Look Who's Talking: Deregulated Signaling in Colorectal Cancer

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Abstract. *Background: The phenotypic expression of any given heterogeneous tissue type is the composite of interactions between individual cell types governed principally by inherent, cell-specific molecular mechanisms. Materials and Methods: Using a combination of laser-capture microdissection, oligonucleotide microarrays, bioinformatic and statistical tools, we analyzed colorectal cancer tissues in order to spot the significant pathways that were differentially deregulated between the epithelial and stromal compartments and compared these alongside the ones of whole tissue dissection. Results: The stromal pathway profiles were very similar to the ones of whole tissue, in contrast to the epithelial input, with stroma emerging as the major determinant of the cancer phenotype. Differentially expressed genes in the epithelial compartment correlated significantly with the carbohydrate antigen 19-9 tumor marker. Conclusion: The accurate interpretation of data arising from the analysis of heterogeneous tissue structures lends itself to inherent biases of its constitutive components with each component presenting explorable analytical advantages.*

In colorectal cancer, as well as in several other cancer types, the quest for gene signatures and molecular markers that unambiguously define a given phenotype, stage or prognosis still remains a challenge. To date, several expression profiling data have been published and even though these data are not entirely discordant, the lack of a clear consensus remains glaring (6). The reasons have been attributed not only to differences in methodologies, techniques or expression platforms, but largely to tissue constitution and composition (6, 11, 12). To circumvent these problems, several groups have exploited pure cell isolation techniques inclusive of, but not limited to, laser capture microdissection (LCM) (9, 12, 21, 27, 31) and fluorescence-activated cell sorting (FACS) (28), and elucidated pure population signatures. These signatures have, to an extent, identified the pre-envisaged differences between tumor stroma and epithelium in the context of differentially regulated genes; however, it has become increasingly evident that genes do not act in isolation but concertedly through pathways, in order to mediate cellular mechanisms and processes (37). Such an in-depth look, with particular reference to colorectal cancer, is presented here.

In our study, on a genome-wide basis, we compared the individualized epithelial and stromal expression signatures, as well as active pathways, and concomitantly contrasted these with whole-tissue profiles from the same set of patients, as would have been obtained from routine expression analyses. Whereas the p38 and the peroxisome proliferator-activated receptor alpha/ retinoid X receptor, alpha (PPARα/RXRα) pathways were featured prominently in tumor epithelium, endothelin, the key determinants in the stroma signaling were interferon and chemokine (C-X-C motif) receptor 4 (CXCR4). The genes which were common to all compartments did not fall into a singular functional class nor strongly characterized a given pathway, but spanned across a broad range of interesting phenotypes inclusive of, but not limited to, electron transport molecules and regulators of transcription and proliferation.

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Key Words: Laser-capture microdissection, differential gene expression, epithelium, stroma, pathway analysis.

Materials and Methods

Patient material. Tumor and corresponding normal tissue samples of patients with colorectal cancer were obtained from the tumor bank of the University Hospital Mannheim, Germany. The project was sanctioned by the Ethical Committee of the Medical Faculty, University Hospital Mannheim, and informed consent was obtained

from patients or their spouses when the former were deceased. All together, 35 paired tumor and normal samples from patients who had undergone surgery between 2002- 2008 were utilized. Tumor and normal sample sections were prepared by pathologists prior to snap freezing and subsequent storage in liquid nitrogen.

LCM. LCM was carried out on a PALM MicroBeam system (PALM Microlaser Technologies, Munich, Germany). Epithelial and stromal cells from tumor and normal sections were catapulted separately into the PALM adhesive caps with four samples corresponding to tumor epithelium (TE), tumor stroma (TS), normal epithelium (NE) and normal stroma (NS) generated from each patient. Stromal dissection was random and not cell-type specific. LCM was employed for samples from only 10 out of the 35 patients.

RNA isolation from whole dissected tissue and LCM generated samples. Total RNA was isolated from whole-tissue and LCM samples with RNeasy mini and micro kits (Qiagen, Hilden, Germany), respectively, in line with the manufacturer's instructions. RNA quality was ascertained with Bio-Rad Experion™ (Bio-Rad, Hercules, CA, USA) using Standardsens and HighSens chips for whole tissue and LCM samples accordingly. In all cases, samples with an RNA quality index (RQI) ≥7 were utilized.

In vitro transcription and amplification. The process was performed with the TargetAmp 2-Round Biotin-aRNA amplification kit 3.0 (Epicentre Biotechnologies, Madison, WI, USA) in keeping with the appended protocol. The quality and quantity of the produced messenger RNA was subsequently evaluated with Bio-Rad Experion™ before hybridization.

Microarray hybridization and data analysis. Anti-sense RNA was hybridized onto Illumina human sentrix-8 chips (Illumina Inc, San Diego, CA, USA), washed, blocked and then stained with streptavadin Cy3 (Amersham Biosciences, Buckinghamshire, UK) with resultant detection of the fluorescence emission. Data extracted from the microarrays were analyzed using three different bioinformatic tools: Bead Studio 3 (Illumina Inc, San Diego, CA, USA), Chipster software for DNA microarray data analysis (Center for Science, Espoo, Finland) and the linear model for microarray data (Limma) (29). The array data have been deposited in NCBI's Gene Expression Omnibus and are accessible *via* the GEO Series accession number GSE31279.

