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Novel bioinformatic identification of differentially expressed tissue-specific and cancer-related proteins from the Human Protein Atlas for biomarker discovery

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ABSTRACT. Identification of cancer-associated and tissue-specific proteins is important for research on carcinogenesis mechanisms and biomarker discovery. Here we performed a new strategy to identify candidate cancer proteins by mining immunohistochemistry protein profiles. Proteins with quantitative values from 14 normal tissues and their corresponding cancer tissues were compared and analyzed using bioinformatics. The final results included identification of tissue-specific proteins and differentially expressed proteins in different cancer types that are primarily involved in energy metabolism and cell invasion. From the tissue-specific proteins, secreted and membrane proteins were further screened and functionally clustered. These primarily belonged to the gene families of endogenous ligands, cluster of differentiation molecules, and solute carriers, and were mainly involved in the processes of cell motility, hormone metabolism, adhesion, and transport. Further studies are warranted to validate the candidates identified herein and substantiate the suggested enriched functions. The results

from this study might provide a reliable resource to study underlying carcinogenesis mechanisms and discover potential cancer targets for the development of therapeutic targets and of early diagnosis and disease response markers.

Key words: Cancer; Bioinformatics; Immunohistochemistry; Biomarkers

INTRODUCTION

Carcinogenesis is a complex and unpredictable process that involves many molecular alterations leading to deficits in multiple cellular signaling pathways (Capaccione and Pine, 2013). Molecules involved in this process may be used as potential biomarkers to reflect disease state and reveal its underlying pathogenesis (Liu et al., 2012a,b). Therefore, cancer biomarker discovery is crucial for progressive research in cancer biology and clinical application. Biomarkers may include DNA, RNA, or protein molecules, with proteins being considered the most promising (Polanski and Anderson, 2007). Biomarkers in each tissue should have differential expression levels or activities between different disease states, thus serving as a measured or evaluated profile for reflecting normal biological processes, pathogenic processes, or responses to treatment (Prassas et al., 2012).

With the advent and development of proteomic biotechnologies, many potential protein biomarkers have been identified by differential proteomic techniques in various cancer types (Brinton et al., 2012; Honda et al., 2013). Some of these proteins were clustered and manifested in different databases, such as dbDEPC (http://lifecenter.sgst.cn/dbdepc/index.do), which describes differentially expressed proteins in human cancers, GeMDBJ Proteomics (https://gemdbj.nibio.go.jp/dgdb/DigeTop.do), which includes an integrated proteome database for cancer research, CanProVar (http://bioinfo.vanderbilt.edu/canprovar/), which was designed as a human cancer proteome variation database, and the Human Protein Atlas (HPA, http://www.proteinatlas.org/), which contains quantified immunochemistry results of normal and cancer tissues based on antibody proteomics. By comparing and re-analyzing these data, we can obtain new insight into the research of underlying mechanisms of carcinogenesis and discovery of cancer biomarkers. However, due to the heterogeneity of experimental methods and specimen preparation between laboratories (Issaq et al., 2011; Heckman-Stoddard, 2012), the results obtained from proteomic analyses lack good reproducibility and require further verification and validation before they can be used in clinical detection and to explain underlying mechanisms.

Alteration of protein expression can lead to disturbances in molecular function or pathways regulating cell growth, survival, or metastasis (Polanski and Anderson, 2007). Molecules that exhibit changes in a specific cancer can be used as biomarkers for detection, diagnosis, or prognosis. To perform a reliable functional analysis and identify potential cancer biomarkers, the proteomic variation data were normally verified by Western blot or immunohistochemistry. Immunohistochemistry plays vital role in histological diagnosis, and the immunohistochemical markers could be used for estimating prognosis and predicting therapy response (Ordóñez, 2013). The HPA is built based on immunohistochemistry data, and provides a reliable proteomic resource for biomarker discovery (Pontén et al., 2011). It is a powerful platform not only to provide immunohistochemical mapping but also to provide quantitative protein expression profiles across different tissues. The information generated using the HPA

