Molecular Determinants of Retinoic Acid Sensitivity in Pancreatic Cancer

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Abstract

**Purpose:** To identify a predictive molecular "signature" for sensitivity to retinoic acid in pancreatic cancer.

**Experimental Design:** Fourteen patient-derived, low-passage pancreatic ductal adenocarcinoma (PDAC) lines with varied expression of fatty acid–binding protein 5 (FABP5) and cellular retinoic acid–binding protein 2 (CRABP2) were used to evaluate the response to all-trans retinoic acid (ATRA). Cell proliferation, apoptosis, and migration/invasion assays were used to measure the in vitro response. Tumor growth was monitored in subcutaneous xenografts in athymic nude mice for 4 weeks.

**Results:** Response to ATRA was observed to be dependent upon differential expression of FABP5 versus CRABP2. Thus, elevated FABP5 expression was associated with minimal cytotoxicity and tumor growth inhibition and a paradoxical increase in migration and invasion. Conversely, CRABP2 expression in the absence of FABP5 was associated with significant tumor growth inhibition with ATRA, even in gemcitabine-resistant tumors. The ATRA-resistant phenotype of FABP5highCRABP2null cells could be circumvented by ectopic expression of CRABP2. Alternatively, reexpression of endogenous CRABP2 could be enabled in FABP5highCRABP2null PDAC lines by exposure to decitabine and trichostatin A, thereby relieving epigenetic silencing of the CRABP2 gene promoter. Immunohistochemical staining for FABP5 in archival human tissue microarrays identifies a subset of cases (13 of 63, ~20%) which are negative for FABP5 expression and might be candidates for ATRA therapy.

**Conclusions:** The widely used agent ATRA deserves a "second look" in PDAC, but needs to be targeted to patient subsets with biopsy-proven FABP5-negative tumors, or be combined with a chromatin-modifying agent to reexpress endogenous CRABP2.

Introduction

Pancreatic cancer or pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal of human cancers. Of 44,030 new cases of pancreatic cancer in the United States, 37,660 are estimated to die in 2011 (1). The 5-year overall survival rate of 4% has been steady over the last few decades, despite exhaustive efforts at developing new and improved therapeutic strategies (2). With a better understanding of cellular signaling pathways, it may be possible to salvage abandoned or failed drugs by identifying subgroups of patients which are likely to respond favorably to the targeted therapy.

The naturally occurring retinoid, all-trans retinoic acid (ATRA) or tretinoin, is approved in the clinic for the treatment of acute promyelocytic leukemia, and is currently being tested in clinical trials in additional tumor types (3). Prior studies in PDAC cell lines and xenografts have reported the ability of ATRA to induce cell-cycle arrest, apoptosis, and epithelial differentiation, although a closer parsing of the data confirm a highly variable sensitivity to retinoids (4–6). In fact, in some instances, retinoids can induce an increase in PDAC cell migration through upregulation of intracellular oncogenic pathways (7). Nonetheless, based on the weight of preclinical evidence, 2 pilot phase II clinical trials have been conducted using the related retinoid, 13-cis retinoic acid, in patients with advanced PDAC, but the results were disappointing (8, 9).

ATRA typically causes cellular differentiation, growth arrest, and apoptosis in most epithelial cell types through...
activation of retinoic acid receptors (RAR). But, in cell types like keratinocytes, ATRA can promote cell survival and hyperplasia (10). Recent seminal studies have elucidated the mechanistic bases for these paradoxical effects of retinoic acid on cell growth and survival (11, 12). Specifically, it is now established that the "orphan" nuclear receptor peroxisome proliferator–activated receptor β or δ (PPARβ/δ) also binds to, and is activated by, exogenous retinoids. Upon appropriate ligand-dependent activation, PPARβ/δ or RAR bind to their indispensable dimerization partner, retinoid X receptor (RXR), and it is the corresponding heterodimer that targets DNA at PPARβ-response element (DRE) or retinoic acid response element (RARE), respectively, and modulates transcription. Whereas expression of "classical" RAR-dependent genes leads to apoptosis and growth arrest, activation of the PPARβ/δ receptor initiates a transcriptional program that promotes proliferation, cell survival, and tumor growth. The partitioning of ATRA between PPARβ/δ versus RARs is regulated by the relative levels of 2 critical intracellular ligand–binding proteins, fatty acid–binding protein 5 (FABP5) and cellular retinoic acid–binding protein 2 (CRABP2). Depending on their relative abundance in the cell, FABP5 and CRABP2 deliver exogenous retinoids from the cytosol to either nuclear PPARβ/δ or RARs, respectively, thereby selectively enhancing the transcriptional activity of the cognate nuclear receptors (10).