Quantitative real-time PCR validation. Quantitative RT-PCR was carried out on a Light Cycler 480 system (Roche Applied Science, Germany) using Quantitect Primer assays (Qiagen) and SYBR green detection system. The delta –delta– CT algorithm was utilized using three housekeeping genes: succinate dehydrgenase (SDHA), tata-box binding protein (TBP) and beta-2 microglobulin (B2M) for normalization, as previously elaborated by Vandesompele *et al*. (36). Twelve out of the 44 commonly expressed genes were validated in all 35 patients.

Bioinformatic analysis. To appraise the spectrum of biological functions, canonical pathways and networks involving our candidate genes in the three compartments (whole tissue, stroma and epithelium), we used the Ingenuity Pathway Analysis (Ingenuity Systems Inc, Redwood City, CA, USA), software tool, supported in part by the DAVID ease (16;17) and Pathfinder (14) software tools. The annotated lists of significantly deregulated genes together with their fold changes were imported into the core analysis tool. The generated pathways were edited to allow only those above the threshold *p*-value (0.05) and those with cancer related affiliations. Two factors were key in determining if the pathways were significant, namely the number of genes in the data set compared to the entire number of genes in a given pathway, and a right-tailed Fischer's exact test to rule out the probability that such a combined occurrence of genes was by chance. The set cut-off *p*-value of 0.05 corresponds to a negatively log-transformed value of 1.3, and the higher this log-transformed value, the more significant the association.

Results

Patient profile. Of all samples taken into account, 8 had (UICC) stage 1 disease, 11 had stage 2, 4 had stage 3 and 12 had stage 4 disease with documented metastasis. Of the total population, 24 patients were males and 11 were females Details of the patient demographics are given in Table I.

Gene expression profile. The Limma data set was selected for all analyses. All values were log 2-transformed and genes with a value of $p<0.1$ and which were at least 2.5-fold up- or down-regulated were considered significant. Using these criteria, we found 2217, 211 and 1644 genes to be significantly deregulated between tumor and normal samples in the whole tissue, epithelial and stromal compartments, respectively. When we compared these, we discovered that 44 of these genes were common for all compartments, 373 were shared between whole tissue and stroma, 73 by whole tissue and epithelium and 16 by epithelium and stroma. A total of 78 genes were found to be exclusively expressed in the epithelium and 1,211 genes exclusively in the stroma (illustrated in Figure 1; genes common to all compartments are listed in Table II).

Comparison of expression data with meta-analysis of gene expression profiling in colorectal cancer. To further validate our gene expression data, we compared our compartmentalized profile, as well as the whole-tissue expression profile with the meta-analysis conducted by Chan *et al*. (8). Out of the 22 genes most commonly reported as being up-regulated in cancer *versus* normal tissue, there existed 15 in our whole tissue list, 10 in our stromal list and 4 in our epithelial list, with 3 genes: transforming growth factor, beta 1 (*TGFB1*), interferon induced transmembrane protein 1 (*IFITM1*) and P-cadherin (placental) (*CDH3*) common to all lists. There were five out of eight genes amongst the most reported down-regulated genes in 6 or more studies, with creatine kinase, brain (*CKB*) common to all compartments (Table III).

Compartmental pathways. Differential whole-tissue expression: Here, we considered pathways that were generated from genes that were differentially expressed between tumor and normal

Table I. *Demographic details of patients whose samples were used for the microarray experiments.*

Case no	Gender	Age	Tumor location	TNM	Tumor grade	CEA (μg/l)	CA19-9 (kU/l)
1	M	59	Rectosigmoid	pT2/N0/M0	G2	12.3	3
2	F	49	Rectum	pT2/N0/M0		28.4	19.9
3	M	62	Rectum	pT2/N1/M0	G3	0.7	15
4	F	41	Rectum	pT3/N2/M1	G2	6.8	19
5	M	49	Rectum	pT4/N0/M0	G2	440	150
6	M	71	Sigmoid colon	pT3/N1/M0	G2	898	5590
7	M	48	Sigmoid colon	pT4/N2/M1	G3	10.6	185
8	F	68	Transverse colon	pT3/N2/M1	G3	110.7	227
9	F	45	Rectosigmoid	pT3/N0/M0	G2	6.1	638
10	M	57	Left colonic flexure	pT2/N0/M0	G2	2.1	91
11	M	57	Caecum	pT2/N0/M0	G2	0.3	9
12	F	39	Colon	pT2/N0/M0	G2	<1	2
13	M	55	Sigmoid colon	pT2/N0/M0	G2	1	NA
14	M	54	Rectum	pT2/N0/M0	G2	525	2304
15	M	74	Rectum	pT2/N0/M0	G1	6.6	72
16	M	79	Colon	pT4/N0/M0	G3	<1	<1
17	M	78	Colon	pT3/N0/M0	G2	12	NA
18	F	74	Colon	pT3/N0/M0	G2	4	NA
19	M	66	Colon	pT3/N0/M0		1	<2
20	M	80	Colon	pT3/N0/M0	G2	NA	NA
21	M	76	Cecum/ascending colon	pT3/N0/M0	G2	0.6	NA
22	M	67	Colon	pT3/N0/M0	G2	1.7	11
23	M	72	Cecum	pT3/N0/Mx		8.81	NA
24	F	76	Colon	pT3/N0/M0	G2	NA	NA
25	F	61	Colon	pT3/N0/Mx	G2	NA	NA
26	F	81	Rectum	pT3/N0/Mx	G3	4.3	24
27	M	63	Rectum	pT3/N0/M0	G2	NA	22
28	M	59	Colon	pT3/N1/M0	G2	2.8	18
29	F	60	Rectum	pT3/N1/M0	G2	2.7	4
30	M	83	Right colonic flexure	pT4/N2/M1	G2	15.6	NA
31	M	70	Colon	pT4/N2/M1	G2	11.8	6
32	M	74	Colon	pT3/N1/M1	G2	16.7	132
33	M	72	Colon	pT4/N1/M1	G2	1	9
34	M	64	Rectum	pT3/N2/M1	G3	41.4	50
35	F	66	Colon	pT3/N2/M1	G3	3.7	<1

