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Identification of novel pancreatic adenocarcinoma cell-surface targets by gene expression profiling and tissue microarray

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ABSTRACT

Pancreatic cancer has a high mortality rate, which is generally related to the initial diagnosis coming at late stage disease combined with a lack of effective treatment options. Novel agents that selectively detect pancreatic cancer have potential for use in the molecular imaging of cancer, allowing for non-invasive determination of tumor therapeutic response and molecular characterization of the disease. Such agents may also be used for the targeted delivery of therapy to tumor cells while decreasing systemic effects. Using complementary assays of mRNA expression profiling to determine elevated expression in pancreatic cancer tissues relative to normal pancreas tissues, and validation of protein expression by immunohistochemistry on tissue microarray, we have identified cell-surface targets with potential for imaging and therapeutic agent development. Expression profiles of 2177 cell-surface genes for 28 pancreatic tumor specimens and 4 normal pancreas tissue samples were evaluated. Expression in normal tissues was evaluated using array data from 103 samples representing 28 organ sites as well as mining published data. One-hundred seventy unique targets were highly expressed in 2 or more of the pancreatic tumor specimens and were not expressed in the normal pancreas samples. Two targets (TLR2 and ABCC3) were further validated for protein expression by tissue microarray (TMA) based immunohistochemistry. These validated targets have potential for the development of diagnostic imaging and therapeutic agents for pancreatic cancer.

1. Introduction

Pancreatic adenocarcinoma (PanAdo) is increasing in incidence and is associated with a high mortality rate, which is largely due to late stage diagnosis and a paucity of treatment options [1]. Surgical resection is the only effective therapy for PanAdo [2]. Due to the high incidence of metastasis at the time of diagnosis, surgery is rarely a viable option [3]. Endoscopic ultrasound and other imaging modalities are used for detection of pancreatic cancer in symptomatic patients [4]. However, the ability to non-invasively detect this disease with high specificity and sensitivity could increase treatment options and decrease mortality. Novel agents that detect pancreatic cancer relative to normal tissues have potential for use as diagnostic imaging or therapeutic agents. Such agents could follow tumor response to therapy through non-invasive imaging, aid in diagnosis through molecular characterization of the specific tumor type, selectively deliver therapy to tumor relative to normal tissues and provide more treatment options to patients with inoperable tumors or disseminated disease. To date, few cellular targets have been identified that are specific for pancreatic adenocarcinoma (PanAdo) [5–7].

Targeting imaging agents, such as synthetic antibodies or peptides, to the tumor cell-surface holds considerable promise and is an active area of research. Agents have been developed that bind cell-surface targets and have generated promising results in animal models and, in some cases were successfully tested in

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humans. RGD peptide-based ligands bound to a variety of proteins, peptides, small molecules, nucleic acids and radiotracers were used to deliver therapeutic and imaging agents to tumor vasculature [8]. An ^{18}F-Galacto-RGD ligand was tested in humans, showing desirable pharmacokinetics, and was used to visualize αvβ3-integrin expression via PET imaging [9,10]. Radiolabeled monoclonal antibodies that target cell-surface antigens were approved for the treatment of B-cell non-Hodgkin's lymphoma [11]. Unfortunately, antibody conjugates have yet to demonstrate similar success when used to treat solid tumors [11,12]. Agents targeting a single surface marker may be as simple as a small peptidomimetic ligand conjugated to an imaging moiety with a small linker molecule (monovalent), or as complex as a nanoparticle with imaging contrast agent or therapeutic agent encapsulated internally and functionalized externally with numerous ligands (multivalent).

Targets suitable for imaging alone, for the purpose of following therapy and for characterizing the tumor molecular phenotype do not have the strict requirement of being expressed in the tumor tissue only. For imaging, it is important for a target to be expressed in the tumor, but not in the organ of origin of that tumor. It is also desirable that targets are not highly expressed in vital organs or organs involved in clearance. Using mRNA expression profiling of human tissue samples, we have identified >170 cell-surface gene products that exhibit elevated expression in pancreatic cancer relative to normal pancreas. From this list, targets were prioritized based on the breadth of coverage amongst the PanAdo tumor samples tested, the degree of elevation in tumor relative to normal pancreas, the pattern of expression amongst normal tissues and by, for the purpose of ligand development, what is known about ligand-target structure activity relationships (SAR). Two high-priority targets, ABCC3 and TLR2, were further validated for expression in tissue microarrays, in cultured pancreatic cancer cells, and by reviewing expression profiles presented in public datasets.

