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Microarray-based analysis of COL11A1 and TWIST1 as important differentially-expressed pathogenic genes between left and right-sided colon cancer

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Abstract. Colonic cancer has become a main reason of mortality associated with cancer; however, left and right-sided colonic cancer have diverse outcomes in terms of epidemiological, histological, clinical parameters and prognosis. We aimed to examine the discrepancies between these two types of colon cancers to identify potential therapeutic targets. In the present study, three gene expression profiles (GSE44076, GSE31595, GSE26906) from Gene Expression Omnibus (GEO) database were downloaded and further analyzed. A PPI (protein-protein interaction) network of the differentially-expressed genes (DEGs) of GSE44076 between tumor and normal was established with the Search Tool for the Retrieval of Interacting Genes database. Then, the DEGs of these two colon cancers (left, right) samples were identified. Subsequently, the intersection of DEGs of left and right-sided colon cancer samples obtained from three databases, and DEGs of tumor and normal samples were analyzed. Collagen type XI α1 chain (COL11A1), Twist family bHLH transcription factor 1 (TWIST1), insulin-like 5 and chromogranin A were upregulated proteins, while 3β-hydroxysteroid dehydrogenase was downregulated protein in right colon cancer than in left-sided tumor samples. Through further experimental verification, we revealed that COL11A1 and TWIST1 were significantly upregulated at the mRNA and protein levels within right-sided colon cancer compared with in left-sided colon cancer samples ($P<0.05$), consistent with bioinformatical analysis. Furthermore, a positive correlation between COL11A1 and TWIST1 protein expression was observed ($P<0.0276$). Collectively, our data showed that COL11A1 and TWIST1 may be potential prognostic indicators and molecular targets for the treatment of right-sided colon cancer.

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Introduction

Colonic cancer has become the most common cancer, as well as a general cause of human mortality from cancer. Mortality rates from colonic cancer are predicted to increase substantially in Costa Rica, Australia, the United States, Ireland and Canada, with the mortality rate expected to rise by 60% until 2035 (1). It was also identified that left and right-sided colon cancer differs significantly with reference to histological, epidemiological and clinical parameters (2,3). In addition, patients suffering from right-sided colonic cancer usually have worse prognosis than those with the other types of cancer (4). These variations may be due to genetic differences that induce distinct carcinogenic and biological behaviors. The impact of these findings on screening and treatment remains to be elucidated (2). Thus, it is crucial to further investigate the disparity between two types of colon cancers, which may be beneficial in suppressing the spread of primary cancer.

In order to further identify the variable genetic expression of the two colon cancers, the GSE44076 (5), GSE31595 (6) and GSE26906 (7) datasets were downloaded from the database of Gene Expression Omnibus (GEO). We then analyzed the data to screen differential expression of genes (DEGs) and construct a protein-protein interaction (PPI) network. Through database analysis, we found several genes that were significantly upregulated or downregulated, respectively, in left and right-sided colonic cancer.

Several lines of evidence indicated that COL11A1 was upregulated in various cancers, including colorectal (8-10), pancreatic (11), non-small cell lung (12), breast (13), ovarian (14), and head region and neck squamous cell cancer (15), suggesting

an oncogenic role for COL11A1. Regarding the formation and progression of tumors, COL11A1 was confirmed to facilitate the progression of ovarian, and head and neck squamous cell carcinoma (14-16). It was also reported as a possible biomarker of colon cancer (12,17).

The expression of TWIST1, a key factor in the conversion of epithelial-mesenchymal transition (EMT), is closely associated with the high probability of tumorigenesis, chemotherapeutic resistance, poor prognosis and metastasis (18-20). Furthermore, TWIST1 was be found to be overexpressed in various malignant tumors, such as cancer of the thyroid (21), esophagus (22), breast (23), lung (24), stomach (25), pancreas (26), hepatocytes (27), colon (28) and cervix (29). Thus, the expression of TWIST1 is often used as a potential target for cancer treatment (27,30-32).

Through database analysis, we determined COL11A1, TWIST1, insulin-like 5 (INSL5) and chromogranin A (CHGA) to be hub proteins for upregulation, and 3β-hydroxysteroid dehydrogenase (HSD3B2) was a hub protein for downregulation. To further verify the importance of these genes in left- and right-sided colon cancer, we selected and determined the expression levels of COL11A1 and TWIST1 in various tissue samples obtained from our department. Through the combination of database and experimental analyses, the present study is the first to identify genetic dissimilarities between these two colon cancer types to the best of our knowledge. Our findings also revealed that the mRNA and protein expression levels of COL11A1 and TWIST1 were upregulated in right-sided colonic cancer than in left-sided tumor samples. This suggested that COL11A1 and TWIST1 may be potential prognostic markers and/or molecular targets for the treatment of right-sided colon cancer.

Materials and methods

Ethics statement. The present study was approved by the Ethics Committee of Xiamen University. In total, 17 left-sided colonic cancer and 13 right-sided colonic cancer samples were collected from the Zhongshan Hospital of Xiamen University between August 2014 and August 2015 (the patients and samples are characteristics in Table I). This research was conducted in accordance with the Declaration of Helsinki (33), the International Ethics Standards for Human Biomedical Research (34) and the relevant provisions of the National Natural Science Foundation of China, jointly supported by the World Health Organization and the International Council of Medical Science Organizations (35).

Gene expression profile data. We downloaded and used three datasets from the GEO database and the data of patients enrolled in this study. The inclusion criteria were set as follows: i) Patients with clinical information, including the site of resection or biopsy; and ii) patients with complete cancer data and normal data.

GSE44076 is a dataset containing 246 samples provided by Solé *et al* (5), utilizing Affymetrix Human Genome U219 Array platform. The dataset comprised 66 colon resected left-sided colonic cancer samples and 38 right-sided samples from colonic carcinoma tissues, as well as 98 normal samples for colonic tissues.

The GSE31595 dataset constituted 37 samples provided by Thorsteinsson *et al* (6), utilizing the Affymetrix Human Genome U133 Plus 2.0 Array platform. This dataset comprised 14 colon resected left-sided colonic cancer samples and 23 right-sided samples from colonic carcinoma tissues.

The GSE26906 dataset contained 90 samples provided by Birnbaum *et al* (7), utilizing Affymetrix Human Genome U219 Array platform. The dataset comprised 65 colon resected left-sided colonic cancer samples and 25 right-sided samples from colonic carcinoma tissues.

