ORIGINAL RESEARCH

The role of PEG3 in the occurrence and prognosis of colon cancer

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Purpose: Imprinted genes are often identified as key players in the etiology and prognosis of many tumors; however, the role they play in colon cancer remains unclear. Along with the development of big data analysis came the discovery of a wealth of genetic prognostic factors, like microsatellite instability for colon cancer, which need to be taken into consideration when evaluating new biomarkers for the disease.
Methods: We systematically mined public databases to find recurrence free survival (RFS)-related imprinted genes for colon cancer patients on the mRNA level by univariate and multivariate survival analyses. We then investigated the association of methylation status and microRNA expression of the targeted imprinted genes with survival rate of colon cancer patients. Lastly, in a clinical study we used qRT-PCR and immunohistochemistry to quantify mRNA and protein expression of the imprinted genes that related to RFS in our bioinformatics screening, respectively, in 20 tumor tissues compared to paired adjacent tissues.
Results: The results show that paternally expressed gene 3 (PEG3) is the only imprinted gene related to colon cancer patient prognosis on the mRNA level in our datasets, and high mRNA expression of PEG3 is associated with a poor prognosis. Furthermore, the methylation beta value of cg13960339, as well as the expression of 4 microRNAs, negatively correlated with PEG3 mRNA level and were correlated with the prognosis of colon cancer patients. Moreover, the expression of PEG3 mRNA in colon cancer is significantly lower, but PEG3 protein expression is significantly higher compared to that in normal tissues.
Conclusion: PEG3 is likely associated with the progression and prognosis of colon cancer.
Keywords: colon cancer, prognosis, imprinted gene, PEG3

Introduction

Colon cancer is a public health problem and is a major cause of morbidity and mortality worldwide. Colon cancer is the third most common cancer diagnosis among men and women in the United States.[1,2] Although surgical resection is a relatively effective method for the treatment of colon cancer, metastasis and recurrence often occur after surgery, which greatly shortens the survival time of patients. Along with the development of big data analysis came the discovery of a wealth of genetic prognostic factors, like microsatellite instability for colon cancer, which need to be taken into consideration when evaluating new biomarkers for this disease.

Imprinted genes usually express only one of the homologues from the parents, and are regarded to be important in mammalian growth, development, and cell differentiation.[3,4] Monoallelic expression ensures that the levels of the proteins expressed by these genes are tightly regulated. Loss of imprinting is an epigenetic

modification involving loss of parental allele-specific expression due to changes in the methylation status of imprinted genes, where the repressed allele becomes active and the tight regulation of protein expression from this gene is lost. Loss of imprinting is seen frequently and precociously in a large variety of human tumors.[5] For example, loss of imprinting of insulin-like growth factor 2, leads to overexpression of the protein and is associated with a poor prognosis and resistance to chemotherapy in many cancers.[6,7] Additionally the paternally imprinted gene, neuronatin, is a biomarker for breast cancer because high expression is associated with poor prognosis.[8]

CpG-rich regions, termed CpG islands, in the promoters of imprinted genes have allele-specific methylation patterns that govern monoallelic expression.[9] Epigenetic instability of imprinted domains on these CpG islands is often regarded as a biomarker for human cancers.[10,11] Additionally, the mRNA expression of most imprinted genes is downregulated in primary tumors compared to normal tissues and is typically accompanied by DNA methylation level changes in the imprinted domains; examples of genes that undergo these changes include PEG3, mesoderm-specific transcript, and stimulatory GTPase α.[12] MicroRNA is another master epigenetic regulator that alters more than 30% of human gene expression. These molecules are a class of evolutionarily conserved, small non-coding RNAs, which exist widely in eukaryotes and do not encode proteins but function in RNA silencing and post-transcriptional regulation of gene expression. By base-pairing with complementary sequences within mRNA molecules, miRNAs silence translation by either cleavage of the mRNA strand, or through binding to the 3′ untranslated region.[13] MicroRNAs affect many physiological and pathological processes, such as tumor development.[14] It is reported that microRNA expression is associated with survival prognosis of colon cancer patients.[15] However, few studies have systematically investigated the independent role that microRNAs play in colon cancer survival prognosis together with multiple covariates.

