BIOMARKERS, GENOMICS, PROTEOMICS, AND GENE REGULATION

Tumor Vessel Up-Regulation of *INSR* Revealed by Single-Cell Expression Analysis of the Tyrosine Kinome and Phosphatome in Human Cancers

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The tyrosine kinome and phosphatome harbor oncogenes and tumor suppressor genes and important regulators of angiogenesis and tumor stroma formation. To provide a better understanding of their potential roles in cancer, we analyzed the expression of 85 tyrosine kinases and 42 tyrosine phosphatases by *in situ* hybridization 48 human normal and 24 tumor tissue specimens. Nine-tenths of the assessed transcripts had tumor cell expression concordant with expression array databases. Further, pan-cancer expression of *AATK*, *PTPRK*, and *PTPRU* and expression of *PTPRS* in a subset of tumors were observed. To demonstrate tumor subcompartment resolution, we validated the predicted tumor stroma-specific markers *HTRA1*, *HTRA3*, *MXRA5*, *MXRA8*, and *SERPING1* *in situ*. In addition to known vascular and stromal markers such as *PDGFRB*, we observed stromal expression of *PTK6* and *TNS1* and vascular expression of *INSR*, *PTPRF*, *PTPRG*, *PTPRU*, and *TNS1*, of which *INSR* emerged as a tumor-specific vessel marker. This study demonstrates the feasibility of large-scale analyses to chart the transcriptome *in situ* in human cancers and their ability to identify novel cancer biomarkers. (*Am J Pathol* 2015, 185: 1600–1609; <http://dx.doi.org/10.1016/j.ajpath.2015.02.019>)

Certain gene families, such as the tyrosine kinases and tyrosine phosphatases, are known to be frequent targets of mutations in human cancers.^{1–3} Tyrosine phosphorylation, governed by the balanced action of tyrosine kinases and phosphatases, is essential to many cellular processes that regulate transcription, cell cycle progression, proliferation, differentiation, and apoptosis.^{4,5} The tyrosine kinome harbors several proto-oncogenes, such as *ABL*, *ERBB2*, *FGFR1*, *KIT*, *NTRK3*, and *PDGFRB*, and is the focus of intense drug discovery efforts.^{6–11} Similarly, somatic loss-of-function mutations in tyrosine phosphatases were identified in several common human cancers, for example, *PTPN3*, *PTPN13*, *PTPN14*, *PTPRF*, *PTPRG*, and *PTPRT* in colorectal cancers.² Kinases and phosphatases such as vascular endothelial growth factor receptors, *PDGFRB*, fibroblast growth factors, *TIE*, *BMX*, and *PTPRB* also play important roles in angiogenesis, an essential process in tumor development.^{12–17} This explains the intense interest in drug discovery to target kinases and phosphatases and the need to systematically map their expression in human normal and malignant tissues.

Advances in sequencing technologies have enhanced our ability to find putative cancer genes, leading to a surge in the number of genes mutated in 1% to 5% of patient tumors.^{18–20} To unambiguously identify these genes as bona fide cancer genes they must be linked to tumor phenotypes by functional studies. However, few universal experimental approaches enable characterization of large gene sets, leading to a substantial investment in each gene studied. One essential aspect is the gene expression pattern, which can help prioritize genes that are expressed in the malignant cells for further studies. Expression array studies and transcriptome analyses have generated extensive knowledge on the gene expression levels of the human transcriptome in normal and diseased tissues. Several different resources such as MediSapiens offer aggregated and normalized data from large sets of gene expression arrays that can provide a reference for whole

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organ or whole tumor gene expression profiles.²¹ Further, initiatives such as the Allen Human Brain Atlas have increased the understanding of human brain function and the impact of genetic disorders on brain structures.²² These efforts provide excellent references to validate *in situ* expression analyses of human genes.

Although systematic charting of the transcriptome *in situ* was performed in the developing mouse, no such effort was performed in the context of human tissues despite the inherent scalability and specificity of *in situ* hybridization (ISH).²³ To enable such efforts, we have adapted automated mRNA ISH for expression analysis of large gene sets in human formalin-fixed, paraffin-embedded (FFPE) tissue arrays.²⁴ To better understand which cell types of normal tissues and tumors express different tyrosine kinases and phosphatases, we analyzed their *in situ* expression pattern in 48 different normal human tissues and tumors of the colorectum, breast, prostate, kidney, bladder, and testis. In combination with existing large-scale expression data sets, this provides a comprehensive and validated expression map of the human tyrosine kinome and phosphatome and enables the discovery of novel compartment-specific cancer biomarkers.

