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The low-abundance transcriptome reveals novel biomarkers, specific intracellular pathways and targetable genes associated with advanced gastric cancer

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Studies on the low-abundance transcriptome are of paramount importance for identifying the intimate mechanisms of tumor progression that can lead to novel therapies. The aim of the present study was to identify novel markers and targetable genes and pathways in advanced human gastric cancer through analyses of the low-abundance transcriptome. The procedure involved an initial subtractive hybridization step, followed by global gene expression analysis using microarrays. We observed profound differences, both at the single gene and gene ontology levels, between the low-abundance transcriptome and the whole transcriptome. Analysis of the low-abundance transcriptome led to the identification and validation by tissue microarrays of novel biomarkers, such as LAMA3 and TTN; moreover, we identified cancer type-specific intracellular pathways and targetable genes, such as IRS2, IL17, IFNγ, VEGF-C, WISP1, FZD5 and CTBP1 that were not detectable by whole transcriptome analyses. We also demonstrated that knocking down the expression of CTBP1 sensitized gastric cancer cells to mainstay chemotherapeutic drugs. We conclude that the analysis of the low-abundance transcriptome provides useful insights into the molecular basis and treatment of cancer.

Key words: gastric cancer, low-abundance transcriptome, novel biomarkers and therapeutic targets, specific intracellular pathways, CTBP1 and chemotherapy sensitization
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Each year, almost one million people worldwide are diagnosed with gastric cancer, and more than 700,000 people die of this disease, representing ∼10% of cancer mortality globally.[1] Despite advances in diagnostic imaging that have improved the early detection of gastric cancer, the advanced stages of the disease still has a poor prognosis.[2] Recent advances in stratified medicine have improved therapeutic responses in advanced HER2-positive gastric cancer patients treated with trastuzumab[3]; however, even in these cases, resistance develops rapidly, the benefit is transient, and most of these individuals eventually progress, mainly due to the selection of nonexpressing malignant cell clones.[4]

A challenge for the application of functional genomics to cancer research is to identify the expression profile of the low-abundance transcriptome as a potential source of tumor-specific genes useful as biomarkers and targets. The screening of human cancerous tissues by whole gene expression analyses has identified mainly transcripts related to cell–ECM interactions and the cell cycle and other gene ontology groups that clearly represent highly abundant transcripts.[5] Although RNA-Seq can provide an unbiased profile of a transcriptome, the broad dynamic range of gene expression

What's new?
The present study aimed to identify novel markers and targetable genes and pathways in advanced human gastric cancer through analysis of the low-abundance transcriptome. Aberrant cancer-specific intracellular pathways such as the wnt/hedgehog and the PI3K and genes like *CTBP1* were identified. Most of the differentially expressed low-abundance transcripts were not detected when the whole transcriptome was analyzed. *CTBP1* was further identified as a novel target for sensitization of gastric cancer cells to chemotherapeutic drugs that have shown limited effectiveness in the clinics. The study of the low-abundance transcriptome might help to improve response to mainstay treatments and develop novel therapies.

levels still necessitates considerable over-sequencing to effectively sample the differentially expressed transcriptome.[6]

Yet, a useful alternative is the use of a PCR-based suppressive subtractive hybridization that can equalize the abundance of cDNAs within the target samples enriching low-abundance and rare transcripts, followed by gene expression analysis.[7,8] This approach has been used in very few studies to profile the low-abundance transcriptome in human hepatoma,[8,9] breast and nasopharyngeal carcinomas[10] and identify genes potentially associated with breast cancer progression.[11] Here, we applied this procedure to identify low-abundance transcripts that were differentially expressed in human gastric adenocarcinomas compared with their paired adjacent noncancerous tissues. The vast majority of the differentially expressed low-abundance transcripts was not detected when the whole transcriptome was analyzed, leading to the identification of novel biomarkers, specific intracellular pathways and gene targets, that were aberrantly expressed in the cancer tissue. Further functional studies identified CTBP1 as a novel target for sensitization of gastric cancer cells to chemotherapeutic drugs.

Material and Methods

Clinical samples

The samples were obtained with the prior approval of the Ethics Committee of the Hospital Temuco Tumor Bank from patients who signed informed consent or deceased patients after an anonymization step. The cancerous and adjacent noncancerous tissues were obtained from 12 patients with advanced gastric adenocarcinoma who did not receive adjuvant therapy. The collected tissues were preserved immediately in RNALater (Ambion Inc., Austin, TX, USA) and stored at −80°C. Before RNA extraction, the samples were histologically analyzed to confirm malignancy. Human Universal Reference RNA and normal gastric RNAs were purchased from Clontech (Palo Alto, CA, USA).

Suppression subtractive hybridization

Total RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA, USA). Purified Total RNA (1 μg) was used for first strand synthesis with the Super SMART PCR cDNA Synthesis kit (Clontech, Palo Alto, CA, USA) and SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). Subtractive hybridization was performed with the aid of the Clontech PCR-Select cDNA Subtraction Kit (Clontech, Palo Alto, CA, USA) following the supplier's protocol. A customized primer was designed to preserve the T7-promoter region in the 5′ end of the subtractive amplicon (5′- CTAATACGACTCACTATAGGGCTCGAGCGGCC-3') in the secondary PCR.