allows the screening for differential protein profiles across different tissues. In the present study, we identified differentially expressed cancer proteins and tissue-specific proteins by re-analyzing HPA datasets. Our strategy compared proteins quantitatively between normal and cancer tissues by selecting up- or down-regulated proteins in cancers, and compared the proteins across different tissues for the selection of proteins highly specific to or strongly expressed in a single tissue. Secreted and membrane proteins were further selected to prioritize candidates for future validation and verification. The results might provide new insight into cancer biology research, leading to a better understanding of cancer progression and facilitating cancer biomarker discovery.

MATERIAL AND METHODS

Data collection

Staining profiles for proteins in normal and cancer tissues were downloaded from the HPA. The normal and cancer tissues included breast, cervix, colon, larynx, liver, lung, ovary, pancreas, prostate, kidney, stomach, testis, thyroid gland, and urinary bladder samples. The expression level of each protein was then graded into four levels: strong: >75%; moderate: 25-75%; weak: <25%, and negative: 0% for use as retrieval parameters. The differentially expressed proteins were defined as those that exhibited a change in expression of more than two levels between the previously described groups. The resulting proteins in each cancer type were grouped into up- and down-regulated proteins. The specifically expressed proteins referred to proteins with higher expression of more than two levels across different tissue types.

Functional annotation clustering analysis of differentially expressed proteins

The protein identifiers were uploaded to the Database for Annotation Visualization and Integrated Discovery (DAVID; http://david.abcc.ncifcrf.gov/) and enrichment analyses of the Gene Ontology (GO) terms, including the biological process and molecular function, were performed using the functional clustering annotation tools. The default options with high classification stringency were used. Finally, the cluster names were extracted from the most biologically relevant GO term that was assigned to that cluster.

Over-representation analysis of specifically expressed proteins

The over-representation analyses of GO terms, including biological processes and molecular function, were performed using the functional tool of ConsensusPathDB-human (http://cpdb.molgen.mpg.de/CPDB), which is a molecular functional interaction database. The GO level -2 and -3 categories were selected, and we set the P value cutoff at 0.01.

Analysis of secreted and membrane proteins

The secreted and membrane proteins were screened through tools in LOCATE (http://locate.imb.uq.edu.au/), which is a curated database for describing membrane organization. The membrane proteins included types I, II, and III proteins.

RESULTS

Identification of differentially expressed proteins in different cancer types

By comparing the quantitative immunohistochemistry results across normal and cancer tissues with a 2-level difference, up- and down-regulated proteins in different cancers were identified. As displayed in Figure 1, the largest number of up-regulated proteins were identified in ovarian cancer (1671 proteins), followed by renal cancer (1015 proteins), and the largest number of down-regulated proteins were identified in stomach cancer (4370 proteins), followed by testis (3673 proteins), lung (3055 proteins), and pancreatic (3046 proteins) cancers.

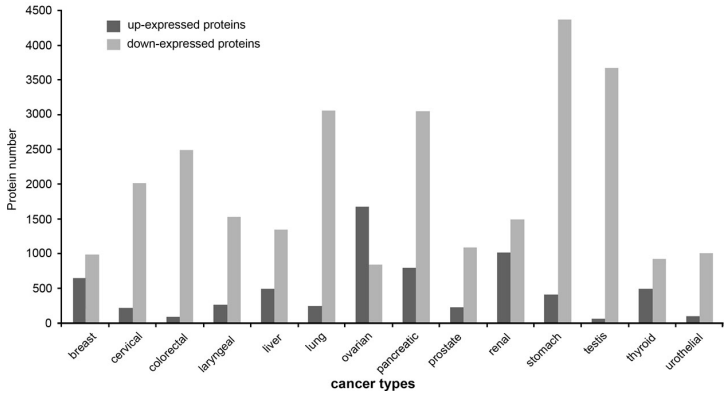

Figure 1. Distribution of up-regulated and down-regulated proteins in different cancer types.

