides.

IHC staining. Immunohistochemical staining of HMGA1 was conducted at Cincinnati Children’s Hospital Medical Center.
HMGA1 is elevated in pancreatic cancer PanIN cells

The HMGA1 antibody (ab129153, Abcam, Cambridge, MA, USA) was used for staining on a fully automated Ventana Discovery XT system (Ventana Medical Systems, Inc., Tuscon, AZ, USA) and following the manufacturer’s protocol. The slides were deparaffinised and pretreated in citrate for 32 min. Sections were treated with the HMGA1 antibody in a dilution of 1:2000 for 5 min, followed by treatment with a rabbit secondary antibody (DISCOVERY UltraMap anti-Rb HRP (RUO) 760-4315, Roche, Tuscon, AZ, USA) for 12 min. The HRP secondary antibody was used to develop a signal through the use of 3,3’-Diaminobenzidine (DAB) binding to the antigen. The substrate for the HRP was DAB. The Roche OmniMap anti-Rb HRP kit was used and slides were processed using the Discovery XT instrument. Slides were then placed into hematoxylin for 8 min, followed by bluing reagent (Ventana Medical Systems, Inc. (Ventana) Bluing Reagent, 760-2037, Roche) for 4 min. The HMGA1 antibody dilution was 1:2000 from a stock of 100 ml at 1.631 mg ml$^{-1}$. Slides were digitally scanned at 20 $\times$ using the Aperio AT2 scanner (Leica Biosystems). Uploaded images were annotated in Aperio eSlide Manager to include regions of interest and the nuclear algorithm was used to conduct the automated analysis of the HMGA1 protein. The specificity of the commercially available HMGA1 antibody has been previously demonstrated (O’Day et al., 2015).

Aperio analysis. The HMGA1 is an architectural transcription factor that is localised to the nuclei of all cells. Therefore, quantisation of HMGA1 levels was restricted to cellular nuclei. Nuclei quantities and areas were selected by following the instructions in the Aperio IHC Nuclear Image Analysis User’s Guide. Internal controls were used to ensure identical IHC staining for control tissue and transformed PanIN tissues. In younger mice that still contained significant amounts of acinar tissue, 1000 nuclei (±5 nuclei) were circled from within the acinar tissue to serve as an internal control using Aperio software (Figure 1A and B). In transformed sections of the pancreata, 1000 nuclei (±5 nuclei) were circled using Aperio software focussing only on PanIN structures (Figure 1E and F). The PanINs, originally thought to be derived from duct cells, were found to be derived from acinar cells with the transformation process being referred to as acinar-to-ductal metaplasia (ADM) (Schmid, 2002; Kopp et al., 2012; Prévot et al., 2012; Liou et al., 2013). In the mouse model used in this study, PanINs and tumours develop from ADM (Hingorani et al., 2003). In some of the 15-month images, normal untransformed acinar tissue was absent, or present at amounts that yielded <1000 nuclei (±5), and therefore fibroinflammatory stroma cells surrounding the PanINs were selected to serve as the internal control nuclei to fulfill the internal control nuclei count (Figure 1C and D). The reactive stroma of the pancreas used as a region to select internal control cells is sometimes referred to as fibroinflammatory stroma that has been described as a transitional tissue that occurs before the display of PanINs (Hruban et al., 2001). Although the appearance of reactive stroma or fibroinflammatory stroma is widely present in carcinoma, and pancreatitis, it can also be displayed in normal disease-free pancreatic tissue (Luttges, 1999). After selection of internal control and PanIN nuclei, the nuclear algorithm within the Aperio software was executed and the attributes tables were generated containing the nuclear output layer data (Figure 2). The percent positive nuclei were determined and denoted into four predefined levels in the attributes table (Zarrella et al., 2016). The levels were represented as follows: none (0) blue, weak (1+) yellow, moderate (2+) orange, and intense (3+) red (See Figure 1F for an example). The standard nuclear algorithm parameters were used in the program. Algorithm parameters, results, and intensity thresholds were examined by pathologist Dr Stringer at CCHMC to ensure correct analysis.

| Name                  | Value    |
|-----------------------|----------|
| Percent positive nuclei| 71.3287  |
| Intensity score        | 2        |
| (3+) percent nuclei    | 28.7712  |
| (2+) percent nuclei    | 24.5754  |
| (1+) percent nuclei    | 17.982   |
| (0+) percent nuclei    | 28.6713  |
| Average positive intensity | 168.468 |
| Average negative intensity | 232.0   |
| (3+) nuclei            | 288      |
| (2+) nuclei            | 246      |
| (1+) nuclei            | 180      |
| (0+) nuclei            | 287      |
| Total nuclei           | 1001     |
| Average nuclear RGB intensity | 152.37  |
| Average nuclear size (pixels) | 127.188 |
| Average nuclear size (µm$^2$) | 32.0008  |
| Area of analysis (pixels) | 704420  |
| Area of analysis (mm$^2$) | 0.1772   |

