Hypoxia predicts aggressive growth and spontaneous metastasis formation from orthotopically-grown primary xenografts of human pancreatic cancer

Qing Chang¹, Igor Jurisica¹,², Trevor Do¹, and David W. Hedley¹,²,³

¹Ontario Cancer Institute/Princess Margaret Hospital and the Campbell Family Institute for Cancer Research, 610 University Ave. Toronto, Ontario, Canada
²Department of Computer Science and Department of Medical Biophysics, University of Toronto, 1 King's College Circle, Toronto, Ontario, Canada
³Department of Medical Oncology and Hematology, Princess Margaret Hospital, 610 University Ave. Toronto, Ontario, Canada

Running title: Hypoxia in pancreatic cancer.

Key words: hypoxia; pancreatic cancer; xenograft models; metastasis; proliferation.

Grant Support: This work was supported by John and Esther Loewen Family Fund and a research grant from the Ontario Institute for Cancer Research. Computational analyses and infrastructure were supported in part by Ontario Research Fund GL2, Canada Foundation for Innovation, Canada Research Chair program, and IBM. This research was funded in part by the Ontario Ministry of Health and Long Term Care. The views expressed do not necessarily reflect those of the OMOHLTC.

Corresponding Author/Requests for Reprints: David W. Hedley, Department of Medical Oncology and Hematology, Princess Margaret Hospital, 610 University Avenue, Toronto, ON M5G 2M9 Canada. Tel: 1-416-946-2262; Fax: 1-416-946-6546; Email: david.hedley@uhn.on.ca

Disclosure of Potential Conflicts of Interest: No potential conflicts of interest were disclosed.
Abstract

Hypoxia in solid tumors is associated with treatment resistance and increased metastatic potential. Although hypoxia has been reported in pancreatic cancer patients, there is little direct evidence that this contributes to their overall poor prognosis. To address this, we examined the associations between hypoxia and biological aggression in a series of patient-derived xenografts grown orthotopically. Early passage xenografts were established from 16 patients undergoing surgery for pancreatic cancer, and maintained in the pancreas of immune-deprived mice. Hypoxic cells were labeled using the 2-nitroimidazole probe EF5, and stained for immunofluorescence microscopy of tissue sections, or as cell suspensions for flow cytometry. Bromodeoxyuridine uptake, microvessel density, cleaved caspase-3, and the differentiation markers E-cadherin, cytokeratin 19 and vimentin were analyzed in relation to hypoxia. Orthotopic implants closely resembled the histology of the original surgical samples. The 16 primary xenografts showed a wide range in their growth rates and metastatic potential, reminiscent of the spectrum of behavior seen in the clinic. EF5 labeling, tumor growth rates and metastatic patterns were highly consistent within replicates, indicating a significant transmissible (genetic or epigenetic) component. Hypoxia was highly correlated with rapid tumor growth, increased bromodeoxyuridine uptake, and with spontaneous metastasis formation. mRNA expression analysis showed increased expression of genes involved in cell survival and proliferation in the hypoxic models. The results suggest that hypoxia is a major adverse prognostic factor in pancreatic cancer patients and support the introduction of techniques to measure hypoxia directly in patients, and the development of treatment protocols to target hypoxia.
Introduction

Hypoxia occurs in solid tumors when the consumption of oxygen exceeds its delivery by the vascular system (1). In most large clinical studies the outcome is worse for patients with hypoxic tumors, consistent with the long-held idea that hypoxia confers both radiation and chemotherapy resistance (2-4). Unexpectedly, high levels of tumor hypoxia were also found to predict for metastasis development in patients treated by surgery alone, pointing to biological effects beyond those linked to drug and radiation resistance (5, 6).

The hypoxic microenvironment is hostile to cancer cell survival. Hypoxia tolerance can occur due to genetic changes such as p53 loss that enhance cell survival (7), or to cellular responses to hypoxia, driven for example by hypoxia-inducible factors (HIFs) or the unfolded protein response, that modify gene expression, cell phenotype, and metabolism (8, 9). In a recent paper describing the genetic evolution of pancreatic cancers, it was suggested that cancer cells adapted to hypoxia could undergo additional genetic changes that enhance metastasis formation (10). In other words, hypoxia might be a cause of genetic change, as well as a consequence.