In the current study, we first systematically mined public databases to find prognosis-related imprinted genes for colon cancer patients based on mRNA level by univariate and multivariate survival analysis. We then investigated whether an association exists between methylation status, predicted regulator microRNA level of the target imprinted gene, and prognosis of colon cancer patients. Our study describes the independent roles imprinted genes may play in colon cancer.

Materials and methods
Patients and samples
In this study, colon cancer datasets from TCGA (The Cancer Genome Atlas, https://cancergenome.nih.gov/) were downloaded and processed by GDCRNATools.[16] An independent colon cancer dataset GSE39582 was downloaded from GEO (Gene Expression Omnibus). Clinical data including age, sex, histology, tumor stage, grade, tumor location, etc. are available in both datasets. IC_{50} value of 5-fluorouracil (5-fu) in different colon cancer cell lines was downloaded from the Genomics of Drug Sensitivity in Cancer (GDSC) database (www.cancerRxgene.org). The work flow of mining data is shown in Figure 1.

Twenty pairs of primary colon cancer and adjacent tissue were obtained from Xiangya Hospital, Central South University. The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the Institute of Clinical Pharmacology of Central South University (registration no. CTXY-150001-2). In addition, prior to entering into the study, written informed consent was obtained from all the participants. Clinical data collected included gender, age, familial history, Histological type, lymphatic metastasis, stage, tumor location and differentiation. Staging was based on the TNM (tumor, node, and metastasis) classification system of the International Union against Cancer 2009 (UICC).[17]

Survival and statistical analysis
Recurrence free survival (RFS) is the period from resection to the first radiological evidence of tumor recurrence.[18] The Kaplan-Meier method and multivariate Cox's proportional were used to assess the prognosis of colon cancer patients for univariate and multivariate analyses, respectively, and the log-rank test was used to compare the curves among high-risk and low-risk groups. The above two analyses were done through the survival package in R language, covariates include age, stage, sex, tumor location, and MMR (mismatch repair) status. Hazard ratio (HR) and 95% confidence interval (CI) were used to describe the relative risk. Paired Student's t-tests were used to compare the expression of PEG3 in primary tumor to that in normal tissues by GraphPad Prism 6.0 (Graphpad Inc., San Diego, CA, USA). Associations between PEG3 expression and immunostaining results were tested by chi-square or Fisher's exact test by SPSS 23.0 (SPSS Inc., Chicago, IL, USA). Spearman's correlation analysis was performed to calculate the coefficient of correlation
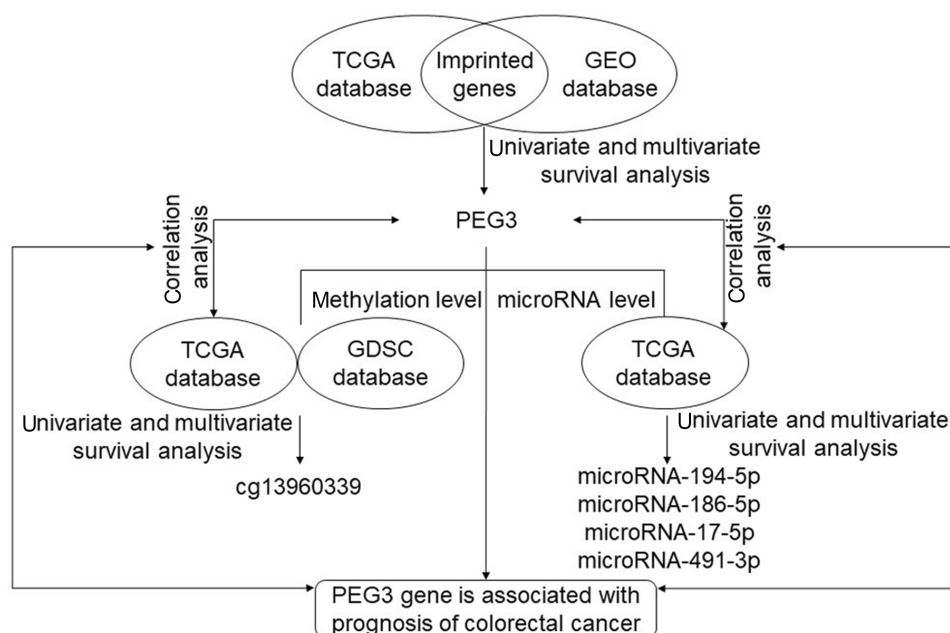
Figure 1 The flow chart of how we screened imprinted genes and related expression to prognosis.
Abbreviations: GDSC, Genomics of Drug Sensitivity in Cancer; GEO, Gene Expression; PEG, Paternally expressed gene; TCGA, The Cancer Genome Atlas.

