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Rapid Characterization of Candidate Biomarkers for Pancreatic Cancer Using Cell Microarrays (CMAs)

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Abstract

Tissue microarrays have become a valuable tool for high-throughput analysis using immunohistochemical labeling. However, the large majority of biochemical studies are carried out in cell lines to further characterize candidate biomarkers or therapeutic targets with subsequent studies in animals or using primary tissues. Thus, cell line-based microarrays could be a useful screening tool in some situations. Here, we constructed a cell microarray (CMA) containing a panel of 40 pancreatic cancer cell lines available from American Type Culture Collection in addition to those locally available at Johns Hopkins. As proof of principle, we performed immunocytochemical labeling of an epithelial cell adhesion molecule (Ep-CAM), a molecule generally expressed in the epithelium, on this pancreatic cancer CMA. In addition, selected molecules that have been previously shown to be differentially expressed in pancreatic cancer in the literature were validated. For example, we observed strong labeling of CA19-9 antigen, a

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Supporting Information Tables containing a list of cell lines used to generate pancreatic cancer CMA and a summary of the scoring of immunocytochemical labeling. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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prognostic and predictive marker for pancreatic cancer. We also carried out a bioinformatics analysis of a literature curated catalog of pancreatic cancer biomarkers developed previously by our group and identified two candidate biomarkers, HLA class I and transmembrane protease, serine 4 (TMPRSS4), and examined their expression in the cell lines represented on the pancreatic cancer CMAs. Our results demonstrate the utility of CMAs as a useful resource for rapid screening of molecules of interest and suggest that CMAs can become a universal standard platform in cancer research.

Keywords

microarrays; immunocytochemistry; biomarkers; immunohistochemistry

INTRODUCTION

Immunohistochemistry (IHC) has become a key technique to assess expression as well as subcellular localization of proteins of interest in tissues.^{1,2} IHC plays an essential role both in clinical medicine and in basic research. Tissue microarrays (TMAs) have been successfully incorporated as a high-throughput platform for cancer research.³ For instance, Kampf et al. generated TMAs, which contained 48 normal human tissues along with 216 tumors representing the 20 most common cancer types.⁴ TMAs are useful because they can provide diagnostic information on a large number of patient samples while minimizing the number of slides to be processed.

Biomedical studies are often carried out on model systems employing cell lines to address the functional importance of molecules of interest. However, it still remains difficult to select rapidly and accurately appropriate cell line(s) for functional studies. In 2005, Ferrer et al. developed and immunophenotyped a panel of paraffin-embedded cell lines using five human prostate cancer cell lines and a human cervical adenocarcinoma cell line.⁵ Waterworth et al. published a cell array containing 23 cancer cell lines, of which two were derived from pancreatic cancer (MiaPaCa and Panc-1).⁶ In 2006, Andersson et al. described cell TMAs (cells and tissues) as a tool for antibody-based proteomics using 46 frequently used cell lines in addition to 12 patient cell samples.⁷ Therefore, microarrays generated from cultured cell lines which are formalin fixed and embedded in paraffin can serve as a platform for *in vitro* analysis of protein expression profiles. To our knowledge, no cell microarrays (CMAs) have yet been reported that were designed to evaluate protein expression and subcellular localization for a single cancer type in a comprehensive fashion using a panel of a large number of cell lines.

As a pilot, we choose pancreatic cancer, which is the fourth leading cause of cancer-related deaths in the United States, with a 5-year survival rate <6%.^{8,9} A number of studies have reported potential biomarkers for this deadly disease. We have previously developed a compendium of potential biomarkers in pancreatic cancer cataloguing altered genes curated from the published literature.¹⁰ Despite the availability of this long list of potential biomarkers, no systematic studies have yet been reported to validate them across frequently