Materials and Methods

Reference Data Sets

To obtain reference gene expression data with cell type resolution during organism development and to obtain expression data from human organs and tumors, we i) collated annotated GenePaint database (<http://www.genepaint.org>; last accessed February 8, 2015) ISH data (release July 5, 2012), ii) mined the literature for ISH gene expression studies, and iii) summarized phenotypes and expression patterns from published gene-targeting studies in the mouse. Further, we used the MediSapiens database (<http://ist.medisapiens.com>, last accessed February 8, 2015) that encompasses 157 normalized gene expression array studies with 9783 human tissue specimens from 43 normal tissue types and 68 tumor types to obtain bulk tissue gene expression data from the adult human²¹ (Supplemental Tables S1–S3). We were unable to find annotated, publicly available developmental gene expression data at cell type resolution for one-third of tyrosine kinases and phosphatases.

Large-Scale ISH on FFPE Human Tissue

Forty-eight arrayed normal adult human tissues in triplicate and 24 cancers each of breast, colorectum, prostate, kidney, bladder, and testis were used for each gene. All tissues were obtained from Uppsala University Hospital (Uppsala, Sweden), placed in neutral buffered (pH 7.4) 4% formaldehyde after the surgical procedure. The time between ligation of blood flow and fixation was estimated to be 15 minutes to 1 hour, and the fixation time was 1 to 4 days. After fixation, the tissue was cut into pieces, dehydrated,

infiltrated with paraffin, and assembled on tissue microarray blocks. These blocks were cut in 4- μ m sections and used for ISH and immunohistochemistry (IHC).²⁵

ISH on tissue arrays was performed as described.²⁴ Essentially, FFPE tissue arrays were deparaffinized in xylene and permeabilized by proteinase K treatment. PCR primers for generation of approximately 500-nt products were designed for each gene. The PCR products were used to generate digoxigenin-labeled antisense RNA probes by *in vitro* transcription. The tissue sections were incubated with the RNA probes (Supplemental Table S4) for 4 hours. After hybridization, high-stringency washes and several blocking steps were performed to remove unbound probe and to block endogenous enzyme activities. The bound RNA probes were detected by chromogenic anti-digoxigenin IHC. Whole slide images were digitized at 40-fold magnification by using an Aperio ScanScope CS Slide Scanner System (Leica Biosystems, Buffalo Grove, IL). For each tissue, gene expression was annotated to tumor cell compartment, vascular, connective tissue stroma, and inflammatory cells as either strong, intermediate, or absent. Previously, we evaluated the use of sense RNA probes as negative controls and independent antisense RNA probes for validation of gene expression patterns.²⁴ We concluded that the best approach here would be to use publicly available resources and databases such as PubMed, GenePaint, and MediSapiens and also independent RNA probes for validating the gene expression of the tyrosine kinases and phosphatases.

Comparisons between MediSapiens and ISH

By comparison with MediSapiens data, we estimated that a MediSapiens signal intensity of 200 arbitrary units is likely to produce a signal also in the ISH analysis. ISH expression patterns were considered concordant with MediSapiens expression patterns if the tumor type with the highest ranked expression level in MediSapiens also showed positivity in one or more of such tumors in ISH, the rank order was conserved between MediSapiens and ISH such that tumor types that had higher levels in MediSapiens had ISH expression, and when tumor types with lower expression rank lacked ISH expression. Genes with median MediSapiens expression level ≤ 200 in all but three or fewer examined tissues were considered not expressed/expressed at low levels. We defined genes with outlier expression levels in MediSapiens as having a group of observations with at least twice the median expression level and an expression level ≥ 1000 in one or more forms of cancer.

In Silico Prediction of Novel Tumor Stroma-Specific Genes

Serial analysis of gene expression (SAGE) libraries for transcripts enriched in breast cancer-associated myofibroblasts compared with normal myofibroblasts were obtained from

Allinen et al²⁶ through the Cancer Genome Anatomy Project.²⁷ The Digital Gene Expression Display tool (<http://cgap.nci.nih.gov/tissues/gxs>, last accessed October 31, 2014) was used to identify SAGE tags with at least fourfold enrichment in the cancer myofibroblast library. Duplicate gene entries and genes with tag count <20 were removed, and the remaining genes were annotated with UniProt (<http://www.uniprot.org>, last accessed February 8, 2015). Entries with subcellular location *secreted* or *transmembrane* and no previous information on involvement in cancer in the literature were investigated further. Secreted or transmembrane proteins with potential growth factor activity, enzymatic activity, or receptor activity were selected for ISH validation.