Microarray data processing and statistical analysis

The subtractive amplicons were purified with an E.Z.N.A Cycle Pure kit (Omega Bio-Tek, Norcross, GA, USA). The aRNA were synthesized and labeled using the SuperScript Indirect RNA Amplification System and Alexa Fluor dyes (Invitrogen, Carlsbad, CA, USA). The aRNA generated were used for hybridization to the 48.5K Exonic Evidence Based Oligonucleotide (HEEBO) arrays purchased from Microarray Inc. (Nashville, TN, USA). The slides were scanned using the VersArray ChipReader scanner (Bio-Rad, Hercules, CA, USA), and the signal intensity was evaluated using SpotReader Software (Niles Scientific, Portola Valley, CA, USA). The raw data were deposited in the Gene Expression Omnibus (GEO), under accession number GSE38940 and subseries GSE38932 and GSE38939.

qRT-PCR and pathway PCR Array analysis

To synthesize cDNA, 1 μg of RNA was reverse-transcribed using the AffinityScript qRT-PCR cDNA Synthesis Kit (Stratagene, La Jolla, CA, USA). The quantitative expression analysis was performed using an oligonucleotide primer for the specific sequences of the transcripts (Supporting Information Table1) and the Brilliant II SYBR Green qRT-PCR Master Mix (Stratagene, La Jolla, CA, USA). The QARS, POLR2L and TFCP2 genes were used as internal controls. The reactions were quantified in a real-time thermocycler Mx3000p, and the amplification data were analyzed using the MxPRO software (Stratagene, La Jolla, CA, USA).

For PCR array, we selected four pairs of gastric cancer samples (1, 5, 7 and 12) and their corresponding noncancerous tissues. Human angiogenesis, PI3K/AKT, custom Wnt/hedgehog and B and T cell activation PCR array primers sets were used according to the manufacturer's specifications (Real Time Primers, Elkins Park, PA, USA). To increase the robustness of the data, normalization was performed with nine different control genes ACTB, β2M, G3PDH, HPRT1,

PGK1, PP1A, RPL13A, QARS and POLR2L, which were previously analyzed in Genorm software.[12]

Cell culture, reagents and antibodies

The human gastric cancer cell lines AGS, SNU-1, SNU-16, N87 and KATO-III were obtained from the American Type Culture Collection (ATCC) and were maintained according to the supplier's instructions. The cells were cultured for less than 3 months from the time that were received from the ATCC, and during this period, RNA was extracted for validation of gene expression levels. AGS cells were reauthenticated before performing the *in vitro* studies combining siRNAs and the chemotherapeutic drugs 5-FU, cisplatin and epirubicin. FlexiTube siRNAs targeting CTBP1 that included SI03211201 (FlexiTube siRNA), SI04142082 (FlexiTube siRNA), SI04301325 (FlexiTube siRNA) and SI04347749 (FlexiTube siRNA), and the AllStars negative control siRNA were obtained from Qiagen (Valencia, California, USA). Immunohistochemical staining on tissue microarrays was performed using anti-TTN and anti-LAMA3 (Sigma-Aldrich, St. Louis, MO, USA) primary antibodies. Other antibodies used included anti-CTBP1 (Santa Cruz Biotechnology, California, USA), anti-α-tubulin, HRP-conjugated goat anti-mouse secondary antibody (Invitrogen, Carlsbad, CA, USA) and HRP-conjugated goat anti-rabbit (Millipore, Billerica, MA, USA).

Tissue array and immunohistochemical analysis

The gastric BC01114 tissue microarrays (TMAs) were purchased from US Biomax Inc. (Rockville, MD, USA). Deparaffinization and staining was performed according to supplier's protocol. Primary antibodies were incubated with the slides and detected with HRP polymer secondary antibody conjugate Super Picture Polymer (Invitrogen, Carlsbad, CA, USA) and Nova Red Substrate (Vector Lab, Burlingame, CA, USA). The immunohistochemical grading was obtained by multiplying the percentage of positive cells (P) by the intensity (I). Positive cells = 0: <10%; 1+: 10–25%, 2+: 25–50%; 3+: 50–75%; 4+: >75%. Intensity staining = 1: Weak; 2: Moderate; 3: Strong. A final staining score of 0 was classified as negative, 1–3 as low, 4–6 as moderate and 8–12 as high.

Western blot analysis

Protein samples were lysed in RIPA buffer, supplemented with a protease inhibitor cocktail (Proteo-block, ***Fermentas*** Glen Burnie,. MD, USA). The protein concentration was determined by BCA assay (Pierce, Rockford, Illinois, USA), and the proteins were resolved by SDS-PAGE on a 12% acrylamide gel. Antibody-bound proteins were detected using an EZ-ECL chemiluminescence detection kit (Biological Industries, Israel) and radiographic film.

Migration assay

Cell migration assays and quantification were performed using 48-well chemotaxis chamber as described previously.[13] AGS cells (2×10^4) were allowed to migrate for 12 hr through 8 μm polycarbonate membranes (Neuro Probe, Cabin. John, MD, USA) embedded in 0.1% gelatin using 10% fetal bovine serum in the lower chamber as attractant.

Clonogenic assay

Clonogenicity assay was performed as previously described.[14] Cells were seeded at 200 cells per well in six-plaques and was incubated for ~2 weeks to allow colony formation.

Functional validation for chemotherapeutic sensitization

For CTBP1 and chemotherapy drug studies, cells were transfected with siRNA against CTBP1 or a siRNA control (5 nM) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Twenty-four hours later, transfected cells (1.0×10^4) were plated in 96-well plates and treated with varying doses of 5-fluorouracil (5-FU), cisplatin and epirubicin, kindly provided by Laboratorio KAMPAR (Santiago, Chile). Cell viability was determined 72 hr after drug addition using CellTiter Aqueous One Cell Proliferation assay an MTS-based assay (Promega, Madison, WI, USA).

Statistical analysis

The Wilcoxon matched pairs test was used to assess the qRT-PCR and immunohistochemical results. The mRNA expression and the functional effects of CTBP1 knockdown were examined by the Student's *t*-test. $p < 0.05$ was considered statistically significant. All statistical analysis was performed using GraphPad Prism version 5.0 for Windows (San Diego, CA, USA).

Results

Validation of the subtractive procedure before microarray analysis

The differentially expressed list of the low-abundance transcripts of 12 gastric samples was compared with the transcripts obtained from the analysis of the whole transcriptome of the same samples. The HEEBO microarrays contained 44,544 70-mer oligonucleotide probes, representing ~30,718 unique genes. The cDNAs from each cancer sample or its paired adjacent noncancerous tissue were used separately as the tester, whereas cDNAs obtained from normal gastric or colon tissue RNA were used as the driver. To allow comparison between the whole- and the low-abundance transcriptome, we hybridized all the samples (cancer and noncancerous) against an aRNA obtained from a universal reference (Supporting Information Fig. 1).