studied cell lines. In this study, a comprehensive CMA for human pancreatic cancers was generated that contains all pancreatic cancer cell lines available from the American Type Culture Collection (ATCC, <http://www.atcc.org>) in addition to several others that were generated at Johns Hopkins. We have tested these pancreatic cancer CMAs by immunocytochemical labeling for Ep-CAM (epithelial cell adhesion molecule) to determine the expression of this epithelial marker. To address the general applicability of the pancreatic cancer CMA, we also performed immunocytochemical labeling for CA19-9 (carbohydrate antigen 19-9) and CD44, molecules that are known to be overexpressed in some pancreatic cancers.¹¹ In addition to these, we also examined two potential biomarkers, HLA class I and TMPRSS4 (transmembrane protease, serine 4), and observed strong labeling of the large majority of the pancreatic cancer cell lines present on the CMA. Overall, our results indicate that CMA is a high-throughput platform for antibody-based screening in cancers for rapid characterization of cell lines for antigens of interest.

MATERIALS AND METHODS

Cell Lines

A total of 40 cell lines derived from pancreatic adenocarcinoma and two normal pancreatic cells were used to construct the pancreatic cancer CMAs. The human pancreatic cancer cell lines Hs700T, Hs766T, MiaPaCa-2, Capan1, Capan2, Panc-1, CFPAC1, BxPC3, SW1990, Su8686, HPAF II, AsPC1, Panc2.03, and HPAC were purchased from ATCC, while the remaining cell lines were contributed by investigators at Johns Hopkins (Pa01C, Pa02C, Pa03C, Pa04C, Pa07C, Pa08C, Pa09C, Pa13C, Pa14C, Pa15C, Pa16C, Pa17C, Pa18C, Pa20C, Pa21C, Pa28C, Pa29C, PK8, PK9, PL5, A2.2, A2.4, JD13A, A38.41, A38.44, and Panc184). Two normal pancreatic cells, HPNE and HPDE, were from ATCC and from Dr. Ming-Sound Tsao (Ontario Cancer Institute, Toronto, ON, Canada), respectively. All cells were cultured in the appropriate media in accordance with the recommended guidelines. The cells were cultured until they were ~70% confluent; then they were serum-starved overnight, washed thrice with phosphate buffered saline, and fixed in 10% formalin in phosphate buffered saline for 10 min. The formalin fixed cells from eight 150 mm plates were collected in a 50 mL tube by scraping cells directly and subjected to centrifugation at 500g at 4 °C. Cell pellets were transferred into 1.5 mL microcentrifuge tubes containing 2% agarose, centrifuged again at 500g for 5 min, and stored at 4 °C for 2 days. Formalin-fixed paraffin-embedded blocks were then prepared for generating the cell microarray slides.

Immunocytochemical Staining

The immunocytochemical staining procedure was carried out as described previously for immunohistochemistry for tissues.¹² In brief, slides were incubated at 60 °C for 2 h, followed by deparaffinization in xylene. Sequential rehydration using 100%, 95%, 70%, and 50% ethanol for 2 min each time was carried out before incubating the arrays in preheated antigen retrieval buffer. After treatment with peroxidase, the sections were blocked with 5% goat serum before overnight incubation with primary antibodies at 4 °C. Antibodies against Ep-CAM (1:100, mouse monoclonal, C# 2929, Cell Signaling Technology), CA19-9 (1:100, mouse monoclonal, C# CA1003, EMD Chemicals), pan-HLA class I (1:20, mouse monoclonal W6/32, C# 311423, BioLegend), TMPRSS4 (1:20, rabbit polyclonal, C# HPA#006238, Sigma-Aldrich), and CD44 (1:20, mouse monoclonal, C# 3570, Cell Signaling Technology) were diluted using antibody diluent (Dako, S0809). Harris hematoxylin was used as a counterstain and then quenched in dilute ammonium hydroxide. Cell lines with hematoxylin and eosin staining were individually observed on Nikon DS-Fi1, with a microscope operated using an NIS-Elements F package, while immunolabeled cells were scanned using an Aperio ScanScope CS. The immunocytochemical labeling was assessed by an experienced pathologist (RHH) at Johns Hopkins University by scoring as 0

to 3. Normal tissues, including normal colon and bladder, were included in the CMAs to help with core localization, and these normal tissues served as positive internal controls for the grading of the intensity of labeling.