IHC

Tissue sections were deparaffinized, hydrated in graded ethanol, and blocked for endogenous peroxidase activity. Heat-induced antigen retrieval was performed by boiling the glass slides in citrate buffer, pH 6.0, for 4 minutes at 125°C. Automated IHC was performed as described previously.²⁸ Briefly, tissue sections were blocked and rinsed in wash buffer. Primary affinity-purified polyclonal antibody was incubated on the slides, followed by incubation with a secondary anti-rabbit antibody conjugated to a horseradish peroxidase-labeled polymer. The signal was developed with diaminobenzidine mixed with chromogen. The tissues were counterstained with Mayers hematoxylin.

Results

Gene Expression Reference to the Kinome and Phosphatome

To obtain a comprehensive reference on tissue expression, we compiled gene expression data from prior ISH efforts in the mouse, literature on knockout characterization and developmental studies in the mouse, and normalized transcriptome analyses of human tissues and tumors for 85 genes of the tyrosine kinome and 42 genes of the tyrosine phosphatome (Supplemental Tables S1–S3). The tyrosine kinome has been more extensively studied than the phosphatome, which is revealed by the numbers of publications per gene (median, 487; range, 8 to 29,077 for the kinases versus median, 61; range, 1 to 9207 for the phosphatases). For these genes, we obtained normalized human array expression data (96% versus 95% of genes), annotated GenePaint mouse developmental expression patterns (22% versus 17%), developmental gene expression literature (56% versus 55%), and published mouse knockouts (96% versus 64%) (Supplemental Tables S1–S3).

In Situ Analysis of Gene Expression in Arrayed Human Normal and Tumor Tissues

We next generated ISH probes to all genes of the tyrosine kinome and phosphatome (median length, 515 nucleotides;

Figure 1 Summary of compartment-specific gene expression analysis of 127 genes of the tyrosine kinome and phosphatome. The *in situ* gene expression pattern of 85 PTKs and 42 PTPs were evaluated in human normal tissues and cancers of the breast, colorectum, prostate, bladder, kidney, and testis. Six genes had expression patterns discordant with reference gene expression data. Further, 31 genes could not be validated because no reference data were available or their observed expression levels and reference database expression levels were low. Ninety genes had clear expression signals in accordance with validation data sources. Of these, 75 displayed epithelial or tumor cell expression, 18 vascular expression, 16 stromal expression, and 36 inflammatory cell expression, in normal or tumor tissues. N, normal; PTK, protein tyrosine kinase; PTP, protein tyrosine phosphatase; T, tumor.

range, 255 to 595 nucleotides) (Supplemental Table S4). Valid RNA probes could not be generated for the tyrosine kinases *FGFR3*, *LTK*, and *SRMS* and for the tyrosine phosphatases *MPPED1*, *PTPN5*, *PTPN7*, and *PTPRR* because of multiple splice variants and/or short sequences, which led to their exclusion from analysis. Chromogenic ISH of arrayed FFPE tissue specimens was performed, comprising 48 arrayed normal adult human tissues in triplicate and 24 cancers each of the breast, colorectum, prostate, kidney, urinary bladder, and testis for each of the 127 genes. The stained tissue specimens were scored for expression in the epithelial or tumor cell compartments and in the vascular, stromal, and inflammatory cell compartments (Supplemental Tables S5 and S6). By comparison with published literature and databases, we sought to determine the validity of gene expression patterns (Figure 1). One-fifth of genes showed expected low or undetectable expression in normal tissues and the tumor tissues studied here (tyrosine kinases *ABL2*, *ALK*, *BMX*, *DDR2*, *EPHA3*, *EPHA5*, *EPHA6*, *EPHA10*, *FER*, *FRK*, *INSRR*, *JAK2*, *LMTK2*, *MUSK*, *NTRK1*, *NTRK2*, *ROS1*, *TEC*, and *TXK* and tyrosine phosphatases *MTM1*, *MTMR3*, *PTPDC1*, *PTPN2*, *PTPN14*, *PTPN21*, *PTPN22*, *PTPN23*, and *PTPRH*), which was confirmed by largely negative ISH analyses. In addition, no reference data were available for *EPHB1*, *ERBB4*, *JAK3*, *PTPRA*, and *PTPRM*. For 4% of the tyrosine kinases and 7% of the tyrosine phosphatases, the ISH expression pattern was discordant with validation data