The subtraction efficiency was confirmed by an average decrease of >50% in the expression levels of 100 selected housekeeping probes (Supporting Information Table 2*a*). In addition, we confirmed the decreased expression of glicerol-3-phosphate dehydrogenase (G3PDH), glutaminyl-tRNA synthetase (QARS), TATA box binding protein (TBP) and ubiquitin-conjugating enzyme E2D 2 (UBE2D2) by

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quantitative real-time PCR (qRT-PCR) (Supporting Information Table 2*b*). Most of the differentially expressed low-abundance transcripts (Supporting Information Table 3) exhibited expression values near zero in the whole transcriptome analyses, confirming the enrichment of low-abundance transcripts (Supporting Information Fig. S2).

Identification of low-abundance cancer-associated transcripts

To establish a threshold for analysis, a probe was considered positive if it was present in at least two pairs of samples. The differentially expressed genes included those with an absolute fold change ≥ 1.32 and p-values < 0.05. A total of 278 differentially expressed genes were detected by microarray analysis of the whole transcriptome. Of these, 114 were upregulated, and 164 were downregulated. On the other hand, 530 differentially expressed genes were identified in the low-abundance transcriptome of gastric cancer, including 213 upregulated and 317 downregulated genes. Only 12 differentially expressed genes were identified in common between the low-abundance and the whole gastric cancer transcriptome (Fig. 1*a*). The clustering analysis demonstrated that the sets of differentially expressed genes (either from the whole or the low-abundance transcriptome) were able to distinguish cancer samples from noncancerous samples with perfect accuracy (Supporting Information Fig. S3).

Several of the differentially expressed genes were further analyzed by qRT-PCR. These analyses validated 91.3% (21/23) of the genes obtained from the whole transcriptome analysis (Fig. 1*b*). Moreover, qRT-PCR analyses validated with statistical significance 70.0% (14/20) of the genes found differentially expressed in the low-abundance transcriptome (Fig. 1*c*). Four more genes, SRGAP1, ANXA13, GPR68 and AGR2 showed the same tendency of the microarrays since they were overexpressed in gastric cancer samples compared to the adjacent tissue; while we were unable to validate MAST3 and SAMD9 expression (Fig. 1c and Supporting Information Fig. S4) Overall, the high rate of validation by qRT-PCR (18/20 genes) indicates that the data obtained from the low-abundance transcriptome studies, was robust.

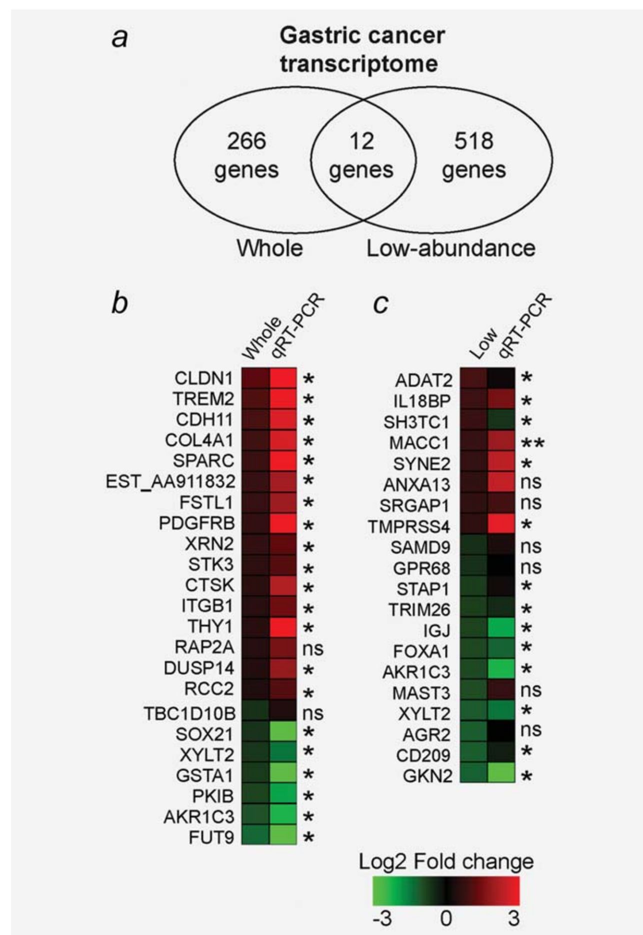

Figure 1. The use of a subtractive hybridization step for low-abundance transcriptome analyses led to the identification of novel cancer-associated transcripts. (*a*), Venn diagram showing the overlapping genes identified in the whole and the low-abundance transcriptome. (*b*, *c*), qRT-PCR analysis was performed on selected differentially expressed genes. The mRNA levels were assessed in at least five samples of gastric cancer and their paired noncancerous tissue. The red color represents upregulation, and the green color represents downregulation. qRT-PCR significance *p*-values: $*p < 0.05$, $**p < 0.01$. ns, non-significant by Wilcoxon matched pairs test.

TMA validation of differentially expressed transcripts

In searching for potential novel biomarkers we performed TMAs studies of two genes that were selected since, (a) none of them was previously reported to be associated with gastric cancer; (b) according to the Human Protein Atlas (http://www.proteinatlas.org/) both appeared to be expressed in the epithelial cells and (c) antibodies for immunohistochemical analyses were available. LAMA3 (laminin alpha 3) is one of the subunits (together with LAMB3 and LAMC2) of Laminin-332 (LM-332, formerly termed laminin-5) with an essential role in cell adhesion and motility.[15] The expression of LAMA3 was analyzed in 37 primary gastric cancerous tissues paired with their respective adjacent noncancerous tissues and five normal gastric tissues. Eighty-four percent of malignant samples (31/37) displayed increased intracytoplasmic LAMA3 staining compared to their noncancerous counterparts (Figs. 2*a* and 2*b*). Of note, 95% of the noncancerous samples and all of the normal gastric samples exhibited negative staining, whereas almost 60% of the cancerous tissues exhibited moderate to high levels of staining (Fig. 2*a* and *b*; Supporting Information Fig. S5*a*). On average, the gastric cancer samples exhibited 17-fold increase in the expression levels of LAMA3 compared to adjacent noncancerous tissues ($p < 0.0001$; Fig. 2*c*).