The increased metastatic potential of hypoxic cancers might result from changes in cell biology that are mediated by cellular responses to hypoxia (11-13). For example, growth under hypoxic conditions may reprogram epithelial cells towards a more mesenchymal phenotype due to the activation of E-cadherin transcriptional repressors (12, 14, 15). Cells undergoing epithelial/mesenchymal transition (EMT) show reduced cell/cell interaction via adherens junctions, and increased extracellular matrix interactions via integrins/focal adhesion kinase. Consequently, they are more motile and able to invade
and metastasize (16, 17). Successful development of a metastasis presumably requires at least one cancer initiating cell. Pancreatic cancer stem cells have been reported to show EMT features (18, 19), and it has also been proposed that hypoxia provides a niche that favors the survival of cancer cells with stem-like properties (20-22).

Although pancreatic cancers are often considered as being hypoxic, most publications have used histological surrogate markers for hypoxia that might be compromised by surgical ischemic time, or by sampling error due to the heterogeneous distribution of hypoxia in tumor tissue (23, 24). Direct evidence that pancreatic cancers are hypoxic is limited to a small study in which intra-operative pO₂ measurements were made by inserting needle electrodes into the exposed tumor (25). To address the question of whether hypoxia plays an important role in pancreatic cancer patients, we studied a series of early passage xenografts established from pancreatectomy samples, and grown orthotopically. The results show striking associations between hypoxia and aggressive growth and metastasis formation.

**Materials and Methods**

**Establishment of orthotopic primary pancreatic cancer xenografts**

Animal experiments were carried out using protocols approved by University Health Network Animal Welfare Committee. The primary pancreatic cancer xenografts were established as previously described (26-29). Fresh pancreatectomy samples that were superfluous to diagnostic needs were obtained from the University Health Network Tumor Tissue Bank according to institutional human ethical guidelines. Primary xenografts were established at the orthotopic site of 4- to 5-week-old severe combined
immunodeficiency mice by attaching tumor fragments to the surface of the exposed pancreas by a small incision in the upper left abdomen under general anesthesia. 16 orthotopic primary pancreatic cancer xenografts, designated as Ontario Cancer Institute Pancreas (OCIP) 16, 18, 19, 20, 21, 23, 24, 28, 30, 34, 36, 37, 38, 40, 51, and 52 were used in this study. A minimum of 6 replicate tumors was used for each model.

**Detection of tumor Hypoxia and proliferation**

To allow assessment of the hypoxic regions within tumors, mice were injected i.v. and i.p. with the 2-nitroimidazole hypoxia marker agent EF5, 30 mg/kg, 4 h prior to sacrifice, as described previously (30, 31). They were then injected i.p. with 100 mg/kg 5-bromo-2’-deoxyuridine (BrdU) (Sigma, St. Louis, MO) dissolved in PBS 30 min prior to sacrifice. Tumors without EF5 and BrdU injection were used as negative controls for each model. Tumors were then rapidly excised and bisected. One half was fixed and processed for paraffin embedding, and the other half disaggregated to a single-cell suspension for flow cytometry analysis.

**Flow cytometry analysis**

Single cell suspensions from tumors were prepared by an enzymatic technique (Collagenase XI, Protease, and DNase I cocktail) for flow cytometry as described previously (32, 33). Aliquots of single-cell suspensions were then fixed either in 80% ethanol to allow DNA denaturation for anti-BrdU labeling, or in 4% formaldehyde for 10 min to optimize intracellular protein staining. For dual EF5 and BrdU labeling, cells were acid denatured, neutralized, and then stained with anti-EF5 monoclonal antibody (Cy5 conjugates, ELK3-51, obtained from Dr. Cameron Koch, University of Pennsylvania), anti-BrdU (PRBQD-Alexa 488, Phoenix Flow Systems, San Diego, CA) and 1 μg/ml 4’,
6-diamidino-2-phenylindole (DAPI). Anti-vimentin and anti-cytokeratin 19 (both from Abcam, Cambridge, MA) were also used for staining. Samples were analyzed using an LSRII flow cytometer (BD Biosciences, San Jose, CA). Single parameter DNA content histograms were analyzed using ModFit LT™ 3.0 (Verity, Topsham, ME). The percentages of EF5 and BrdU positive cells were analyzed using WinList™ 6.0 (Verity, Topsham, ME).