between the levels of mRNA expression and methylation as well as microRNA, respectively, using R. All tests were two-tailed and $P<0.05$ was deemed as statistically significant.

Quantitative real-time reverse transcription PCR

For the quantitative real-time RT-PCR assay, 20 paired primary colon cancer and non-tumorous tissues were obtained and ground into powder in liquid nitrogen. Total RNA was extracted from the powder and cDNA synthesized using PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (Takara, Shiga, Japan) according to the manufacturer's protocol. Expression of mRNA was quantified by qRT-PCR with a LightCycler 480 (Roche Diagnostics, Basel, Switzerland) using SYBR® Premix DimerEraser™ (Takara). The primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China) and the sequences of the primers were as follows: PEG3 forward: 5′-CCTACCCAAGCACCAGTC G-3′, and PEG3 reverse: 5′-GGAACTGCGTGACACATC CT-3′; PPIA forward: 5′-GTGGTATAAAAGGGGCGGGA G-3′, and PPIA reverse: 5′-GTGGGGTTGACCATGGCTAA TAGTA-3′; B2M forward: 5′-ATGCCTGCCGTGTGAACCA TGTGA-3′, and B2M reverse: 5′-TCCAAATGCGGCATCTT CAAACCTC-3′. Amplification conditions were denaturation for 30 sec at 95°C and then 40 cycles of 92°C for 5 sec, 55°C for 30 sec, and 72°C for 30 sec. The mRNA expression of

PEG3 was normalized to the average of the expressions of B2M and PPIA.[19]

Immunohistochemistry

Immunohistochemistry was performed as previously described.[20] Paraffin embedded tissue sections were cut to 4 μm thick and blocked with endogenous peroxidase. The autoclave technique was used to achieve heat-induced antigen retrieval. Sections were then incubated overnight at 4°C with rabbit polyclonal antibody anti-PEG3 (PA5-51298; Thermo Fisher Scientific, Inc.) at a 1:40 dilution. Subsequently, the sections were washed three times with phosphate buffer saline, and then incubated with a biotin-labeled goat anti-rabbit antibody. Sections were then stained with peroxidase-conjugated streptavidin (Boster, Wuhan, China) with 3, 3′-diaminobenzidine tetrahydrochloride as the chromogen. Finally, the sections were dyed with hematoxylin. Each slide was evaluated using light microscopy and the staining was scored semiquantitatively by assessing the intensity (on a 0–3 scale: 0, no staining of tumor cells; 1, weak; 2, moderate; 3, strong staining) and by estimating the percentage of positive cytoplasmic or membranous staining cells (on a 1–4 scale: 1, 1–25% staining; 2, 26–50% staining; 3, 51–75% staining; or 4, >75% staining). Intensity score ≥2 and ≥25% cells were defined as being positive for levels of protein expression in this study.[21]

Results

PEG3 is the only imprinted gene related to colon cancer prognosis

Clinical information and mRNA expression data were downloaded from the TCGA database (280 colon cancer patients) and GEO database (GSE39582, 566 colon cancer patients). The characteristics of patients including sex, age, tumor location, stage, distant metastasis and adjuvant chemotherapy are presented in Table 1. We included 100 imprinted genes found in the human genome in our analyses.[12] Data obtained from TCGA indicate that higher mRNA expression of PEG3, PEG10, SGCE and TFPI2 were significantly associated with shorter RFS in univariate and multivariate analyses.