2. Materials and methods

2.1. Gene expression analysis and target identification using DNA microarrays

Collection of tissue specimens and RNA samples, RNA isolation and generation of DNA microarray expression data used in this study were described previously [13]. Microarray data are published in the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/projects/geo/index.cgi), data series GSE11838. A list of genes encoding proteins with cell-surface epitopes was previously compiled [13] and was used in this study to select and identify targets.

To identify targets, we selected cell-surface genes that are expressed as mRNA in at least 2 of the pancreatic tumor samples surveyed, but are not expressed in any of the normal pancreas samples. To determine expression versus non-expression, normal and tumor cutoff values were used that were previously reported for these data [13]. Briefly, we binarized expression of the cell-surface genes to "not expressed" or "expressed" for each tissue sample by plotting the expression distribution histograms for the pancreatic tumor samples and normal tissue samples, and selecting relative cutoff values based on the area under the curve. This process imposes the highest possible stringency to avoid identification of false targets. A coverage flag was assigned to each tumor sample for each possible target. If a tumor sample expressed a given target and none of the normal pancreas samples express the target, it is assigned the flag '1', otherwise, it is assigned '0'. Retained targets were then ranked by the percent coverage for all tumor samples. Thus, the highest ranked targets were predicted to cover the most tumors and have little or low avidity to normal pancreas tissue.

2.2. Tissue microarray construction and immunohistochemistry

As previously described [13], formalin-fixed paraffin embedded tissues were first examined with H&E staining using whole sections to identify pathologically distinct areas of interest (tumor, adjacent normal and normal). Two TMAs were used for this analysis, one composed of 42 samples of PanAdo and 4 samples of normal pancreas, the other composed of 282 samples from 66 normal tissues inclusive of 12 normal pancreases. TMAs were constructed by punching 1.5 mm discs using a tissue arrayer (Chemicon, Temecula, CA) and re-embedding them into a new paraffin block [14–17]. Each TMA block was cut into 5 μm sections and H&E staining was performed on every 50th section to assess retention of desired pathologies. TMA slides for normal tissues in 0.6 mm spot sizes (Version CHTN2002N1) were provided by the Cooperative Human Tissue Network (CHTN) which is funded by the National Cancer Institute (NCI). Other investigators may have received specimens from the same subjects.

To optimize staining conditions, antibodies were titrated against regular tissue sections and 'tester' TMA slides containing a variety of tumor and normal tissues. TMA slides were first subjected to antigen retrieval by heating at 100 °C in citrate buffer (0.1 M, pH 6.0) for 5–30 min, depending on the antibody. Slides were then incubated with primary antibodies at optimal dilutions for 30 min at room temperature. Biotinylated secondary antibodies were applied, followed by streptavidin-peroxidase complex (Vision BioSystems, Novell, MA) and resolved with diaminobenzidine chromogen for 12 min. Stained slides were evaluated using light microscopy and scored (0 = negative to 3+ = intensely positive) by a board-certified pathologist (G.H.). The primary antibodies and dilutions used were: ABCC3 (MRP3) (R&D Systems), 1:75; and TLR2 (Abcam), 1:500.

The percentage of cells staining positive for TLR2 in normal pancreas tissues was determined using digital image analysis. The normal tissue microarray slide stained with TLR2 antibody, with core diameters of 0.6 mm, was scanned using the Aperio ScanScope XT digital imaging system at 200× magnification. Micrographs were analyzed using customized algorithm macros created for commercial use by Aperio Technologies (positive pixel count v9.1), and adapted to accurately portray the staining produced by each marker (hue_value = 0.08; hue_width = 0.55; color_saturation_threshold = 0.04; Iwp_high = 210; Iwp_moderate = 165; Iwp_low = 100). Pancreas tissue microarray (TMA) cores were identified, and the regions of interest were selected by an expert pathologist for each core. The algorithm was applied to all normal pancreatic tissue for 12 total core regions and reported as percent positive with strong, moderate and weak positive data subsets.