Data pre-processing and DEG analysis. We used the Affy package (36) to preprocess the three separate datasets, correcting their background and transforming them from probe level into gene symbol. Robust multiarray average was used to normalize the values of probe level intensity and the signal estimates of generated probe set. Subsequently, the DEGs between left and right-sided colonic cancer of three separate datasets were conducted by utilizing the limma package (37) in R. We calculated the fold-change (FC) of relative genetic expression, and the threshold criteria of DEG selection were $P<0.05$ and $|\log2FC|\geq1$. Subsequently, on the basis of limma package in R, we selected DEGs between colon cancer and normal samples with the dataset of GSE44076 (37). We also calculated the FC in relative gene expression. This threshold criteria used for DEG selection were $P<0.05$, $|\log2FC|\geq2$.

PPI network construction of the DEGs between colonic cancers and normal tissue samples. The potential interactions among the DEGs between colonic cancers and normal tissue samples in the GSE44076 dataset was predicted by the Search Tool for the Retrieval of Interacting Genes (STRING) database (version 10) (38,39). The proteins from database were entered in STRING. With the criterion of a combined score of >0.4, and as visualized with Cytoscape (version 3.2.1), only interactions which contained at least one DEG were selected to establish the PPI network (40).

Identification of the hub gene between left and right-sided colonic cancer. We obtained the intersection of total DEGs of left-sided compared with right-sided colonic cancer in three separate datasets, and the DEGs of GSE44076 between colon cancers and normal tissues with the criterion of a combined score of >0.4. The DEGs contained in the intersection were identified as the hub gene.

Prognoscan database analysis and literature review. We utilized the PrognoScan database (http://www.abren.net/PrognoScan/) to analyze potential associations between DEGs and survival in colon cancer using Kaplan-Meier analysis (41). Furthermore, the patients were compared on the basis of high and low expression to analyze survival according to the PrognoScan database. In order to select oncogenes for further analysis, a literature search of PubMed was performed for each gene using the term 'GENE NAME]', 'cancer'.

Tumors. In the present study, mRNA Droplet Digital™-PCR was run on the pane of 17 fresh-frozen left-sided colonic cancer tissues and 13 right-sided samples collected at

Table I. Clinicopathological characteristics in colon cancer samples.

Clinicopathological parameters	Tumor location		P-value
	Left	Right	
Age, years			0.269
≥50	10	5	
<50	7	8	
Sex			0.004
Male	13	3	
Female	4	10	
TNM stage			0.633
Tis +I	2	0	
II	10	8	
III	4	4	
IV	1	1	
Histology grade			0.432
Well + moderate	12	10	
Poor	3	3	
Mucinous adenocarcinoma	2	0	

TNM, tumor-node-metastasis; Tis, carcinoma *in situ*.

the Zhongshan Hospital of Xiamen University between 2014 and 2015 (Table I). All samples were frozen at -80˚C immediately after collection by surgeons who directly took part in the study and removed the surgical specimens. The mean age of the 17 patients was 64.82 years (ranging from 43-83 years-old) and the maximum tumor diameter ranged from 2.7-12 cm (mean, 5.476 cm) in left sided colon cancer. The mean age of the 13 patients was 61.15 years (ranging from 39-82 years-old) and the maximum tumor diameter ranged from 2-10 cm (mean, 5.362 cm) in right sided colon cancer. There were no significant differences between the two groups in age (P=0.4281) and maximum tumor diameter (P=0.8824).

Cell culture and transfection. The colon cancer HCT116 cell line was purchased from the Institute of Cell Biology and cultured in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Excell Bio, Shanghai, China), 100 U/ml penicillin and 100 μg/ml streptomycin at 37˚C in a humidified incubator containing 5% CO_2. In total, 5 μg Flag-TWIST1 or COL11A1 plasmids (Public Protein/Plasmid Library) were transfected into HCT116 cells; after 36 h, the cells were collected. TWIST1 or COL11A1 expression was analyzed using a Flag-antibody. The expression levels of E-cadherin and N-cadherin or inhibitor of NF-κB (IKKβ) were determined using reverse transcription-quantitative (RT-q) PCR, and the expression levels were normalized to GAPDH. The results are presented as the mean ± SD of three independent experiments. Plasmid DNA transfections were performed with Turbofect reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions.

Western blotting. The colon cancer samples were lysed with cell lysis buffer 20 mM Tris-HCl (pH 7.5), 20 mM β-glycerophosphate, 150 mM NaCl, 1 mM sodium orthovanadate, 1 mM PMSF, 10 mM NaF, 10 μg/ml leupeptin, 2 μg/ml aprotinin,1% Triton X-100 and 1 mM EDTA]. Protein concentration was determined using the bicinchoninic acid method and 80 μg of total protein was used for western blotting. Protein lysates were separated using 12% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked for 1 h in 5% BSA and incubated with primary antibodies overnight at 4˚C, washed, and incubated with secondary antibodies (1:5,000) for 1 h. The membranes were then washed and the protein bands were visualized using the Immobilon Western Chemiluminescent HRP Substrate Kit (EMD Millipore).

Western blotting was performed with the following primary antibodies: actin monoclonal antibody (1:5,000; cat. no. A5441; Sigma-Aldrich; Merck KGaA), Twist1 (1:1,000; cat. no. 46702; Cell Signaling Technology, Inc.) and COL11A1 (1:1,000; cat. no. 42818; Cell Signaling Technology, Inc.). The horseradish peroxidase-conjugated secondary goat anti-rabbit and goat anti-mouse (1:5,000; cat. nos. 31466 and A16078; Thermo Fisher Scientific, Inc.). densitometry was performed using Quantity One software (Bio-Rad Laboratories, Inc.).

RNA isolation, reverse transcription-quantitative PCR (RT-qPCR). In the present study, total RNA was isolated using the RNA Simple Total RNA Kit (Biotek, Inc.), according to the manufacturer's instructions, and first-strand cDNA was obtained by utilizing First-strand cDNA Synthesis Kit (Biotek, Inc.). The RT reaction was performed at 42˚C for 60 min and 85˚C for 5 min, the cDNA was stored at -20˚C. We amplified cDNA samples via qPCR and the SYBR Green Real-Time PCR Master Mix (Toyobo Life Science) utilizing an ABI 7500 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) with the following primers: GAPDH forward, 5'-TCTCCTCTGACTTCAACAGCGA-3' and GAPDH reverse, 5'-GTCCACCACCCTGTTGCTGT-3'; TWIST1 forward, 5'-GGCCAGGTACATCGACTTCC-3', TWIST1 reverse, 5'-TCCAGACCGAGAAGGCGTAG-3'; COL11A1 forward, 5'-CAACTCAGCCATCCTGACTT-3', COL11A1 reverse, 5'-GTCTCACCACCAGATGTGAA-3'. The thermocycling conditions for the qPCR were as follows: 15 min at 95˚C, followed by 10 cycles of 30 sec at 94˚C, 90 sec at 72˚C; and 20 cycle of 30 sec at 94˚C, 90 sec at 58˚C and 60 sec at 72˚C, with a final incubation for 60 min at 72˚C.