Significance of these four genes in colon cancer prognosis was further validated in an independent cohort from GEO. The results showed that only PEG3 mRNA expression was correlated with colon cancer prognosis in the two databases (Table 2). Due to these findings, the imprinted gene PEG3 was chosen for further study. In both TCGA and GEO databases, univariate and multivariate survival analysis showed that the expression of PEG3 is a risk factor in colon cancer prognosis (HR>1), and the high-expression group had a shorter RFS compared to the low-expression group (Table 2). Combining the above results from the TCGA and GEO databases, we conclude that, of the imprinted genes we evaluated, PEG3 is the only one that affects the prognosis of patients with colon cancer, and it is a disadvantageous factor for prognosis on mRNA level.

Methylation of cg13960339 is an advantageous prognostic factor and negatively correlates with PEG3 mRNA level

In the TCGA database, 292 cases of colon cancer data containing both clinical prognostic information and genome-wide methylation data based on Illumina Methylation 450K chip were evaluated. Univariate and multivariate analyses were performed to identify methylation sites that affect prognosis. Among all the methylation sites associated with PEG3, cg13960339 was the only one associated with RFS of colon cancer in the TCGA dataset, the results of the univariate and multivariate survival analyses showed that high cg13960339 methylation levels significantly increased the RFS of colon cancer patients (Figure 2A).

We used spearman's correlation analysis to identify the relationship between methylation and mRNA expression. Degree of methylation was estimated using beta values (between 0 and 1, with 0 being unmethylated and 1 fully methylated). In the TCGA-Colon Adenocarcinoma (COAD) dataset, a lower methylation beta value of cg13960339 was negatively correlated with PEG3 mRNA level (Figure 2B) and was significantly associated with poor prognosis (Figure 2A). The results of this multivariate analysis are consistent with the above results (P=0.027, HR=0.481, 95% CI=0.251–0.919). These results suggest that methylation of cg13960339 may be a biomarker of patient prognosis and regulate the expression of PEG3.

In order to confirm whether the cg13960339 methylation level is indeed an indicator of prognosis, IC_{50} values in colon cancer cells to 5-fu therapeutics in the GDSC database were evaluated. IC_{50} values were divided into two groups by median: high or low. The beta value in the low IC_{50} group was 0.7387, and in the high IC_{50} group was 0.5989 (P=0.0068), which suggests that higher cg13960339 methylation could increase drug sensitivity and inhibit the survival of cancer cells. The correlation analysis between the beta value of cg13960339 and IC_{50} data showed that they are significantly correlated (Spearman r=−0.298, P=0.0468). The negative correlation indicates that methylation of PEG3 reduces the IC_{50} of 5-fu, which indicates that methylation at this methylation site may be beneficial to the therapeutic effect of 5-fu on patients with colon cancer, and suggests it is beneficial to the prognosis of patients.

Expression of 4 microRNAs associate with the prognosis of colon cancer patients and negatively correlate with PEG3 mRNA level

Prognostic data and microRNA expression data downloaded from the TCGA website were matched by patient ID. Clinical prognostic information from 441 cases of colon cancer and microRNA expression data were included. The results of the log-rank and Cox regression analyses are shown in Table 3. Patient prognosis was affected by the expression of 8 microRNAs.

Spearman's correlation analysis was conducted to compare microRNA expression data with PEG3 mRNA expression data from TCGA. In 280 COAD patients, only 5 of the 8 microRNAs were significantly associated with PEG3 mRNA level (P<0.05). However, for one of

Table 1 Patient demographics and clinical characteristics

Variables	mRNA		DNA methylation	microRNA
	TCGA	GEO	TCGA	TCGA
Platform	Agilent 224k G4502A_07 RNASeq, level3	Affymetrix U133 Plus 2.0 GSE39582		
Number of patients	280	566	292	441
Sex				
Male	153	310	154	227
Female	124	256	130	206
N/A	3	0	8	8
Age (years)				
<60	90	150	93	118
≥60	187	415	191	315
N/A	3	1	8	8
Tumor location				
Left	117	342	99	166
Right	156	224	181	262
N/A	7		12	13
Clinical stage				
Stage I+II	151	297	156	239
Stage III+IV	117	265	120	185
Stage 0	0	4	16	0
N/A	12	0		17
Tumor size (cm)				
T1+T2	51	56	51	86
T3+T4	225	486	232	345
T0	0	1	0	0
NA	3	20	8	9
TIS	1	3	1	1
Reginald lymph node involvement				
N0+N1	231	436	236	353
N2+N3	45	104	47	79
N+	1	6	1	1
NA	3	20	8	8
Distant metastasis				
M0+M1	185	543	228	375
MX	88	3	51	51
NA	7	20	13	15
Adjuvant chemotherapy				
Yes	84	233	89	
No	15	316	15	
NA	181	17	188	