We undertook this study to evaluate the sensitivity to retinoids in a large panel of genetically characterized patient-derived PDAC cell lines. We hypothesized that differences in the FABP5 and CRABP2 expression accounts for the variable therapeutic responses to retinoic acid in pancreatic cancer and that altering this ratio to favor partitioning of retinoids from PPARβ/δ to RAR would result in improved therapeutic efficacy. Our studies establish that PDAC cells indeed show strikingly variable responses to ATRA based on the relative intracellular levels of FABP5 and CRABP2. Cancer cells that are FABP5<sup>high</sup>/CRABP2<sup>null</sup> are typically resistant to ATRA-induced growth inhibition <i>in vitro</i> and <i>in vivo</i> and show an enhancement in migration and invasion phenotypes upon ATRA exposure. In contrast, PDAC cells that are FABP5<sup>null</sup>/CRABP2<sup>high</sup> retain their <i>in vitro</i> and <i>in vivo</i> sensitivity to ATRA. The CRABP2 promoter is epigenetically silenced in PDAC and enforced expression in cells with otherwise high endogenous FABP5 expression mitigates the paradoxical enhancement in migration and invasion phenotypes observed with ATRA. Finally, by an immunohistochemical assay, we identify that a minor subset of primary PDACs (~20%) completely lack FABP5 expression (<i>i.e.</i>, tissue equivalent of FABP5<sup>null</sup>), and this cohort might represent an enriched ATRA-sensitive population for therapy using retinoids. In light of the recent demonstration that ATRA can induce quiescence of pancreatic stellate cells and thereby reduce cancer cell proliferation in a paracrine manner (13), our data underscores the need for a "second look" with retinoids in a rationally selected PDAC patient cohort.

Materials and Methods

Cell lines and reagents

The patient-derived, low-passage cell lines used in this study were generated at our institute (14). All cell lines were maintained in Dulbecco's Modified Eagle's Medium (catalogue no. 11965-092; Invitrogen) supplemented with 10% FBS and 1% Pen-Strep. Cultures were routinely tested negative for mycoplasma presence by MycoAlert Mycoplasma detection kit (Lonza). In <i>vitro</i> treatment with ATRA (Catalogue no. R2625; Sigma) were done in 0.5% FBS-containing media. CRABP2 expression vector was constructed using Gateway technology (Invitrogen). Pa20C cell line was stably transfected with either pDest26-CRABP2 (Pa20C-CRABP2) or the empty vector (Pa20C-Vector), and stable clones were maintained in presence of 500 μg/mL G418.

<i>In vitro</i> cell growth assay

<i>In vitro</i> cell growth was determined by the means of CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega). After 2 days of growth, cells were treated with indicated doses of ATRA in dimethyl sulfoxide (DMSO) or DMSO alone for another 3 days before cell growth was measured, as per manufacturer's instructions.

Apoptosis assay

Dissipation of mitochondrial transmembrane potential (ΔΨm) was used to assess apoptosis, as measured by flow cytometry, with tetramethylrhodamine methyl ester perchlorate (TMRM) dye (Catalogue no. T668; Invitrogen;
ref. 15). Briefly, cells in culture were treated with ATRA for 3 days before TMRRM was added during the last 20 minutes of culture at a final concentration of 100 nmol/L. Cells were trypsinized, washed, and resuspended in flow cytometric buffer and ran on FACSCalibur (BD Biosciences) instrument. Data were analyzed with the CellQuest Pro (BD Biosciences) software.

**Semiquantitative real-time reverse transcriptase PCR**

RNA from cultured cells was extracted by RNaseasy kit and reverse transcribed with the QuantiTect Reverse Transcription Kit, both from Qiagen. Semiquantitative real-time reverse transcriptase PCR (qRT-PCR) for human FABP5 and CRABP2 transcripts was carried out by the Fast SYBR Green Master Mix on StepOnePlus Real-Time PCR System (Applied Biosystems). Expression levels were determined with the 2^(-ΔΔCt) method and values normalized to β-actin as endogenous control. Sequences of the primers used are available upon request.

