Published in final edited form as:

Pancreas. 2011 July ; 40(5): 730–739. doi:10.1097/MPA.0b013e31821ae25b.

Increased expression of DNA repair genes in invasive human pancreatic cancer cells

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Abstract

Objective—Pancreatic cancer was the fourth leading cause of cancer death in the United States in 2009. Recurrence of disease following resection occurs due to neoplastic cell survival. To better understand these highly aggressive cells, gene expression microarrays were performed.

Methods—Using the established lines HPAC and PANC1 and a Matrigel assay, genome expression arrays were performed to analyze patterns between invasive and total cells.

Results—Significant increases in the expression of genes related to DNA repair were observed. A number of the same genes also demonstrated an increase in expression when comparing bulk cells to a putative tumor initiating cell (TIC) population. The TIC population was isolated using the spheroid technique, and compared to bulk cells, spheroid cells functionally repair breaks in DNA faster after challenged with the drug gemcitabine. Finally, using OncoPrint, we observed a significant increase in DNA copy number of *BRCA1* and *RAD51* in tissue isolated from metastatic pancreatic cancer compared to tissue isolated from the primary site.

Conclusions—From these data we conclude that the most invasive cells within a pancreatic tumor are able to thrive due to their increased genomic stability. These cells have also been linked to the TIC population in a tumor.

Keywords

Pancreatic cancer; invasion; DNA repair; gemcitabine; tumor initiating cells

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Supplemental digital contents are available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the Journal's Web site (www.pancreasjournal.com).

Introduction

The American Cancer Society estimated that 43,140 Americans will be diagnosed with pancreatic cancer in 2010 in the United States [1], and the disease remains the fourth leading cause of cancer death overall. An even more striking statistic is that in the same year, 36,800 people are predicted to die as a result of this disease [1]. Surgical resection is the best method for long-term survival in patients, however, only about 15% of the patients are diagnosed early enough to be eligible for surgery, and furthermore, 80% of those patients who will undergo surgery will have a recurrence of the disease within two years [1]. This percentage remains high due to the aggressive nature of pancreatic cancer and the ability of highly invasive cells to resist current treatment regimes [2–4]. These highly invasive cells are then able to survive and metastasize to other vital organs, which is uniformly fatal in patients [2]. A vast majority of the patients diagnosed with pancreatic cancer receive the nucleoside analog drug gemcitabine as part of their treatment regime [5]. In the last few years, however, it has become increasingly evident that combination therapy with gemcitabine is much more effective at increasing patient survival [6]. This finding is due to the survival and expansion of gemcitabine resistant populations of cells [6].

We sought to investigate the gene expression profile of a rare population of very invasive cells using an *in vitro* Matrigel assay and defined media [7]. Previous work using prostate cancer cell lines demonstrates that these highly invasive cells have a stem-like phenotype [7], have undergone an epithelial to mesenchymal transition (EMT) during the process of invasion, and are also highly tumorigenic when injected into mice. Within the last 6 years, it has been well documented that only a small fraction of epithelial tumor cells, termed tumor initiating cells (TIC), have the ability to form colonies *in vitro* or to initiate a new tumor upon injection into a host *in vivo* [8]. TICs share some properties which are similar to a true stem cell, such as the ability to undergo self-renewal, they exhibit higher rates of proliferation and under the right conditions can undergo differentiation.

To date, TICs have been isolated from almost every solid tumor type including those from the bladder, brain, breast, colon, head/neck, liver, lung, ovary, pancreas, prostate, and skin [9–13]. In the past few years, TICs have been isolated from pancreatic cancers by FACS for expression of CD44⁺/CD24⁺/ESA⁺ as well as CD133⁺ [14]. The CD133⁺ subpopulation of cells were exclusively tumorigenic and highly resistant to treatment with gemcitabine [15]. Additionally, TICs can be isolated by generating pancreatic spheroids using specialized culture conditions and highly defined media called stem cell media (SCM) [16–19]. The pancreatic spheres express higher levels of the stem cell markers CD44 and CD133, and demonstrate higher tumorigenic potential in animals compared to total cells [18, 19].