Statistical analysis. Data are presented the mean ± SD of at lowest three individual experiments. All statistical tests were performed using Graphpad Prism 6.0 (GraphPad Software, Inc.). One-way ANOVA followed by a Bonferroni's post-test for manifold comparisons, and pair-wise comparisons with Student's test (two-tailed) were conducted. The correlation analyses were proceeded with Pearson's test. $P<0.05$ was considered to indicate a statistically significant difference.

Confirmed by continually changing the cut-off values in the analysis of all our results, receiver operating curves (ROC) curves were established by all potential specificity/sensitivity pairs with an particular detection method (42). In our study, ROC curves were used to identify the hub genes that would be

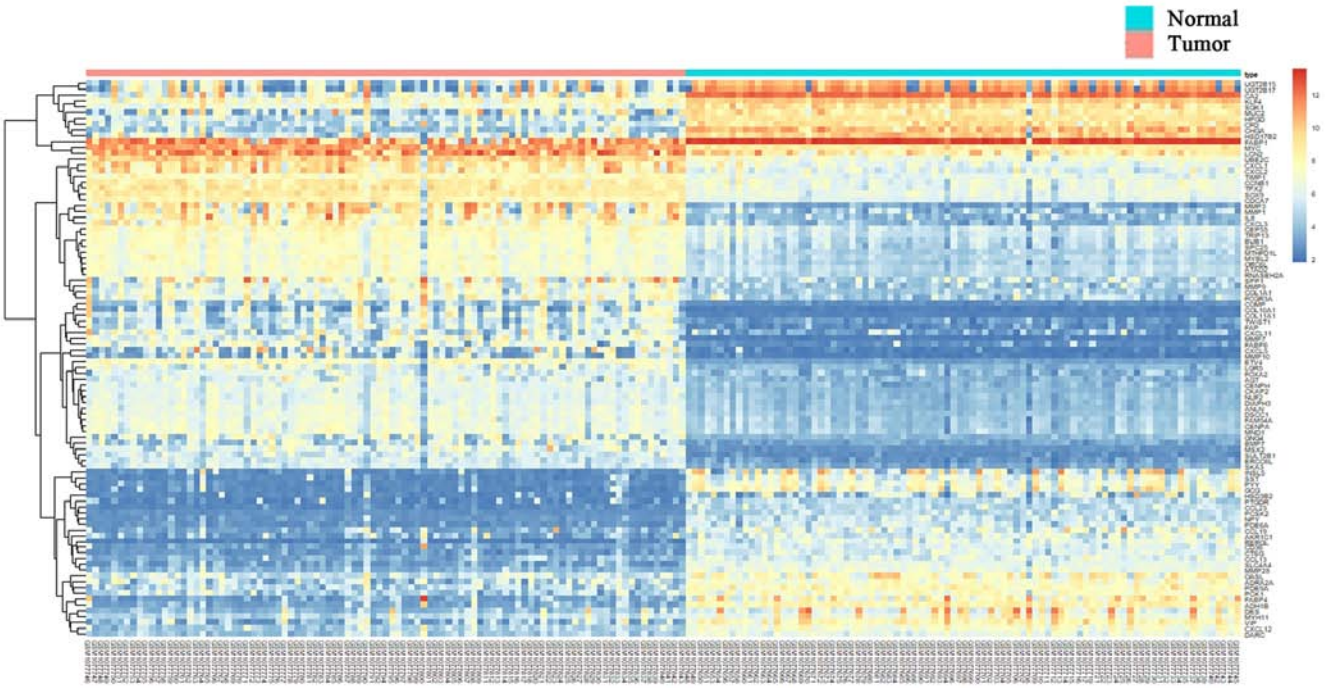

Figure 1. Screening of differentially-expressed genes. The GSE44076 gene expression datasets were downloaded from the Gene Expression Omnibus database and the upregulated DEGs and downregulated DEGs between tumor and normal tissues were identified. DEGs, differentially-expressed genes.

highly predictive of the tissue samples and could distinguish between left and right-sided colon cancer tissues.

Functional enrichment analysis. To investigate the functions that may be changed by the DEGs identified, functional Gene Ontology (GO) enrichment analysis was performed using Database for Annotation, Visualization, and Integrated Discovery (version 6.8; https://david.ncifcrf.gov/) (43) to determine the biological functions of these DEGs between tumor and normal tissues. An enriched gene count >2 and cut-off criteria were selected based on $P<0.05$ in our study.

Results

Screening of DEGs. To further identify the differential gene expression between these two types colonic cancers, we selected and downloaded the GSE44076, GSE31595 and GSE26906 gene expression datasets from the GEO database for DEG screening (Fig. 1). The GSE44076 dataset, amounting to 48 DEGs, revealed 20 upregulated and 28 downregulated DEGs. The GSE31595 dataset, amounting to 46 DEGs, contained 21 upregulated and 25 downregulated DEGs. The GSE26906 dataset, amounting to 34 DEGs, revealed 23 upregulated and 11 downregulated DEGs. Additionally, these DEGs of tumor and normal tissues were employed for further analysis using the GSE44076 dataset (Table II).

PPI network. A PPI network with these DEGs between tumor and normal tissues was constructed from pre-processing of the GSE44076 dataset, via analysis with STRING and Cytoscape software. Proteins and related interactions were symbolized by nodes and lines respectively in the PPI network, which consisted of GSE44076, including 228 proteins and 683 interactions (Fig. 2). In the PPI network, the hub proteins should be the nodes with an average connective degree ≥5. From PPI network analysis, we could identify network of various proteins in colon cancer.

Analysis the association between DEGs and survival. Subsequently, we obtained the intersection of DEGs between left and right-sided colonic cancers obtained from the three aforementioned databases and DEGs of tumor and normal samples (Fig. 3A). COL11A1, TWIST1, INSL5 and CHGA were determined to be upregulated proteins, while HSD3B2 was downregulated protein in right-sided colonic cancer samples than left-sided tissues. Among them, COL11A1, TWIST1 and HSD3B2 were in accordance with the DEG profiles of tumor and normal tissue. We plotted ROC curves for these proteins. The closer to upper-left corner the ROC curves are, the more accurate the test is. The genes we screened exhibited a typical trend (Fig. 3B). In addition, through Kaplan-Meier analysis, upregulated COL11A1 and TWIST1 was associated with poor survival rates in colon cancer. By contrast, the downregulation of HSD3B2 exhibited generally poor survival rates in colon cancer. INSL5 and CHGA were not associated with a statistically significant difference in survival rates (Fig. 3C). We performed a literature search of PubMed for each gene using the terms 'GENE NAME]', 'cancer'. Of the five genes, according to a previous study COL11A1, and TWIST1 had been described as being candidate oncogene (44); therefore, in subsequent analyses, we selected two of them (COL11A1 and TWIST1) for the further verification.