**Western blotting**

Protein extracts were prepared with RIPA buffer (Sigma) and quantified by BCA protein assay (Thermo Scientific). Fifty micrograms of protein was resolved on 4% to 20% Tris-Glycine gel (Invitrogen), transferred to nitrocellulose membranes, and blotted with primary antibodies against FABP5 (MAB3077; R&D Systems) and CRABP2 (sc-10065; Santa Cruz Biotech) at a dilution of 1:1,000. Anti-actin antibody was used as an internal control for protein loading.

**Migration and invasion assay**

A modified Boyden chamber was used to conduct cell invasion assay (16). Briefly, 20,000 cells in 0.5% FBS media (with DMSO or ATRA) were seeded per BD Matrigel–coated inserts, placed in a 24-well plate with media containing 10% FBS, and cultured for a period of 12 to 48 hours, depending on the cell line. Invaded cells were stained with hematoxylin and eosin (H&E) and imaged with a phase contrast microscope (Olympus BX51). For analysis, cells were counted in 10 randomly selected fields (20× objective lens) and their means plotted after normalizing to values from MTS assay on the same number of cells grown in parallel in the same plate.

For the wound-healing assay of cell migration, a scratch was made with a 200 μL sterile pipette tip in a confluent layer of cells, washed with media 3 times before returning them to incubator. Images were taken after a 30 minutes recovery and after 1 to 2 days of ATRA treatment, with an inverted phase contrast microscope (Nikon Eclipse TE2000-S) with Spot Advanced software. Six images were taken per wound, at 10× objective lens magnification. The percentage wound area was measured with ImageJ software (version 1.44v; NIH).

**Subcutaneous xenograft model**

Five million of actively growing cells in 1:1 ratio of PBS and BD Matrigel were injected subcutaneously on each flank of 9 athymic nude mice per cohort. When the average tumor volume was approximately 250 mm³, mice were randomized to receive either vehicle control or ATRA (15 mg/kg in corn oil administered intraperitoneally from Monday to Friday for 4 weeks). Mice were routinely inspected physically for any abnormal changes and their body weight taken weekly to monitor any ATRA-related cytotoxicity. Tumor volumes were measured at indicated intervals during the course of treatment and tumor weight taken at the end of the experiment. A portion of each removed tumor was preserved in formalin to be used later in the immunohistochemical analysis. Gemcitabine (catalogue no. AP-1025; NetQem), 50 mg/kg in saline was given twice a week (Tuesday and Thursday), intraperitoneally. For the combination therapy arm, both treatments were administered concomitantly.

**Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling staining**

DeadEnd Fluorometric TUNEL System (Promega) was used for terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining in formalin-fixed, paraffin-embedded tissue sections as per manufacturer’s instructions.

**Immunohistochemistry**

Briefly, formalin-fixed, paraffin-embedded tissue sections were deparaffinized with xylene and hydrated by a graded series of ethanol washes. Antigen retrieval was accomplished by heating the slides in citrate buffer (pH 6.0) at 100 °C for 20 minutes. Endogenous peroxidase activity was quenched by 0.3% H₂O₂ in methanol. Nonspecific binding was blocked with 2.5% normal goat serum before incubation with the primary antibody. Chromogenic detection was enabled using Poly-HRP anti-Rabbit (Leica Biosystems) or iMmPRESS Anti-Rat Ig (Vector laboratories) with 3,3’-diaminobenzidine (DAB+) substrate (Dako) as chromogen. Slides were counterstained with Harris hematoxylin solution. Primary antibodies were as follows: anti-FABP5 (dilution 1:200, MAB3077; R&D Systems) for staining human tissue microarrays and anti-Ki-67 (dilution 1:200, clone SP6; Cell Marque) for measuring cell proliferation. Quantification was done by counting Ki-67–positive cells in 10 randomly selected fields (40× objective lens magnification) of phase contrast microscope (Olympus BX51), using 5 independent xenografts per treatment condition. Only neoplastic cells were evaluated, and stromal cell staining was excluded from the counting process. Fields containing areas of extensive necrosis were also excluded from evaluation.