The established pancreatic cell lines HPAC and PANC1 were used in our *in vitro* Matrigel assay and microarray analysis was performed in order to compare gene expression profiles of invasive to non-invasive cells. In the invasive cells, we observed significant increases in gene expression in a large number of genes related to DNA repair, specifically genes involved in the BRCA1-mediated DNA repair. Additional genes identified are classified as members of hereditary breast cancer susceptibility signaling including *ATM*, *RAD50* and *RAD51*, *PTEN* and a number of genes belonging to the Fanconi anemia family which have previously been linked to DNA repair mechanisms [20, 21]. Similar genes were found to be increased in the putative pancreatic tumor initiating cell (TIC) population isolated from spheroid cultures compared to the total cell lines used. The spheres were generated using both established PANC1 cells and a cell line generated from primary patient material termed Panc4.14. Functionally, we also observed an increase in the ability of the TICs to repair DNA after challenge with gemcitabine compared to the total population of cells.

We have demonstrated that an invasive population of pancreatic cancer cells have a generalized increased expression of DNA repair genes, including BRCA1. This observation was further extended to the tumor initiating cell model of pancreatic cancer.

Materials and Methods

Cell Lines and Reagents

HPAC and PANC1 human pancreatic cancer cell lines were obtained from ATCC (Manassas, VA, USA) and both were cultured according to the manufacturer's instructions. (Cell Line Verification Test Recommendations, ATCC Technical Bulletin No. 8 (2008)). The primary patient line Panc4.14 was obtained as a kind gift from Dr. Elizabeth Jaffee at Johns Hopkins University, Baltimore, MD and cultured accordingly [22]. Stem cell media (SCM) was prepared using DMEM/F12 with human supplementation of 10 ng/mL bFGF, 20 ng/mL EGF and 5 µg/mL along with 0.4% BSA (each from Sigma, St. Louis, MO, USA) and insulin-transferrin-selenium (ITS) (Sigma). Gemcitabine was diluted in DMSO and obtained from Selleck Chemicals, Houston, TX, USA.

Matrigel Invasion Assay

Matrigel-coated 24-well inserts (8 µm pore size) and non-coated control inserts purchased from BD Biosciences Clontech (Palo Alto, CA, USA) were used as previously described [7]. The HPAC and PANC1 cell lines were used. For staining, the Diff Quick staining kit was used according to the manufacturer's instructions (Dade Bearing, Inc., Newark, DE, USA).

Microarray analysis

RNA was isolated and labeled as previously described [17], with the following modifications. Reverse transcriptase was heat inactivated at 65°C for 10 minutes followed by RNase A RNA degradation at room temperature for 30 min. Universal Reference RNA (Stratagene, La Jolla, CA, USA) was labeled with Cy3-dUTP and experimental samples were labeled with Cy5-dUTP. Samples were hybridized to an Agilent whole genome gene expression array following manufacturer's directions. Arrays were scanned using a GenePix 4000B scanner (Molecular Devices, Sunnyvale, CA, USA) and analyzed using mAdb software (National Cancer Institute, Bethesda, MD, USA - <http://madb.nci.nih.gov/>). The arrays were analyzed using Cluster and Treeview offered by Michael B. Eisen as freeware (<http://rana.lbl.gov/EisenSoftware.htm>). The gene lists were imported to the Ingenuity Software for further analysis (Ingenuity Systems, Redwood City, CA, USA - <http://www.ingenuity.com/>).

Immunofluorescence

labeling of invasive or non-invasive cells was performed directly on the Matrigel membrane as previously described [7] using the BRCA1 antibody (Santa Cruz, Santa Cruz, CA, USA) and secondary goat anti-rabbit 800 channel (Molecular Probes, Carlsbad, CA, USA). Images were analyzed using the Zeiss LSM5 Image Browser and further prepared in Adobe Photoshop CS (version 4). Increased levels of nuclear BRCA1 are seen in invasive cells compared to the non-invasive cells on top of the membrane (20X). Zoomed cell from invasive overlay panel where a number of BRCA1 foci are seen (40X). Staining was performed in duplicate on separate days to verify the results.

Proliferation Assays

Cells were seeded overnight in a 96 well plate in 100 µL of regular media at a density of 2000 cells per well. Cell proliferation was measured using the CellTiter-Glo assay from Promega (Madison, WI, USA) after 24 hours or 5 days using 100 µL of reagent and an

incubation time of 20 minutes. The relative luciferase units (RLU) were quantified using a Tecan Infinite 200 plate reader. Samples were performed using an N of 8.