Verification of the DEGs (COL11A1 and TWIST1) between left and right-sided colonic cancer with western blotting analysis and RT-qPCR. In order to estimate the levels of COL11A1 and TWIST1 expression that are implicated in these two sorts of colon cancer, we performed RT-qPCR analysis

Table II. DEGs were identified from three profile datasets, including up-regulated genes and down-regulated genes in which the colon cancer tissues compared with normal colon tissues and right-sided colon cancer tissues compared to left-sided colon cancer.

DEGs	Upregulated genes	Downregulated genes
GSE44076 tumor and normal	IL8, MYC, MMP9, CCNB1, AGT, BUB1, CENPA, CXCL1, TPX2, CEP55, UBE2C, NUF2, CXCL11, CXCL5, GNG4, TIMP1, SPP1, MMP3, MND1, CXCL2, TRIP13, MMP7, ATAD2, MMP1, CXCL3, COL1A1, ANLN, ERCC6L, CENPH, SKA3, DSCC1, SPC25, SOX9, LGR5, CDCA7, CKAP2, BMP7, RNASEH2A, FOXA2, FAM54A, FCGR3A, MMP10, DIAPH3, COL11A1, TWIST1, ETV4, LCN2, MYBL2, SULT2B1, COMP, COL10A1, FABP6, MTHFD1L, ORC6L, MSX2, FAP	CXCL12, SST, NPY, PYY, CCL19, GCG, ADRA2A, INSL5, CD36, CA2, CHGA, FABP4, CCL13, HSD3B2, VIP, KLF4, CCL23, PCSK2, PDE6A, FABP1, MUC2, PDE9A, HPGD, OASL, CTSG, RERGL, SGK1, AKR1C1, CFD, SLC4A4, DES, MMP28, HSD17B2, DARC, PCK1, UGT2B15, UGT2B17, ADH1B, MYH11, PTGDR
GSE44076 right and left	TTPA, TRIM54, PNLIPRP2, ELAVL2, G0S2, RBP2, MSLN, EREG, ZNF43, TNNC1, PRAC, ODAM, QPRT, PCP4, COL9A3, LY6G6D, BEX2, SLC26A3, GAL, CKMT2	MB, HOXC6, HOXB6, ARX, AIM2, SLC28A3, B3GNT6, L1TD1, DMRTA2, GABRP, GBA3, ART3, CLDN2, FOXA1, CYP4X1, KRT6B, CA9, HEPACAM2, FOXD1, HOXB8, MS4A8B, C4BPA, DMBT1, MT1H, MUC1, IDO1, KLK1, PIGR
GSE31595 right and left	FGD1, PRAC1, C11orf70, HS3ST3A1, PKIA, PI3, INSL5, HOXB13, PHACTR3, ASB9, SYNE4, RLN2, FLJ41455, TWIST1, CKMT2, SLC35D3, CCDC113, COL11A1, PPBP, EPYC, CPE	IL10, USP30-AS1, SLC51A, HAR1A, CRTAM, NKG7, NPY6R, NR1H4, TREH, BLNK, MEP1B, GDPD3, PITX2, CYP2C18, AIM2, PP7080, CD69, GBA3, GRAMD1C, CCL8, HSD3B2, CCL5, HMGCS2, RARRES3, SLC20A1
GSE26906 right and left	PRAC1, CLDN5, SERPINE2, PCDH19, CHGA, PRELP, SERPINA6, PLA1A, SCNN1B, STMN2, MAP7D2, CHRDL1, MUC12, PCP4, HSD17B6, MN1, ARMCX1, CHRDL2, CLDN10, CCL11, AOC3, CLC, BEX2	PLA2G3, RIBC2, CAPN6, PIWIL1, SLC28A3, GMPR, TMEM171, REG3A, L1TD1, FOXD1, CLDN2

Upregulated genes, represented genes that were upregulated in right-sided colon cancer tissues than in left-sided colon cancer tissues, while downregulated genes represented those that were downregulated in right-sided colon cancer tissues than in left-sided colon cancer. DEGs, differentially expressed genes.

of 17 fresh frozen left-sided colonic cancer samples and 13 right-sided ones obtained from our department (Table III). It was shown that the levels of COL11A1 and TWIST1 expression in cell samples of right-sided colonic cancer samples were significantly higher than that in the left side samples (Tables III and IV, Fig. 4A and B). The protein expression levels of COL11A1 and TWIST1 in the two types of cancer tissues were detected via western blotting analysis. In accordance with the RT-qPCR results, significantly increased levels of COL11A1 and TWIST1 expression were observed in right-sided colon cancer tissues than in left-sided tumor samples (Tables III and IV, Fig. 5A-C). We also analyzed the correlation between the expression of COL11A1 and TWIST1 proteins in colonic cancer samples. The expression of COL11A1 and TWIST1 proteins exhibited a positive correlation (Figs. 4C and 5D), indicating that the two proteins could serve key roles during the formation and progression of these two types colonic cancers.

Functional enrichment analysis. The GO analysis suggested that COL11A1 was involved in 'proteinaceous extracellular matrix', 'collagen trimer' and 'chondrocyte development', and TWIST1 was involved in 'osteoblast differentiation', transcription factor activity of RNA polymerase II, 'positive regulation of transcription from RNA polymerase II promoter', 'sequence-specific DNA binding' and 'nucleus' (Table V).

Discussion

In 1990, Bufill was the first to report that distinct biological pathways may be associated with left and right colonic carcinoma (45). Since then, many studies have revealed significant