**Statistical analysis**

All in vitro experiments were repeated a minimum of 3 times to ensure reproducibility. Data presented in figures are from one representative experiment that was qualitatively similar in the replicate experiments. Statistical significance was determined with 2-tailed Student t test, one-way ANOVA, and Mann–Whitney U tests with Prism (GraphPad Software Inc.) version 5.01. A value of P < 0.05 was regarded as statistically significant.
Results

Expression profile of FABP5 and CRABP2 in PDAC lines

We conducted in silico analysis of the “Deep Serial Analysis of Gene Expression (Deep SAGE)” data from the recent exomic and transcriptomic analysis of 24 pancreatic adenocarcinoma samples (14) for the expression of various components of retinoic acid signaling pathways. There was a notable pattern of reciprocal differential expression for FABP5 and CRABP2 transcripts in these lines (Supplementary Fig. S1A). We confirmed this reciprocal expression pattern by qRT-PCR for mRNA (Fig. 1A) and Western blotting for protein expression (Fig. 1B). Notably, only 3 of 13 (23%) PDAC lines coexpressed the 2 proteins at a detectable level. On the basis of the relative expression of FABP5 and CRABP2, we selected a panel of 4 cell lines which were either FABP5nullCRABP2high (Pa01C, Pa03C), FABP5nullCRABP2null (Pa04C, Pa14C) or FABP5nullCRABP2high (Pa03C, Pa01C), which we predicted to be ATRA resistant or sensitive, respectively, for further functional studies.

Differential in vitro responsiveness to ATRA

As predicted, on the basis of relative FABP5 and CRABP2 expression, we determined that FABP5 highCRABP2null cells (Pa04C and Pa14C) were resistant, whereas FABP5nullCRABP2high cells (Pa01C and Pa03C) were sensitive to ATRA at concentrations up to 10 μmol/L on MTS assays (Fig. 1C). We then assessed apoptosis in these cells by flow cytometry, using TMRM probe to measure loss of mitochondrial transmembrane potential. Both FABP5nullCRABP2high lines (Pa01C, Pa03C) showed an increase in apoptosis even at 1 μmol/L ATRA at 72 hours treatment, consistent with their observed ATRA sensitivity, whereas FABP5nullCRABP2null cells (Pa04C, Pa14C) showed no increase in apoptosis even up to 10 μmol/L ATRA (Fig. 1D).

PDAC cells were also observed microscopically for morphologic changes upon ATRA treatment at 48 hours. In published studies, ATRA-sensitive PDAC cells were reported to increase in size and develop dendrite-like processes, indicative of cellular differentiation (17). Compared with vehicle-treated cells, only the FABP5nullCRABP2high Pa01C showed morphologic features consistent with differentiation upon ATRA exposure, whereas FABP5nullCRABP2null Pa04C cells seemed to have reduced dendrite-like processes (Supplementary Fig. S1B). Finally, analysis of DNA content by propidium iodide after 3 days of ATRA treatment confirmed an increase in apoptosis even up to 10 μmol/L ATRA in the FABP5 nullCRABP2high Pa01C line (Fig. 1E, left). In contrast, significantly more cells invaded through Matrigel upon ATRA exposure of FABP5nullCRABP2null Pa04C cells (Fig. 1E, right). In a wound-healing assay for cell migration, FABP5nullCRABP2high Pa01C cells failed to exhibit migration upon ATRA treatment even in the absence of any demonstrable cell death at the endpoint of experiment (Fig. 1F, top). Notably, FABP5nullCRABP2null Pa04C cells exhibited significantly greater percentage of wound closure (i.e., increased migration) upon ATRA treatment than the DMSO control (Fig. 1F, bottom).

Differential in vivo responsiveness to ATRA

Having confirmed the contrasting in vitro effects of ATRA in PDAC cells with differential expression of FABP5 and CRABP2, we assessed therapeutic efficacy for this compound in vivo, using xenograft models. Mirroring the in vitro data, ATRA significantly inhibited the tumor growth of FABP5nullCRABP2high Pa01C xenografts (Fig. 2A, left, P < 0.0001), whereas the FABP5nullCRABP2null Pa04C xenografts were completely resistant (Fig. 2A, right; both tumor volume and tumor weights were measured as parameters of growth with equivalent results). The excised xenografts from the control and treatment arms were stained and analyzed for the cell proliferation marker Ki-67 and for apoptosis using TUNEL. There was a significant reduction in the numbers of Ki-67 positive nuclei, in conjunction with a massive increase in the numbers of TUNEL-positive nuclei, in ATRA-sensitive FABP5nullCRABP2high Pa01 xenografts as compared with the vehicle control (Figs. 2B and C). In striking contrast, there was a modest but statistically significant increase in Ki-67–positive nuclei upon ATRA treatment in the ATRA-resistant FABP5nullCRABP2null Pa04C xenografts; minimal TUNEL-positive nuclei were observed in the treated xenografts. We observed a comparable outcome with ATRA therapy in an independent pair of xenografts predicted to be either ATRA sensitive (FABP5nullCRABP2high Pa03C), or ATRA resistant (FABP5nullCRABP2null Pa14C), based on differential expression of FABP5 and CRABP2 (Supplementary Fig. S2).