**Immunohistochemical and immunofluorescent staining**

Serial sections were cut from paraffin-embedded tumor tissue, and stained with H&E, Masson’s Trichrome, and for α-smooth muscle actin by immunohistochemistry (Dako A/S, Glostrup, Denmark). The remaining sections were labeled for fluorescence microscopy using primary antibodies against BrdU (Caltag, Burlingame, CA), CD31 (Santa Cruz Biotechnology, Santa Cruz, CA), cleaved caspase-3 (Cell Signaling Technology, Danvers, MA), E-cadherin (Vector Laboratories, Burlingame, CA), vimentin (American Research Products, Belmont, MA), respectively, and then dual labeled with an anti-EF5 Cy3 conjugate. Secondary Cy5-conjugated anti-mouse or anti-rabbit antibodies were used for indirect immunofluorescence staining (Jackson Laboratories, West Grove, PA). Secondary antibodies were used alone to control for nonspecific background. Sections were counterstained with 1 μg/ml DAPI to outline the nuclear area.

**Image capture**

Entire immunofluorescence-stained sections were imaged at 1 μm resolution using a laser scanning system (TISSUEscope™, Biomedical Photometrics, Waterloo, ON, Canada), and composite images of regions of interest were imaged at higher resolution (20 × 257x11 on May 28, 2017. © 2011 American Association for Cancer Research. Cancer Res Published OnlineFirst on February 22, 2011; DOI: 10.1158/0008-5472.CAN-10-4049. Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.
magnification) using a conventional fluorescence microscope and scanning stage (BX50, Olympus Corporation, Center Valley, PA). Uncompressed TIFF images (8-bit) were acquired for analysis.

**Image analysis**

H&E images were reviewed to generate masks of viable tumor areas for individual images of immunofluorescence using image analysis tools developed in-house based on IDL 6.3 programming language. Fluorescence of debris and other artifacts was omitted. Measurements of fluorescence intensity in viable tumor areas were done using Image-Pro Plus 6.1.0 (Media Cybernetics, Bethesda, MD). Immunofluorescence intensity was visually inspected and EF5 positive labeling was represented by intensities above the 75th percentile (the 3rd quartile, Q3), as previously described (34). The integrated optical density and fractional labeled area were measured in viable tumor areas using the Q3 threshold. The product of integrated optical density and fractional labeled area was calculated to represent relative protein abundance. Ratios of protein abundance in EF5 positive region and non-EF5 region of individual tumors were used to determine group medians. The percentages of positive stained nuclei were measured for BrdU and cleaved caspase-3 analysis, based on the binary images. Researchers were blinded to sample identity during analysis.

**Reverse transcription and quantitative PCR (RT-qPCR)**

Total RNA was isolated from 3 frozen tumors of each model by homogenization in TRIzol reagent and acid phenol-chloroform extraction, purified by RNeasy mini kit (Qiagen, Mississauga, ON, Canada). RT was completed at 42°C with SuperScript II RNase H reverse transcription kit (Invitrogen, Burlington, ON, Canada). qPCR was
performed with 10 ng of the first-strand cDNA synthesis mixture as a template and individual primer sets (See supplementary information) using the SYBR Green PCR Master Mix and an Applied Biosystems 7900 HT instrument (Foster City, CA). Primers were designed using Primer Express 3.0 (Applied Biosystems, Foster City, CA). Gene expression levels in the samples were calculated relative to control using the comparative C_T method (35): \( \Delta \Delta C_T = \Delta C_{T, \text{sample}} - \Delta C_{T, \text{control}} \), fold change = \( 2^{-\Delta \Delta C_T} \). The geometric mean of GAPDH, \( \beta \)-actin, TATA binding protein, and \( \beta \)-2-microglobulin were used to normalize the expression data (\( \Delta C_T \)).

**Protein-protein interaction analysis**

Individual genes were mapped to proteins using Interologous Interaction Database (I2D) ver. 1.9 (36). Only direct interactions of target proteins and direct interactions among their interactors were considered. The protein-protein interaction network was visualized and annotated in NAViGaTOR ver. 2.2.1 (37).

**Statistical methods**

The statistical significance of differences in numerical data was evaluated with a Student t test, the statistical significance of correlation between different factors was evaluated with Pearson correlation analysis, and the statistical significance of comparison of survival curves was evaluated with Logrank test. A \( P \) value of less than 0.05 was considered to be statistically significant. All statistical tests were two-sided.