TMM: Tumor node metastasis; CEA: carcinoembryonic antigen; CA19-9: carbohydrate antigen 19-9.

whole-tissue samples. Seven cancer-related pathways were established to be significant and included those of the role of CSK homologous kinase (CHK) proteins in cell cycle checkpoint control; cell cycle G_1/S checkpoint regulation; polyamine regulation in colon cancer; ataxia telangiectasia mutated (ATM) signaling; p53 signaling; hypoxia-inducible factor, alpha subunit 1 (HIFA1) signaling; and, as would be expected, molecular mechanisms of cancer (Figure 2C).

Epithelial expression. In the epithelial compartment, the active pathways were not as enriched as were for the whole-tissue compartment, obviously due to there being fewer deregulated genes, but nonetheless had a completely different profile. In the context of gene numbers in relation to all genes known for the specific pathways, no statistically significant differences were found. However, data for differences in eight pathways were found to be significant, with *p*-values ranging from 0.5 to 0.005 (minus-log *p*-value of 1.25-2.25). These were p38 MAPK, PPARα/RXRα, B-cell activating factor, toll-like receptor, tight junction and endothelin signaling and the pathways active in xenobiotic metabolism by cytochrome P450 (Figure 2A).

Stromal expression. The operational pathways in the stromal compartment were strikingly similar to those of the whole-tissue profile, indicating that most whole-tissue expression profile is greatly affected by the stromal composition. Additionally, CXCR4 and interferon signaling were detected, which were not evident in the whole-tissue profile (Figure 2B).

Functional classification of compartmentalized expression. Functional classification serves to cluster genes into operational functional categories. In all three compartments, cancer was the most significant attributable process, but

interestingly, the level of significance for enrichment in the stroma was 3.5- and 2.7-log fold higher than in epithelium and whole tissue respectively, suggesting that the stroma is a much stronger classifier of cancer than are epithelial cancer cells (Figure 3).

Network analysis. As the interactions between genes become more thoroughly investigated, canonical pathways become either reinforced or newly established. Networks represent such developing associations, and provide a strong scaffold for the discovery of other interactions. We generated a separate network for the three compartments considered here and, without pre-selection bias, acquired a significant similarity between the stromal and whole-tissue networks (Figure 4) indicating again how whole-tissue expression analysis is greatly influenced by the stromal component.

Correlation of expression data with clinico-pathologic variables. As with all data involving patient samples, we sought to establish a significant correlation of the differential compartmentalized and whole-tissue expression data with the matched clinical variables of the corresponding patients. We performed a global statistical test using a logistic model and set the level of significance at $p \leq 0.05$. We evaluated the tumor size (pT1-T2 *vs*. pT3-T4), lymph node status (pN0 *vs*. pN1/N2), presence or absence of distant metastasis (pM0/Mx *vs*. pM1) and carcino-embryonic antigen (CEA) set at ≤5 μg/l for negative values and >5 μg/l for positive values. Carbohydrate antigen 19-9 (CA 19-9) was also included, where a value of ≤40 kU/l was considered negative. For the tumor markers, only values obtained before surgery were used. Considering that our patients spanned across all stages of colorectal cancer and with a total population of 35 patients, the power was too low to establish any significant association, with the exception of CA 19-9 in the epithelial compartment, where we obtained a significance level of $p=0.028$. Contributing molecules to this significance were, leucine zipper-like transcription regulator (*LZTR1*), transmembrane protein 201 (*TMEM201*), homeobox D1 (*HOXD1*), lysophosphatidic acid receptor 1 (*LPAR1*) and the DNA-directed RNA polymerase II (*POLR2D*). Out of these, *LZTR1, HOXD1* and *LPAR1* were significantly up-regulated in samples with a CA 19-9 value less than 40 kU/l, and *TMEM201* and *POLR2D* were significantly higher in those samples where CA 19-9 was greater than 40 kU/l. These findings are summarized in Table IV.