RESULTS

We have previously established a compendium of candidate biomarkers in pancreatic cancer from manual curation of over 5200 published articles.¹⁰ In all, 2516 genes were cataloged as potential biomarkers because they were reported to exhibit 2-fold or greater overexpression at the mRNA and/or protein level. Importantly, ~70% of these were known to be upregulated only at the mRNA level, with no evidence at the protein level. To rapidly test the expression of molecules of our interest in pancreatic cancer cell lines by an antibody-based labeling method, we developed a CMA for pancreatic cancer.

Development of Cell Microarrays for Pancreatic Cancer

We collected two normal human pancreatic cell lines along with 40 human pancreatic cancer cell lines from ATCC and from the Johns Hopkins Hospital. Details of the cell lines, including patient age and sex, whether derived from primary tumor or metastasis, and mutation status of certain genes, if available, are provided in Supporting Information Table 1. Of these, 19 were derived from primary tumors, while 21 were collected from metastatic lesions. Of the metastatic cell lines, eight were derived from liver, three from lung, two from peritoneum, two from ascites, and one each from bone, lymph node, bile duct, and spleen. The genomes of 15 of the cell lines have been sequenced previously.¹³ The CMA was constructed in the same fashion as TMAs with duplicate spots for each cell line. To observe the cytological pattern of cells on the CMA, we carried out hematoxylin and eosin staining (Figure 1). The right panel in Figure 1 shows a higher power magnification of one of the cell lines in the CMA, showing the architecture of structurally intact cells.

Testing of Individual Molecules by Immunocytochemical Labeling of CMAs

Several antigens, including Ep-CAM, CA19-9, CD44, HLA class I, and TMPRSS4, were selected to screen the expression levels on the CMAs. The extent of immunocytochemical labeling of the various cell lines and their origin are provided in Supporting Information Table 2.

Ep-CAM

As a proof of concept, we first immunolabeled for Ep-CAM, a molecule known to be expressed in the epithelial cells of many organs. The epithelial cell adhesion molecule, Ep-CAM, also known as CD-326, ESA, KSA, M4S1, MK-1, DIAR5, EGP-2, EGP40, KS1/4, MIC18, TROP1, EGP314, HNPCC8, and TACSTD1, is a 40 kDa type 1 transmembrane glycoprotein with intracellular, transmembrane, and extracellular domains from the Human Protein Reference Database (HPRD; <http://www.hprd.org>).^{14,15} The top left panel in Figure 2 shows its molecular domain structure with a THYRO domain and a TM domain. Ep-CAM is also expressed in normal pancreatic tissue, but its expression is enhanced in the large majority of pancreatic adenocarcinomas.^{16–18} In a large scale quantitative transcriptomic study, Ep-CAM was shown to be overexpressed in 12 out of 14 (86%) pancreatic cancer cell lines analyzed and in 11 out of 15 (73%) tumor samples derived either from xenografts or from patients.¹³ As shown in the left bottom panel in Figure 2, we observed positive labeling for Ep-CAM in 78% (22 with a score of “3”; 6 with a score of “2”; 4 with a score of “1”) of pancreatic cancer cell lines in the CMA. Ep-CAM labeling of four representative cores is shown in the right panel in Figure 2. These findings confirm that immunocytochemical labeling of CMAs can be applied for determining protein expression in a rapid fashion. In addition, Ep-CAM showed a higher expression in two liver metastatic

cell lines (A2.1 and A2.2) but almost no expression in another metastatic cell line (TSO111), indicating that Ep-CAM might play a role in pancreatic cancer metastasis.