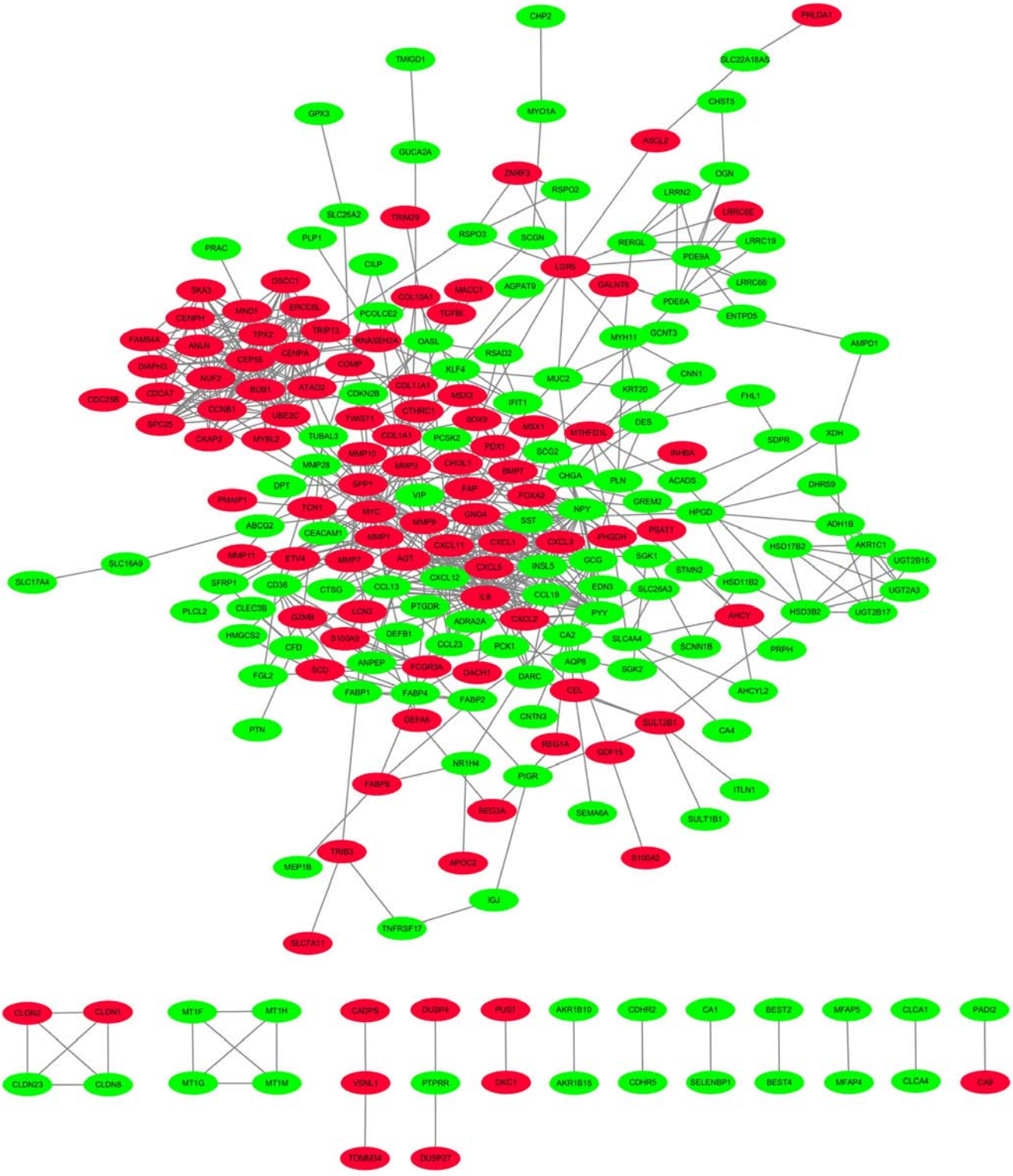

Figure 2. PPI network. A PPI network was constructed for the differentially-expressed genes of tumor and normal with pre-processing of the GSE44076 dataset. PPI, protein-protein interaction. Genes that were upregulated are shown in red and genes that were downregulated are shown in green.

differences in epidemiological, clinical and histological parameters between the two regions of colonic cancers (2-4,46). Irrespective of age and gender, synchronous/metachronous, the level of BRAF, microsatellite instability (MSI) and consensus molecular subtype (CMS), the location of a primary tumor is considered as an important individual prognostic factor for colonic cancer (47). Patients with left-sided colonic cancer usually have better prognoses than those with right-sided colonic cancer (4). Furthermore, there are varying responses in palliative chemotherapy, as well as cetuximab and bevacizumab owing to tumor location (47-50). Primary left-sided colon cancer patients may benefit from the chemotherapy with additional cetuximab in both first and second-line treatments for metastatic colorectal cancer (49). It has been reported by Venook *et al* (47) that in treating patients with right-side colonic cancer, bevacizumab was more effective than