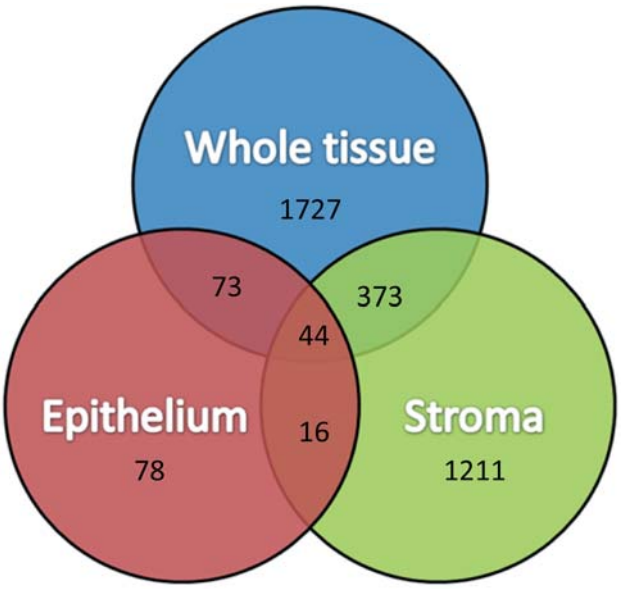

Figure 1. *Venn diagram showing significantly deregulated genes in whole-tissue, epithelial and stromal compartments.*

Discussion

In the process of understanding tumor initiation, progression and dissemination, it is clear that certain genetic and epigenetic events modify tissue behavior and endow cells with newly acquired capacities of proliferation, apoptosis evasion, extravasation and metastasis, mediated mainly through concerted interventions in signal transduction pathways, culminating in the destabilization of normal tissue homeostasis (37).

The interaction of an epithelial tumor entity with its surrounding stroma was described by Paget as akin to that of seed and soil, and for a tumor to grow and intravasate, it needs the 'permissiveness' of the stromal compartment (25). In order to dissect this process on all fronts, we laser micro-dissected epithelium and stroma from tumor, as well as normal, tissues and by using bioinformatic tools, we compared the gene expression profiles of the different compartments.

The epithelial and stromal pathways were distinctly different with the exception of endothelin 1 signaling, which was significantly active in both compartments. The endothelin axis is able to trigger a highly interconnected signaling network that ultimately activates the hallmarks of cancer, including aberrant cell proliferation, adhesion, migration, invasion, angiogenesis and antiapoptotic activity (2). The tumor epithelial cells must also initialize angiogenesis themselves, without which process, the tumor would fail to develop, as local diffusion for transport of nutrients and removal of waste products from the tumor site would suffice for only very small tumors (32). This is perhaps why this signaling pathway was found to be active in both compartments.

Accordingly, the most active pathways in the epithelial compartment are the ones that modulated cell proliferation and differentiation (p38 MAPK) (38), gene transcription (PPARα) (20), tumor immune evasion (toll-like receptor signaling) (15), and loss of cell contact inhibition, polarity