Figure 2 Expression of tyrosine kinases and phosphatases in normal and cancer tissues. **A:** Expression of known cancer genes. *ERBB2/HER2* is strongly expressed in a subset of breast cancers, but expression is also noted in normal breast epithelium, normal colon epithelium, and colorectal cancers. Kinases and phosphatases with strong epithelial staining across several tumor types include *DDR1* and *PTPRF* in bladder and bladder cancer. **B:** Expression of genes with unknown role in cancer. *AATK* is strongly expressed in a subset of cancers, exemplified here by colorectal cancer, and intermediate expression is observed in normal colon. For *PTPRK* and *PTPRU* intermediate-to-strong epithelial expression is seen in all cancer types, shown here for bladder cancers, and expression is also observed in normal bladder. *PTPRS* is strongly expressed in cancers of the bladder and testis, and normal bladder, whereas normal testis is negative. **Arrows** indicate gene expression in the epithelium. Original magnification: $\times 8$ (**A** and **B**), except $\times 10$ (**A**, normal bladder for *DDR1* and *PTPRF*; **B**, bladder cancer for *PTPRK*). N, normal; T, tumor.

sources (*AXL*, *EPHA4*, *YES1*, *MTMR2*, *MTMR6*, and *PTPN11*) (Figure 1 and Supplemental Tables S1–S3 and S5–S7). Positive expression patterns consistent with prior knowledge were observed for 71% of the genes. The genes *CSK*, *DDR1*, *ERBB3*, *JAK1*, *PTP4A2*, *PTPRF*, and *PTPRK* would be predicted to be expressed across the tumor tissues studied here, which was also the case (Supplemental Table S3). From microarray analyses, several tyrosine kinases and phosphatases were expressed predominantly in hematologic tissues (*BTK*, *FGR*, *FLT3*, *HCK*, *ITK*, *LCK*, *MATK*, *PTK2B*, *ZAP70*, *MTM1*, *PTPN6*, and *PTPRC*), whereas others were precluded from such tissues (*ABL1*, *EPHB3*, *ERBB3*, *FGFR2*, *PDGFRA*, *PDGFRB*, *PTPN3*, *PTPN13*, *PTPRD*, and *PTPRF*), which was confirmed by ISH (Supplemental Tables S1–S3 and S5–S7).

Translocations and amplifications of driver genes may be reflected in elevated gene expression levels in an affected subset of patient tumors.²⁹ Twenty kinome expression outliers (see *Materials and Methods* for definition) in gene expression array data included *DDR2*, *EPHB3*, *ERBB2*, *FGFR1-2*, *FLT3*, *INSR*, *KIT*, *NTRK2*, *PDGFRA*, *PTK6*, *PTK7*, *RET*, *TIE1*, *TNK1*, and *YES1* in breast cancers; *EGFR*, *ERBB2*, *PTK6*, and *PTK7* in bladder cancers; *EPHA2*, *ERBB3*, *PDGFRB*, *PTK6*, *PTK7*, and *TNK1* in colorectal cancers; *ERBB3*, *INSR*, and *TIE1* in renal carcinomas; and *FGFR1* and *TNK1* in prostate cancers. Eight phosphatome gene expression outliers were identified, namely *DUSP23*, *PTP4A1*, *PTPN3*, *PTPN12*, and

PTPRT in breast cancers; *PTPN13* in testicular cancers; *PTPN18* in prostate cancers; and *PTPRK* in colorectal cancers (Supplemental Table S3). Although a partial explanation for this pattern may be derived from intertumoral variations in cell type composition, note that the frequently amplified oncogenes *ERBB2* and *EGFR*, along with genes known to take part in oncogenic translocations (*ERBB3*, *FGFR1*, *FLT3*, *KIT*, *PDGFRs*, *RET*, and *PTPN3*), are represented (Mitelman Database of Chromosome Aberrations and Gene Fusions in Cancer, <http://cgap.nci.nih.gov/Chromosomes/Mitelman>, last accessed May 15, 2014). Of these outlier genes in MediSapiens, 10 of 20 kinases (*EGFR*, *EPHA2*, *EPHB3*, *ERBB2*, *ERBB3*, *INSR*, *PTK6*, *PTK7*, *RET*, and *TNK1*) and seven of eight phosphatases (*DUSP23*, *PTP4A1*, *PTPN3*, *PTPN12*, *PTPN13*, *PTPN18*, and *PTPRK*), were observed by ISH (Supplemental Table S5).