2.3. Quantitative real-time RT-PCR

Quantitative real-time RT-PCR (qRT-PCR) was performed as previously described [18] with the following changes. Primer sets were designed to generate cDNA and perform RT-PCR from ACTB (β-actin), ABCC3 and TLR2 mRNA. PCR conditions were determined so that maximum yield without spurious priming was achieved. RT-PCR was conducted using a Smart Cycler® (Cephid, Sunnyvale, CA) and the QuantiTect SYBR Green RT-PCR Kit (Qiagen). As controls, a no-RT reaction was run for each extract and a no-template reaction was included during each experiment. Melt curves yielded a single melt peak for all template reactions and a minimal melt peak for the no-template control reaction. Raw mRNA expression values were determined as being 2^{-CT}, where CT is the second derivative of the fluorescence curve.

Table 1
Pancreatic cancer targets identified by DNA array expression profiling and selected for TMA validation.

Target	Gene name	DNA array tumor coverage %	Tumor samples covered, $n = 28$
ABCC3	ATP-binding cassette, subfamily C, member 3	75	1–3,5,8,9,11–13,15,17–25,27,28
TLR2	Toll-like receptor 2	64	2,3,9,10,12,15–25,27,28

2.4. Public datasets

Expression data for promising targets were complied through searches of the current literature (PubMed) and expression profiles accessed using the NCBI Expression Omnibus (GEO) Repository. The study encompassed 1781 tumor samples representing a variety of histological tumor classes from several tissue sites and 212 normal samples derived from more than 70 different tissues. The CEL files for the tumor samples were downloaded from the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/projects/geo/index.cgi), data series GSE2109. This is the Expression Project for Oncology carried out by the International Genomics Consortium (IGC). The normal samples came from the GEO data series GSE7307, Human Body Index, which was a transcriptional profiling project carried out by Neurocrine Biosciences, Inc. The CEL files were analyzed using the MAS 5.0 algorithm (Affymetrix Corp.) and then screened through a rigorous quality control panel to remove samples with low percentages of probesets called present by the MAS 5 algorithm, indicating problems with the amplification process; high scaling factors, indicating poor transcript abundance during hybridization; and poor 3′/5 ratios, indicating RNA degradation either prior to or during processing. The remaining samples were normalized to the trimmed average of 500 in the MAS 5 algorithm. The resultant data were then smoothed using the 2D smoother algorithm, Sigma Plot 11.0, and expression signal distributions plotted as percent tissue samples versus log2 normalized intensity.

3. Results

3.1. Identification of targets for development of imaging or therapeutic agents

Currently, there are no targeted imaging or -therapeutic agents available for detection, diagnosis and treatment of pancreatic cancer. For imaging primary pancreatic tumors, it is only necessary to identify targets that are differentially expressed in cancer relative to normal pancreas tissue. Since targets identified this way may also be expressed in other tissues at peripheral organ sites, agents that specifically bind these targets may not be suitable for delivery of systemic therapy, but could be used for local therapy, e.g. intraoperative. Ideal targets will have limited expression in vital organs, or organs involved in the clearance of such agents, e.g. heart, liver, kidney. To begin this work, a list of cell-surface genes was curated and used to filter DNA expression array data that were generated from 28 pancreatic tumor samples and 103 normal tissue samples from 28 organ sites [13]. Following this, the analysis required a binarization of expression data (0 = not expressed and 1 = expressed). Using the highest possible stringency, threshold cutoffs were established for normalized array intensity values, below which genes were considered "not expressed" in normal tissues, and above which genes were considered "expressed" in tumor tissues [13]. For this work, the highest stringency that still yielded a workable number of genes (i.e. 100–200) was used. The normal cutoff value of 0.45 median normalized expression, below which genes were categorized as not expressed, and a tumor threshold value of 0.75 percentile, above which genes were considered expressed, were applied. These thresholds yielded 170 target candidates ranging from 4% to 68% tumor coverage while not being expressed in any of the normal pancreas samples. From this list, the top 12 targets were selected that had the broadest coverage amongst tumor samples and the highest array intensity values above background. These were evaluated for non-expression in other normal tissues and for known structure activity relationships to determine the potential for binding ligand development. Expression in vital normal tissues was evaluated for each of these 12 prospective targets by individually checking the normal tissue DNA microarray expression data and expression data from public databases. Further consideration was given to targets that had known ligand-binding structure activity relationships (SAR). From this analysis, two high value targets were selected for further validation: ABCC3 and TLR2 (Table 1).