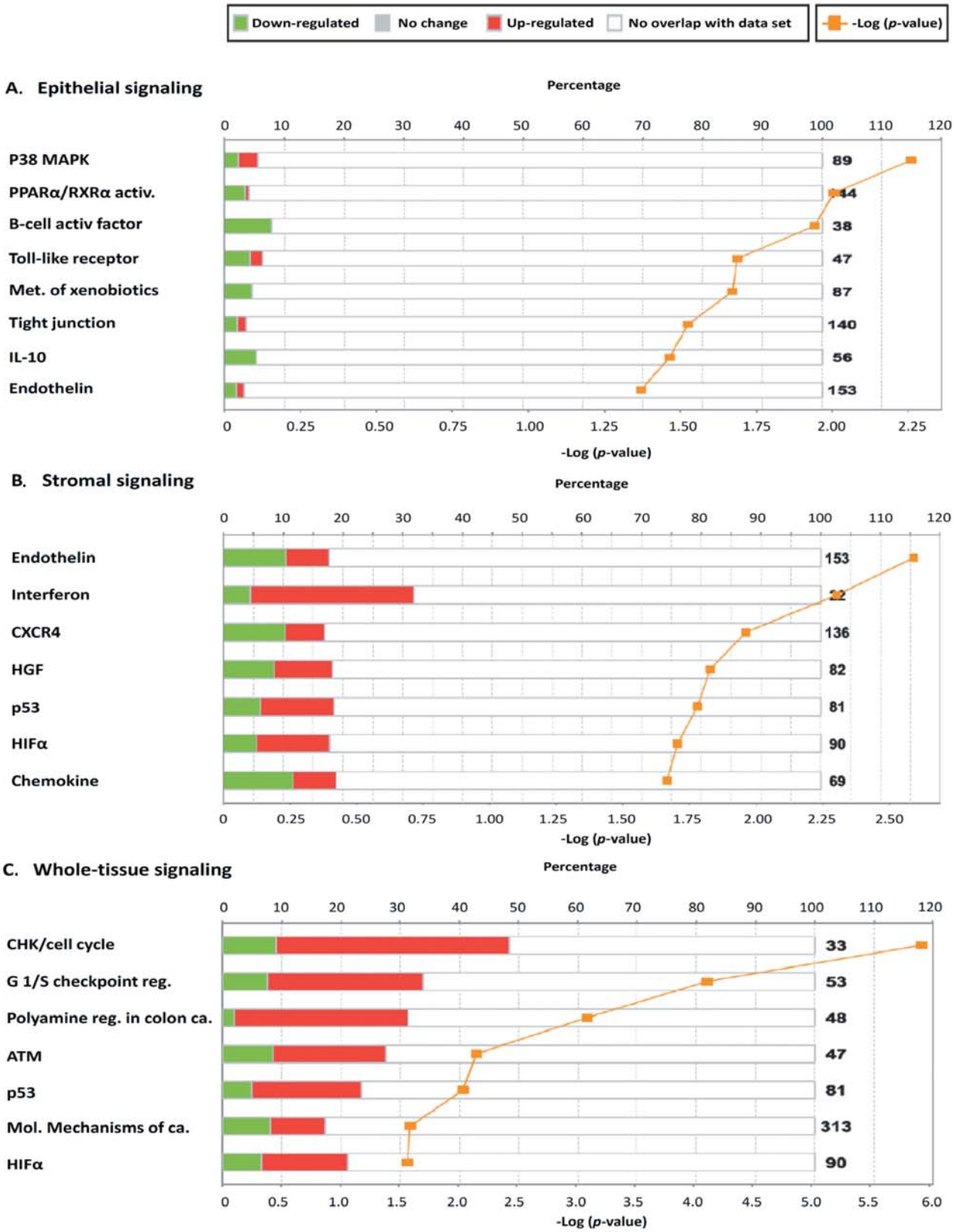

Figure 2. *Active canonical pathways in the epithelial (A), stromal (B) and whole-tissue (C) compartments.*

Table II. *Deregulated genes common to all compartments.*

Genbank_accession number	Gene name	Fold change (T/N)
NM_000717	Carbonic anhydrase IV	6
NM_022343	17-KDa fetal brain protein	7
NM_019025	Spermine oxidase	4
NM_003641	Interferon induced transmembrane protein 1 (9-27)	4
NM_005063	Stearoyl-coA desaturase (delta-9-desaturase)	–5
NM_013332	Hypoxia-inducible protein- 2	–4
NM_018945	Phosphodiesterase 7B	–4
NM_000063	Compliment component 2	4
NM_001823	Creatine kinase, brain	–6
NM_000358	Transforming growth factor, beta-induced, 68KDa	8
NM_021101	Claudin 1	–4
NM_005170	Achaete-scute complex-like 2 (Drosophila)	8
NM_017899	Tescalcin	–4
NM_001986	ETS Variant gene 4 (e1A enhancer binding protein , E1AF)	–5
NM_000849	Glutathione S-transferase M3 (brain)	8
NM_001793	Cadherin 3, type 1, P-cadherin (placental)	6
NM_001024074	Histamine N-methyltransferase	7
NM_173583	Hypothetical protein FLJ33790	5
NM_022059	Chemokine (C-X-C motif) ligand 16	–4
NM_033260	Forkhead box q 1	–3
NM_001401	Endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor, 2	–6
NM_015429	ABI gene family, member 3 (NESH) binding protein	5
NM_002474	Myosin, heavy polypeptide 11, smooth muscle	–5
NM_006988	ADAM metallopeptidase with thrombospondin type 1, motif 1	6
NM_013261	Peroxisome proliferative activated receptor, gamma, coactivator 1, alpha	–4
NM_144650	Alcohol dehydrogenase, iron containing, 1	6
NM_021158	Tribbles homolog 3 (Drosophila)	6
NM_001008406	B-cell receptor-associated protein 29	7
NM_199169	Transmembrane, prostate androgen induced RNA	–4
NM_201441	TEA domain family member 4	6
NM_002612	Pyruvate dehydrogenase kinase, isozyme 4	5
NM_147168	Chromosome 9 open reading frame 24	7
NM_003254	TIMP metallopeptidase inhibitor 1	8
NM_007168	ATP-binding cassette, sub-family A (ABC1), member 8	4
NM_016354	Solute carrier organic anion transporter family, member 4A1	–4
NM_006262	Peripherin	4
NM_018689	Hypothetical protein IR2155535	7
NM_007350	Pleckstrin homology-like domain, family A, member 1	5
NM_000422	Keratin 17	–5
NM_021021	Syntrophin, beta 1 (dystrrophin-associated protein A1, 59KDa, basic component 1)	–6
NM_000603	Nitric oxide synthase 3 (endothelial cell)	–5
NM_025130	Hexokinase domain containing 1	6
NM_133443	Glutamic pyruvate transaminase (alanine aminotransferase) 2	5
NM_153267	MAM domain containing 2	6

and growth control (tight junction signaling) (22). Invariably, the coordinated interplay of these pathways endows tumor cells with the appropriate machinery for tumorigenesis and sets the stage for the subsequent phases of invasion, intravasation and metastasis, which must involve the stroma.

The stromal pathways, on the other hand, converge to mediate enhanced invasion, migration, angiogenesis and metastasis in stark contrast to epithelial pathways that are mainly proliferation driven.