CA19-9

CA19-9, also known as cancer antigen 19-9 or sialyl Lewis (a) antigen, is a tetrasaccharide carbohydrate consisting of NeuAc α 2-3Gal β 1-4(Fuca1-3)GlcNAc, as depicted in the top left panel of Figure 3. Although variable, CA19-9 is upregulated in carcinomas of the pancreas.^{11,19} If CA19-9 levels are elevated in patients with pancreatic cancer prior to therapy and/or surgery, a decrease in the levels of CA19-9 indicates regression of cancer while rising levels indicate recurrence or metastatic disease.¹⁹ Because of the reported variability of CA19-9 levels in pancreatic cancer, it was expected that immunocytochemical labeling would reveal a variable expression in the cell lines on the CMA. Since this antigen is related to blood group antigens, individuals with the Lewis a- b- blood group do not produce detectable amounts of CA19-9 antigen, since they do not express the glycosyltransferase which sialates the Lewis antigen.²⁰ Approximately 5% of individuals are Lewis type negative, and thus, patients with pancreatic cancer with this blood group cannot be monitored by CA19-9 screening.^{20,21} As shown in Figure 3, we observed that 63% (14 with a score of “3”; 5 with a score of “2”; 6 with a score of “1”) of pancreatic cancer cell lines on the CMA showed positive labeling of CA19-9 with no detectable labeling of the two normal pancreatic cell lines. Mucin-1 is known to be modified by this carbohydrate.²² It has also been reported that several serum proteins including MUC5AC and MUC16 can also be post-translationally modified by sialyl Lewis (a) antigen and labeled with antibodies to CA19-9.²³ This CMA result on CA19-9 not only showed the practicability of screening of molecules other than proteins but also opens a possibility for other carbohydrates to be screened as novel biomarkers for pancreatic cancer.

CD44

CD44 is a widely expressed type 1 transmembrane glycoprotein that is involved in cell–cell interactions, cell adhesion, and migration, and it is primarily a receptor for hyaluronic acid, collagens, osteopontin, and matrix metalloproteases.²⁴ CD44 contains four protein domains: a hyaluronic acid binding domain, a variable domain, a transmembrane domain, and a cytoplasmic region.²⁴ The variable domain corresponds to nine exons in the gene, which can be transcribed to generate various isoforms of CD44, resulting from alternative splicing.²⁵ CD44 is associated with vessel invasion and tumor metastasis. Tumor cells expressing CD44 along with CD24 and CD133 often define cancer stem cells and have the ability to metastasize and initiate epithelial to mesenchymal transitions.²⁶ Because CD44 has a number of isoforms, we used a pan-CD44 antibody for immunocytochemical staining of the pancreatic cancer CMA. Several studies have shown CD44 to be overexpressed in pancreatic cancer cell lines, intraductal papillary mucinous neoplasm, as well as pancreatic cancer tissues at the RNA and protein levels.^{27–29} We found that the large majority (19 with a score of “3”; 10 with a score of “2”; 4 with a score of “1”) of cell lines expressed CD44 as shown in Figure 4. In a recent study, it was found that stemlike CD24+CD44+ cells in the Panc-1 cell line showed a higher metastatic activity.³⁰ We could also recapitulate this high level of expression of CD44 in Panc-1 cells using our CMA platform (Figure 4). Interestingly, a poorly differentiated lung metastatic cell line (A38.5) showed a higher level of expression of CD44 when compared to a cell line derived from peritoneal metastasis from the same patient (A38.41).