Identification of specifically expressed proteins across different normal or cancer tissues

The unique proteins that were highly specific to or strongly expressed in certain cancer or normal tissues were identified (Table 1). The largest number of unique cancer proteins was identified in the thyroid gland (103 proteins), and the largest number of unique normal tissue proteins was identified in the testis (123 proteins). Urinary bladder had few unique proteins in either normal or cancer tissues.

Secreted and membrane proteins

We found that secreted and membrane proteins from the specifically expressed proteins appeared to be the most promising biomarkers. By retrieving the subcellular localization database, secreted and membrane proteins in each tissue were identified as displayed in Table 2. In cancer tissues, a total of 45 secreted proteins and 93 membrane proteins were identified. Many of the secreted proteins (13) were present in pancreatic cancer, and many of the membrane proteins were present in thyroid cancer. A total of 59 secreted proteins and 89 membrane proteins were selectively identified in normal tissues, and the largest number of secreted (11) and membrane (19) proteins were present in the testis. These secreted proteins primarily belonged to the gene family of endogenous ligands (6) and cluster of differentiation (CD) molecules (5). The membrane proteins were mainly from the gene families of CD molecules (16) and solute carriers (15).

Table 1. Number of proteins specifically expressed in different tissues.

Tissue	Specific proteins (N)	
	Cancer tissue	Normal tissue
Breast	14	19
Cervix	3	26
Colon	35	60
Kidney	25	12
Larynx	36	10
Liver	49	42
Lung	2	34
Ovary	5	5
Pancreas	77	51
Prostate	38	18
Stomach	14	30
Testis	28	123
Thyroid gland	103	10
Urinary bladder	2	6

Table 2. Secreted and membrane proteins specifically expressed in different normal *vs* cancer tissues.