Taken together, 91% of investigated transcripts had tumor cell *in situ* expression patterns in agreement with array database gene expression profiles, thus supporting the feasibility, sensitivity, and specificity of large-scale ISH to map gene expression in human FFPE tissues (*Materials and Methods*) (Figure 1 and Supplemental Tables S5 and S6).

Compartment-Specific Gene Expression Analysis in Human Cancers

Gene expression analyses of bulk tissue specimens will reflect the average gene expression in the different cells that constitute

Figure 3 Expression of kinases and phosphatases in stromal and inflammatory cells. **A:** Expression in stroma. The cancer-associated fibroblast markers *PDGFRA* and *PDGFRB* are expressed in stromal compartments in cervix, endometrium and in tumors of the bladder and colorectum, respectively. Expression of *PTK6* is seen in the connective tissue stroma of breast and bladder cancers. *TNS1* shows stromal expression in a subset of normal tissues and in cancers, exemplified here by prostate and testicular cancers. **B:** Expression in inflammatory cells. Expression of *CSF1R* is observed in macrophages in colorectal cancers and normal colon. *PTK2B* is expressed in inflammatory cells of normal colon and lymph nodes. The lymphoid cell-specific kinase *ZAP70* is expressed in inflammatory cells of normal colon and in many lymphatic tissues such as tonsil. Expression of *PTPRC* is seen in inflammatory cells of appendix and tonsil. **Arrows and asterisks** indicate gene expression in stromal and inflammatory cells. Original magnification: $\times 8$ (**A**, *PDGFRB*, *PTK6*, and *TNS1*; **B**, *PTK2B* in normal lymph nodes and *PTPRC*); $\times 10$ (**B**, *PTK2B* in normal colon and *ZAP70*); $\times 20$ (**A**, *PDGFRA*; **B**, *CSF1R*).

the sample but will not reveal gene expression in subcompartments of a tissue. To identify novel biomarkers and therapeutic targets in cancer, we sought to further locate gene expression to the tumor cells, vascular, connective tissue stroma, and inflammatory cells. We first mined SAGE libraries for transcripts enriched in breast cancer-associated myofibroblasts compared with normal myofibroblasts²⁶ to ensure that stromal gene expression could be detected by ISH. A total of 397 SAGE tags at least fourfold enriched in cancer myofibroblasts, of which 326 corresponded to known genes, were identified. From 143 genes with ≥ 20 SAGE tags, UniProt was used to identify 11 secreted and 7 transmembrane proteins, with evidence of ligand, receptor, or enzymatic activity, which were selected for ISH validation (Supplemental Figure S1). Of these, five genes (*HTRA1*, *HTRA3*, *MXRA5*, *MXRA8*, and *SERPING1*) showed tumor stroma-specific expression in cancers of the breast or colorectum (Supplemental Figure S2). Thus, the ISH approach can detect gene expression also in stromal cells.

Tumor Cell Compartment

Three-fifths of the studied genes were expressed in epithelial or tumor cell compartments (Figure 1). The spectrum of expressed

tyrosine kinases and phosphatases varied between cancers of the colorectum (39% versus 50%), testis (35% versus 55%), bladder (38% versus 50%), breast (31% versus 31%), prostate (18% versus 26%), and kidney (15% versus 19%). A selection of genes expressed in tumor cells is shown in Figure 2. The collagen-binding receptor *DDR1* was shown to be involved in various cancers, such as those of the brain,³⁰ and mRNA expression was seen in all epithelial tumors studied here (Figure 2A and Supplemental Table S5). Expression of the proto-oncogene *ERBB2* was observed in cancers of the breast, colorectum, and bladder and in normal bladder, breast, and colon (Figure 2A and Supplemental Table S5), which is relevant in the context of the observation of *ERBB2* amplifications in 5% of colorectal cancers.²⁰ *PTPRF*, found to be up-regulated in breast cancer,³¹ showed strong mRNA expression in normal epithelia and cancers of the bladder, breast, colorectum, prostate, and testis (Figure 2A and Supplemental Table S5). Several kinases and phosphatases were identified that were not previously associated with cancer. The kinase *AATK*, which is expressed in the developing mouse brain,³² was strongly expressed in a subset of colorectal, kidney, bladder, and testicular cancers, and intermediate expression was observed in breast and prostate cancers and normal colon (Figure 2B and