Figure 2. Example of a table of nuclear output data generated by the Aperio software. An example of the nuclear algorithm output generated from an image of a 15-month-old mouse. The percent nuclei, specifically the 3+, 2+, and 1+ values, within this algorithm are used to establish a H Score for each image.
As expected in the Ptf1a-Cre; LSL-KrasG12D mouse model, the number of PanINs in mouse pancreata increased in the order 5 months, 11 months, and 15 months (Figure 3). Based on visual inspection, mouse pancreata at 5 months of age were mostly composed of normal acinar tissue with scattered pockets of transitioned PanIN tissue (Figure 3A). At 11 months of age, mouse pancreata displayed significantly more PanINs compared with 5-month tissues (Figure 3B). By 15 months of age, most of the acinar tissue in the pancreata had transitioned to PanINs and significant cellular fibroinflammatory stroma was apparent surrounding the PanINs (Figure 3C).

Pancreata IHC stained for HMGA1 from six female mice from each age category were quantified using the Aperio software. Figure 4A–C shows examples of harvested internal control nuclei from untransformed acinar tissue from three different mice from the 5-month-age group. Figure 4D–F shows the resulting scores for each nucleus harvested in the circled region. Nearly all of the internal control nuclei had undetectable levels of HMGA1 indicated by the blue colouring of the processed nuclei. Figure 4G–I shows examples of harvested nuclei from PanINs present in the same three 5-month-old mice. Figure 4J–L shows the same, circled regions processed by the Aperio algorithm, illustrating significant numbers of yellow, orange, and red nuclei, indicating that the PanINs contained weak, moderate, and intense expression levels compared with the negative internal control nuclei. These representative data illustrate the difference in HMGA1 levels observed in nuclei of untransformed acinar cells compared with nuclei of transformed PanIN cells. Supplementary Figure S1A–C shows representative harvested internal control nuclei from untransformed acinar tissue from three different mice from the 11-month-age group. Supplementary Figure S1D–F shows the resulting scores for each nucleus harvested in the circled region. Again, internal control nuclei had mostly undetectable levels of HMGA1 indicated by the blue colouring of the processed nuclei. Supplementary Figure S1G–I shows harvesting of nuclei from PanINs in 11-month-old mice. Finally, in Supplementary Figure S2, 15-month-old mice were typically devoid of untransformed acinar tissue. Therefore, regions of cellular fibroinflammatory stroma were selected as internal controls. Supplementary Figure S2A–C shows examples of harvested internal control nuclei from cells from the stroma from three different mice from the 15-month-age group. Supplementary Figure S2D–F shows the resulting scores for each nucleus harvested by the circled region. Again, nuclei from fibroinflammatory stroma internal control cells had mostly undetectable levels of HMGA1 indicated by the blue colouring of the processed nuclei. Supplementary Figure S2G–I shows harvesting of nuclei from PanINs present in same three 15-month-old mice. Supplementary Figure S2J–L shows the same regions processed by the Aperio software, indicating elevated HMGA1 levels in PanIN epithelia, consistent with that observed in PanIN nuclei in pancreata harvested from 5- and 11-month-old mice.

Figure 5 shows a boxplot analysis of the raw data for the internal controls compared with the PanINs for each age category. The boxplots indicate large separation between the H Scores of internal control nuclei and the H Scores of PanIN nuclei for all age groups compared (Figure 5A). Closer examination of the internal controls for the three groups indicated a slight increase in HMGA1 levels in the nuclei of internal control stroma cells in comparison with nuclei of normal acinar tissue (Figure 5B). Statistical analysis of the H Scores for all comparison between age groups is summarised in
Table 1. Based on the results of a Shapiro–Wilks test for each group, all six groups were found to have normal distributions. Because of the differences in variance between the control and study groups, a one tailed Welch’s $t$-test was conducted between each age category’s control and study populations. For the 5-month-age group (comparing the control with the study), the $P$-value was found to be 0.00025. The observed effect size was 6.50 between the control and study 5-month-age groups. Based on the