Tissues	Cancer		Normal	
	Secreted proteins	Membrane proteins	Secreted proteins	Membrane proteins
Breast	FBLN1, MTTP, STT3B, SCGB2A2	PCDH19, SLC15A3	PIP, CHSY1, CCL25	DPP6, PRLHR, ZDHHC16
Cervix	-	-	SPINK5, Slpi, CELA2A	KRT6C, IQCF1, KRT6B, PLVAP,
Colon	DPEP1, CDHR5, SIRPB1	SIRPB1, SLC17A6, GPA33, Gpr82, CDHR5, FASLG	CDHR5, CA4, PYY, REG4, CLCA1, vWF, UMOD, ZG16	GGT5, MUC13, UGT2B10, UGT2A3, UGT2B28, UGT2B7, SGCD, CNTNAP2, SELE, GPR61, PIGO, ENTPD1, CADM3, RTN3, LPHN2
Larynx	CD99L2, ANGPTL3	KIRREL, KRT6B, CYB561D1, SLC5A7, RHBDD2, SLC10A6	GLB1L, NTN3, SFRP2, RNASE2	TMEM117, MANBAL
Kidney	VCAN, CST7	CDH16, CST7, GGTLC2, GGT4P, NIM1	APOA1, APOB, PTPRO,	KIRREL, CRIM1, PODXL, ITGA8, PTPRO, NPHS2, KIAA0317
Liver	LRP1, F2, LYZL4, LOXL3, MUC15, PRSS38	MUC15, LRP1, SLC27A2, SGCZ, CCR3, CREB3L2, TFR2, KCND1, S1PR5, ZDHHC1, OR56B1	CRP, PON3	S1PR5, TFR2, ABCC2, SLCO1B3, GPR12, CYP1A2
Lung	-	FAM57B	LECT2, CTSG, ITGB2, SFTPA1	MPO, ITGAM, ITGB2, MRC1, NUP205, DOCK5, PAQR5, CD163, CYBA, ALOX5AP
Ovary	-	WT1, ADIPOR2	CD55	CD55
Pancreas	COL4A2, DCN, PIP, CSH1, FGF18, COL6A3, SPARCL1, ACE, AGTR1, TPSAB1, TFF2, CALR3, RNASE2	PLLP, MS4A1, CD19, OR4F21, SLC22A1, CD34, CACNA2D1, EMB, LYZL2, B3GNT4, SGCB, CMTM5, RGMB, CPNE9	DBC1, REG1A, CPA1, IAPP, TFF3, CPA2, GCG	CCDC107, DGCR2, TFF3, LOC150763, PIGM, BEST2, MBOAT1, LPPR1, SV2A, TMEM145
Prostate	CPE, PLAT, KLK2, ACPP, HEXB, KLK3, PLAT	KIAA1324, PLAT, ACPP, HEXB, FOLH1, SLC3A1, AQP8, OR2AK2, RAP1GAP	KLK4, KLK2, ACPP, HTRA4, KLK3	ACPP, FOLH1, TMPRSS13, CD38
Stomach	BMP4, VEGFB	GPR15, DISP1	C1RL, TGFB1, PGC, TFF2, GIF, TMEM178	LINGO1, IGSF3, TCTN3, SLC36A2, TMEM178
Testis	-	ALPL, DSCAM, TPRA1	ACRV1, ACR, ACRBP, PTN, BMP8B, GLB1L, INSL6, INHA, LYZL6, SPINK2, FMR1NB	TMEM132D, TEX264, TTC21A, TMEM102, CYP19A1, TBC1D9, SUN5, SLC2A8, TEX101, MYCBP2, PTCHD3, NKAIN3, ADAM2, KCNG4, OPRM1, SLC35E4, MYCBP2, CLDN11, SLC35E4
Thyroid gland	C2ORF40, TPO, AMELY, MAMDC2, TG	CLSTN3, SUSD1, CD3G, TPO, PTPRH, PEAR1, HEPACAM2, ACVRL1, MRC2, ADAM19, CLSTN3, NBEA, TMPRSS9, HS6ST2, NOX4, PCDHB5, CABP7, C17ORF68, BCL2, SLC23A1, ADRA1A, TMEM204, RETSAT, CNIH2, SLCO2A1, ADRA1A, CMKLR1, KCNK6, UPK1A, LRRC55, NKAIN2, LPCAT2, SLC25A31	TPO, TG, CPQ	TPO, CDH16, PGCP, SLC17A2
Urinary bladder	FAT2	KCNE1	-	UPK3A

Comparison of normal testis-specific proteins with cancer-specific proteins

The specifically expressed testis proteins selected in this study were compared with specifically expressed cancer proteins. ANKIB1 was specifically expressed in breast cancer, and TEX264 was specifically expressed in colorectal cancer. SPATA7 was commonly highly expressed in breast and ovarian cancers. Notably, except for HSPA4L in pancreatic cancer and MBD3L1 in stomach cancer, 24 proteins were commonly highly expressed in pancreatic and stomach cancers.

Ontological analysis

To map the major functional categories, the up-regulated proteins in every cancer type were grouped into several functional clusters using the functional annotation clustering tool DAVID (Table 3). Certain enriched functional clusters were common in more than three cancer types, including cell cycle, mitochondrion, cytoskeleton, cell adhesion, protein localization, and tight junction proteins. These prominent functional clusters were primarily involved in the biological processes of energy metabolism and cell invasion.

Table 3. Enriched functional clusters of up-regulated proteins in different cancers.