Figure 4 Expression of tyrosine kinases and phosphatases in normal and tumor vessels. **A:** Vascular expression of the known vessel markers *FLT1*, *PTPRB*, and *PTPRM* in testicular cancers; *KDR* in kidney cancer; *TEK* and *TIE1* in endometrium; *PDGFRB* in bladder cancer; and *PTPRJ* in normal testis. **B:** Vascular expression of genes with previously unknown role in angiogenesis. *PTPRF* shows vascular expression in spleen, *PTPRG* in colorectal cancer, and *PTPRU* and *TNS1* demonstrated vascular expression in testicular cancers. Original magnification: $\times 20$ (**A**, *PDGFRB*); $\times 40$ (**A**, remaining images, and **B**).

Supplemental Table S5). For the candidate tumor suppressor genes *PTPRK* and *PTPRU*,³³ epithelial expression in normal bladder and all studied cancers was observed (Figure 2B and Supplemental Table S5). Expression of the transmembrane tyrosine phosphatase *PTPRS*, a potential tumor suppressor in head and neck cancers,³⁴ was found in bladder and testicular cancers (Figure 2B and Supplemental Table S5). As expected, the tumor cell ISH expression pattern was largely in accordance with the whole tissue expression predicted by normalized gene expression array data (Supplemental Tables S3 and S5).

Stromal and Inflammatory Cells

The cancer-associated fibroblast markers *PDGFRA* and *PDGFRB*¹⁵ were expressed in stromal compartments in tumors of the bladder and colorectum, and *PDGFRB* was also expressed in breast, kidney, and testicular cancers (Figure 3A and Supplemental Table S6). Strong expression was also observed in connective tissue of endometrium (Figure 3A). *PTK6* is involved in regulating epithelial cell differentiation, and expression was observed in gut and skin and in cancers of the breast, colorectal, and prostate.³⁵ Expression was also seen here in the connective tissue stroma of bladder and breast cancers (Figure 3A and Supplemental Table S6). *TNS1* showed stromal expression in normal testis and endometrium, and in cancers of the

bladder, prostate, and testis (Figure 3A and Supplemental Table S6). Expression of the colony-stimulating factor 1 receptor (CSF1R), an important regulator of macrophage homeostasis,³⁶ was observed in macrophages in breast and colorectal cancers and in normal breast and colon (Figure 3B and Supplemental Table S6). *PTK2B*, with a role in macrophage cell migration,³⁷ was expressed in inflammatory cells of the normal colon, lymph nodes, tonsil, appendix, and spleen (Figure 3B and Supplemental Table S6). Inflammatory cells of testicular cancer, normal colon, and tissues such as lymph nodes, spleen, tonsil, appendix expressed the lymphoid cell-specific kinase *ZAP70* (Figure 3B and Supplemental Table S6). *PTPRC*, expressed in hematopoietic cells,³⁸ demonstrated expression in inflammatory cells of appendix, lymph nodes, and tonsil (Figure 3B and Supplemental Table S6).

Blood Vessels

One-third of the tyrosine kinases and tyrosine phosphatases, were previously found (see Reference Data Sets) to be expressed in endothelial cells or vessel walls during development or in the adult (Supplemental Tables S1 and S2). We observed vascular expression of the known vessel markers *EPHA4*, *EPHB4*, *FLT1*, *FLT4*, *KDR*, *MET*, *PDGFRB*, *PTK2*, *TEK*, *TIE1*, *PTPRB*, *PTPRJ*, and

Figure 5 *INSR* transcript and protein is expressed in tumor vessels but not in normal vessels. **A:** *INSR* mRNA expression in vessels is observed in all evaluated tumor types but prostate cancer, with strong expression seen in kidney cancers. **B:** The protein expression shows similar tumor-specific staining patterns, with strong expression in kidney cancers. CD34 expression in vessels is used as a positive control. N, normal; T, tumor. Original magnification: $\times 20$ (**B**); $\times 40$ (**A**).