Pancreatic Sphere Formation Assays

PANC1 cells and Panc4.14 were seeded 1000 cells per mL in SCM supplemented with (insulin, transferring and selenium) ITS (Sigma) and 1% KO-Serum Replacement (Gibco, Carlsbad, CA, USA) in non-adherent 6 well plates coated with Hydrogel (Corning Life Sciences, Chemsford, MA, USA). The pancreatic spheres were generated for 14–18 days and then harvested for RNA isolation using Trizol (Invitrogen, Carlsbad, CA, USA).

Florescent Activated Cell Sorting/Analysis

PANC1 cells were washed in FACS buffer (PBS plus 1–2% BSA and 5 mM EDTA) and incubated at 4 °C for 15 minutes in the dark with 10 μ l/10⁶ cells of anti-CD133 conjugated APC (Miltenyi Biotec, Auburn, CA, USA). Cells were washed 3X in FACS buffer, resuspended at 1 $\times$ 10⁶ cells/ml, and surface expression was determined using the Canto (BD, San Jose, CA, USA). Cells were gated using a live-dead stain (propidium iodide-PI from BD) on the X-axis and dead cells in P3 were excluded from the percentage of CD133 positive cells. The assay was performed in duplicate and similar percentages were obtained.

Comet Assay

Alkaline comet assays were performed according to the manufacturers' instructions (Trevigen, INC., Gaithersburg, MD, USA). Data analysis was performed using TriTek Comet Score Freeware v1.5 software (TriTek, Corp. Sumerduck, VA, USA). Images were acquired using an Olympus IX70 inverted microscope and a camera using MagnaFire SP software (version 1.0 $\times$ 5) at 20X. Samples from 2 individual experiments were scored.

Quantitative real time polymerase chain reaction (qRT-PCR)

Total RNA was isolated using TRIzol (Invitrogen Corporation, Carlsbad, CA, USA). RNA from non-invasive cells was isolated using a cell pellet acquired from trypsinizing cells from one membrane after invasive cells were removed with a cotton swab. Conversely, RNA from the invasive cells was isolated by combining three membranes where the non-invasive cells were removed using a cotton swab. The membranes were pooled and placed in TRIzol for 10 minutes at room temperature, and the conventional procedure for isolation of RNA was then followed. To increase the yield of RNA, 5 μ g of linear acrylamide (Ambion, Austin, TX, USA) was added prior to precipitation of RNA with isopropanol. Additionally to increase overall yield, 100ng of RNA was amplified using the MessageAmp aRNA Amplification Kit (Ambion, Austin, TX, USA). cDNA was prepared using the SuperScript®III First-Strand Synthesis System (Invitrogen Corporation, Carlsbad, CA, USA). Quantitative real time polymerase chain reaction (qRT-PCR) analysis was performed using a StepOne Real-time PCR machine (Applied Biosystems, Foster City, CA, USA) with TaqMan Gene Expression Assay reagents and probes (Applied Biosystems). A total of 4 μ L of cDNA was used in a 20 μ L reaction resulting in a 1:5 dilution. The following FAM labeled human probes were used: BRCA1 (Hs00173237_m1), RAD51 (Hs00153418_m1), CD44 (Hs00174139_m1), CD24 (Hs00273561_s1), CD133 (Hs01009260_m1), CXCR4 (Hs00237052_m1), ESA (Hs00158980_g1) and 18S rRNA (Hs99999901_s1). Relative fold induction of mRNA was compared between non-invasive and invasive cells using the Delta-Delta CT method of quantitation, and 18S rRNA was used as a loading control. Samples are representative of 3 individual experiments.

Meta-analysis on patient databases

Oncomine 4.2 database analysis tool is available with a subscription at <http://www.oncomine.org>. Selected data was compared for DNA copy number in pancreatic primary tumor samples as well as their respective metastatic specimens.