**Results**

**Characterization of orthotopic primary pancreatic cancer xenografts**
Clinicopathological features and K-ras and p53 mutation status of the 16 patient donors are shown in Table 1. Histological examination of the H&E sections showed that the orthotopically-grown primary xenografts were typically moderately-well differentiated, mucin-secreting adenocarcinomas that closely resembled the original surgical specimen (Fig. 1). They were organized into glandular structures, with a prominent fibrovascular stroma replete with α-smooth muscle actin-positive mesenchymal stromal cells (Supplementary Fig. 1). Cellular DNA content analysis by flow cytometry confirmed that in many of these tumors, normal mouse cells accounted for >80% of the total cell population (Supplementary Fig. 2).

**Growth rates correlate with BrdU uptake**

Since the tumors were grown at the orthotopic site, their sizes were estimated by palpation and the passage time was used as a surrogate marker for the growth rate. Tumor growth rates were defined by the number of days between orthotopic implantation and growth to ~1cm diameter. The primary xenografts showed a wide range in growth rates (i.e. passage times of 4-26 weeks), which correlated with BrdU incorporation assessed by immunofluorescent staining analysis (Pearson r = -0.7643, P = 0.0006, Fig. 2A).

**Tumor growth and metastasis rate**

Mice were examined for the presence of metastases at the time of tumor resection, with histological confirmation of suspicious lesions. As shown in Fig. 2B, the gross metastasis patterns were similar to those seen in pancreatic cancer patients, and by microscopy they were also organized into glandular structures surrounded by stroma, similar to the appearance of the primary tumor. Spontaneous metastases to liver (OCIP23, 28, 36, 37, 38, 51, and 52) and lung (OCIP28) occurred. Malignant ascites also developed in six of
the ten fast growing models (OCIP21, 23, 28, 34, 36, and 52). The development of spontaneous metastases from the orthotopic site was highly correlated with the tumor growth rate ($P = 0.0002$) and BrdU incorporation ($P = 0.0003$), shown in Fig. 2C.

**Hypoxia measurements**

Representative plots of EF5 labeling vs. DNA content for the 16 primary xenografts are shown in Figure 3A. EF5 staining was low or absent in OCIP16, 18, 19, 24, 30 and 40 (top panel), whereas OCIP20, 21, 23, 28, 34, 36, 37, 38, 51, and 52 showed a heterogeneous distribution of positive staining similar to that previously reported (33, 38). Positive staining was seen both in the aneuploid cancer cells, and in the normal mouse stroma. The percentages of EF5 positive cells were highly consistent within individual models. The tumors appeared to segregate into high and low EF5 labeling at a cut-off value of 5% positive EF5 labeling, with little overlap between the two groups ($P < 0.0001$; Fig. 3B).

**Hypoxia and BrdU labeling**

Dual immunofluorescence staining for EF5 and BrdU showed a striking loss of BrdU incorporation in the EF5 positive regions, indicating that hypoxia causes cell cycle arrest in these models (Fig. 4A). Interestingly, hypoxia associated with cell cycle arrest was also seen in small (<1mm) liver metastases from the hypoxic models, suggesting that this might cause resistance to cell cycle-active agents like gemcitabine when used as adjuvant treatment (Fig. 4B). However, as shown in Figure 4C, overall the extent of BrdU labeling in the hypoxic models was significantly greater than that seen in the low EF5 tumors ($P = 0.0002$). This apparent paradox is accounted for by higher BrdU labeling in the better oxygenated regions of the hypoxic models, as seen in Fig. 4A. Hypoxia was also
associated with rapid growth ($P = 0.0002$) and with spontaneous metastasis formation ($P = 0.0003$).

**Hypoxia and apoptosis**

As shown in Supplementary Fig. 3A, cleaved caspase-3 labeling of individual cells showed a heterogeneous distribution that appeared similar in the EF5 negative and positive areas. However, regions surrounding necrotic areas were often densely populated by cleaved caspase-3 positive cells, as has been previously reported (7). When these regions were excluded, cleaved caspase-3 staining in viable tumor tissue of hypoxic models was significantly higher than EF5 low models ($P = 0.0005$, Supplementary Fig. 3B).

**Hypoxia and microvessel density**

Consistently in these models, blood vessels were organized in the stroma surrounding the tumor nests and did not invade into masses of tumor tissue (Supplementary Fig. 4A), as has been observed by our group and others using cell line-derived xenografts (39, 40). Microvessel density and the percentage of CD31 positive pixel density obtained from the replicate tumors of 16 models are shown in Supplementary Fig. 4B. No significant correlation between hypoxia and either marker of blood vessels was observed in these models.