PTPRM in at least one normal or tumor specimen (Supplemental Table S6). Weak staining in vessels of colorectal, kidney, and testicular cancers was observed for FLT1, which is believed to be important for development of embryonic vasculature³⁹ (Figure 4A and Supplemental Table S6). The kinase insert domain receptor (KDR), a receptor for vascular endothelial growth factor,⁴⁰ had vascular expression in bronchus, endometrium, pancreas, and small intestine and in cancers of the kidney and colorectum (Figure 4A and Supplemental Table S6). Vascular PDGFRB expression was observed in placenta and in cancers of the kidney, testis, and bladder (Figure 4A and Supplemental Table S6). The receptors TEK and TIE1, which are expressed in developing vascular endothelial

cells, were found in endometrium where angiogenesis occurs regularly during the reproductive life of females⁴¹ (Figure 4A and Supplemental Table S6). PTPRB, previously found to be involved in maintaining and remodeling blood vessels,¹² was expressed in vessels in cancers of the kidney and testis (Figure 4A and Supplemental Table S6). For PTPRJ, vascular expression was demonstrated in several normal tissues such as testis but also in testicular cancer (Figure 4A and Supplemental Table S6). Staining in vessels of colorectal, kidney, and testicular cancers was observed for PTPRM (Figure 4A and Supplemental Table S6).

Further, we observed vascular expression of several genes not previously linked to angiogenesis. These included *INSR*, *PTPRF*, *PTPRG*, *PTPRU*, and *TNSI* (Figures 4B and 5A and Supplemental Table S6). The insulin receptor (*INSR*) was implicated in hypoxia-induced angiogenesis in the retina, but few studies have explored its role in disease.^{42,43} We observed strong mRNA expression of *INSR* in tumors, predominantly of the kidney and bladder but also in breast, colorectum, and testis but not in prostate cancer (Figure 5A and Supplemental Table S6). No vascular staining was observed in any of the evaluated normal tissues (Supplemental Table S6). The expression pattern of *INSR* was validated with an independent RNA probe (Supplemental Table S4). The protein expression of *INSR* showed similar staining patterns for the epithelial cancers studied here, with strongest expression in kidney cancer (Figure 5B). *PTPRF*, a potential biomarker for prostate cancer,⁴⁴ was expressed in vessels of spleen and lymph nodes (Figure 4B and Supplemental Table S6). For *PTPRG*, a candidate tumor suppressor gene in ovarian, lung, and breast cancers,⁴⁵ vascular expression was observed in cancers of the bladder, colorectum, kidney, and testis and in the placenta (Figure 4B and Supplemental Table S6). Expression in vessels of the cerebellum, normal bladder, and testicular cancer was shown for *PTPRU* (Figure 4B and Supplemental Table S6). Tensin-1 (*TNS1*), which is suggested to play a role in mesangial extracellular matrix production,⁴⁶ was expressed in vessels in a subset of the cancer types analyzed and in a wide panel of normal tissues, including bladder, colon, kidney, prostate, and testis (Figure 4B and Supplemental Table S6).

Finally, several kinases and phosphatases were predominantly expressed in the central nervous system (Supplemental Table S7). Examples of such expression patterns are available in Supplemental Figure S3. Expression of NTRK3, PTPRN, PTPRS, and PTPRU was seen in Purkinje cells of the cerebellum but also in the granular layer for NTRK3 and PTK2B. Furthermore, NTRK3, PTK2B, PTPRN, PTPRS, and PTPRU were expressed in neuronal cells in the cerebral cortex, hippocampus, and lateral ventricle.

Discussion

Gene expression in cells that constitute a minor fraction of a tissue may not be detectable in analyses of bulk tissue transcriptomes. Systematic *in situ* gene expression analyses

could therefore reveal important but previously overlooked gene expression in subcompartments of the tumor. This work represents the first comprehensive *in situ* gene expression analysis of the tyrosine kinases and phosphatases in normal tissues and epithelial cancers of the breast, prostate, colorectum, kidney, bladder, and testis. A prime advantage of this approach is high specificity, demonstrated by correspondence to previously known gene expression patterns (see [Comparisons between MediSapiens and ISH](#)) for 91% of analyzed genes, whereas the main limitation is decreased sensitivity because of the effects of fixation and embedding on tissue samples. The approach enables screening of large sets of genes to identify targets with expression patterns of interest.