The relative coverage of the 28 pancreatic tumor samples based on the two targets identified in this study was evaluated using the DNA microarray expression data. The analysis revealed distinct differences in tumor coverage amongst the two targets. ABCC3 covered the broadest range of tumors and many, but not all (2, 10, 16) overlapped with tissues expressing TLR2 (Table 1). Hence, in combination, the two targets cover 79% of the tumor samples surveyed. Notably, in unsupervised analysis, 5 of these 28 tumor samples clustered with the normal pancreas samples based on their expression of cell-surface genes [13]. This implies that either these 5 samples represent a subtype of pancreatic tumors with normal-like cell-surface expression, or that the specimens were significantly contaminated with normal pancreas tissue, despite the fact that samples were rejected when histopathology showed >30% normal tissue. Three of these five tumor samples were not covered by these two tumor markers, meaning that the percentage of pancreatic cancer coverage by two markers could be as high as 87%.

3.2. Validation of targets using tissue microarray (TMA)

To validate targets, we performed TMA-based immunohistochemistry to examine protein levels of the targets in tissue samples. We constructed a TMA using pancreatic cancer and normal pancreas tissues, and have obtained a normal tissue TMA (see Section 2). The pancreatic cancer array contains 42 cases of PanAdo, each of which is represented by 3 cores (1 core from adjacent normal region and 2 cores from the tumor region). It also includes samples from 2 cases of islet cell tumor, 4 cases of pancreatitis and 4 cases of normal pancreas from individuals with healthy pancreas. The normal tissue TMA contains 282 cores representing 66 different normal tissue types. Commercial antibodies for the target proteins were obtained, immunostaining was performed using optimized conditions and results were scored by a board-certified pathologist (G.H.).

Representative staining of the targets in ductal pancreatic adenocarcinoma tissues and normal ductal pancreatic tissues are shown in Fig. 1. Table 2 summarizes the pancreatic cancer tissue microarray results for ABCC3 and TLR2. TLR2 had strong staining in most tumor tissues and minimal staining in adjacent normal tissues. A large proportion (70%) of tumor samples scored with ≥2+ staining versus 10% staining in adjacent normal, respectively. The differential in expression was not as compelling for ABCC3, with (≥2+) staining in only 37% in tumor versus 29% staining in adjacent normal respectively. Staining for TLR2 and ABCC3 was also performed on normal pancreas tissue samples and for both targets there was no ≥2+ staining of normal pancreas. However, there was

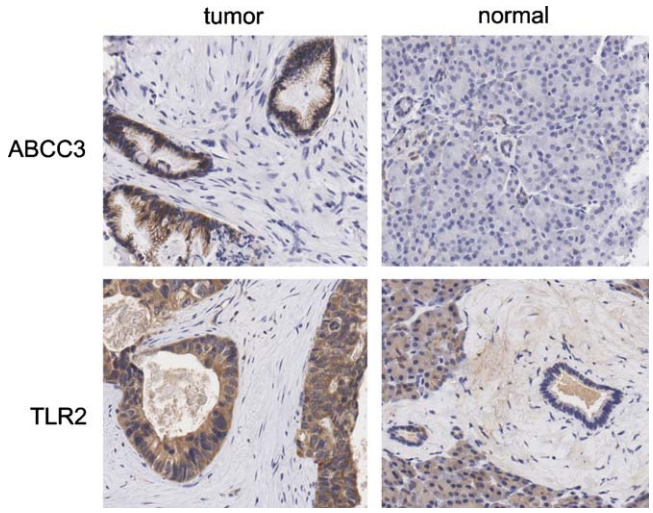

Fig. 1. Representative immunostaining results of ABCC3 (top) and TLR2 (bottom) in pancreatic adenocarcinoma (left) and normal pancreas (right).

one normal sample with +1 staining for TLR2. It is possible that some of these targets may be expressed in adjacent tissues due to field effects and inflammation. For example, ABCC3, which appeared to be expressed in 29% of adjacent normal samples, was completely negative on the four normal pancreas samples.

The percentage of positive (≥1+) staining for ABCC3 was equal to the percentage of tumor coverage calculated from the DNA microarray data, 75% in both cases. But the percentage of positive staining for TLR2 was much higher (98%) compared to DNA microarray coverage (64%). The percentages of 2+ or higher cases for TLR2 was also high (70%), and in close agreement with the DNA microarray coverage amount. ABCC3 had considerably lower 2+ or greater cases (37%). For further validation, immunostaining of the normal tissue microarray was also performed for the two targets to identify potential sites of cross-reactivity. ABCC3 had generally the lowest staining of normal tissues amongst the two targets. Weak staining (1+) was observed in normal colon and gastric mucosa samples. ABCC3 staining of the gall bladder samples ranged from 1+ to 2+. Although prominent staining was observed in normal tissue adjacent to pancreatic cancer, ABCC3 staining was negative in the 16 samples of normal pancreas included on the TMAs.