Contributing to the stromal expression cascades was interferon signaling, which promotes the transcription of more than 300 interferon-stimulated genes (5, 10, 30). Type I and II interferon-mediated signals are shown to be regulated by the p38 MAPK pathway (34, 35), the epithelial cross-talk of which likely represents how this cascade in the stroma may have been activated. Other active pathways included those of chemokines and their receptors, including CXCR4, a superfamily of small secreted molecules that

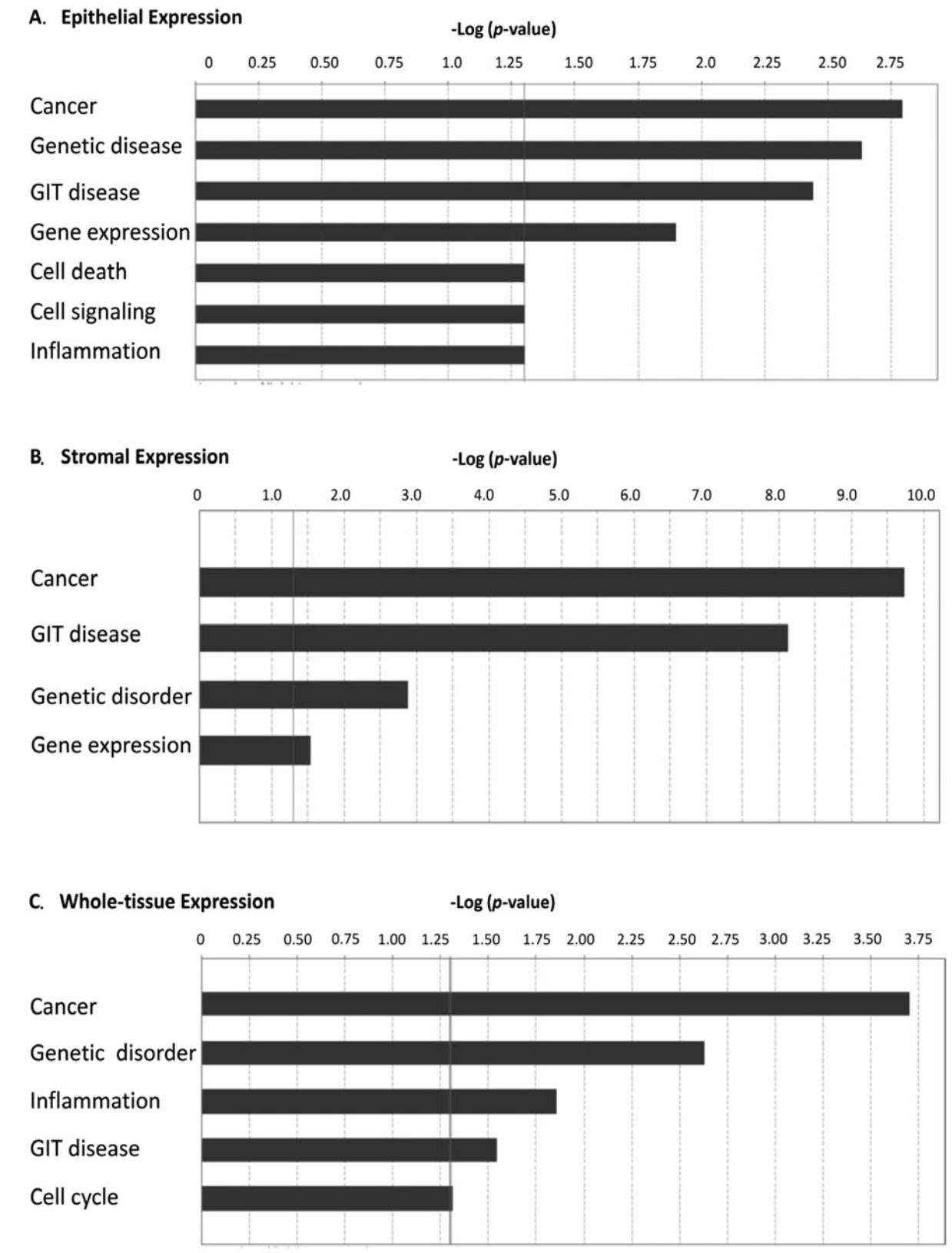

Figure 3. *Biological functions significantly associated with deregulated genes in the epithelial (A), stromal (B) and whole-tissue (C) compartments. Brown longitudinal lines represent the cut-off (-Log) p-value (see text).*

Table III. *Differentially regulated genes overlapping with the most consistently reported deregulated genes from the meta-analysis of colorectal cancer gene expression profiling performed by Chan et al. (14).*