Results

The HPAC and PANC1 cell lines were used to perform the *in vitro* Matrigel assay and to isolate a population of highly invasive cells [7, 23]. The defined media (SCM) was used as a chemoattractant and after 24 hours both the non-invasive and invasive populations of cells were collected and their RNA isolated. Microarray analysis was performed and genes which demonstrated more than a 1.8-fold increase (log-transformed value of 0.8 or higher) between non-invasive and invasive cells were selected for further analysis. To determine if the assay was selecting for a population similar to that seen in the prostate model, we confirmed an increase in expression of the traditional ‘stemness genes’ such as CD44, CD24, ESA, Nanog, SOX2 and OCT3/4 (see Supplementary Figure 1, Supplemental Digital Content 1, <http://links.lww.com/MPA/A53>). Further analysis of the microarray data determined that a number of the genes demonstrating an increase in expression within the invasive cells belong to DNA repair pathways (see Supplementary Table, Supplemental Digital Content 2, <http://links.lww.com/MPA/A55>; see Supplementary Table, Supplemental Digital Content 3A and 3B, <http://links.lww.com/MPA/A56>). When inputting our gene list to the Ingenuity pathway analysis software the top functional pathways included genes regulating the cell cycle and DNA replication, recombination and repair (Figure 1A). The canonical pathways demonstrating the most gene members from our list included the role of BRCA1 in DNA damage response, hereditary breast cancer signaling and the role of CHK proteins in cell cycle checkpoint control (Figure 1B). Further examination of the BRCA1 pathway demonstrates increases in not only BRCA1 itself in the invasive cells, but also an increase in a significant number of its direct binding partners including RAD50 and RAD51, MSH2 and MSH6, as well as a number of members belonging to the Fanconi anemia gene family (FANCA, FANCE and FANCG), and the protein kinase cell cycle regulators ATM and ATR (Figure 2). Immunocytochemistry (ICC) of BRCA1 foci best confirms increased BRCA1 expression in the invasive cells compared to non-invasive population (Figure 3). Real-time quantitative PCR (qRT-PCR) was also performed, but only very slight changes were observed (data not shown) and protein lysates did not demonstrate very high expression of nuclear BRCA1, even in control samples (data not shown). We attribute this to our low cell numbers, further solidifying our rationale for using ICC.

This is of significant interest because these invasive cells are being linked to emerging tumor initiating cell (TIC) hypothesis, making their patterns of regulation very attractive for biomarker analysis and therapeutic development in further studies [7, 23–25]. Microarray analysis was performed comparing gene expression of total PANC1 cells and a primary patient cell line termed Panc4.14 to their TIC populations isolated via a sphere formation assay (Cabarcas et al., unpublished)¹. Pancreatic spheres have been previously shown to express higher levels of the stem cell markers CD44 and CD133, and demonstrate higher tumorigenic potential in animals compared to total cells [18, 19].

A number of the same genes were increased in both the invasive populations and the TIC fraction isolated from PANC1 cells (see Supplementary Table, Supplemental Digital Content 4, <http://links.lww.com/MPA/A58>), including an average of 1.28-fold increase in

¹Stephanie M. Cabarcas, Lesley A. Mathews, Suneetha B. Thomas, Elaine M. Hurt, Elizabeth M. Jaffee, Ralph H. Hruban and William L. Farrar. Characterization of a unique population of pancreatic cells from primary patient material.

BRCA1 and 4.94-fold in MSH2. When comparing microarray data of total Panc4.14 cells and spheres, there was also a 2.09-fold increase of BRCA1 expression (unpublished, Cabarcas et al.) 1. This further supports that the BRCA1-mediated pathway is playing a critical role in regulating the TICs population, not only from established cell lines, but also from primary patient tissue.

In order to determine if the spheroid cells actually did in fact have an increased ability to repair DNA, we wanted to challenge the cells and quantify the amount of repair occurring. To first ensure that our cultures were behaving in a manner similar to previously published reports, we cultured total cells in the drug gemcitabine for 5 days and measured the expression of the putative pancreatic TIC marker CD133. It has been well documented that culturing total cells in gemcitabine for an extended period of time results in the expansion of a population of gemcitabine-resistant cells expressing higher levels of CD133 [3, 26, 27]. As seen in Figure 4, sub-lethal doses of gemcitabine (near the IC50s of each line used) resulted in an increase in the expression of CD133 as measured by FACS analysis for both HPAC and PANC1 cells (Figure 4A and 4B, respectively). Higher doses lead to a decrease in the expression in the HPAC cells compared to lower the lower dose, and we believe this was due to the onset of cell death (Figure 4A). PANC1 cells, however, which have an even higher IC50, had a further increase in CD133 expression.