Next, we tested the ATRA-sensitive line Pa01C in a subcutaneous xenograft model using the combination of ATRA and gemcitabine. The parental xenograft is known to be resistant to gemcitabine, and as expected, no significant tumor growth inhibition was observed with monotherapy (Fig. 2D, both tumor weight and tumor volume are depicted). In contrast, the combination of gemcitabine and ATRA resulted in significant tumor growth inhibition, although the efficacy was only marginally improved over the considerable efficacy of ATRA alone. Nonetheless, these results underscored that, in the appropriate biomarker profile predicting retinoid responsiveness, ATRA might be useful as a second-line treatment in gemcitabine resistant tumors.

Using a modified Boyden chamber Matrigel invasion assay, we found almost complete abrogation of invasion in the FABP5nullCRABP2high Pa01C line (Fig. 1E, left). In contrast, significantly more cells invaded through Matrigel upon ATRA exposure of FABP5nullCRABP2null Pa04C cells (Fig. 1E, right). In a wound-healing assay for cell migration, FABP5nullCRABP2high Pa01C cells failed to exhibit migration upon ATRA treatment even in the absence of any demonstrable cell death at the endpoint of experiment (Fig. 1F, top). Notably, FABP5nullCRABP2null Pa04C cells exhibited significantly greater percentage of wound closure (i.e., increased migration) upon ATRA treatment than the DMSO control (Fig. 1F, bottom).

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Figure 1. Expression profile of FABP5 and CRABP2 in PDAC cell lines and in vitro responsiveness to ATRA. A, real-time qRT-PCR with 14 patient-derived, low-passage PDAC cell lines showing relative expression levels of FABP5 and CRABP2 normalized to β-actin. Mean of 3 experiments done in triplicates plotted with error bar denoting ±SEM. B, Western blot analysis showing protein expression of FABP5 and CRABP2 in a panel of 13 PDAC lines with actin as protein loading control. Three of 13 (23%) coexpressed both proteins, whereas 10 of 13 (76%) express one or the other binding protein. C, ATRA treatment reduces in vitro growth of FABP5-high CRABP2-high (Pa01C, Pa03C) cells, whereas FABP5-high CRABP2-null (Pa04C, Pa14C) are completely resistant even at the highest dose. Values are plotted relative to the DMSO-treated control and represent mean of 3 independent experiments, done in triplicates. D, apoptosis assay measuring mitochondrial transmembrane depolarization with TMRM dye and measured by flow cytometry, plotted as mean of 3 independent experiments. Results from 2 FABP5-high CRABP2-null ATRA-resistant (Pa04C, Pa14C) and 2 FABP5-null CRABP2-high ATRA-sensitive (Pa01C, Pa03C) lines are shown. E, invasion assay using modified Boyden chamber and (F) wound-healing assay for Pa01C and Pa04C. ATRA exposure significantly blocks invasion (E) and migration (F) in the FABP5-null CRABP2-high Pa01C cells while actually enhancing both parameters in the FABP5-null CRABP2-high Pa04C line. Scale bar is 100 and 250 μm in (E) and (F), respectively. Bottom panels summarize mean of 3 independent experiments done in triplicates (after normalizing to growth in MTS assay). Error bar denotes ±SEM. ***, P < 0.01; **, P < 0.001.
Figure 2. In vivo therapeutic response to ATRA is dependent on relative expression of FABP5 and CRABP2. Athymic nude mice were injected subcutaneously with either Pa01C (FABP5nullCRABP2high) or Pa04C (FABP5highCRABP2null) and treated with either ATRA (red line) or oil as control (black line). A, mean of tumor volumes measured at indicated time intervals plotted for Pa01C (left) and Pa04C (right). Bar denotes mean of tumor weight (in mg) at the end of study. Significant tumor growth inhibition is observed in the FABP5nullCRABP2high Pa01C xenografts, whereas the FABP5highCRABP2null Pa04C xenografts are completely resistant. B, Ki-67 staining on excised xenografts confirms significant reduction in proliferation in FABP5nullCRABP2high Pa01C xenografts and a modest but significant increase in Ki-67 labeling in the FABP5highCRABP2null Pa04C xenografts. Representative light microscopic images shown at 40× objective lens magnification. Scale bar is 40 μm. Right bar denotes values plotted as mean of number of positive nuclei from 10 fields per section (5 sections per group). Error bar denotes ±SEM. **P < 0.05; ***P < 0.001. C, TUNEL staining on excised xenografts shows pronounced apoptotic nuclei in the ATRA-treated FABP5nullCRABP2high Pa01C xenografts and minimal staining in the ATRA-treated FABP5highCRABP2null Pa04C xenografts. Representative images shown at 20× objective. Scale bar is 100 μm. D, efficacy of single-agent ATRA and combination therapy in Pa01C, a gemcitabine-resistant xenograft. Left, mean of tumor volumes measured at various time intervals plotted, treated with indicated drug combinations. Gemcitabine (Gem; 50 mg/kg) in saline was given twice a week. Middle, representative images of tumor-bearing mice from each group. Right, mean of tumor weight (in mg) at the culmination of study. Error bar denotes ±SEM.
Modulation of CRABP2/FABP5 ratio in ATRA-resistant PDAC