these microRNAs (miR-181b-5p) the correlation coefficient is very small and the correlation is weak. The other 4 microRNAs showed higher and negative correlation with PEG3 expression (Table 4). Additionally, higher expression of these 4 microRNAs in colon cancer patients was associated with longer RFS (Table 3).

Table 2 Four imprinted genes associated with RFS in TCGA and validated in GEO by univariate and multivariate survival analysis

Gene Name	TCGA database				GEO database			
	Univariate		Multivariate		Univariate		Multivariate	
	HR (95% CI)	P-value	HR (95% CI)	P-value	HR (95% CI)	P-value	HR (95% CI)	P-value
PEG3	2.608(1.455–4.708)	0.001	2.278(1.247–4.162)	0.007	1.376(1.022–1.854)	0.035	1.385(1.017–1.886)	0.039
PEG10	2.199(1.288–3.938)	0.008	2.073(1.147–3.748)	0.016	1.342(0.966–1.808)	0.053	1.369(1.006–1.863)	0.046
SGCE	2.779(1.534–5.034)	0.001	2.520(1.371–4.630)	0.003	1.139(0.848–1.529)	0.389	1.07(0.795–1.462)	0.628
TFPI2	1.910(1.061–3.438)	0.031	1.938(1.059–3.544)	0.032	0.946(0.705–1.271)	0.714	0.926(0.680–1.261)	0.626

Abbreviations: SGCE, epsilon-sarcoglycan gene; TFPI2, Tissue Factor Pathway Inhibitor 2.

Expression of PEG3 mRNA is significantly lower but protein is significantly higher in colon cancer compared to adjacent tissue

To investigate the potential role of PEG3 in the etiology of colon cancer, the mRNA expression level of PEG3 in tumors and adjacent tissues of patients was explored in the TCGA dataset using R language, and validated in samples from Xiangya Hospital. In 33 types of different cancers from the TCGA database, the expression level of PEG3 was compared between the primary tumor and adjacent tissue. As shown in Figure 3A, in colon cancer patients, lower PEG3 mRNA expression was observed in tumors compared to adjacent tissue ($P<0.05$), which suggests that PEG3 may act as a tumor suppressor rather than act as an oncogene.

Subsequently, mRNA expression of PEG3 in 20 paired tissues were tested by qRT-PCR that confirmed our findings from the TCGA database. The patient information is shown in Table 5. PEG3 mRNA expression in colon cancer tissue was significantly lower compared to the adjacent tissue ($P<0.01$). The mean ± SD of colon cancer and para-cancer tissue of PEG3 is 0.025±0.0230 and 0.045 ±0.022, respectively (Figure 3B).

We performed immunohistochemical staining on the 20 pairs of colon cancer tissues and adjacent tissues, which were also used in our mRNA expression studies. Our immunohistochemical results showed that PEG3 protein expression is higher in cancer tissues than in adjacent tissues ($P=0.016$). The PEG3 protein is mainly distributed in the cytoplasm in colon cancer tissues, whereas very little protein was detected in the para-cancer tissue (Figure 4).

Discussion

This is the first study to identify PEG3 as the only imprinted gene that affects the prognosis of colon cancer. Additionally, we discovered that lower methylation levels of cg13960339 within the PEG3 promoter is associated with the prognosis of colon cancer. And lastly, several microRNAs that regulate PEG3 expression, are also associated with the outcome of colon cancer.