To determine differences in DNA repair, alkaline based comet assays were performed using PANC1 total cells and cells isolated from spheroids. The cells were treated with 500 nM gemcitabine for only 24 hours, harvested and run using the Trevigen Comet Assay kit (Figure 5C–F). To ensure that the cells would still be viable, a cell proliferation assay was conducted at the 24 hour time point, as well as after long term culture for 5 days (Figure 5A and 5B, respectively). It is clear that the drug does take longer than 24 hours to have drastic effects on cell survival and number. With regards to the comets, they appear much longer in total (adherent) cells compared to the putative TICs isolated from the spheroid cells (Figure 5C). Some background damage was observed when treating with DMSO alone in both groups of cells, but less in the spheres (Figure 5C). Untreated cells were also run and little to no comets were observed (data not shown). Quantification of the assays determined that although the amount of DNA did not change between spheres and total cells (Figure 5D), the olive tail moment (Figure 5E) and tail area (Figure 5F) was much higher in the adherent population, yet only significant differences were observed for the olive tail moment. A number of the adherent samples did not demonstrate very large tails and as a result the software scores the area as zero (Figure 5F). Furthermore, treating the spheres with high amounts of gemcitabine for 24 hours actually resulted in even higher levels of the DNA repair gene BRCA1 and the TIC markers of pancreatic cancer CD44, CXCR4, CD133 and ESA (Figure 6).

Finally, to determine if our observations had merit in human specimens of pancreatic cancer we interrogated the Oncomine analysis software. Data was selected from a study conducted by Harada et al. because this study was an integrated molecular profiling of DNA copy number in pancreatic cancer samples[28]. In this work, a significant increase in the DNA copy number of *BRCA1* ($p=0.007$), *FANCI* ($p=0.018$) and *RAD51* ($p=0.033$) were found to correlate with the aggressiveness of the sample (see Supplementary Figure, Supplemental Digital Content 5, <http://links.lww.com/MPA/A59>). We also compared our gene findings to a panel of genes identified as a ‘death-from-cancer’ signature[29] and these genes did not demonstrate significant increases in DNA copy number within metastatic pancreatic tissue (see Supplementary Figure, Supplemental Digital Content 5, <http://links.lww.com/MPA/A59>).

Overall, we demonstrate some very compelling data demonstrating that the invasive pancreatic cancer cells have increased levels of a number of DNA repair genes, including BRCA1. These invasive cells have been linked to the tumor initiating cell/cancer stem cell hypothesis. These TICs are enriched in culture upon treatment with the front line pancreatic cancer drug gemcitabine. Our data demonstrates that this could be supported by their ability to rapidly repair breaks induced in the DNA, leading ultimately to their survival in patients.

Discussion

Pancreatic cancer is one of the most lethal of all of the solid malignancies. The lethality of this disease occurs because most patients are only first diagnosed with late stage disease. Although treatment regimes are advancing, and clinicians are gaining information from basic research on how to better attack pancreatic neoplasms, the biology regulating the most aggressive cells within the tumor remains largely unknown. It is thought that these aggressive cells are also the most invasive cells, and are able to survive and metastasize to other vital organs, leading to fatality in patients [2–4]. In order to investigate the molecular pathways that could be regulating these highly aggressive cells, we performed gene expression arrays on a sub-population of highly invasive cells isolated from two established pancreatic cancer cell lines.

From the microarray analysis, we have identified that invasive pancreatic cancer cells have a significantly elevated level of DNA repair genes. We verified this observation using a BRCA1 foci assay (Figure 3) since only slight increases were observed using qRT-PCR (data not shown). The foci assay is more accepted for verification of BRCA1 activity in the DNA repair field [30, 31]. This observation was interesting due to the recent identification of germline mutations in the DNA repair gene partner and localizer of BRCA2 (*PALB2*) in families with pancreatic cancer [32]. This data would suggest that mutations in DNA repair genes could possibly lead to the onset of pancreatic cancer due to inefficient maintenance of genomic stability. Our data suggests that the most invasive and aggressive cells within a pancreatic tumor actually have more efficient DNA repair ability due to elevated levels of genes in the major repair pathways.

Likewise, when comparing the gene expression profiles of the invasive cells to the putative TICs pool, (isolated using the spheroid method from the total population of cells), a number of the same genes were elevated (see Table, Supplemental Digital Content 4). Pancreatic spheres are an accepted model for the isolation of putative TICs and have been previously shown to express higher levels of the stem cell markers CD44 and CD133, and demonstrate higher tumorigenic potential in animals compared to total cells [18, 19].