**Hypoxia and cell differentiation**

Hypoxia has been reported to promote transition towards a more mesenchymal phenotype, associated with increased potential for metastasis (12, 15). We therefore searched for features of EMT in the hypoxic models. Representative dual immunofluorescence staining for EF5 and E-cadherin is shown in Supplementary Fig.
5A. In all models tested, the cells within the hypoxic regions showed epithelial features similar to the non-hypoxic regions, including maintenance of apical/basal polarity, surface expression of E-cadherin, and negative staining for vimentin. Single cell measurements by flow cytometry showed a striking heterogeneity in vimentin expression in the cancer cell population of some models, indicating a degree of plasticity towards expression of a mesenchymal marker (Supplementary Fig. 5B). However, vimentin expression was predominant in EF5 negative cells, indicating that it was not directly linked to hypoxia. Interestingly, vimentin-positive cells co-expressed the epithelial marker cytokeratin 19 (Supplementary Fig. 6). The significance of this finding is not known.

**Gene expression profiling of hypoxic vs. non-hypoxic models**

As shown in Fig. 4, there is a strong association between hypoxia, proliferation, and metastasis formation. Since rapid proliferation can cause hypoxia simply by pushing cells away from blood vessels, this might be sufficient to explain the more aggressive behavior of the hypoxic models, as illustrated in Schematic I, Fig. 5A. Alternatively, hypoxia tolerance might be acquired by some primary xenografts, allowing the accumulation of hypoxic cells that then undergo further reprogramming towards a more invasive or metastatic phenotype (Schematic II, Fig. 5A). To explore these alternatives, we selected a panel of genes involved in proliferation, survival, and adaptation to hypoxia that have been previously studied in pancreatic cancers, and compared the expression levels in the hypoxic vs non-hypoxic models. As shown in Fig. 5B, the hypoxic models showed highly significant increases in the expression of genes coding for proteins involved in cell cycle regulation (Aurora kinases A and B, Cyclin B1; $P<0.0001$, $P=0.0012$, $P=0.0002$, ...
respectively), consistent with their greater BrdU incorporation. However, we also found increased expression of genes involved in cell survival (e.g. Bcl-2, XIAP, and Survivin), DNA damage repair (BRCA2 and Rad51), and HIF-1α, which would favor the acquisition of hypoxia tolerance. Protein-protein interaction analysis also shows strong linkages between hypoxia, proteins involved in cell cycle regulation, and apoptosis (Fig. 6). Ten of the target proteins have genome maintenance function, and seven are cellular fate and organization. CASP3 directly connects to 6 other target proteins, while BCL2 connects to 5. Despite fewer direct connections, SMAD4 and MYC are the most central to the network (as analyzed by all-pairs-shortest-paths in NAViGaTOR). From the target proteins, the highest degree node in the network is MYC (438 interacting partners), HIF1A (296 interacting partners), and TGFBR1 (231 interacting partners).

**Discussion**

In this paper we identified striking associations between hypoxia and the aggressive features of rapid growth and high metastatic potential in a series of orthotopically-grown primary pancreatic cancer xenografts. The highly consistent results within replicate samples indicate that the underlying mechanisms have a transmissible (genetic or epigenetic) component. This suggests that they reflect to some extent biological characteristics of the cancers from which they were derived.

Deficiencies in neovascularization have been linked to the development of hypoxia in some xenograft models, and pancreatic cancers have long been known to show reduced perfusion with radiological contrast agents (41). However, we did not see a correlation between microvessel density and hypoxia in the primary pancreatic cancer xenografts,
suggesting that the failure to develop a vascular system is not directly linked to hypoxia in these tumors.

Within EF5 positive regions the uptake of BrdU was greatly decreased, indicating that hypoxia causes cell cycle arrest in these tumors. Although there are many previous reports showing this effect in other *in vivo* models (42, 43), we observed a striking increase in the uptake of BrdU in the EF5 negative regions of the hypoxic models, relative to the low hypoxia models, that correlated with their more rapid growth and greater metastatic potential. The hypoxic models also showed a highly significant increase in the number of cells expressing cleaved caspase-3, in both EF5-positive and -negative regions, suggesting that the more rapid cell proliferation was to some extent counterbalanced by increased cell loss to apoptosis.