Gene symbol	Accession number	Gene description	Involved compartments
Up-regulated			
TGFBI	NM_000358	Transforming growth factor-beta induced	Epithelium/stroma/whole-tissue
IFITM1	NM_003641	Interferon-induced transmembrane protein 1	Epithelium/stroma/whole-tissue
GDF15	NM_004864	Growth differentiation factor 15	Stroma/whole-tissue
CXCL1	NM_001511	Chemokine (C-X-C motif) ligand 1	Stroma/whole-tissue
CDC25B	NM_212530	Cell division cycle 25 homologue B (*Schizosaccharomyces pombe*)	Stroma/whole-tissue
IFITM2	NM_006435	Interferon-induced transmembrane protein 2	Whole-tissue
COL1A2	NM_000089	Collagen, type I, alpha 2	Whole-tissue
CKS2	NM_001827	Cell division control 2 protein homolog kinase regulatory subunit 2	Whole-tissue
TOP2A	NM_001067	Topoisomerase (DNA) IIα	Whole-tissue
UBE2C	NM_181803	Ubiquitin-conjugating enzyme E2C	Stroma/whole-tissue
CDH3	NM_001793	Cadherin 3, type 1, P-cadherin (placental)	Epithelium/stroma/whole-tissue
SLC12A2	NM_001046	Solute carrier family 12 (sodium/potassium/chloride transporters), member 2	Whole-tissue
MMP11	NM_005940	Matrix metallopeptidase 11 (stromelysin 3)	Stroma/whole-tissue
CSE1L	NM_001316	Chromosome segregation 1-like (yeast)	Whole-tissue
COL3A1	NM_000090	Collagen, type III, α1 (Ehlers-Danlos syndrome type IV, autosomal dominant)	Stroma/whole-tissue
COL4A1	NM_001845	Collagen, type IV, α1	Epithelium
Down- regulated			
CA2	NM_000067	Carbonic anhydrase II	Epithelium/whole-tissue
MALL	NM_005434	Mal, T-cell differentiation protein-like	Whole-tissue
SLC26A2	NM_000112	Solute carrier family 26 (sulfate transporter), member 2	Whole-tissue
ACADS	NM_000017	Acyl-coenzyme A dehydrogenase, C-2 to C-3 short chain	Whole-tissue
CKB	NM_001823	Creatine kinase, brain	Epithelium/stroma/whole-tissue
CA1	NM_001738	Carbonic anhydrase I	Epithelium/whole-tissue
KRT20	NM_019010	Keratin 20	Whole-tissue

control the migration of many cell types in the body, and their mediation in tumor cell growth, migration and metastatic spread has not only been observed, but found to be of prognostic significance (39). Hepatocyte growth factor (HGF) signaling mediates, amongst others, epithelial-mesenchymal transition, angiogenesis, cell motility, invasiveness and metastasis (1, 4), accordingly we found MET, the primary ligand for the HGF on average 3-fold up-regulated in our tumor samples. Further reinforcing the metastasis disposition of the stroma was HIF signaling, playing critical roles on tumor cell expansion by regulating energy metabolism and the induction of angiogenesis (7, 24). HIF activation correlates with metastasis in multiple tumors and can promote metastasis through the regulation of factors governing tumor cell metastatic potential including CXCR4 and stromal factor-derived factor 1 (13).

The commonly expressed pool of genes begs the question why two different cell types with different functions should have a common expression. Plausible explanations include a robust signature that cuts across cell types, or an expression switch transiting one cell phenotype to another, as is observed in the epithelial to mesenchymal transition. We were able to confirm that *TGFB1, CDH3, IFITM1* and *CKB* all belonging to the list of genes reported as most commonly deregulated in expression profiling studies of cancer *versus* normal tissue (8) and several of these genes have also been shown to partake in epithelial to mesenchymal transition (18, 19, 23, 33). These facts are further supported by the study conducted by Sheehan *et al*. (26) found by proteomic microarrays that signaling molecules expressed in stroma and epithelium appear more similar to each other in tumors than in normal colon, also hinting at epithelial to mesenchymal transition .

We found that deregulation of *LZTR1, TMEM201, HOXD1, LPAR1* and *POLR2D* genes correlated significantly with the level of CA 19-9 tumor marker, suggesting that these genes could individually or collectively be used to evaluate disease state and/or progression. A multicenter trial with larger patient numbers would be necessary to confirm if this is indeed the case, but the limitation would be that only pure epithelial cells can be used for this assessment. Ideally, for a feasible clinical application, a marker combination should be robust against stromal contamination of tumor tissues of patients consequently our common gene signature set might have been suited as a marker pattern, indicative of a particular prognosis, course of disease and