TTN (titin), also known as connectin, is responsible for the passive elasticity of muscle and has been reported as a potential melanoma biomarker.[16,17] The expression of TTN was validated also by TMA in 35 primary gastric cancer tissues, their respective adjacent noncancerous tissues and five normal

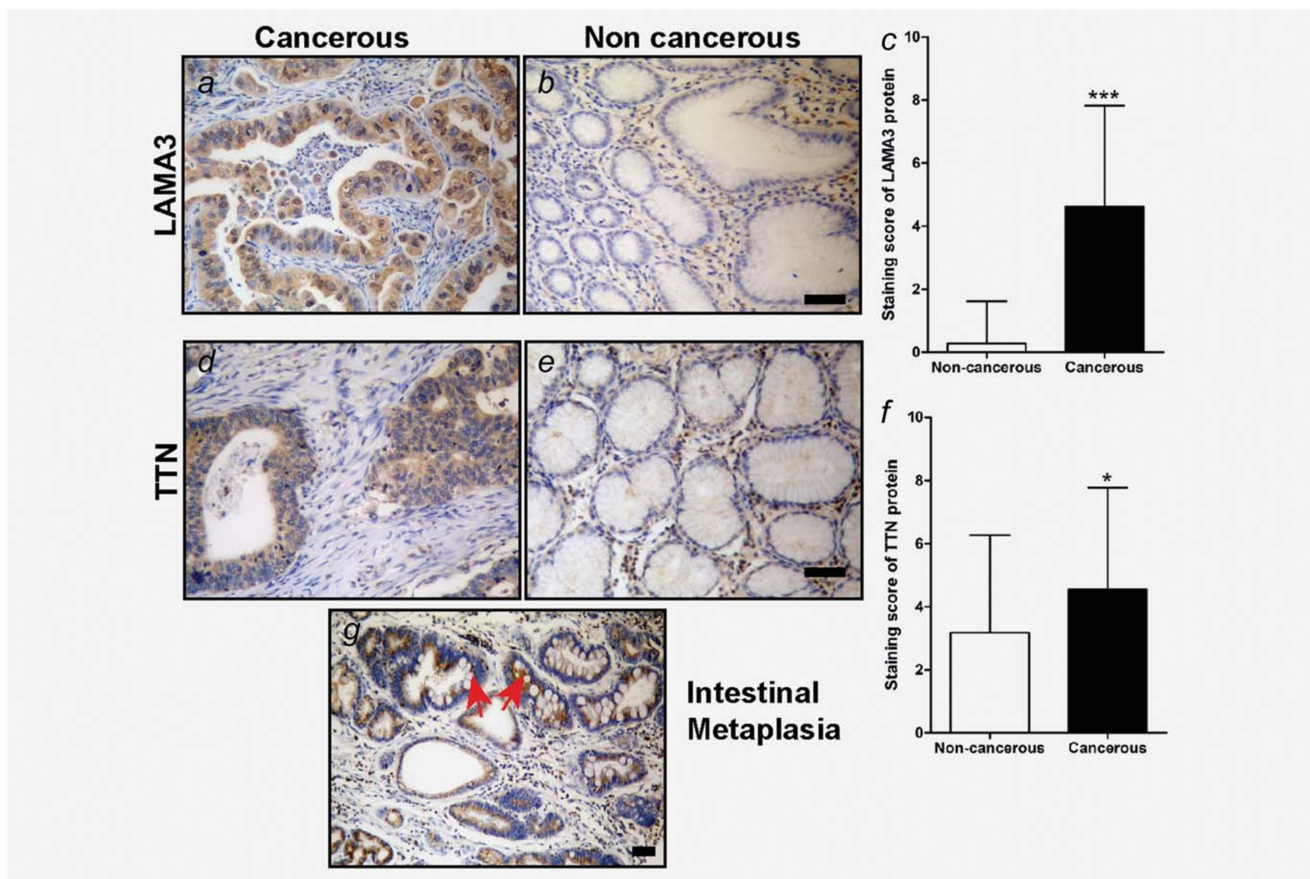

Figure 2. Tissue microarray analyses of LAMA3 and TTN in gastric cancer. (*a*,*b*), LAMA3 staining in gastric cancer samples (*a*) and adjacent noncancerous tissue (*b*). (*c*), quantification of LAMA3 expression in gastric cancer and adjacent noncancerous tissue ($n = 37$). (*d-f*), TTN staining in gastric cancer samples (d) and adjacent noncancerous tissue (*e*). (*f*), Quantification of TTN expression in gastric cancer and adjacent noncancerous tissue ($n = 35$). (*g*) The red arrows indicate the strong TTN staining in intestinal metaplasia. The data are expressed as the mean ± SD ; ***$p < 0.001$; * $p < 0.05$ by Wilcoxon matched pairs test. Scale bars, 100 µm.

gastric samples. A moderate to high intensity of TTN staining was observed in more than 60% of the cancer samples compared to less than 40% of the noncancerous adjacent tissues (Figs. 2*d* and 2*e*; Supporting Information Fig. S5*b*). In addition, 5 of 6 normal tissue samples displayed low or completely absent TTN staining (data not shown). Moreover, 63% of the malignant gastric samples (22/35) showed increased TTN expression compared to their respective adjacent noncancerous tissues ($p < 0.05$; Fig. 2*f*). Most importantly, the moderate and strong TTN staining intensity in the adjacent noncancerous tissues was observed mainly in areas of intestinal metaplasia, a premalignant lesion involved in gastric carcinogenesis (Fig. 2*g*). Thus, we were able to validate at the protein level two of the differentially expressed genes identified in the low-abundance transcriptome of gastric cancer.