Cell survival is compromised under hypoxic conditions. Hypoxia tolerance can occur due to mutation of genes such as p53 that enhance survival, or through metabolic adaptation in response for example to HIF transcription factors, or the unfolded protein response. Cells adapted to survive hypoxia might then undergo further alterations towards a phenotype with stem cell-like or invasive properties such as EMT that would favor metastasis. Although our results indicate that hypoxia is insufficient to produce EMT in pancreatic cancers, we cannot exclude the possibility that it may play a role under certain conditions.

The association between hypoxia and increased metastatic potential of pancreatic cancer is novel, and of major clinical relevance. However, the extent to which hypoxia is driving metastasis, rather than being the consequence of rapid proliferation, remains uncertain.

Rapid proliferation pushes cells away from blood vessels, so that they become hypoxic,
and eventually necrotic. Therefore it is possible that hypoxia is a bystander effect, and that biological aggression seen in the hypoxic models is being driven by proliferation. However, our preliminary analysis, shown in Fig. 5, appears to favor the acquisition of hypoxia tolerance, since the hypoxic models show increased expression of genes involved in survival and DNA damage response, as well as HIF1A whose transcriptional activation promotes hypoxia tolerance (8). Furthermore, in preliminary experiments we found that hypoxia persisted during treatment with a MEK inhibitor that potently inhibits cell cycle progression (29), suggesting that some degree of hypoxia tolerance exists in these models (Supplementary Fig. 7).

Detailed investigation of the possible genetic mechanisms linking hypoxia to aggressive growth is ongoing, including DNA sequencing as part of the International Cancer Genome Consortium Pancreatic Cancer Genome Project (44).

Clinical Implications

Our results predict that the presence of hypoxia in patients undergoing surgical resection for pancreatic cancer carries a high risk of recurrence. Although the follow up data are limited, we observed a trend for longer survival in the patient donors of the low hypoxia tumors (Supplementary Fig. 8). However, it should be noted that TNM stages for patients enrolled in this study are higher than is typical for patients undergoing pancreatectomy (45), probably because insufficient material for xenografting was available from early stage tumors, which may account for the relatively poor survival. In order to determine the prognostic significance of hypoxia in pancreatic cancer patients, we have started a clinical trial administering the nitroimidazole probe pimonidazole to patients undergoing pancreatectomy (46, 47). Because primary xenografts are being established from these
patients, this study will also allow us to determine the extent to which hypoxia in the patient samples is correlated with hypoxia measurements made in the corresponding primary xenografts.

Regardless of whether hypoxia plays a significant role driving metastasis, or is simply the consequence of rapid growth, BrdU negative cells in hypoxic regions are expected to be resistant to gemcitabine, the mainstay of adjuvant chemotherapy protocols, since this drug is incorporated during S-phase. Significantly, cell cycle arrest in relation to hypoxia occurs in early liver metastases from orthotopic primary pancreatic cancer xenografts (Fig. 4B). If these non-cycling cells are able to re-enter the cell cycle following the destruction of proliferating, better-oxygenated cells, then the tumor would be expected to repopulate. The overall effect of adjuvant treatment might therefore be to delay cancer recurrence, rather than eliminate all residual cancer cells, and results from randomized clinical trials suggest that this might indeed be the case (48). If so, a logical strategy would be to combine classical chemotherapy drugs like gemcitabine with bioreductive agents that show selective toxicity towards hypoxic cells (49, 50).

In conclusion, results shown in this paper point towards important mechanisms that link the development of hypoxia to biological aggression in pancreatic cancer patients. Elucidation of the underlying mechanisms is likely to suggest novel therapeutic interventions to improve patient outcome. However, if the results seen in the orthotopic primary xenografts are confirmed in pancreatectomy patients, they predict that improved patient outcome might be achieved by incorporating hypoxia-targeted agents into existing adjuvant or neoadjuvant treatment protocols, regardless of the underlying molecular mechanisms.
Acknowledgments

We thank Dr. Richard P. Hill, Dr. Steven Gallinger and Dr. Ming Tsao for many helpful discussions. We also thank Pinjiang Cao, Lucy Zhang, Jing Xu, Ni Liu, and Emin Ibrahimov for their excellent technical assistance.