TLR2 stained +1 in normal secretory endometrium, colon and gastric mucosa, seminal vesicle, and ependymal cell samples. TLR2 stained 2+ in normal salivary gland, bladder transitional epithelium and the kidney cortex. Expression in the kidney is in agreement with the DNA microarray data which indicates moderate expression levels in the 4 normal kidney samples. Expression of TLR2 was not detected in normal pancreas tissue by DNA array and TLR2 antibodies did not stain 3 of the 4 normal pancreas samples in the pancreatic cancer TMA. However, one normal sample in the tumor TMA scored 1+, and TLR2 staining was observed in a small subset of cells within the 12 normal pancreas samples in the normal tissue microarray (Fig. 2A). The percentage of cells within these normal samples were scored via image analysis with 93.7% of cells scoring negative, 4.3% weak positive, 1.8% staining moderate positive (Table 3). The TLR2 positive subset of cells is morphologically consistent with normal stem cells (round cells with central nucleus and abundant clear cytoplasm, "fried egg" appearance) (Fig. 2B). These cells were also seen as single TLR2 positive cells amongst the ring of TLR2 negative epithelial cells in a duct (Fig. 2C). It is possible that TLR2 is a marker for pancreatic stem cells.

ABCC3 and TLR2 exhibited moderate to strong staining in the normal adrenal gland samples (Fig. 3). However, only ABCC3 exhibited a clear cell-surface staining pattern. This is somewhat in agreement with the DNA microarray data which had strong expression of ABCC3 and moderate expression of TLR2 in adrenal tissue.

3.3. Validation of targets in pancreatic cancer cell lines

Since targets were validated in tumor tissue samples that have a heterogeneous mixture of cell types, determination of target

Table 2
IHC scoring of pancreatic cancer TMA.

Target	Sample classification	Score					% of cases with ≥2+
		0	1+	2+	3+	N/E	
ABCC3	Normal	4	0	0	0	0	0
	Adjacent Normal	12	10	8	1	7	29
	Tumor	13	18	13	5	3	37
TLR2	Normal	3	1	0	0	0	0
	Adjacent Normal	13	13	3	0	9	10
	Tumor	1	13	25	8	5	70

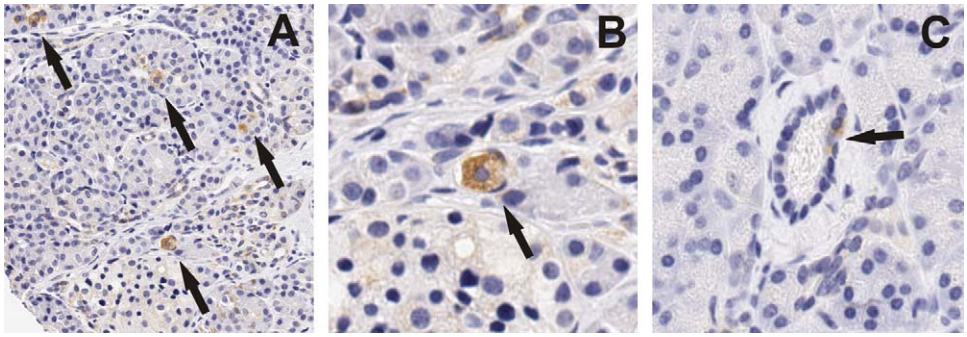

Fig. 2. Representative immunostaining results of TLR2 staining in normal pancreas samples from the normal tissue microarray (CHTN2002N1). Panel A, arrows indicate subset of positive staining cells. Panel B, arrow indicates a positive cell with possible "fried egg" stem cell morphology. Panel C, arrow indicates a single positive cell in duct.

Table 3
Percentage of cells scoring positive for TLR2 expression in normal pancreas tissue samples in the normal tissue microarray.