Figure 4. The 5-month nuclear algorithm analysis. (A–C) Examples of internal control acinar tissue circled before running the algorithm. (D–F) The same internal control acinar tissue regions depicted in (A–C), but post nuclear algorithm analysis. (G–I) Example of PanINs circled before the nuclear algorithm analysis. (J–L) The same PanINs as depicted in (G–I), but post the nuclear algorithm analysis. All images were stained for HMGA1 and captured at $\times 20$ in Aperio.
P-value and Cohen’s $d$ value, it could be concluded that the control group was significantly different and did not belong to the same population as the study group. For the 11-month-age group (comparing the control with the study), the $P$-value was found to be 0.000084. The observed effect size was 6.36 between the control and study 11-month-age groups. Based on the $P$-value and Cohen’s $d$ value, it could be concluded that the control group was significantly different and does not belong to the same population as the study. Finally, for the 15-month-age group (comparing the internal control population with the study population), the $P$-value was found to be 0.00016. The observed effect size was 7.37 between the control and study 15-month-age groups. Based on the $P$-value and Cohen’s $d$ value, it could be concluded that the HMGA1 levels in nuclei from the internal control cells of the stroma cells were significantly different compared with that in PanIN cells, and did not belong to the same population of nuclei as the nuclei from the PanIN group of nuclei.

DISCUSSION

The HMGA1 is generally expressed at low levels in normal healthy tissue and HMGA1 expression levels have been shown to be elevated in cancerous tissue (Fusco and Fedele, 2007; Hristov et al, 2010). This study was designed to specifically examine HMGA1 levels in the pancreas over the course of development in the Ptf1aCre; LSL-KrasG12D mouse model for PC. The expression levels of HMGA1 were examined at 5, 11, and 15 months to characterise how HMGA1 expression levels varied in the pancreas tissue at early, intermediate, and late stages of precancerous PanIN development. At 5 months of age, the pancreas was mostly composed of normal acinar tissue with scattered pockets of PanINs. At 11 months of age, mice pancreata displayed a significant increase in transformed PanINs compared with pancreatic tissue at 5 months of age, with PanINs typically comprising more than half of the pancreas tissue accompanied by an increasing amount of fibroinflammatory stroma. By the age of 15 months, pancreata were typically entirely composed of PanINs surrounded by a significant amount of fibroinflammatory stroma. It should be noted that the progression from normal healthy pancreas, dominated by exocrine acinar tissue, to a tissue essentially composed entirely of PanINs and fibroinflammatory stroma, proceeds initially by the transformation of acinar cells to PanINs in a process referred to as ADM, where metaplasia refers to the transformation or replacement of one cell type by another, that is, transformation or replacement of acinar cells by PanIN epithelial cells. As the mouse ages, there is increased progression...
from localised replacement of acinar cells by PanINs to a more disorganised tissue structure characterised by wholesale conversion of acinar tissue to PanINs with significant emergence of fibroinflammatory stroma that precedes cancer initiation.

Our results indicated that HMGA1 levels were essentially below detection in normal acinar tissue and in the complex reactive cellular stroma surrounding PanINs, but significantly elevated in nuclei of established PanINs. The pancreatic adenocarcinoma stroma is known to be composed of both cellular and acellular components, including fibroblasts, myofibroblasts, pancreatic stellate cells, immune cells, blood vessels, extracellular matrix, cytokines, and growth factors (Waghray et al., 2013). Once acinar cells transformed into PanIN epithelial cells, we could not detect any further increase in the HMGA1 levels in PanIN cellular nuclei with increasing age. From our data, we cannot make any conclusion regarding whether or not elevated HMGA1 expression levels in acinar cells precede ADM and PanIN formation, but our data clearly show that HMGA1 expression levels are a hallmark and a characteristic of PanIN epithelial cells once they have been established by the process of ADM. Although HMGA1 expression levels are generally not elevated in the fibroinflammatory stroma surrounding established PanINs, a significant increase in HMGA1 content was observed in the stroma in the 15-month-old mice in comparison with the 5- and 11-month-old mice, reflecting HMGA1 expression in an increasing population of PC stellate cells in the stroma as the pancreas tissue approaches a transition to adenocarcinoma.

Although the precise role that HMGA1 plays in PC progression and the precise mechanism that elevated HMGA1 levels play in acinar to ductal metaplasia remains unknown, it is well established that HMGA1 expression levels are elevated in the cells of many human cancers. Therefore, elevated HMGA1 expression levels are considered a hallmark of many human cancers. Consequently, HMGA1 is receiving growing attention as a cancer biomarker and as a potential target for cancer therapy and treatment. Given the growing appreciation of the importance of HMGA1 in cancer progression, and the heavy reliance on mouse models for PC research, the results presented here validate the use of the Ptf1aCre; LSL-KrasG12D mouse model for studies investigating the role that HMGA1 plays in cancer progression, and for research targeting HMGA1 as a potential anticancer therapeutic target (Liau and Whang, 2008; Shah and Resar, 2012).

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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