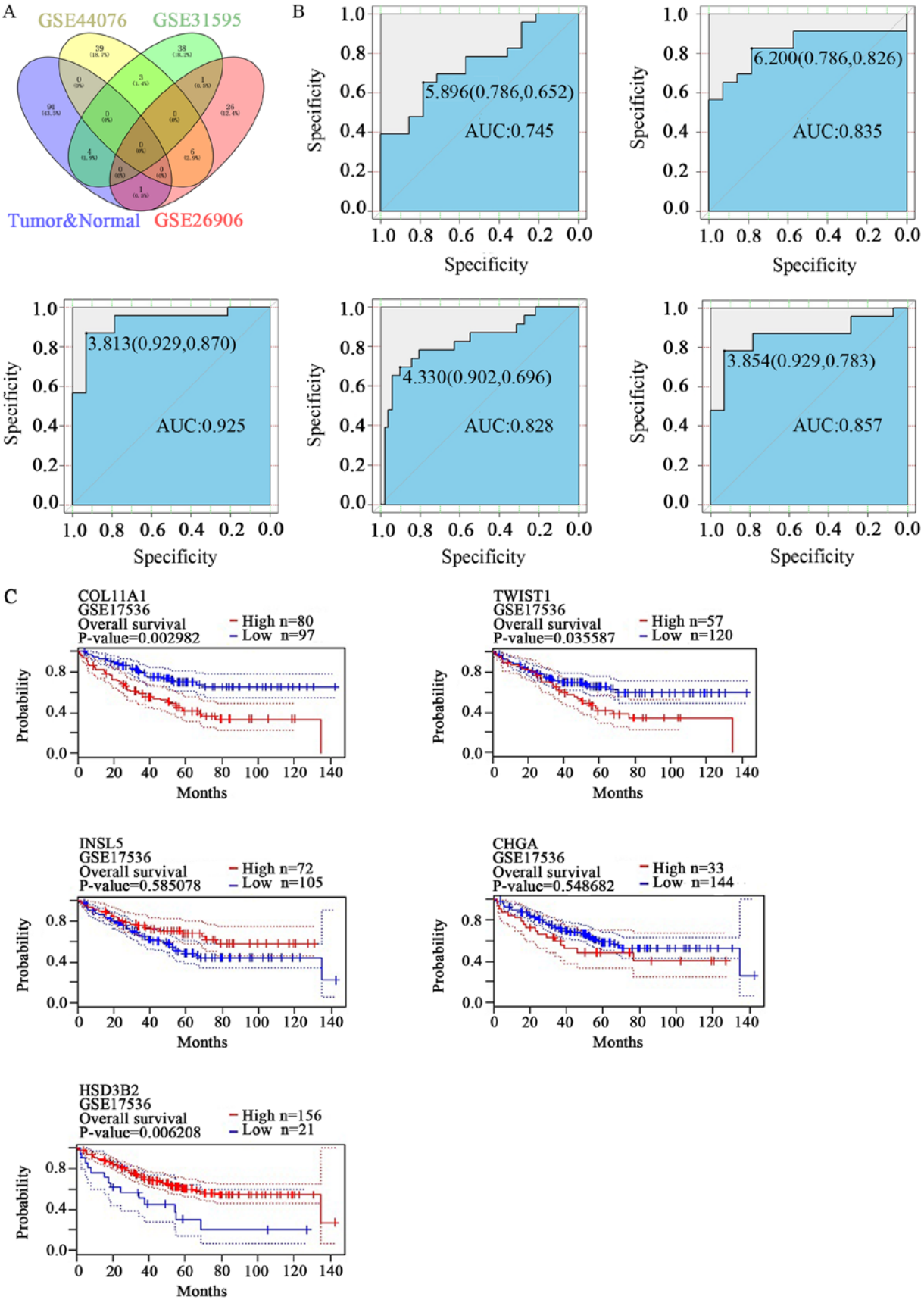

Figure 3. Analysis of the association between DEGs and survival. (A) Identification of aberrantly differentially expressed genes in gene expression datasets (GSE44076, GSE31595 and GSE26906) obtained between left-sided colon cancer and right-sided colon cancer, and gene expression datasets (GSE44076) obtained between colon cancer and normal tissues. (B) Receiver operating curves for intersection of DEGs between left and right-sided colonic cancers obtained from the aforementioned datasets and DEGs of tumor and normal samples. (C) Five-year overall survival. Kaplan-Meier curves stratified by the indicated mRNA levels and analyzed by a log-rank test. PrognoScan database was used to analyze the potential correlation between DEGs and survival in colon cancer. AUC, area under the curve; CHGA, chromogranin A; COL11A1, collagen type XI α1 chain; DEGs, differentially-expressed genes; HSD3B2, 3β-hydroxysteroid dehydrogenase; INSL5, insulin-like 5; TWIST1, Twist family bHLH transcription factor 1.

Table III. Clinicopathological characteristics and COL11A1 mRNA expression in colon cancer samples.

Clinicopathological parameters	No. of cases	COL11A1 expression ($2^{-\Delta Cq}$, mean)	P-value
Tumor location			
Left	17	1.1993	<0.0001
Right	13	2.7947	
TNM stage			
Tis + I	2	1.1530	0.7026
II	18	1.9950	
III	8	1.9223	
IV	2	1.5617	
Histology grade			
Well + moderate	22	1.8974	0.4455
Poor	6	2.1369	
Mucinous adenocarcinoma	2	1.0769	

COL11A1, collagen type XI α1 chain; TNM, tumor-node-metastasis.

Table IV. Clinicopathological characteristics and TWIST1 mRNA expression in colon cancer samples.

Clinicopathological parameters	No. of cases	TWIST1 expression ($2^{-\Delta Cq}$, mean)	P-value
Tumor location			<0.0001
Left	17	1.1546	
Right	13	2.3867	
TNM stage			0.7767
Tis + I	2	1.2984	
II	18	1.7548	
III	8	1.7536	
IV	2	1.2214	
Histology grade			0.3601
Well + moderate	22	1.6988	
Poor	6	1.9118	
Mucinous adenocarcinoma	2	0.9055	

TNM, tumor-node-metastasis; TWIST1, Twist family bHLH transcription factor 1.

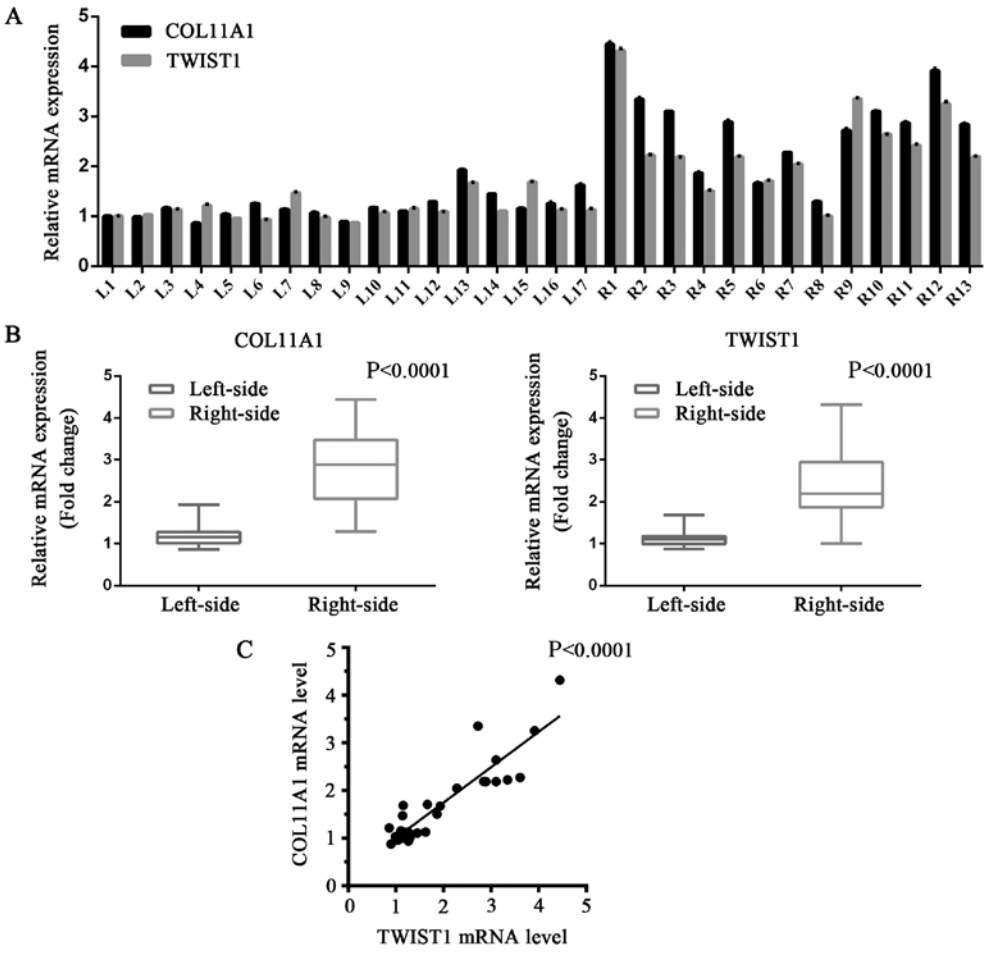

Figure 4. mRNA expression of COL11A1 and TWIST1 in colon cancer clinical samples. (A) Reverse transcription-quantitative polymerase chain reaction assay. The mRNA expression levels of COL11A1 and TWIST1 in 30 clinical samples were examined by qPCR assay. (B) The statistical data from COL11A1 and TWIST1 mRNA levels in various colon cancer clinical samples. COL11A1 and TWIST1 were upregulated in right-sided tumors than left-sided tumors. (C) Positive correlation between the expression of COL11A1 and the expression of TWIST1 mRNA in two different colon cancer clinical samples. Results are representative of three independent experiments, and the error bars represent the standard deviation. COL11A1, collagen type XI α1 chain; TWIST1, Twist family bHLH transcription factor 1.