Sample	% 0 (Negative)	% 1+ (Weak positive)	% 2+ (Moderate positive)	% 3+ (Strong positive)	% Positivity (sum of 1+ through 2+)
1	94.4%	1.5%	3.5%	0.7%	5.6%
2	97.6%	1.8%	0.6%	0.0%	2.4%
3	95.9%	2.4%	1.6%	0.1%	4.1%
4	91.2%	4.3%	3.7%	0.8%	8.8%
6	92.4%	5.9%	1.6%	0.1%	7.6%
8	90.6%	7.1%	2.2%	0.0%	9.4%
9	94.4%	3.8%	1.7%	0.2%	5.6%
10	96.9%	2.7%	0.4%	0.0%	3.1%
11	92.0%	6.5%	1.4%	0.0%	8.0%
12	94.1%	4.7%	1.1%	0.1%	5.9%
Mean	93.7%	4.3%	1.8%	0.2%	6.3%
St Dev	2.3%	1.9%	1.0%	0.3%	2.3%

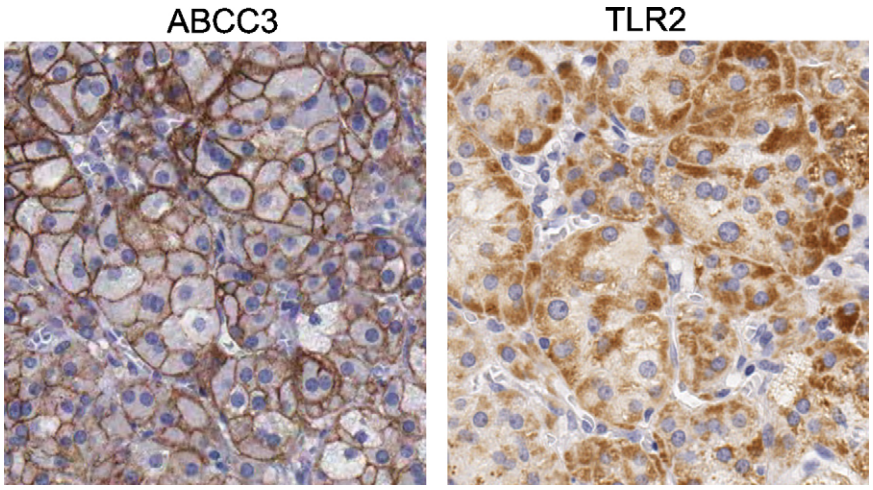

Fig. 3. Representative immunostaining results of ABCC3 and TLR2 in normal adrenal tissue from the corresponding region of adjacent sections from the normal tissue microarray (CHTN2002N1).

expression in existing pancreatic cancer cell lines can potentially serve as further validation. However, it is understood that lack of expression in a cell line does not invalidate an in vivo derived target. Microarray data were generated for 17 pancreatic adenocarcinoma cell lines. From these data, 5 lines (AsPC-1, Capan-1, HPAFII, PSN-1 and SU86.86) were identified as expressing both of these target genes. Expression of ABCC3 and TLR2 at the mRNA level was detected in these cells and determined quantitatively by qRT-PCR (Table 4). From qRT-PCR, the relative target expression levels were higher for ABCC3, which is in agreement with the mean of the median normalized DNA array measurements amongst the five cell lines with dual expression: ABCC3 = 1.6 > TLR2 = 1.3; amongst all 17 PanAdo cell lines: ABCC3 = 8.9 > TLR2 = 1.4; and the DNA microarray of patient PanAdo tumor samples: ABCC3 = 2.7 > TLR2 = 2.4. Median normalized DNA microarray values for normal pancreas tissue samples were considerably lower for both targets: ABCC3 = 0.64 and TLR2 = 0.55.

Table 4
Expression (mRNA) of validated targets in pancreatic cancer cell lines by qRT-PCR.

Target cell line	ABCC3 (SEM)[a]	TLR2 (SEM)[a]
AsPC-1	7.4 (2)	0.004 (0.0002)
Capan-1	7.4 (1)	0.31 (0.1)
HPAFII	0.8 (0.2)	0.004 (0.002)
PSN-1	0.053 (0.005)	0.14 (0.07)
SU86.86	0.2 (0.06)	0.95 (0.3)

[a] Normalized to β-actin (ACTB) expression [(target gene 2^{-CT}/ACTB 2^{-CT}) × 1000]. Data are the mean of 3 samples and error values are the standard error of the mean (SEM). n.d. = not detected by primer sets that have proven to detect transcript in control samples.

The two markers are broadly expressed amongst the 17 cell lines surveyed by DNA microarray. This observation is in agreement with the broad expression detected in patient tumor samples (Table 1). ABCC3 was expressed in 82% of the 17 cell lines compared to 75% of patient samples and TLR2 was expressed in 47% of the cell lines compared to 64% of patient tumors.