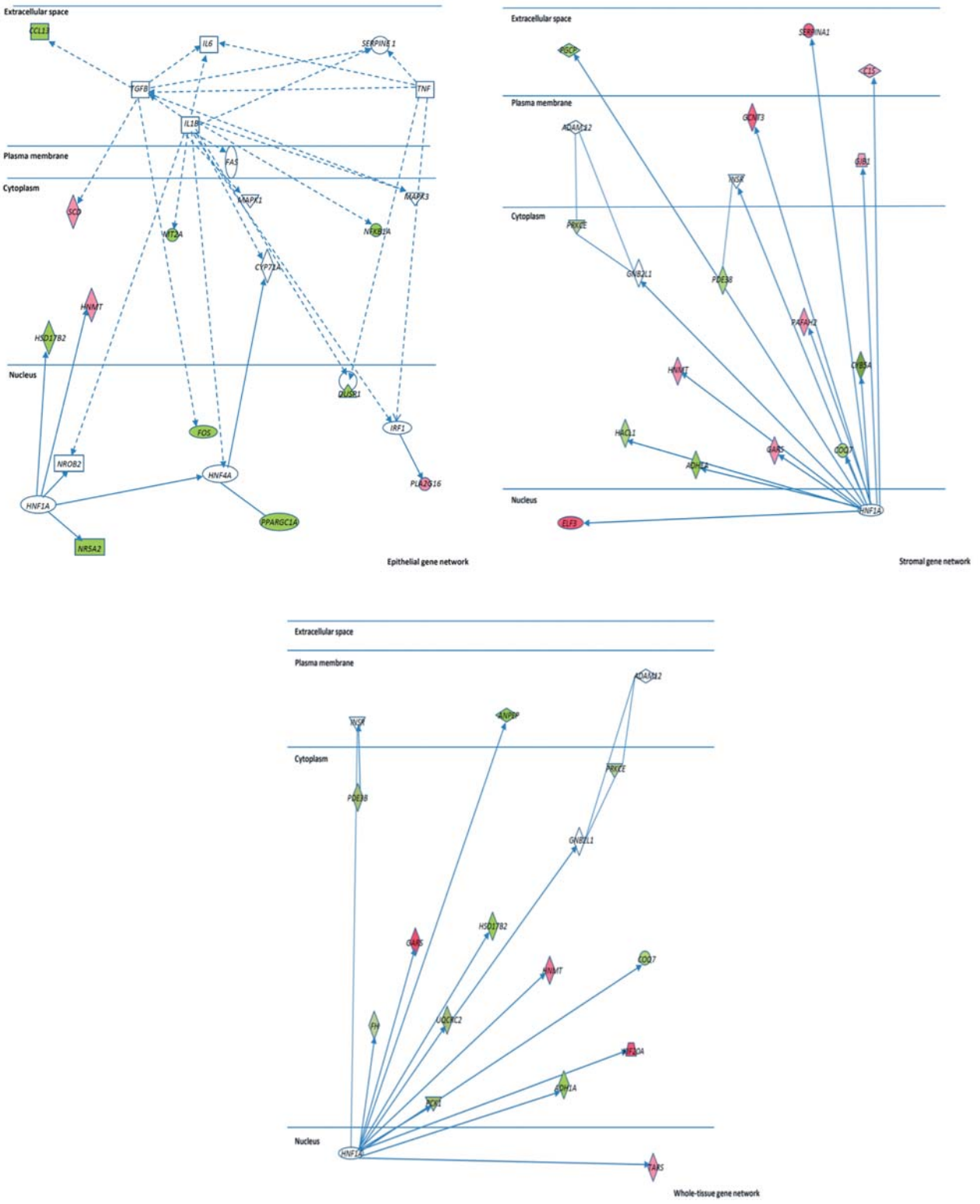

Figure 4. *Networks of interacting genes identified using Ingenuity Pathway Analysis. Red indicates overexpressed and green underexpressed genes. Direct and indirect interactions are symbolized by solid and dashed lines respectively. Node shapes represent functional classes of gene products: rectangles with solid lines for cytokines, rectangles with dotted lines for growth factors, upright triangles for phosphatases, inverted triangles for kinases, truncated triangles for transporters, diamonds for enzymes, rhomboids for peptidases , latitudinal ovals for transcriptional regulators or modulators, longitudinal ovals for transmembrane receptors and circles for other molecules.*

Table IV. *Clinicopathologic associations with expression data using the global test statistic.*

Whole-tissue	*p*-value	Contributing genes
T1/T2 *versus* T3/T4	0.588	-
N0 *versus* N1/N2	0.402	-
M0 *versus* M1	0.759	-
CEA	0.285	-
CA 19-9	0.378	-
Epithelium		
T1/T2 *versus* T3/T4	0.143	-
N0 *versus* N1/N2	0.875	-
M0 *versus* M1	0.250	-
CEA	0.964	-
CA 19-9	0.028	*LZTR1, TMEM201, HOXD1, LPAR1, POLR2D*
Stroma		
T1/T2 *versus* T3/T4	0.295	-
N0 *versus* N1/N2	0.484	-
M0 *versus* M1	0.808	-
CEA	0.533	-
CA 19-9	0.057	-

response to a certain type of therapy, amongst other possible applications. Unfortunately, with our low patient numbers, we were unable to define a significant pattern of gene expression across compartments that could be applied to any of these clinically important questions. Additionally, the molecular cross-talk between tumor epithelium and stroma is simply not reflected by analyzing whole-tissue extracts, and therefore, a differential assessment of compartments and a separation of cell types of patient tumors will also be necessary in the future to help answer clinical questions. We anticipate that an answer to this issue might be given by the ongoing worldwide Cancer Genome Project.

In conclusion, our findings strongly suggest that the accurate interpretation of data arising from the analysis of heterogeneous tissue structures lends itself to inherent biases of its constitutive components and that while whole-tissue samples are very important sources of information, each constitutive component presents its own analytical advantage. The latter point is supported by the recent finding, in colorectal carcinomas, that stromal cells did not share *KRAS* mutations that the epithelial compartment harbored (3).

Conflict of Interest

No conflicts declared.

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