Our data nicely coincide with the observation that the most invasive cells are being linked to the TIC populations many groups have been isolating and observing [33]. With regards to the TIC hypothesis, as early as 1950, Klein and coworkers determined that serial transplantation of cells *in vivo* could give rise to primary tumors and metastases (reviewed in [34]). This was one of the first observations that a heterogeneous population of cells within a tumor has stem-like plasticity and multipotency. Within the last number of years, however, it has been well documented that only a small fraction of epithelial tumor cells have the ability to form colonies *in vitro*, or to initiate a new tumor upon injection into a host *in vivo* [8, 35–39]. These cells have been termed the tumor-initiating cells (TICs) or cancer stem cells (CSCs) within the tumor. To date, TICs have been isolated from almost every solid tumors type including those from the bladder, brain, breast, colon, head/neck, liver, lung, ovary, pancreas, prostate, and skin [9–13].

Several markers and methods exist to identify pancreatic TICs. For example, expression of CD44, CD24, CD133, epithelial specific antigen (ESA) and flow cytometry-based 'side-populations' are all associated with pancreatic CSCs reviewed in [4, 40, 41]. To date, the best FACS based protocol used to separate these cells from human tissue has been to harvest the CD44⁺, CD24⁺ and ESA⁺ triple positive populations [39]. These cells only represent about 0.2–0.8% of the total pancreatic cancer cells, yet when as few as 100 were injected into a xenograft mouse model they were able to generate tumors [39]. Additionally, in human and mouse models, CD133 has also been used to isolate and identify pancreatic TICs [15, 42, 43]. This is not surprising given that CD133 positivity has been previously used to isolate TICs from the breast, brain, liver, colon and prostate [40]. In our model of invasion, we see an increase in expression of a handful of these markers (Supplemental Figure 1), and it has been well documented that spheroid cultures isolated from PANC1 cells do as well [14, 27]. Using qRT-PCR we did not see a significant increase in CD133 expression in spheres compared to adherent cells unless the spheres were challenged with gemcitabine (Figure 6B). This observation is not in line with the mouse data mentioned above and is most likely because the assay was only performed after 24 hours. CD133 is more easily detected at lower levels by FACS analysis and not using qRT-PCR or Western blotting.

In line with our data, it is thought that the TIC population possesses a superior DNA repair profile compared to the bulk tumor cells, and thus are able to evade many chemotherapy regimes [44]. The comet assay demonstrates that the spheres had significantly fewer comets after 24 hours of treatment with gemcitabine, compared to the bulk population of tumor cells (Figure 5). Additionally, the observation that treatment of spheres with gemcitabine resulted in an increase in the DNA repair gene BRCA1, as well as the putative pancreatic TIC markers further supports the notion that new therapies besides gemcitabine need to be developed to fully eradicate these cells. This observation is present in other TIC models. For example, it has recently been shown that putative human glioma TICs expressing CD133 demonstrate higher levels of DNA repair and radioresistance compared to cells not expressing CD133 [45]. Furthermore, in regards to normal stem cells, normal multipotent stem cells from the hair follicle bulge have two important mechanisms for increasing their resistance to DNA-damage-induced cell death: higher expression of the anti-apoptotic gene Bcl-2 and transient stabilization of p53 after DNA damage in bulge stem cell compartment [46]. These data further support that both normal and cancer stem cells have evolved a more efficient DNA repair mechanisms to help increase their overall survival. Further investigation using additional patient material will determine how effective the DNA repair signature will be at identifying aggressive cells within a pancreatic tumor.

Finally, the Oncomine data demonstrates a significant increase in the DNA copy number of *BRCA1*, *FANCI* and *RAD51* in metastatic pancreatic specimens compared to primary tumor specimens (see Figure, Supplemental Digital Content 5). This further supports that these DNA repair genes are playing a role in regulating the aggressive nature of this cancer. Compared to our gene list, the 'death-from-cancer' signature [29] did not demonstrate significant increases in DNA copy number within metastatic pancreatic tissue (Supplementary see Figure, Supplemental Digital Content 5). In breast and prostate cancer, this analysis predicts that cancer cells manifesting a stem cell-like expression profile correlate with a decrease in patient survival [29]. Although we did not observe this at the DNA copy level in aggressive pancreatic tissue, it might be present at the gene expression level. Oncomine, however, does not have gene expression data of this nature available at this time to conduct this comparison.