Gene ontology analysis of the low-abundance transcriptome identified cancer type-specific signaling pathways

The lists of differentially expressed genes were further analyzed for the enrichment of gene ontology terms (GO) using the functional annotation clustering classification tool of DAVID Bioinformatics Resources 6.7[18] and PANTHER 7.1 pathway annotation.[19] The main functional GO terms enriched in the whole transcriptome of gastric cancer highlighted processes associated mainly with cell interactions with the extracellular matrix and cell adhesion (Table 1). Interestingly, cell adhesion was also a GO term enriched in the low-abundance transcriptome (Table 1). However, the analysis of the enriched principal pathways maps highlighted completely different pathways when the low-abundance and the whole transcriptome were compared (Table 1). Principal pathways analysis of the differentially enriched pathways in the whole transcriptome of gastric cancer highlighted pathways associated with two heterotrimeric G protein and integrins signaling, and the pyruvate and CoA metabolism (Table 1). Interestingly, the heterotrimeric G protein and integrins signaling, and the pyruvate signaling pathways were also highlighted in the low-abundance transcriptome of gastric cancer (Table 1). However, novel pathways were highlighted in the low-

Table 1. Principal biological annotation and functional terms identified by gene set enrichment analysis in gastric cancer

Analysis	Principal GO term (cellular components, biological process, molecular function)	*p*-value	Principal pathway maps (PANTHER)	*p*-value
Gastric whole transcriptome	GO:0044421~extracellular region part	7.30E-04	P00026:Heterotrimeric G-protein signaling pathway-Gi alpha and Gs alpha mediated pathway	1.05E-02
	GO:0031012~extracellular matrix	3.10E-03	P02772:Pyruvate metabolism	1.06E-02
	GO:0005581~collagen	1.23E-02	P00034:Integrin signaling pathway	1.54E-02
	GO:0007155~cell adhesion	3.33E-02	P02732:Carnitine and CoA metabolism	2.17E-02
	GO:0033764~steroid dehydrogenase activity	4.93E-02		
Gastric low-abundance transcriptome	GO:0043167~ion binding	1.35E-02	P00012:Cadherin signaling pathway	2.89E-03
	GO:0007155~cell adhesion	2.44E-02	P00056:VEGF signaling pathway	4.05E-03
			P00010:B cell activation	6.17E-03
			P00057:Wnt signaling pathway	1.12E-02
			P00036:Interleukin signaling pathway	1.56E-02
			P00053:T cell activation	1.66E-02
			P00048:PI3 kinase pathway	2.77E-02
			P02775:Salvage pyrimidine ribonucleotides	3.16E-02
			P00004:Alzheimer disease-presenilin pathway	3.53E-02
			P00054:Toll receptor signaling pathway	3.53E-02
			P00006:Apoptosis signaling pathway	3.65E-02
			P00005: Angiogenesis	3.74E-02
			P00026:Heterotrimeric G-protein signaling pathway-Gi alpha and Gs alpha mediated pathway	4.81E-02
			P00049:Parkinson disease	4.94E-02
			P02772:Pyruvate metabolism	1.06E-02
			P00034:Integrin signaling pathway	1.54E-02

Gene ontology was performed using DAVID v6.7. The most representative biological functions of each cluster are shown. Pathway enrichment was performed using PANTHER.

abundance transcriptome with even higher statistical significance such as the signaling pathways of VEGF, Wnt, B and T cell activation, PI3Kinase and others (Table 1).

To confirm that the pathways highlighted by the data mining of the low-abundance transcriptome were indeed biologically active in our samples we performed a more detailed analysis of selected pathways. We conducted gene expression studies using custom-designed PCR arrays containing genes involved in the Wnt/hedgehog pathway, the PI3K/AKT pathway, the angiogenesis pathway and the B/T-cell activation pathway. Four samples of gastric cancer and paired adjacent noncancerous tissue were used to assess the mRNA expression levels of the different genes associated with these specific pathways. The relative fold change in each gene in the cancer tissue was expressed in relation to the paired adjacent tissue. Only those genes with an average differential fold expression value > 2.0 were included. The overall data demonstrate that most of the genes in the different pathways were overexpressed in the cancer tissue (Figs. 3*a*–3*d*).

Interestingly, increased expression of the Wnt/hedgehog pathway-associated genes, such as Wnt-1 induced secreted protein 1 (WISP1), protein patched homolog 1 (PTCH), C–terminal of E1A binding protein (CTBP1) and secreted frizzled-related protein 4 (SFRP4), was observed. Moreover, among the members of the family of FZD receptors, we observed increased expression of frizzled family receptor 5