References


Table 1. Primary pancreatic cancer specimens

<table>
<thead>
<tr>
<th>OCIP#</th>
<th>Diagnosis*</th>
<th>Stage†</th>
<th>K-ras mutation‡</th>
<th>p53 IHC§</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>Ductal ADC, MD</td>
<td>T3N1b</td>
<td>K12, GAT</td>
<td>-</td>
</tr>
<tr>
<td>18</td>
<td>Ductal ADC, MD</td>
<td>T3N0</td>
<td>K12, GAT</td>
<td>-</td>
</tr>
<tr>
<td>19</td>
<td>Ductal ADC, MD</td>
<td>T3N1b</td>
<td>K12, GAT</td>
<td>-</td>
</tr>
<tr>
<td>20</td>
<td>Ductal ADC, WD</td>
<td>T3N1b</td>
<td>K12, GTT</td>
<td>+</td>
</tr>
<tr>
<td>21</td>
<td>Ductal ADC, MD</td>
<td>T3N0</td>
<td>wild type</td>
<td>-</td>
</tr>
<tr>
<td>23</td>
<td>Ductal ADC, PD</td>
<td>T3N1b</td>
<td>K12, CGT</td>
<td>+</td>
</tr>
<tr>
<td>24</td>
<td>Ductal ADC, MD</td>
<td>T3N1a</td>
<td>K12, GAT</td>
<td>+</td>
</tr>
<tr>
<td>28</td>
<td>Ductal ADC, WD</td>
<td>T3N0</td>
<td>K12, GTT</td>
<td>-</td>
</tr>
<tr>
<td>30</td>
<td>Ductal ADC, MD</td>
<td>T3N1a</td>
<td>K12, CGT</td>
<td>-</td>
</tr>
<tr>
<td>34</td>
<td>Mucinous Cyst ADC &amp; Inv. ADSQ</td>
<td>T3N1b</td>
<td>wild type</td>
<td>+</td>
</tr>
<tr>
<td>36</td>
<td>Ductal ADC, PD</td>
<td>T2N0</td>
<td>wild type</td>
<td>-</td>
</tr>
<tr>
<td>37</td>
<td>Ductal ADC, MD</td>
<td>T3N1b</td>
<td>K12, GAT</td>
<td>-</td>
</tr>
<tr>
<td>38</td>
<td>Ductal ADC, MD</td>
<td>T3N0</td>
<td>K12, GTT</td>
<td>-</td>
</tr>
<tr>
<td>40</td>
<td>Ductal ADC, MD</td>
<td>T3N1b</td>
<td>K12, GTT</td>
<td>-</td>
</tr>
<tr>
<td>51</td>
<td>Ductal ADC, MD</td>
<td>T4N0</td>
<td>K12, GAT</td>
<td>-</td>
</tr>
<tr>
<td>52</td>
<td>Ductal ADC, WD</td>
<td>T3N0M1</td>
<td>K12, GAT</td>
<td>-</td>
</tr>
</tbody>
</table>

* ADC, adenocarcinoma; ADSQ, Adenosquamous carcinoma; WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated.

† TNM classification of tumors of the exocrine pancreas: T2, tumor limited to the pancreas, more than 2cm in greatest dimension; T3, tumor extends directly into any of the following: duodenum, bile duct, peripancreatic tissues; T4, tumor extends directly into any of the following: stomach, spleen, colon, adjacent large vessels; N0, no regional lymph node metastasis; N1a, metastasis in a single regional lymph node; N1b, metastasis in multiple regional lymph nodes.

‡ mutation occurs in “codon 12, base transition”.

§ p53 IHC, immunohistochemical staining for p53; “+”, positive staining; “-”, negative staining.
Figure Legends

Figure 1. Orthotopic implants closely resemble the histology of the original surgical samples. Histological sections of the original patient sample (left) and the orthotopically grown primary pancreatic cancer xenografts (right) stained with haematoxylin and eosin (H&E). Scale bars = 100 μm.

Figure 2. Growth rates correlate with BrdU incorporation. (A) Tumor growth (defined by number of days between orthotopic implantation and growth to ~1cm diameter) vs. BrdU incorporation (assessed by immunofluorescence analysis) for 16 models. Each data point represents the mean value of 6 replicate tumors. Error bars = 95% confidence intervals. R² = 0.5842; Pearson r = -0.7643; 95% confidence interval = -0.9138 to -0.4324; P = 0.0006. (B) Spontaneous formation of liver metastases (arrowed) from OCIP51, grown at the orthotopic site (left). H&E staining of OCIP51 primary tumor (upper right) and liver metastasis (lower right). Scale bars = 200 μm. (C) Tumor growth (left, P = 0.0002) and BrdU incorporation (right, P = 0.0003) in non-metastatic and metastatic models. Each data point represents the mean value of 6 replicate tumors. Error bars = 95% confidence intervals.