HLA Class I

HLA class I molecules present peptides to CD8+ T cells, while the HLA class II present antigens to CD4+ T cells. Although they have slightly similar function with regard to peptide presentation, peptides originate from intracellularly processed antigens for HLA

class I molecules while peptides for HLA class II are derived from exogenous sources.³¹ HLA class I molecules are expressed on all nucleated cells and allow the immune system to evaluate the intracellular environment of host cells.³² The development and progression of tumors is accompanied by altered protein processing, which also alters peptides displayed by HLA class I molecules on the cell surface, further impacting immune surveillance.³³ Therapeutic intervention strategies exploiting various aspects of immune system–tumor interactions are being evaluated in various cancers, including pancreatic cancer, with the hope of inducing an effective immune response to control localized or metastatic disease.^{34–36} The efficacy of such strategies, however, is partly determined by adequate expression of HLA class I on neoplastic cells. mRNA transcripts of HLA-A, HLA-B, and HLA-C genes have been found to be overexpressed in pancreatic cancer or intraductal papillary mucinous neoplasms.^{37,38} A global transcriptomic analysis of pancreatic cancer also showed that HLA-A and HLA-C were overexpressed in all cell lines ($n = 14$) as well as in 67% and 73% of tumor samples ($n = 15$), respectively.¹³ Labeling of the pancreatic cancer CMA using pan-HLA class I antibodies reveals a broad range of HLA class I expression ranging from low to undetectable expression (5 with a score of “1”; 5 with a score of “0”) to very high expression (26 with a score of “3”; 6 with a score of “2”). Interestingly, some of the cell lines including Pa08C and Hs766T show very high levels of HLA class I molecules on their surface and could be used to identify the peptides being presented for potential use in targeting the neoplastic cells (see Figure 5).

TMPRSS4

Transmembrane protease serine 4, *TMPRSS4*, also known as CAPH2, MT-SP2, and TMPRSS3, is a member of the subfamily of type II transmembrane serine proteases, which are involved in a number of biological processes, and their dysfunction often leads to diseases in humans. Multiple downstream signaling pathways are activated by TMPRSS4, including focal adhesion kinase, extracellular signal regulated kinase, Rac1, Src, and Akt.³⁹ Integrin alpha 5 has been implicated as a molecular mechanism through which TMPRSS4 induces cancer progression and invasion.⁴⁰ TMPRSS4 is weakly expressed in normal tissues but is highly upregulated in pancreatic cancers.⁴¹ TMPRSS4 is also overexpressed in lung and colorectal cancers and has been suggested as a diagnostic marker for malignant thyroid neoplasms.^{40,42} *TMPRSS4* gene has been found to be overexpressed at the mRNA level in intraductal papillary mucinous neoplasm, mucinous cystic neoplasm, and pancreatic cancer.^{38,43–45} The global transcriptomic data showed that 86% of cell lines and 100% tumor samples exhibited overexpression of *TMPRSS4*.¹³ Using the pancreatic cancer CMA, we also confirmed that 90% (7 with a score of “3”; 20 with a score of “2”; 11 with a score of “1”) of cell lines expressed TMPRSS4 (see Figure 6).

DISCUSSION

Here, we have developed a pancreatic cancer CMA and applied this as a platform for rapid screening of expression of proteins and glycans by performing immunocytochemical labeling. Potential biomarkers that had no prior evidence of overexpression in pancreatic cancer at the protein level were among those chosen for immunohistochemical analysis, and their overexpression was confirmed in a subset of pancreatic cancer cell lines. The cell lines could also be screened for expression of molecules that are potential therapeutic targets (e.g., activated kinases) or those that are known or likely to be immunogenic for use in whole cell vaccine approaches. As demonstrated in this study, this platform can also be used for screening carbohydrate epitopes when appropriate antibodies (or similar reagents such as aptamers) are available. In fact, the CMAs can be extended for screening a variety of molecules/entities other than proteins, such as lipids, mutant proteins, splice variants, and post-translational modifications. Unlike TMAs, CMAs have their own unique advantages.

First, they are “renewable”, and second, they can become a universal standard, as other laboratories can generate CMAs using the same cell lines. We expect that the CMAs will become a popular tool for a broad spectrum of biomedical research and clinical uses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS AND ACRONYMS USED THROUGHOUT THE TEXT

CMA	cell microarray
Ep-CAM	epithelial cell adhesion molecule
CA19-9	carbohydrate antigen 19-9
HLA	human leukocyte antigen
TMPRSS4	transmembrane protease, serine 4
IHC	immunohistochemistry
ICC	immunocytochemistry
TMA	tissue microarray
ATCC	American Type Culture Collection

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Figure 1.