To check whether altering the relative endogenous levels of CRABP2 and FABP5 proteins within PDAC cells will alter the effects of ATRA exposure, we ectopically expressed the CRABP2 gene in the Pa20C cell line, which harbors an endogenous FABP5highCRABP2null profile (Fig. 1B). Several stable clones were tested, and one clone that expressed higher CRABP2 than endogenous FABP5 was selected for further experiments. Relative expression of FABP5 and CRABP2 was confirmed by both qRT-PCR (Fig. 3A) and Western blotting (Fig. 3B). We then examined the subcellular localization of endogenous FABP5 in these cells by immunofluorescence upon ATRA treatment. In the vector-transfected Pa20C line, nearly all of the cytoplasmic FABP5 translocated to the nucleus upon ATRA treatment (Supplementary Fig. S3A, Vector). In contrast, ectopic expression of CRABP2 in the Pa20C cell line blocked the ATRA-induced nuclear translocation of FABP5 (Supplementary Fig. S3A, CRABP2). Thus, in the presence of high levels of intracellular CRABP2 than FABP5, ATRA preferentially binds to CRABP2 and gets shuttled to its cognate nuclear receptors. In agreement with this observation, Pa20C-CRABP2 cells displayed diminished in vitro growth (Fig. 3C, \( P < 0.01 \)) and significantly greater apoptosis (Fig. 3D, \( P < 0.001 \)) upon ATRA treatment than the vector-transfected cells. Cell-cycle analysis also showed an increase in the sub-G0 population, indicative of apoptosis, in the Pa20C-CRABP2 clone upon ATRA treatment (Supplementary Fig. S3B and S3C). We then examined the effects of modulating the FABP5 to CRABP2 ratio on the invasion and migration phenotypes. As expected, vector-transfected Pa20C cells showed higher...
Figure 4. Immunohistochemical staining for FABP5 in a human PDAC tissue microarray. Representative images of FABP5 staining representing 63 surgically resected PDAC cases are shown, including negatively-stained normal pancreatic ductal epithelium (left) and PDAC cells (middle, negatively stained; right, positively stained).

Discussion

The vitamin A metabolite retinoic acid regulates multiple biological processes and plays key roles in embryonic development and tissue remodeling in the adult. Retinoic acid displays distinct anticancer activities in certain malignancies like acute promyelocytic leukemia and head and neck tumors, which forms the basis for its therapeutic application in the clinic. Notably, some carcinomas not only fail to become growth inhibited upon treatment with retinoic acid but instead respond to treatment with enhanced proliferation (7, 11, 18). A striking example of the procarcinogenic effect of retinoic acid was observed in the β-Carotene and Retinol Efficacy Trial (CARET), a lung cancer chemoprevention study that was terminated prematurely due to increased cancer incidence in the treatment cohort (19).