TNM staging is a traditionally used prognostic classification system in clinical practice to select patients with colon cancer for adjuvant chemotherapy. However, this system often fails to accurately predict recurrence in many colon cancer patients who undergo surgical therapy. Approximately 10–20% stage II and 30–40% stage III
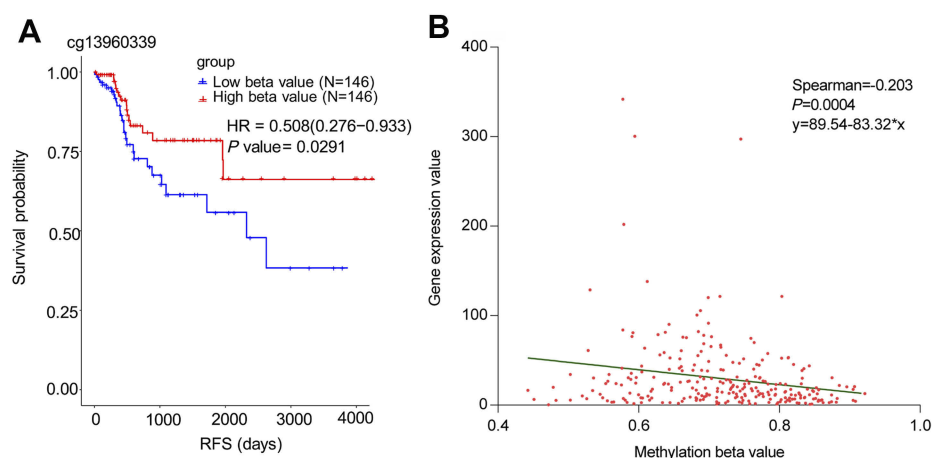
Figure 2 The relationship of cg13960339 methylation and RFS as well as the association with PEG3 mRNA level in colon cancer. (**A**) The Kaplan-Meier survival analysis of cg13960339 methylation in TCGA. (**B**) Correlation between PEG3 mRNA expression and cg13960339 beta value in TCGA.
Abbreviation: RFS, Recurrence free survival.

Table 3 Univariate and multivariate analysis of microRNA expression and RFS in TCGA

microRNA	Univariate		Multivariate	
	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
hsa-miR-194-5p	0.422 (0.247–0.720)	0.002	0.458 (0.267–0.786)	0.005
hsa-miR-186-5p	0.496 (0.296–0.832)	0.008	0.559 (0.329–0.948)	0.031
hsa-miR-17-5p	0.550 (0.328–0.922)	0.023	0.525 (0.311–0.887)	0.016
hsa-miR-491-3p	0.474 (0.284–0.790)	0.004	0.585 (0.346–0.988)	0.045
hsa-miR-181b-5p	2.232 (1.323–3.766)	0.003	1.872 (1.097–3.192)	0.021
hsa-miR-296-3p	0.525 (0.312–0.884)	0.015	0.521 (0.304–0.892)	0.017
hsa-miR-181a-5p	2.165 (1.277–3.670)	0.004	1.937 (1.133–3.313)	0.016
hsa-miR-494-3p	1.820 (1.057–3.133)	0.031	1.783 (1.023–3.109)	0.041

colorectal cancer patients develop recurrence.[22] With the wide spread use of modern biotechnology in research, several somatic mutations, including MMR and KRAS, were proved to influence the outcome and therapeutic effect of colon cancer patients, and thus applied as a basis of medical treatment decisions in routine clinical practices. Therefore, we chose the dataset GSE39582, which has the most comprehensive clinical information, as the validation data set for TCGA. Additionally, the current study includes MMR status as a covariant in the multivariate survival analysis to mine more reliable prognostic signatures for further study; however, the KRAS status was excluded because very few patients had this information in the discovery and validation datasets.

The work flow applied in this study (Figure 1) is effective to identify prognostic biomarkers for colon cancer. Data of PEG3 mRNA expression and promoter region methylation levels correlated with RFS, and the

correlation of mRNA, microRNAs, and methylation changes, will be helpful to determine the mechanism behind the loss of imprinting of PEG3 in colon cancer. Although we weren't able to validate the relationship of microRNA expressions with RFS in TCGA using another method, the overlapping of microRNA expression with solid PEG3 mRNA signatures, as well as the correlation analysis between these two variables, suggest these microRNAs as very promising candidates for further investigation. In addition, we evaluated PEG3 with two bioinformatics websites and forecast the log-rank analysis of prognosis as well as mRNA expression patterns between tumor and para-tumor tissue, and the results were very similar to ours even though different databases and algorithms were used (UCSC Xena and GEPIA, data not shown). Therefore, we provide the first evidence of a prognostic role for PEG3, its methylation sites, and targeting microRNAs in colon cancer.