Table V. Functional enrichment analysis for differentially-expressed genes between tumor and normal contained TWIST1 or COL11A1 based on GO.

Category	Term	Description	P-value	Genes
GOTERM_CC_DIRECT	GO:0005578	Proteinaceous extracellular matrix	0.0022724	MMP9, COMP, COL11A1, MMP1, COL10A1
GOTERM_BP_DIRECT	GO:0001649	Osteoblast differentiation	0.0028232	MSX2, COL1A1, TWIST1, SPP1
GOTERM_BP_DIRECT	GO:0045944	Positive regulation of transcription from RNA polymerase II promoter	0.0096635	CKAP2, FOXA2, ATAD2, BMP7, MYC, ETV4, TWIST1
GOTERM_MF_DIRECT	GO:0000981	RNA polymerase II transcription factor activity, sequence-specific DNA binding	0.0108839	FOXA2, MYBL2, ETV4, TWIST1
GOTERM_CC_DIRECT	GO:0005581	Collagen trimer	0.0135131	COL1A1, COL11A1, COL10A1
GOTERM_BP_DIRECT	GO:0002063	Chondrocyte development	0.04367	MSX2, COL11A1
GOTERM_CC_DIRECT	GO:0005634	Nucleus	0.0498886	FOXA2, NUF2, TPX2, MND1, ATAD2, UBE2C, MYBL2, CENPH, MSX2, SPC25, CDCA7, CENPA, MYC, ETV4, TWIST1

BP, biological process; CC, cellular component; COL11A1, collagen type XI α1 chain; GO, Gene Ontology; MF, molecular function; TWIST1, Twist family bHLH transcription factor 1.

cetuximab. Increasing evidence manifested that molecular profiles of cancers may also differ across these sites (51-54). The incidence rate of CpG island methylator phenotype-high, BRAF mutation, and MSI-high in colorectal cancer increased gradually along the bowel from the rectum to the ascending colon (54,55). Loree *et al* (56) found that, based on comparing the CMS level of an isolated cohort of >600 patients, the sigmoid-rectal region appeared to differ from the other sites, while the transverse colon differs from other right-sided locations. Several studies revealed that distal colon cancer has no significant differences compared with rectal cancer with reference to disease-free survival, somatic alterations and overall survival (57,58). Thus, it is crucial to investigate the molecular variances between two types colonic cancers.

We first reported the differential expression of COL11A1 and TWIST1 between left and right-sided colonic cancers. We observed that COL11A1 and TWIST1 mRNA were upregulated in right-sided colon cancer tissues than in left-sided tissues based on RT-qPCR analysis, which was in accordance with our results of bioinformatical analyses.

COL11A1 and TWIST1 mRNA expression was positively related to tumor location. The results support the notion that upregulated COL11A1 and TWIST1 in right-sided colonic cancer may have notable impact on the invasiveness and worse prognosis of the right-sided colonic cancer.

Furthermore, it has been known that cancer cells have interactions with their surrounding stroma, which was associated with the mechanism of tumor cell invasion (59). There are two chains of type XI collagen involved and one of them is a smaller fibrous collagen and encoded exactly by COL11A1 gene (60). Additionally, the extracellular matrix (ECM) has been considered to be important in tumor behavior, and forms the interstitial matrix conducive to regulating and integrating cell behavior (61-63). Thus, collagens, as major components of ECM, are related to regulation of several important biological processes, including cell proliferation, differentiation and migration (64-66). In addition, the development of the COL11A1 mutations was linked with Marshall syndrome and type II Stickler syndrome (67,68). Based on our results, COL11A1 is mainly enriched in proteinaceous extracellular matrix, collagen trimer and chondrocyte development; COL11A1 was also the predominant node in the PPI network in our study. Proteinaceous extracellular matrix-related genes that could increase the aggression and change the metastatic properties of cells *in vivo* colon cancer contribute to poor prognosis in cancer patients (69). The strong overexpression of COL11A1 has been discovered in various studies for examining diversities between tumor and normal tissues, and linked to the metastasis of tumor and poor prognosis (14). A previous study reported that the expression of COL11A1 of colon tumors is related to anaphase-promoting complex/β-catenin path of colon cancer metastasis (11). We will continue to investigate the mechanism of COL11A1 in the two sides of colon cancer in future work.

According to the results of the GO and PPI analyses, TWIST1 which was enriched in 'osteoblast differentiation', transcription factor activity of RNA polymerase II, 'positive regulation of transcription from RNA polymerase II promoter', 'sequence-specific DNA binding' and 'nucleus', was also the predominant node in the PPI network. Increasing evidence has indicated that cancer cells characterized as cancer stem cells (CSC) or EMT have a strong potential to promote the progression, aggression, metastasis