The recent comprehensive genomic and transcriptomic analysis of 24 pancreatic cancer samples from our group provides us with a unique resource to investigate the presence of a predictive molecular "signature" of retinoid sensitivity in pancreatic cancer (14). Except for one missense mutation in RARB, no other somatic mutation, gene amplification, or duplication of any of the retinoic acid signaling components was found. However, in silico analysis of the Deep SAGE data on these samples showed a differential expression pattern of FABP5 and CRABP2 tags between individual PDAC cell lines. The corresponding retinoid-binding proteins were recently shown to be critical intracellular partitioning factors for either antisurvival or prosurvival effects of retinoic acid (10). Thus, we could test the cell lines with either high FABP5 or high CRABP2 for their responsiveness to ATRA. True to our hypothesis, we found FABP5highCRABP2null PDAC lines to be resistant to ATRA-mediated growth inhibition and apoptosis with increased migration and invasion phenotypes, whereas FABP5nullCRABP2high cell lines retained exquisite ATRA sensitivity. Although not included in our panel, higher FABP5 levels could also explain the previously reported invasion and migration in a Boyden chamber assay upon ATRA treatment (Fig. 3E and Supplementary Fig. S3D), likely due to high endogenous FABP5 expression. In contrast, ectopic overexpression of CRABP2 significantly abrogated the ATRA-induced increase of invasion and migration (Fig. 3E and Supplementary Fig. S3D). Comparable results were observed in wound-healing assays, wherein CRABP2 overexpression mitigated the enhanced migration of Pa20C cells upon ATRA treatment that is observed with the vector-transfected cells (Fig. 3F). This suggests that an endogenous FABP5highCRABP2null ATRA-resistant cell line can potentially be rendered sensitive to ATRA by increasing the intracellular expression of CRABP2.

CRABP2 expression is silenced by promoter methylation in PDAC

Analysis of promoter region of the human CRABP2 revealed a conserved CpG island composed of 129 CpG sites and 1.79 kb in length (Supplementary Fig. S4A, ratio of observed to expected CpG is 0.68). We grew a panel of FABP5highCRABP2null cell lines Pa04C, Pa20C, and Pa08C in the presence of decitabine, an inhibitor of DNA methylation, and trichostatin A, a histone deacetylase (HDAC) inhibitor. We observed a massive increase in the levels of CRABP2 transcripts (20- to 40-fold elevation), mainly upon decitabine exposure, which was augmented by trichostatin A, suggesting epigenetic silencing of the CRABP2 promoter in PDAC cells (Supplementary Fig. S4B), a finding that was confirmed by carrying out methylation-specific PCR (MSP) targeted at the CRABP2 promoter CpG island (Supplementary Fig. S4C).

FABP5 expression in resected PDAC tissues

To identify PDAC subsets that might be predicted to be sensitive to retinoids based on low or absent FABP5 expression, we conducted immunohistochemical staining for FABP5 in an archival human pancreatic adenocarcinoma tissue microarray. Both normal and cancer tissue sections from 63 cases were analyzed. Normal ductal epithelial cells were essentially negative for FABP5 expression (Fig. 4A, left). The expression of intracellular FABP5 was variable in the neoplastic epithelial cells, with 50 of 63 cases (~80%) showing moderate to strongly positive staining (Fig. 4A, right). In contrast, 13 cases (~20%) were completely negative for FABP5 labeling in the neoplastic epithelial cells (Fig. 4A, middle), and the patients from whom these samples were derived might be candidates for retinoid therapy in a first or second line setting. Thus, an immunohistochemical assay in tissue biopsies or resection samples might facilitate the identification of a minority of patients with PDAC (~20%, Table 1) who could benefit from a molecularly-targeted differentiation therapy.

Table 1. Summary of FABP5 staining pattern in the cases analyzed and the possible predicted outcome to ATRA treatment

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<tr>
<th>FABP5 expression</th>
<th>Number (%)</th>
<th>Predicted outcome</th>
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<tbody>
<tr>
<td>Negative</td>
<td>13 (20)</td>
<td>Sensitive</td>
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<tr>
<td>Positive</td>
<td>50 (80)</td>
<td>Resistant</td>
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paradoxical increase in migration/invasion upon ATRA treatment observed in the CAPAN-1 PDAC cells (7). We then confirmed our results in vivo using xenograft models, generated using 4 independent PDAC lines, 2 with a FABP5highCRABP2null profile and 2 with FABP5nullCRABP2high expression. An important caveat in this paradigm is the status of the downstream RARs. Thus, even if CRABP2 is the prominent intracellular carrier protein for exogenous retinoids, the tumor suppressive effects will not be observed unless RARs are expressed. In this regard, we confirmed the expression of RAr and RARβ transcripts in all cell lines in our panel (data not shown). This is consistent with the low frequency of methylation of the corresponding gene promoters in PDAC (20) in contrast to other solid tumors like lung cancer (21).