Table 4 Correlation between selected microRNAs and the expression of PEG3 mRNA

PEG3: microRNA	t	Coefficient	P-value
PEG3: hsa-miR-194-5p	−8.827	−0.468	6.160E-17
PEG3: hsa-miR-186-5p	−7.062	−0.390	6.600E-12
PEG3: hsa-miR-17-5p	−6.229	−0.350	8.660E-10
PEG3: hsa-miR-491-3p	−3.866	−0.226	6.880E-05
PEG3: hsa-miR-181b-5p	−1.669	−0.099	0.048
PEG3: hsa-miR-296-3p	−1.616	−0.096	0.054
PEG3: hsa-miR-181a-5p	−1.087	−0.065	0.139
PEG3: hsa-miR-494-3p	1.736	0.104	0.958
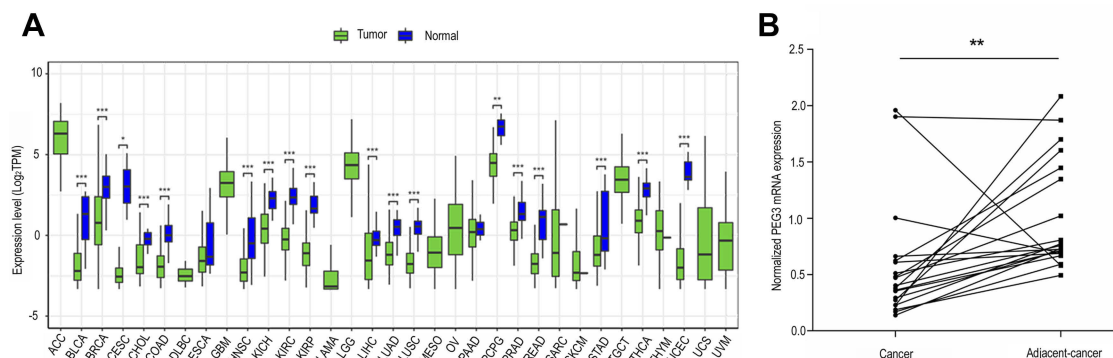
Figure 3 The mRNA expression of PEG3 in cancer and normal tissues. (**A**) PEG3 expression in different cancers from the TCGA database. Green and blue represent PEG3 expression in cancer and normal tissues, respectively. (*P<0.05, **P<0.01, ***P<0.001). (**B**) Expression levels of PEG3 in tumor and normal tissues. The tumor tissues showed lower PEG3 expression than normal tissues (P<0.01).
Abbreviations: ACC, Adrenocortical carcinoma; BLCA, Bladder Urothelial Carcinoma; BRCA, Breast invasive carcinoma; CESC, Cervical squamous cell carcinoma and endocervical adenocarcinoma; CHOL, Cholangiocarcinoma; COAD, Colon adenocarcinoma; DLBC, Lymphoid Neoplasm Diffuse Large B-cell Lymphoma; ESCA, Esophageal carcinoma; GBM, Glioblastoma multiforme; HNSC, Head and Neck squamous cell carcinoma; KICH, Kidney Chromophobe; KIRC, Kidney renal clear cell carcinoma; KIRP, Kidney renal papillary cell carcinoma; LAML, Acute Myeloid Leukemia; LGG, Brain Lower Grade Glioma; LIHC, Liver hepatocellular carcinoma; LUAD, Lung adenocarcinoma; LUSC, Lung squamous cell carcinoma; MESO, Mesothelioma; OV, Ovarian serous cystadenocarcinoma; PAAD, Pancreatic adenocarcinoma; PCPG, Pheochromocytoma and Paraganglioma; PRAD, Prostate adenocarcinoma; READ, Rectum adenocarcinoma; SARC, Sarcoma; SKCM, Skin Cutaneous Melanoma; STAD, Stomach adenocarcinoma; TGCT, Testicular Germ Cell Tumors; THCA, Thyroid carcinoma; THYM, Thymoma; UCEC, Uterine Corpus Endometrial Carcinoma; USC, Uterine Carcinosarcoma; UVM, Uveal Melanoma.