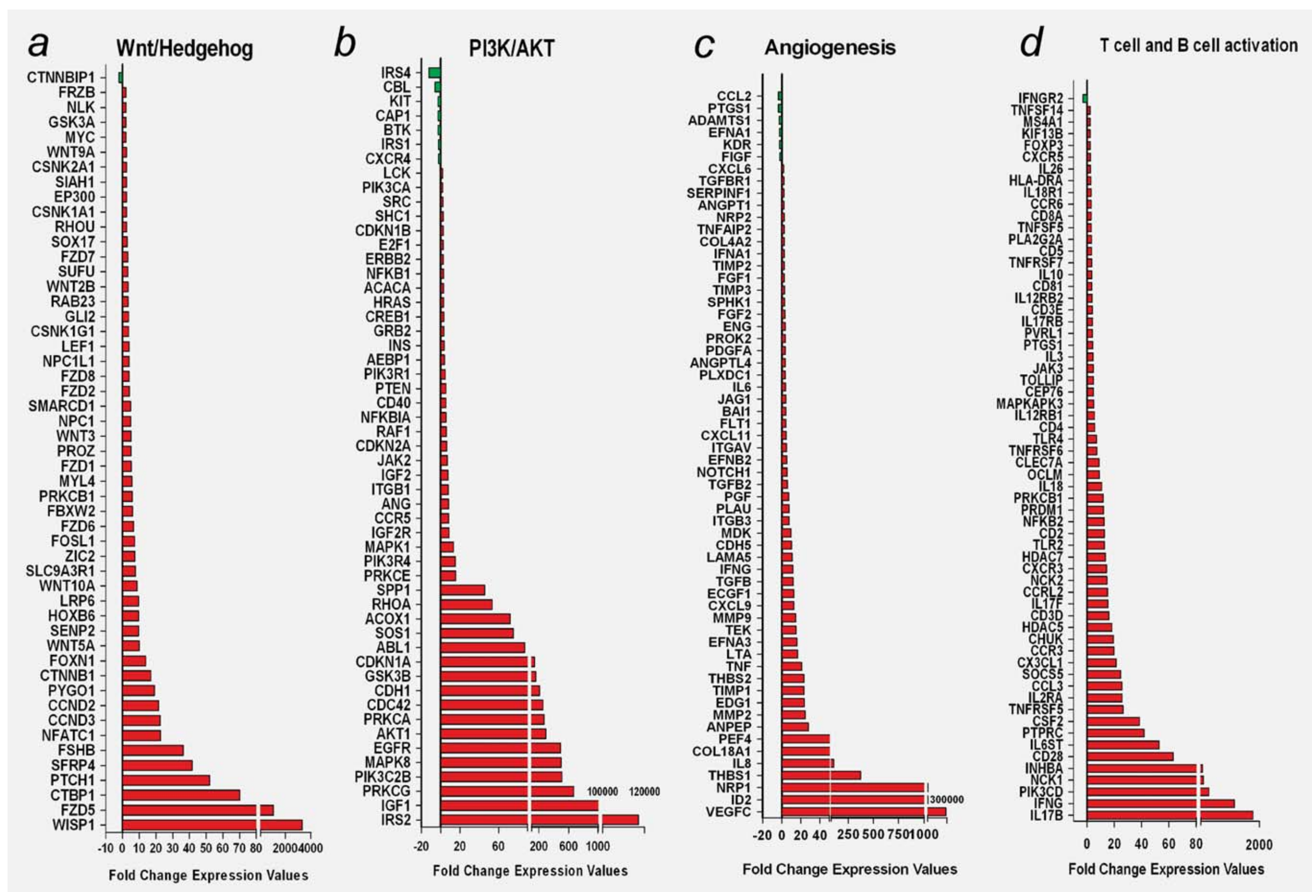

Figure 3. PCR-array analysis highlights overexpressed genes in the different pathways. Expression profiles of genes relevant to the Wnt/ Hedgehog (*a*), the PI3K/AKT pathway (*b*), the angiogenesis pathway (*c*) and the B and T-cell activation pathway (*d*). Each PCR-array contained 87 genes relevant to each pathway as well as 9 housekeeping genes, and the expression values represent the average fold change of gastric cancer samples relative to the paired noncancerous tissues. The data correspond to genes showing fold changes > 2.0.

(FZD5) and its ligand, Wnt5 (Fig. 3*a*). In the PI3K pathway, we observed a striking overexpression of insulin receptor substrate 2 (IRS2) and the downregulation of IRS1 and IRS4. We also observed a clear overexpression of several protein kinases, the protooncogene c-abl oncogene 1, non-receptor tyrosine kinase (ABL1), the insulin-like growth factors IGF1 and IGF2 and the EGFR (Fig. 3*b*). Regarding angiogenesis, the lymphangiogenesis promoter vascular endothelial growth factor C (VEGF–C), interleukin 8 (IL8), inhibitor of DNA binding 2 (ID2) and neuropilin 1 (NRP1) were notably upregulated in gastric cancer samples compared to adjacent noncancerous tissue (Fig. 3*c*). The two genes that exhibited the largest levels of expression in the T-cell and B-cell activation pathway in the gastric cancer tissues corresponded to two inflammatory cytokines, interleukin 17B (IL17B) and interferon gamma (IFNγ) (Fig. 3*d*). Thus, the overall data identified a limited number of specific genes that might be responsible for the aberrant activity of each signaling pathway.

To perform a biological validation of the PCRArrays studies, we next selected all genes with a fold change >10 in the PCRArrays. That included 16 genes of the Wnt/Hedgehog pathways; 20 genes of the PI3K-AKT pathways; 23 genes of the angiogenesis pathway and 29 genes of the B/T cell activation pathways. We next performed a manual search in PubMed and Scirus data bases that included both papers and patents, looking for genes with no previous biological data in gastric cancer. Following this initial search we selected six genes, FZD5, ID2, IRS2, FOXN1, CTBP1 and WISP1. We next assessed mRNA levels of the selected genes in five available gastric cancer cell lines and observed that only CTBP1 that belongs to the Wnt/Hedgehog pathway was highly expressed in all the cell lines (Fig. 4a; Supporting Information Fig. S6); in addition, commercial siRNAs and antibodies were available.

CTBP is a nuclear protein that associates with histone deacetylases and binds to chromatin but may also function as a transcriptional corepressor that interacts with adenoviral E1A.[20] In both cases, CTBP1 is involved in the regulation of the transcriptional status of the cell. Targeting CTBP1 expression with a specific siRNA reduced CTBP1 mRNA and protein levels in gastric cancer cell lines by almost 80% (Figs. 4*b*