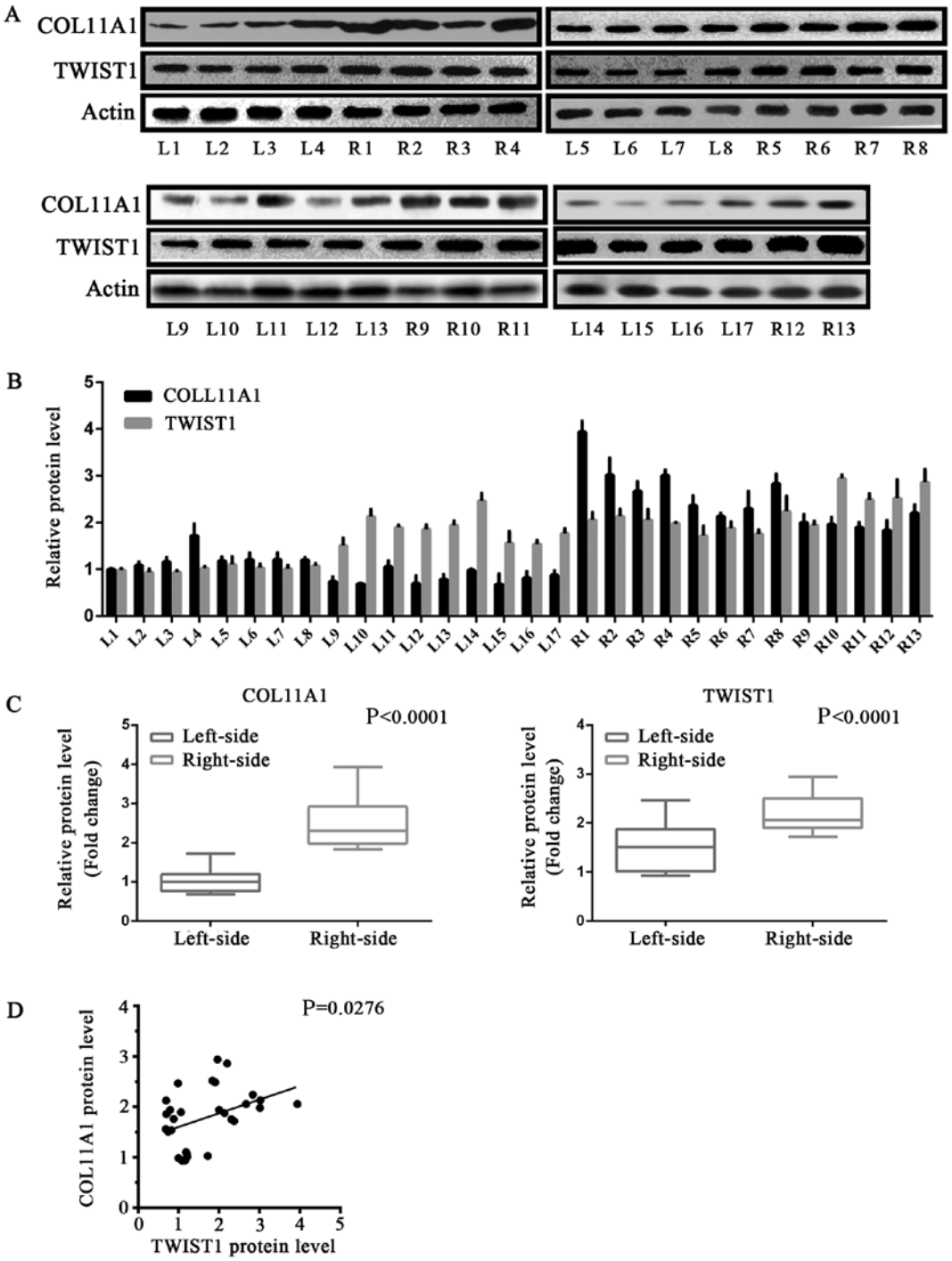

Figure 5. Protein expression of COL11A1 and TWIST1 in colon cancer clinical samples. (A) Western blotting for the expression of COL11A1 and TWIST1 in colon cancer tissues. (B) Densitometry analysis of COL11A1 and TWIST1 protein expression in 30 colon cancer tissue samples. (C) Statistical analysis of COL11A1 and TWIST1 protein levels in two different colon cancer clinical samples. COL11A1 and TWIST1 were determined to be upregulated in right-sided tumors than in left-sided tumors. (D) Positive correlation between the expression of COL11A1 and the expression of TWIST1 protein in two colon cancer clinical samples. Results are representative of three independent experiments, and the error bars represent the standard deviation. COL11A1, collagen type XI α1 chain; TWIST1, Twist family bHLH transcription factor 1.

and chemoresistance of cancer (70-73). It has been shown that the EMT program is associated with the self-renewal ability of cells and may efficiently induce tumors (74,75). It is the essential helix-loop-helix transcription factors (such as TWIST1) that crucial for EMT and CSC; one of these factors (71) suppresses the expression of E-cadherin and promotes EMT during the progression of tumors (76). Specifically, in the promoter of E-cadherin, TWIST1 combines with E-box elements, suppresses the transcription of the cell-cell adhesion molecule expression and promotes EMT, contributing to metastasis during the progression of tumors (77). It has been known that TWIST1 has wide expression in many human cancers, including colorectal cancer (31), and its strong expression has been linked to poor prognosis and chemotherapeutic resistance (27,30-32,78). In colorectal cancer patients, TWIST1 expression has been

associated with chemosensitivity to oxaliplatin and 5-fluorouracil (78). Downregulation of TWIST1 expression induced apoptosis and enhanced the sensitivity of chemotherapy (78). Generally, TWIST1 can promote the chemotherapeutic resistance of cancer cells (23), increase the number of CSCs (74), as well as enhance aggression and metastasis of cancer cells (77,79-85) by suppressing their senescence and apoptosis induced by oncogenes. A study suggested that TWIST1 may be used as a vital clinical biomarker for antiangiogenic therapy as its polymorphisms were related to the survival of patients with metastatic colorectal cancer who were treated with first-line bevacizumab-based chemotherapy (28). The present study proposed the importance of TWIST1 upregulation in right-sided colonic cancer tissues. Patients with right-sided colonic cancer exhibited better outcomes of bevacizumab treatment than those with left-sided colon cancer (86).

Through our results, we also speculated that TWIST1 may associated with COL11A1 to enhance the development of colon cancer. A study showed that the level of TWIST1 mRNA had a positive correlation with that of COL11A1 messenger RNA in ovarian cancers. In addition, COL11A1 could promote the activation of nuclear factor-κB by activating the transcription of IKKβ, and promote the expression of TWIST1, thereby regulating chemoresistance and apoptosis (44). In our experiments, we also demonstrated that TWIST1 could promote the progression of EMT, with the downregulation of E-Cadherin and the upregulation of N-Cadherin, while COL11A may promote the transcription of IKKβ in colon cancer cell (Fig. S1). This suggested the complexity of the regulatory mechanism mediated by these two proteins in left and right-sided colonic cancer. the limitations of our study include the small cohort of patients enrolled for analysis.

In brief, our work suggested that the expression levels of COL11A1 and TWIST1 differ between these two types colonic cancers and may be promising therapeutic targets for treating right-sided colonic cancer. However, further study is also necessary to illustrate the potential molecular mechanisms, and functions of COL11A1 and TWIST1 between these two types of colonic cancer.

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Availability of data and materials

The data of four original microarray datasets GSE44076, GSE31596 and GSE26906 were downloaded from NCBI-Gene Expression Omnibus database (Available online: https://www.ncbi.nlm.nih.gov/geo).

Authors' contributions

JH conceived and designed the experiments. JZ, XH, SY and YJ performed the experiments. CS analyzed the data. CS and JH wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Xiamen University. All samples were collected and gathered at the Zhongshan Hospital of Xiamen University between 2014 and 2015. The research content of this subject strictly follows the Helsinki Declaration, the International Ethics Standards for Human Biomedical Research, and relevant provisions of the National Natural Science Foundation of China, and the World Health Organization and the International Council of Medical Science Organizations.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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