Compartment-specific expression analysis can aid development of diagnostic tests and repurpose tyrosine kinase inhibitor therapies. The specific expression patterns of well-studied compartment-restricted genes such as *ERBB2*, *PDGFRs*, *CSF1R*, and *KDR* indicated that such analyses were possible. Of the 90 genes that had clear expression signals and patterns concordant with validation data sources, 83% displayed expression in epithelial or tumor cells, 18% in stroma, 40% in inflammatory cells, and 20% in cells of vascular structures of normal or tumor tissues. When focusing on tumor tissues, 96% of the genes were expressed in tumor cells, 78% in vascular cells, 44% in stromal cells, and 17% in inflammatory cells. The expression of kinases and phosphatases in tumor cells will contribute the dominant component in expression analyses of bulk tumor tissues, which was confirmed by comparison of *in situ* expression data to gene expression array data. Elevated gene expression caused by gene amplification, such as that of *ERBB2* in breast cancers, is also reflected in strong ISH signals. Of the genes expressed in tumor cells, the tyrosine kinase *AATK* represents a little-studied potential target expressed in many forms of cancer that merits further investigation.

Furthermore, several of the novel cancer-stromal genes discovered with *in silico* design and SAGE libraries could represent potential targets in cancer. The human HTRA family of serine proteases consists of four members, HTRA1 to HTRA4, where HTRA1 was shown to be down-regulated in several cancer forms and suggested to be a tumor suppressor that promotes cell death.⁴⁷ Both HTRA1 and HTRA3 were shown to inhibit transforming growth factor- β signaling by binding to several of its members. In addition, a statistically significant negative correlation between the levels of transforming growth factor- β 1 and HTRA1 and HTRA3, was observed in endometrial cancer, making it possible for both to be involved in inhibition of transforming growth factor- β signaling in endometrial cancer and potentially other types of cancer.⁴⁸ Expression of HTRA3 was recently demonstrated in both epithelial and stromal compartments of colorectal cancer desmoplastic invasion fronts as assessed by IHC.⁴⁹ The matrix remodeling-associated protein MXRA5 was proposed as a novel biomarker in colorectal cancer.⁵⁰ The MXRA8 protein is poorly characterized for cancer, but it was

suggested that it may play a role in the maturation and maintenance of the blood–brain barrier.⁵¹ The gene *SERPINC1* encoding the C1 inhibitor plays an essential role in regulating the complement and contact systems and could therefore be important in the physiologic suppression of inflammation.⁵² The up-regulation of *SERPINC1* seen in the colorectal cancer stroma might be a mechanism to adjust the level of local inflammation and thereby potentiate cancer progression.

By scalable ISH, we confirmed the known stromal cell expression of *PDGFRA* and *PDGFRB* and discovered stromal expression of *PTK6* and *TNS1*. Stromal inflammatory cell expression was limited to the known genes *CSF1R*, *PTK2B*, *ZAP70*, and *PTPRC*. However, several previously unknown vascularly expressed genes were identified, namely *PTPRF*, *PTPRG*, *PTPRU*, *TNS1*, and *INSR*. These genes provide novel potential targets for antiangiogenic approaches. Of these, *INSR* mRNA expression was observed in tumor endothelium but not in endothelium of normal tissues. Interestingly, *INSR* expression was most clearly associated with tumor angiogenesis of all genes assessed in this study, showing vascular expression in 26 tissue specimens of 112 in cancers of the kidney, bladder, breast, colorectum, and testis, including those previously known to be involved in angiogenesis (see [Reference Data Sets](#)). The underlying mechanism remains to be clarified but may reflect a strong demand for glucose in the tumor environment.

The gene families studied here are subject to major drug discovery efforts, and knowing their precise tissue expression in the adult human can guide novel therapeutic applications and can explain physiologic side effects. This study provides a rationale for investigating the insulin receptor as a target for antivascular therapies or as a vascular address for tumor homing. Pervanadate-based specific protein tyrosine phosphatase inhibitors can protect neurons after spinal cord injury⁵³ which motivates further investigations of the neuronal expression of *PTPRN*, *PTPRS*, and *PTPRU*.

By defining the gene expression patterns of 127 genes of known or potential relevance to cancer, this study provides the fundamentals for comprehensive, transcriptome-wide analyses of gene expression in healthy and diseased human tissues. We have demonstrated that transcriptomic data from bulk organs and tumors, along with *in situ* gene expression studies in the mouse, can be used to assess ISH specificity and sensitivity, which is a major advantage to protein level analyses. Moreover, this type of large-scale analysis of gene expression can identify gene candidates with interesting expression patterns and potential clinical utility for further studies. Such efforts will be increasingly valuable with the rapid rate of discovery of novel cancer genes.

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Supplemental Data

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