Figure 3. EF5 measurements by flow cytometry. (A) Representative plots of EF5 labeling vs. DNA content for the 16 primary xenografts. Single-cell suspensions of 16 primary xenografts were fixed, stained with 10 μg/ml EF5-Cy5 conjugates and 1 μg/ml DAPI, and analyzed by flow cytometry. EF5 staining was low or absent in OCIP16, 18, 19, 24, 30 and 40 (top panel), whereas OCIP20, 21, 23, 28, 34, 36, 37, 38, 51, and 52 showed a heterogeneous distribution of positive staining both in the aneuploid cancer
cells, and in the normal mouse stroma. (B) Quantification of EF5 staining by flow cytometry in 6 replicate tumors from 16 primary xenografts.

**Figure 4.** Hypoxia and BrdU labeling. (A) Dual immunofluorescence staining for EF5 and BrdU of OCIP16 (left) and OCIP21 (right). Hypoxic regions stained for EF5 (OCIP21) were morphologically similar to the EF5-negative regions, but the BrdU labeling was much lower. Scale bars = 100 μm. (B) Dual immunofluorescence staining for EF5 and BrdU of OCIP51 primary tumor (left) and liver metastasis (right). Scale bars = 100 μm. (C) BrdU labeling vs. EF5 (left) and tumor growth vs. EF5 (right) for 16 models. Each data point represents the mean value of 6 replicate tumors. Error bars = 95% confidence intervals.

**Figure 5.** Gene expression profiling of hypoxic vs. non-hypoxic models. A. Hypothetical mechanisms to explain the correlations between hypoxia, proliferation, and metastasis. B. mRNA expression of genes involved proliferation, survival, and adaptation to hypoxia by qPCR. P values of comparison of hypoxic (solid black columns) and non-hypoxic models were shown. Each data point represents the mean value of 3 replicate tumors. Error bars = 95% confidence intervals.

**Figure 6.** Protein-protein interaction map of qPCR gene validation set. Network comprises 1,562 proteins and 24,389 interactions (partially transparent to reduce complexity), of which 18 target proteins are connected by 28 direct interactions (thick, black lines). Note that two variants of CDKN2A are listed. Node shape corresponds to GeneOntology biological function as per legend, and 18 target proteins are organized on the circle according to GeneOntology. Node size corresponds to fold difference between hypoxic and non-hypoxic samples based on normalized CT values, as per legend and
methods. Transparency was set to p-value of the difference, as per legend. All other nodes are viewed as small circles, and made transparent to reduce network complexity.
Figure 3

A

OCIP16  OCIP18  OCIP19  OCIP24  OCIP30  OCIP40

OCIP20  OCIP21  OCIP23  OCIP28  OCIP34

EF5

OCIP36  OCIP37  OCIP38  OCIP51  OCIP52

DNA content

B

% Positive EF5 staining

25
20
15
10
5
0

OCIP16  OCIP18  OCIP19  OCIP20  OCIP21  OCIP23  OCIP24  OCIP28  OCIP34  OCIP36  OCIP37  OCIP38  OCIP51  OCIP52

Xenograft models
Figure 4

A

OCIP16  OCIP21

B

OCIP51  Pancreas  OCIP51  Liver

C

[Graph showing growth and DNA content for different samples with markers for metastatic and non-metastatic cases]
Figure 5

A

1. Genetic alteration

Hypoxia; cell cycle arrest

Proliferation

Metastasis

II

Genetic alteration

Hypoxia tolerance; cell cycle arrest

Proliferation

EMT?

stem cell survival

Metastasis

B

Graphical data showing gene expression levels under non-hypoxic and hypoxic conditions in xenograft models.
Hypoxia predicts aggressive growth and spontaneous metastasis formation from orthotopically-grown primary xenografts of human pancreatic cancer

Qing Chang, Igor Jurisica, Tevor Do, et al.

Cancer Res  Published OnlineFirst February 22, 2011.