Breast cancer	Cervical cancer	Colorectal cancer	Head and neck cancer	Liver cancer	Lung cancer	Ovarian cancer
ATP binding	Cell cycle	Cell cycle	Regulation of lipid metabolic processes	RNA processing	Tight junction	Mitochondrion
Protein localization	DNA replication	DNA packaging	Epidermal development	DNA replication	ATPase activity	Ribosome
Cytoplasmic vesicle	Cell junction	Immunoglobulin-like	Cell junction	Ribosome	Mitochondrion	Glycosylation
Proteolysis	Golgi apparatus	Apoptosis	Cyclin	DNA repair	Cytoskeleton	Tight junction
Cell cycle	Cyclin		Regulation of Wnt receptor signaling pathway	Kinase binding	Cell cycle	Cell cycle
Angiogenesis			Kinase binding	Chaperone		Lipid metabolism
Apoptosis				Exonuclease activity		Protein localization
				Cell cycle		ATPase activity
				Protein localization		tRNA processing
				Tight junction		
Pancreatic cancer	**Prostate cancer**	**Renal cancer**	**Stomach cancer**	**Testis cancer**	**Thyroid cancer**	**Urothelial cancer**
Cell adhesion	Transcription factor activity	Mitochondrion	Regulation of response to stimulus	Extracellular region	Cell adhesion	Epidermal development
Extracellular region	Cell cycle	Amine catabolic process	Lymphocyte activation	Response to hormone stimulus	Mitochondrion	Glycoprotein metabolic processes
Cytoskeleton	tRNA processing	Ion transport	Extracellular region	Cell adhesion	ATP binding	Structural molecule activity
Membrane protein	Apoptosis	Glycolysis	Regulation of cell activation		ECM-receptor interaction	Cell cycle
Myofibril assembly	Mitochondrion	Response to metal ions	Cytoskeleton		Muscle contraction	
Cell-matrix adhesion	Sexual reproduction	Carboxypeptidase activity	Fatty acid binding		Amino acid transport	
Peptidase inhibitor activity		ATP binding	Calmodulin binding		Ion transport	
Oxidation reduction		Protein complex assembly	Myofibril assembly		Lipid transport	
Regulation of cell migration		Fatty acid metabolism	Angiogenesis			
		Metallopeptidase activity	Cell adhesion			

Major items with an enrichment score >1.0 were selected.

Over-representation analyses were performed to map the enriched functional terms for specific secreted and membrane proteins. The results showed that specific secreted proteins in cancer tissues mainly functioned as peptidase and peptidase inhibitors, and had growth factor activity, performing the main biological processes of cell motility, response to external

stimulus, and tissue development. Secreted proteins in normal tissues mainly functioned as receptors, peptidases, and lipid transporters, which corresponded primarily to the biological processes of cell motility, cell proliferation, and hormone metabolic processes. Analysis of membrane proteins in cancers showed the enriched functions of transmembrane transporter and signaling receptor activity, which are mainly involved in the processes of cell adhesion and transport. In normal tissues, specific membrane proteins had the enriched molecular functions of peroxidases, cargo receptor activity, and carbohydrate binding, and are involved in hormone metabolic and cellular homeostasis.

DISCUSSION

Cancer biomarkers represent key targets in the field of cancer research, because of their potential utilization in early cancer detection, diagnosis, and monitoring of response to treatment. A comprehensive analysis of human cancer-associated proteins and cancer-specific proteins might facilitate the understanding of the underlying mechanisms of carcinogenesis and the discovery of cancer biomarkers (Liu et al., 2012a,b).

In the present study, we constructed comprehensive differentially expressed protein profiles associated with different cancer types by quantitatively comparing credible immunohistochemistry results between normal and corresponding cancer tissues. Normal and cancer tissue-specific proteins were further screened across different tissue types. The results provided new insights into the research of cancer biology, and useful information for cancer biomarker discovery.