Hematoxylin and eosin staining of CMAs. The panel on the left shows a hematoxylin and eosin stained CMA slide. The panel on the right is a magnified view (20 $\times$) of one of the cell pellets on the CMA that allows one to observe individual cells in greater detail.

Figure 2.

Immunocytochemical staining of Ep-CAM. In the top left panel, the domain structure of Ep-CAM is shown, where the red color indicates the signal peptide and TM refers to the transmembrane domain. The domain colored in green designated as THYRO is thyroglobulin type I repeat, which is found in some glycoproteins. The left bottom panel shows a CMA slide stained with a monoclonal antibody directed against Ep-CAM. The panel on the right shows a magnified view of four cell lines showing different levels of expression of Ep-CAM. The TSO111 cell line showed complete downregulation of Ep-CAM, while two liver metastatic cell lines (A2.1 and A2.2) from one individual and a normal pancreatic cell line (HPNE) showed high expression of Ep-CAM.

Figure 3.

Immunocytochemical staining of CA19-9. In the top left panel, the glycan structure (sialylated Lewis a) recognized by the CA19-9 antibody is shown. The left bottom panel shows a CMA slide stained with a monoclonal antibody directed against CA19-9. The panel on the right shows a magnified view of four cell lines showing varying levels of expression of the CA19-9 target. The lung metastatic cell line (JD13D) showed a lower level of expression of CA19-9 as compared to the primary cell line (JD13A) derived from the same patient. Panc-1 and Panc2.03 cell lines, both derived from primary tumors, showed a distinct expression pattern of CA19-9 as shown.

Figure 4.

Immunocytochemical staining of CD44. In the top left panel, the domain structure of CD44 is shown, where the red color indicates the signal peptide and TM refers to the transmembrane domain. The domain colored in red designated as LINK is a hyaluronan-binding domain found in proteins involved in the assembly of the extracellular matrix and cell adhesion. The black lines with red circles, lightning symbol, or green circles represent sites of phosphorylation, protease cleavage, or glycosylation, respectively. The left bottom panel shows a CMA slide stained with a pan-CD44 antibody. The panel on the right shows a magnified view of four cell lines showing different levels of expression of CD44. Very high expression was observed in a normal pancreatic cell line (HPNE) while moderate expression was observed in a cell line derived from a peritoneal metastasis (A32.1). CD44 expression was observed to be higher in a lung metastatic cell line (A38.5) as compared to a peritoneal metastatic cell line derived from the same patient (A38.41).

Figure 5.

Immunocytochemical staining of HLA class I. In the top left panel, the domain structure of the HLA class I molecule is shown, where the red color indicates the signal peptide and TM refers to the transmembrane domain. The domain colored in yellow designated as IGC is an immunoglobulin domain commonly found in extracellular proteins. The black lines with red circles or green circles represent sites of phosphorylation or glycosylation, respectively. The panel on the right shows a magnified view of four cell lines showing different levels of expression of HLA class I. Two metastatic cell lines from the same patient (A38.41 and A38.5) showed a high expression of HLA class I, although the poorly differentiated cell line (A38.5) expressed a higher level of HLA class I protein. Capan-1, a cell line derived from liver metastasis, showed a higher expression of HLA class I than Capan-2, a cell line derived from a primary tumor, which showed barely detectable expression.

Figure 6.

Immunocytochemical staining of TMPRSS4. In the top left panel, the domain structure of TMPRSS4 is shown, where TM refers to the transmembrane domain. The left bottom panel shows a CMA slide stained with a monoclonal antibody directed against TMPRSS4. The panel on the right shows a magnified view of four cell lines showing different levels of expression of TMPRSS4.