3.4. Verification using public datasets

Expression profiles of promising targets were manually checked for agreement with our data using available existing databases. In recent years, a number of groups have performed gene expression profiling for various tumor and normal tissues, and many have made their data publicly available. These datasets usually vary in array platform, experimental design and quality control and, therefore, are not suitable for the coverage analysis discussed above. However, they are useful for secondary verifications. We have collected datasets from Gene Expression Omnibus (GEO) Repository for a total of 212 normal tissues from more than 70 different tissue types and 1781 tumor tissues representing various histological tumor classes from several tissue sites. All datasets were generated using the Affymetrix DNA microarray platform. We then examined the expression signal intensity distributions in the normal and tumor groups for the genes corresponding to the three targets identified herein.

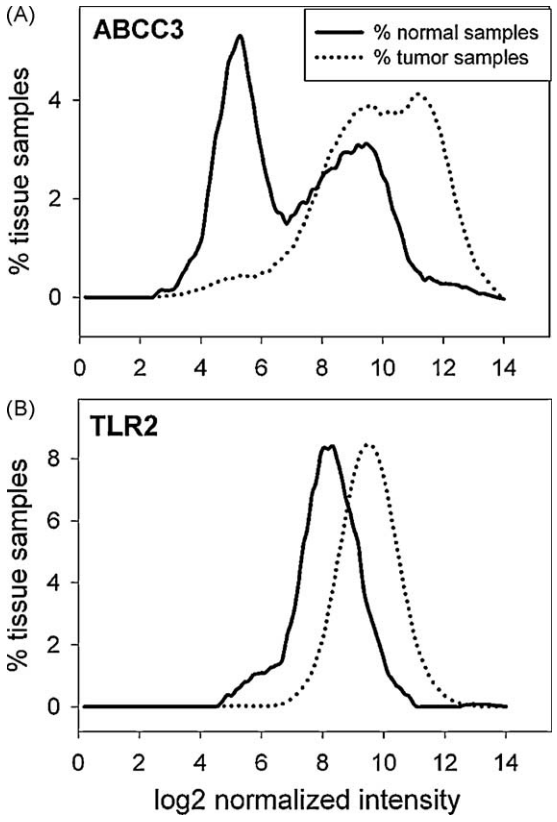

Fig. 4. Expression signal distribution analysis of ABCC3 (A) and TLR2 (B) in normal and tumor tissues from public databases: Normal tissue group of 212 samples from 70 tissue types (solid line); and tumor tissue group of 1781 samples (dotted line) from a variety of histological tumor classes from several tissue sites.

The plot for ABCC3 (Fig. 4A) shows that a large subset of normal tissues do not express ABCC3 as indicated by the solid line peak on the left, and that a subset of normal tissues do express the target, solid line peak on the right. However, the majority of tumors do express ABCC3, dotted line peak on the right, and a greater proportion of the tumors express this target at a higher level than the normal expressing tissues. The plot for TLR2 shows that the majority of normal tissues do not express TLR2 or express at very low levels (Fig. 4B, solid line) while tumor tissues generally express TLR2 (dotted line). It is important to note that these data are plotted in log2 scale and that the seemingly small shift to the left of tumor samples relative to normal samples actually represents a significant increase in expression level. Also, it is notable that the single normal tissue peaks (solid lines) are found at different expression levels relative to one another. This can be deceptive when viewing these data using a log 2 scale as the probes used for each target are not necessarily equivalent in binding target sequence and that differences on the middle and right side of the scale are not great, while differences on the left side of the scale are quite large. It is our interpretation that the narrow solid line peaks represent non- or low-expression of the target. These data are in general agreement with the DNA microarray data for pancreatic tumors where ABCC3 and TLR2 were identified as being highly expressed in a majority of pancreatic tumors relative to normal pancreas.

Although most of the tumor tissues used in the analysis are not pancreatic tumors, this type of distribution analysis still provides valuable information on (1) expression levels of the targets in normal tissues obtained from different individuals and (2) if the targets identified in the pancreatic analyses can be applied to other tumor types. This type of information can help prioritize the targets for agent development.

4. Discussion

Pancreatic adenocarcinoma is generally diagnosed at later stages as disseminated disease. There are no effective treatments for the majority of cases and, thus PanAdo is associated with a high mortality rate [1]. The development of targeted imaging agents that can non-invasively detect, aid in diagnosis, and determine therapy response could improve therapeutic outcomes. As few effective treatment options are available for metastatic pancreatic cancer, the development of targeted therapeutic agents could reduce systemic toxicity, while delivering a higher dosage locally to the tumor, potentially decreasing patient mortality.