Overall, we have demonstrated that an invasive population of pancreatic cancer cells have an increased expression of DNA repair genes, including BRCA1. This observation was

further extended to the tumor initiating cell model of pancreatic cancer, which will greatly impact the development of new therapies being designed to eradicate this disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank Ralph H. Hruban from Johns Hopkins University for providing support with acquiring primary patient material. We would like to also thank Roberta Matthai, Kathleen Noer, and Guity Mohammadi for excellent technical assistance with FACS.

This project has been funded in whole or in part with federal funds from the National Cancer Institute, National Institutes of Health, under Contract No. HHSN261200800001E. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government. This Research was supported in part by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research.

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Figure 1. Ingenuity analysis of gene expression data demonstrating significant changes in A) functional gene pathways and B) canonical signaling pathways in invasive cells

Figure 2. Ingenuity analysis demonstrating significant changes in 'Role of BRCA1 in DNA Damage Response' pathway in invasive cells
Genes highlighted in red demonstrate a significant increase in expression in invasive cells compared to their non invasive counterpart

Figure 3. Increase in BRCA1 protein expression by immunocytochemistry in invasive PANC1 cells

Staining of invasive or non-invasive cells was performed directly on the Matrigel membrane. Cells were incubated with either anti-BRCA1 antibody overnight, washed 3X with PBS and incubated with goat anti-rabbit Alexa-488 for 1 hour at room temperature. Membranes were mounted on glass slides with Vectashield containing DAPI and visualized with a Zeiss-510 L5 confocal microscope. Images were analyzed using the Zeiss LSM5 Image Browser and further prepared in Adobe Photoshop CS. Increased levels of nuclear BRCA1 are seen in invasive cells compared to the non-invasive cells on top of the

membrane (20X). Zoomed cell from invasive overlay panel where a number of BRCA1 foci are seen (40X).

Figure 4. FACS analysis of CD133 expression in HPAC and PANC1 gemcitabine treated cells
Cells were treated for 24 hours with either DMSO, 500 nM or 3 μ M gemcitabine. The cells were stained with anti-CD133 conjugated APC and surface expression was determined using the Canto analyzer. Cells were gated using a live-dead (propidium iodide-PI) stain on the X-axis and dead cells in P3 were excluded from the percentage of CD133 positive cells. **A)** HPAC cells and **B)** PANC1 cells.

Figure 5. Quantification of DNA repair between PANC1 spheres and total cells

A) PANC1 cells were treated with gemcitabine for either 24 hours or **B)** 5 days and proliferation assays (Cell-Titer Glo) were carried out. **C)** Alkaline comet assays (Trevigen kit) were performed on PANC1 spheres or total cells treated with gemcitabine for 24 hours. Images were captured using Olympus IX70 inverted microscope and MagnaFire SP (version 1.0x5) at 20X. Data analysis was performed using TriTek Comet Score Freeware v1.5 software. Differences in the % DNA in the comet Tail were measured (**D**), as well as the overall olive tail moment (**E**) and tail area (**F**). *represents a p-value of < 0.05 comparing DMSO to treated cells. ** demonstrates $P < 0.05$ comparing gemcitabine treated adherent cells to gemcitabine treated sphere cells.

Figure 6. Expression of DNA repair genes and CSC markers in cells

Relative fold induction of mRNA was compared between adherent total cells (A) and spheres (S) treated with or without 3 μ M gemcitabine for 24 hours. Total RNA was isolated using TRIzol and qRT-PCR analysis was performed using a StepOne Real-time PCR machine with TaqMan Gene Expression Assay reagents and probes. Isolation of DNA and cDNA from cells was carried out as previously described in materials and methods. Fold induction was calculated using the Delta-Delta CT method where the adherent cells were set at 1.0 as the control, and 18S rRNA was used as a loading control. Data is shown as transformed log₂. A Two-way ANOVA with a Bonferroni post-test was performed to compare groups and * represents a p-value of < 0.05 comparing adherent to DMSO treated spheres and ** comparing DMSO treated spheres to gemcitabine treated spheres cells.