In our series of PDAC lines, only a minority (3 of 13, 23%) coexpressed both FABP5 and CRABP2. The binding affinity of the CRABP2-RAR pathway for retinoid acid (Kd of 0.1–0.2 nmol/L) exceeds that of the FABP5-PPARβ/δ pathway (Kd of 10–50 nmol/L; refs. 22–24). Thus, in tumors with comparable expression of FABP5 and CRABP2, one might expect to see at least partial tumor suppression effects of ATRA, based on the kinetics of partitioning between nuclear receptors. We simulated this scenario by enforced expression of CRABP2 in a cell line (Pa20C) with an endogenous FABP5highCRABP2null profile. High CRABP2 levels resulted in cytoplasmic sequestration of FABP5, presumably by the preferential binding of ATRA to CRABP2 and subsequent nuclear channeling to RARs. This led to increased apoptosis and mitigation of the paradoxical enhanced migration/invasion phenotype upon ATRA treatment as compared with the resistant parental cell line. This serves as a proof of principle to show that increasing the expression of CRABP2 can make an otherwise resistant cell line sensitive to ATRA treatment. Owing to the absent CRABP2 expression in several PDAC lines and the presence of a 1.7kb CpG island in the putative promoter region of the CRABP2 gene, we suspected the epigenetic silencing of the CRABP2 promoter. Interestingly, the CRABP2 promoter was recently shown to be methylated in head and neck tumors (25). Besides promoter methylation, histone deacetylation also causes lower expression of many genes. Such epigenetically silenced genes are shown to be reexpressed upon treatment with decitabine and trichostatin A in pancreatic cancer cell lines (26). Decitabine (5-aza-2′-deoxycytidine), a cytosine nucleoside analogue which hypomethylates DNA by inhibiting DNA methyltransferases, is already approved by the U.S. Food and Drug Administration (FDA) to treat myelodysplastic syndromes (MDS; ref. 27). Trichostatin A, an inhibitor of class I and II mammalian histone deacetylase enzymes, has also been successfully used in various preclinical cancer models including pancreatic cancer. Exposure to decitabine and trichostatin A in low CRABP2–expressing cell lines resulted in significant reexpression (20- to 40-fold elevation) of CRABP2. This opens up the possibility of combinatorial use of ATRA with decitabine alone, or in combination with HDAC inhibitors, in the treatment of ATRA-resistant tumors. Of note, there is an ongoing phase II clinical trial of decitabine alone or in combination with valproic acid (HDAC inhibitor) and ATRA for treatment of acute myeloid leukemia and a phase I clinical trial of liposomal ATRA and valproic acid for various solid tumors (28). Moreover, the combination of ATRA with trichostatin A showed promising results in a preclinical study on otherwise ATRA-resistant renal cell carcinoma cell lines (29). These recent developments points towards the need of clinical trials involving ATRA with epigenetic modifiers like decitabine and trichostatin A in the treatment of pancreatic cancer.

Although reexpression of epigenetically silenced CRABP2 in tumors with endogenous FABP5 expression is likely to improve the therapeutic efficacy of ATRA, in our series the most unequivocal effects of retinoids were seen in PDAC cells that lacked FABP5 expression altogether. Even a relatively gemcitabine-resistant cell line (Pa01C) with a predicted "sensitive" profile (FABP5nullCRABP2high) showed significant growth inhibition with single-agent ATRA and only minimal additive effect of combination with gemcitabine.

In a prospective clinical trial, the identification of appropriate candidates for molecularly-based therapies is a challenge, especially in patients with advanced PDAC who do not undergo surgical resection, and only limited biopsy material might be available for testing. In this context, immunohistochemical staining for FABP5 provides a relatively simple approach for identifying neoplasms that lack protein expression and is amenable to limited formalin-fixed tissues.

In conclusion, we found retinoid sensitivity of PDAC to be dependent on the relative intracellular expression of FABP5 and CRABP2 proteins. It is possible to identify a subset of tumors that are negative for FABP5, and these are likely to represent an enriched subset of patients that might benefit from retinoid therapy. Such "personalized" patient selection will hopefully ensure a more favorable clinical response using retinoids than an "all-comers" approach.

Disclosure of Potential Conflict of Interest

No potential conflicts of interest were disclosed.

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