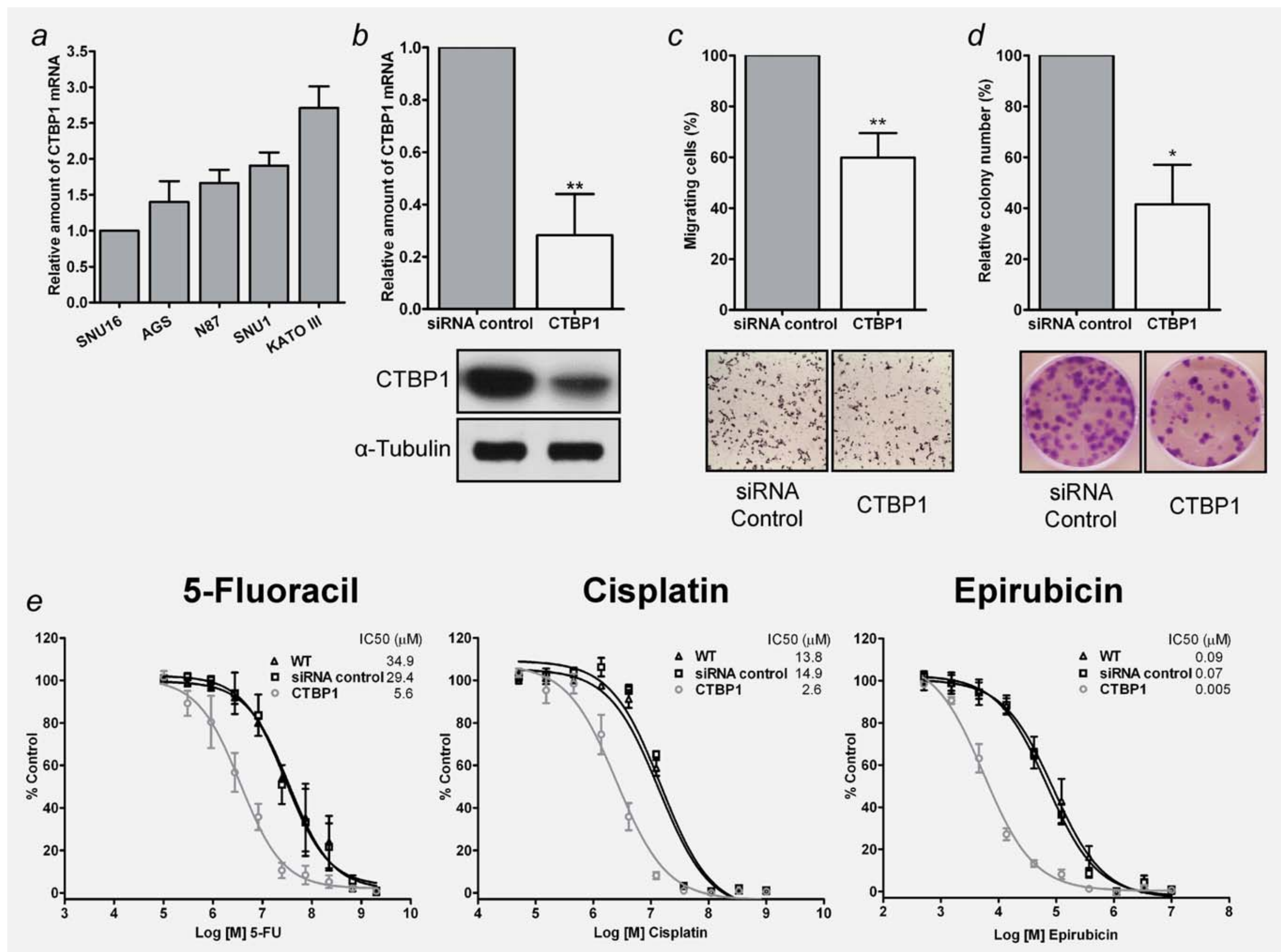

Figure 4. Knock down of CTBP1 expression by siRNA sensitizes gastric cancer cells to chemotherapeutic drugs. (*a*), relative expression of CTBP1 in five gastric cancer cell lines (SNU16, AGS, N87, SNU1 and Kato III). CTBP1 was quantified by qRT-PCR using QARS and TFCP2 as the internal controls. (*b*), CTBP1 knock down was validated by assessing mRNA and protein levels. AGS gastric cancer cells were transfected with CTBP1 specific and control siRNAs. QARS and TFCP2 were used as internal controls, while α-tubulin was used as an internal control for protein loading. (*c*) and (*d*), AGS cells following preincubation for 48 hr with siRNAs transfection. (*c*), Migration analysis. Representative photographs of Giemsa-stained cells are shown. (*d*), Clonogenic capacity following preincubation for 48 hr with an siRNA against CTBP1. Representative photographs of the crystal violet-stained colonies are shown. The data are expressed as the mean ± SD ($n = 3$). ** $p < 0.01$, * $p < 0.05$ by Student's t-test. (*e*), AGS gastric cancer cells were transfected with a siRNA against CTBP1. After 48 hr, cells were treated with varying concentrations of the different chemotherapeutic agents for an additional 72 hr. Cell viability was evaluated with MTS. The data are expressed as the mean ± SD ($n = 3$).

and 4*c*). The decreased expression of CTBP1 following transient siRNA expression in gastric cancer cells inhibited their clonogenic and migration capacities (Figs. 4*d* and 4*e*).

In addition to the regulation of the transcriptional activity, CTBP1 has been shown to sensitize certain malignant cell lines to the genotoxic effects of certain chemotherapeutic drugs through mechanisms associated either with apoptosis or the modulation of multidrug resistance gene 1 (MDR1) levels.[21] Therefore, we decided to target gastric cancer cells with the siRNA for CTBP1 and then expose the cells to chemotherapeutic drugs that are currently used in the treatment of gastric cancer. We observed a highly significant chemosensitizing effect when gastric cancer cells expressing reduced levels of CTBP1 due to siRNA administration were treated with the different drugs. 5-FU had an IC_{50} of 29.4 μM in the presence of a control siRNA in AGS cells lines (Fig. 4*e*). Treatment with the specific anti-CTBP1 siRNA followed by the addition of 5-FU significantly reduced the IC_{50} to 5.6 μM (Fig. 4*e*). The genotoxic agent cisplatin and the anthracycline epirubicin are also part of the standard treatment for gastric cancer. Interestingly, the treatment of AGS gastric cancer cells with anti-CTBP1 siRNA significantly reduced the IC_{50} of cisplatin from 14.9 to 2.6 μM, whereas this siRNA reduced the IC_{50} of epirubicin in AGS cells from 0.07 to 0.005 μM (Fig. 4*e*).