To investigate the underlying mechanisms of cancer development and progression, differentially expressed proteins in each cancer type were identified. Up-regulated proteins in cancers are predicted to play promising roles in understanding tumorigenesis and biomarker discovery. Thus, functional clustering analyses were performed to investigate the underlying common and special functions enriched by up-regulated proteins in each cancer type. Different functional clusters were identified in various cancers, which might indicate the different underlying mechanisms in cancer development and progression. However, several functions were commonly clustered in the cancers, including cell cycle, mitochondrion, cytoskeleton, cell adhesion, protein localization, and tight junction. These functions have previously been shown to be involved in cancer progression (Wallace, 2012; Williams and Stoeber, 2012), invasion (Behrens, 1993), and metastasis (Martin et al., 2011).

Tissue-specific proteins may also be involved in the key pathways associated with cancer development and progression, leading to different functions and biological processes among various tissues. These could be used as a unique tissue/cancer signature to distinguish among different tissue types or reflect disease state (Emig and Albrecht, 2011). In the present study, we screened the normal and cancer tissue-specific proteins among 14 different tissues. Some well-known tissue-specific proteins were also identified, such as ESR1 in breast cancer, shown to be associated with high grade and high proliferation (Moelans et al., 2010), CDX2 in colorectal cancer, used as a highly sensitive and specific marker of adenocarcinomas of intestinal origin (Werling et al., 2003), KLK2 and KLK3 in prostate cancer, utilized as biomarkers (Penney et al., 2011), and ACPP and FOLH1, identified as prostate-specific cancer proteins (Maraj and Markham, 1999). Of the 14 tissues examined, thyroid cancer tissue has the highest number of cancer tissue-specific proteins (103), including the well-known thyroid specific proteins TG and TPO (González et al., 2002). Of the normal tissues, the testis has the largest number of

tissue-specific proteins, which reflects the complex protein expression and regulation in the testis (Liu et al., 2011, 2012c; Hua et al., 2013). These proteins may be potential cancer biomarkers and/or physiologic treatment targets, and further studies are warranted to evaluate their underlying functions.

Secreted proteins can serve as biomarkers for early cancer detection and diagnosis, and membrane proteins may be potentially effective therapeutic targets (Arcinas et al., 2009; Stastna and Van Eyk, 2012). Due to the fact that the highly abundant proteins comprise 99% of the total protein mass in the blood (Loo et al., 2010), the discovery of poorly expressed cancer-specific proteins in the blood by routine biotechnology becomes difficult. It is hypothesized that potential protein biomarkers could be secreted or shed directly into the bloodstream, allowing the evaluation of their concentrations in cancer patients (Prassas et al., 2012), thus discovery and verification of circulating cancer biomarkers by indirect identification and subcellular localization analysis of tissue-specific proteins become effective alternative methods. In the present study, secreted and membrane proteins were screened using Membrane Organization tools (http://locate.imb.uq.edu.au/). Expression levels of these proteins in the blood were verified between normal and cancer patients. Interestingly, these secreted proteins primarily belonged to the endogenous ligand gene family, which could bind to their receptors to trigger signals that affect specific cell development and function. The membrane proteins were primarily solute carriers, which have vital roles in cancer by transporting macromolecules and serving as potential treatment targets (El-Gebali et al., 2013).

As described in our previous studies (Liu et al., 2011, 2012c), some testis-specific proteins could become cancer/testis antigens specifically expressed in certain cancers. These proteins could be used as potential cancer vaccine targets. Notably, 24 proteins with specific testicular expression in the present study were commonly highly expressed in pancreatic and stomach cancers. The results indicated that these two gastric cancers might share certain common underlying mechanisms.

In conclusion, we performed a new strategy to identify candidate cancer-associated and cancer-specific proteins for utilization in future biomarker discovery studies. By comparing protein expression levels of 14 normal tissues with their corresponding cancer tissues, we identified candidate proteins and performed functional analyses. Further studies are warranted to validate the candidates and to substantiate the enriched functions identified herein. The results could be used as a reliable resource to study underlying carcinogenesis mechanisms and discover potential cancer targets for early diagnosis, therapeutic targets, and disease response markers.

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