Our approach has been to identify putative cell-surface markers through gene expression profiling of DNA microarray data generated from patient tumor and unaffected tissue samples. Once identified as being expressed at the level of mRNA, it is essential that expression be validated at the level of protein. For this study, these validations were performed using IHC of tumor and normal tissue microarrays. Cell-surface markers are ideal for targeting since the agent is not required to cross the plasma membrane prior to binding to the target. Once bound to the cell-surface, the agent may be rapidly internalized by receptor-mediated endocytosis or more passively by pinocytosis [19]. Hence, allowing for the intracellular accumulation of imaging contrast or therapeutic payload. It is worth noting that our targeting approach does not require intervening with the biological function of our targets, rather, the cell-surface marker is used more as a landing-pad for attachment and internalization of the targeted agent.

Cell-surface targets (170) were identified as being highly and broadly expressed in PanAdo but not expressed in normal pancreas. Two of these targets, ABCC3 and TLR2, were validated for protein expression. ATP-binding cassette, subfamily C, member 3 (ABCC3) is highly and broadly expressed in PanAdo, and has generally low-expression in normal tissues, except that it is expressed in adrenal tissue (Fig. 3). Expression in adrenal tissue is a concern. However, ABCC3 may be useful for diagnostic imaging of PanAdo using non-toxic imaging agents, or for methods of localized delivery of therapy. ABCC3 is a membrane transport protein that is a member of the MRP multidrug resistance subfamily and is known to mediate taxane resistance in breast cancer [20]. ABCC3 has no known ligands, thus, ligand development will require sophisticated approaches such as the screening of one-bead one-compound random peptide sequence ligand libraries [21], or phage display [22]. Toll-like receptor 2 (TLR2) is expressed in immune cells for the recognition of microbial infections with subsequent immune system activation, and is also expressed in cancer [23]. Palmitoylated synthetic ligands for binding TLR2 are known [24]. In this study we report low to moderate expression in a few additional non-vital normal tissues. However, the moderate TLR2 expression in the kidney cortex and adrenal gland may be of concern for its use in agents with associated toxicity.

A limitation to the sole use of DNA microarray for expression profiling of tissue samples is that the tissue contains a mixture of tumor and host cell types found within a given sample. Or conversely, a determination of non-expression in normal tissue may be misleading if the marker is expressed in a small subset of cell types. An example of this is found in the IHC determination that TLR2 is expressed in a small subset of normal pancreas cells (Table 3), despite the fact that the DNA array data were scored as non-expressing. Some of these cells have stem cell morphologies (Fig. 2). TLR2 has recently been reported as a marker for human renal and mouse embryonic stem cells [25,26]. The percentage of cells expressing TLR2 in normal pancreas is low, with only 2% scoring moderate or strong positive by IHC. Thus, TLR2 is a

potential target for diagnostic imaging of pancreatic tumors and for methods of delivering tumor localized treatment.

A number of strategies are being developed for delivering tumor localized treatment using targets that are also expressed in normal tissues. Toxicity can be decreased by locally activating therapy at the site of the tumor. These approaches may be used without targeting, but targeting to the tumor can increase the local concentration of the agent prior to activation through binding and cellular uptake enhancing the therapeutic effect. An example of this approach includes agents designed for imaging by photoacoustic tomography (PAT) and local delivery of treatment by photothermal ablation [27]. Other examples include agents that are size restricted to remain within the normal vasculature, but are not restricted from leaving the tumor vasculature due to enhanced vascular permeability and retention (EPR) [28]; photodynamic therapy, where light can be directed to the tumor site locally activating therapy [29,30]; and agents that combine ligands for heterologous markers into heterobivalent constructs allowing for specific targeting of tissues that express all markers relative to tissues expressing only a subset of the markers [31].

This study exemplifies the need for thorough characterization and validation of tumor markers prior to expending the considerable time, resources and effort required for development of targeted imaging and therapeutic agents. Quantifying mRNA expression is a useful initial screen, but validation of protein expression is essential. These results also illustrate the difficulty in finding the "ideal" marker that is highly and broadly expressed amongst all cancer of a given type, but is not expressed in vital tissues or organs of drug clearance. It appears that a number of markers, a cocktail, may be required to cover all types of PanAdo, and that development of these markers into imaging and therapeutic agents will require creative approaches to minimize toxicities in normal tissues.

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