Discussion

The identification of the low-abundance transcriptome is of paramount importance in cancer research because subtle

changes in the activity of few genes can lead to malignant transformation and tumor dissemination. In this work, we demonstrated that the analysis of the low-abundance transcriptome permitted the identification of novel genes that could serve as disease markers. Most importantly, this study led to the identification of specific intracellular signaling pathways, their aberrantly leading genes and potential novel druggable targets for improving the treatment of advanced gastric cancer.

Consistent with previous data[5,22] whole transcriptome analysis identified differentially expressed genes with biological functions that were mainly associated with cell adhesion and cell-ECM interactions. Interestingly, less than 5% of the differentially expressed genes were shared between the low-abundance and the whole transcriptome. Further analysis by qRT-PCR validated 90 % of the genes differentially expressed in the low-abundance transcriptome of gastric cancer, demonstrating the robustness of this method. Moreover, studies at the protein level using TMA validated the overexpression of laminin $\alpha 3$ (LAMA3), one of the three subunits of Laminin-322 (Ln-332). There is no evidence in the literature of the involvement of Ln-332 in human gastric cancer; however, more recent studies have demonstrated that gastric cancer cell lines exhibit transcriptional silencing of LAMA3 due to promoter methylation.[23] The LAMA3 staining was located in the cytoplasm of the malignant epithelial cells of gastric cancer samples, showing no evidence of expression silencing suggesting that this process is probably occurring following adaptation of cell lines to *in vitro* culture. The possibility that TTN may be a marker for premalignant lesions is proposed and warrants further investigation. In this regard, recent data suggested that TTN, the largest polypeptide encoded by the human genome might has an oncogenic role;[24–26] TTN is considered a protein kinase, and 63 non synonymous mutations were found in its coding regions in different cancer types of which half might be considered driver mutations.[27]

The analysis of principal pathways in the low-abundance transcriptome highlighted intracellular signaling pathways that differed at a high extent from those obtained following whole transcriptome analyses. Among the intracellular signaling pathways selected for further validation by PCRArrays, only 2, 10, 6 and 5 genes exhibited more than 100-fold overexpression in the Wnt/hedgehog, PI3K/Akt, angiogenesis and B/T-cell activation intracellular pathways, respectively. These genes should be considered as leading the aberrant activities of the enriched pathways and then as preferable targets.

VEGF-C was at the top of the differentially expressed genes. VEGF-C has been associated with lymphatic spread and the invasion capacity of many types of cancer cells, including gastric cancer cells.[28,29] IRS-2, a member of the PI3K/AKT signaling pathway was also highly expressed in gastric cancer samples; the differential abilities of IRS-1 and IRS-2 are intriguing. Although IRS-2 expression has been associated with lymph node metastasis in gastric cancer,[30] loss of IRS-1 expression or function may facilitate tumor progression.[31,32] Interestingly, IGF1, IGF2, insulin-like growth factor 2 receptor (IGF2R) and insulin (INS), all of which are components of the insulin/IGF signaling pathway, were also upregulated.[33] The expression of IL-17 and IFNγ in association with the T/B cell activation pathway has been associated with the activation of the cytotoxic T and NK cell pathways in response to *Helicobacter pylori* infection.[34] Interleukin-17 plays a potential role in the inflammatory response,[35] and in agreement with the present data, its overexpression has been observed in gastric cancer samples.[36] IFNγ is a classic pro-inflammatory cytokine secreted by NK cells and CD8 T-cells that has been associated with different tumor lineages.[37,38] Among the large family of FZD receptors associated with the Wnt signaling pathway, we showed that FZD5 exhibited nearly 1,000-fold overexpression in gastric cancer samples. Its ligand, Wnt5a, also exhibited the largest differential expression among the different Wnt members between cancer and adjacent noncancerous samples. Wnt5a is involved in the activation of canonical and noncanonical Wnt cascades in epithelial cells located at the tumor-stromal interface during invasion and metastasis.[39] Interestingly, pro-inflammatory cytokines such as interleukin 6 (IL-6) and tumor necrosis factor (TNFα) upregulate Wnt5a levels in gastric cancer,[40] and Wnt5a overexpression correlated with a poor prognosis.[41] In coincidence, the largest overexpression in the Wnt pathway was associated with WISP1. Recent studies have shown that WISP1 expression is coordinately regulated by Wnt5a and antagonists of the canonical Wnt pathway.[42]

To functionally validate the data obtained with the low-abundance transcriptome analyses, we decided to perform additional studies with CTBP1. Knocking down the expression of CTBP1 inhibited the clonogenic and migration capacities of the cells, consistent with CTBP1's role as a mediator of hypoxia-induced tumor cell migration.[43] Recent studies have shown that CTBP1 might act as a sensitizer to chemotherapeutic drugs, at least in breast cancer cells.[21] Here, we show for the first time that knocking down the expression of CTBP1 sensitized AGS gastric cancer cells to three different chemotherapeutic drugs, 5-FU, cisplatin and epirubicin, decreasing their IC_{50}s by 5-fold, 6-fold and 14-fold, respectively. The broad chemosensitizing effect of CTBP1 is supported by recent evidence that CTBP1 can promote drug resistance by increasing the expression of MDR1. Downregulation of CTBP1 expression in breast cancer cells renders malignant cells more sensitive to 5-FU and other genotoxic agents such as cisplatin and etoposide.[21, 44]

The development of molecular-targeted drugs for cancer treatment has demonstrated some success. However, even the most successful biological drugs are effective only in a minority of patients; occasionally, less that 10% of patients benefit from treatment. Here, we identified novel biomarkers and leading genes of signaling pathways that are aberrantly activated in gastric carcinomas that might provide the basis for increasingly specific targeted therapies.

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