The function of PEG3 is not completely understood and tends to vary between tissues. The role of PEG3 in survival rates of cancer patients is not fully understood; however, in ovarian cancer, down-regulation of PEG3 mRNA expression was included in a multi-gene prognostic signature for poor outcome of the disease,[23] Additionally, upregulation of PEG3 was reported to be associated with a modest but significant reduction in distant metastases-free survival in breast cancer patients.[24] A bioinformatic-screening study by Kaplan-Meier analyzing survival curves of all cancer patients in TCGA showed that, on the mRNA level, highly expressed PEG3 was related to better clinical outcomes in kidney renal clear cell carcinoma, brain lower-grade glioma, lung adenocarcinoma, pancreatic adeno-carcinoma, pheochromocytoma and paraganglioma, uveal melanoma, and thymoma.[25] Although colon cancer was included in this study no

statistically significant association was found between PEG3 expression and prognosis, and the discrepancy of this study and ours might be due to the different long-term efficacy evaluation index chosen (OS and DFS verse RFS) that altered the patients included in each study. Besides direct investigation of survival rates and PEG3 expression, PEG3-antisense RNA has also been reported to be associated with cancer prognosis. Down-regulation of PEG3-antisense RNA was revealed to be related to poor survival in squamous cell carcinoma of the head and neck, but related to a better prognosis for colorectal cancer patients based on bioinformatic screening of long noncoding RNA expression signatures.[26,27] However, the function of PEG3-antisense RNA is poorly understood, and the idea that it might negatively regulate mRNA and protein levels of PEG3 was only a prediction according to neuron cell line-based transfection experiments.[28] Therefore, the

Table 5 Summary of 20 patients' cohort information

Characteristic	NO.	%
Number of patients	20	100
Sex		
Male	6	30
Female	14	70
Age (years)		
<60	8	40
≥60	12	60
Clinical stage		
Stage II	13	65
Stage III	6	30
Stage IV	1	5
Tumor size (cm)		
T2	1	5
T3	2	10
T4	17	85
Reginald lymph node involvement		
N0	13	65
N1	6	30
N2	1	5
Distant metastasis		
M0	18	90
MX	1	5
NO	1	5

present study only evaluated and validated the independent prognostic role that PEG3 plays in colon cancer, and the mechanism of this needs further investigation.

Deregulation of imprinted genes can happen in somatic cells of adulthood, thus increasing cancer risk.[29] Bioinformatics investigations by others, as well as our observations in this study show decreased mRNA expression of PEG3 in colon cancer.[12,30] However, opposite PEG3 protein to mRNA expression patterns were observed in this study: decreased mRNA but increased protein levels of PEG3 in the cancer tissue. The discrepancy of PEG3 mRNA and protein expression was also found in testicular germ cell tumors.[31] Thus, aberrant overexpression of PEG3 might contribute to the carcinogenesis of colon tissue. PEG3 protein was thought to be located mainly in nuclei,[31] however, the immunohistochemical staining in the current study indicates that almost all the PEG3 protein is in the cytosol with little in the nuclei, which might be due to decreased mRNA expression and lack of protein degradation. PEG3 is quite a unique protein with opposite biochemical properties: it is a growth suppressor at the cellular level

but a growth stimulator at the organismal level.[28] Intriguingly, PEG3 might function as a tumor suppressor in the nucleus; however, accumulation of this protein in the cytosol might inhibit the expression of growth suppressors.-[28] Furthermore, it is reported that PEG3 plays an important role in autophagy and conveys the external signal to induce the formation of autophagosomes in the cell, which might be crucial for protein degradation.[28] Detailed characterization of the role PEG3 plays in colon carcinogenesis with transcriptome, proteomics, and localization studies will be included in our follow-up investigations.

Methylation is essential for genes to remain imprinted, and abnormal methylation levels of the differentially methylated regions are associated with many types of diseases,[9,32] thus we did the preliminary data mining to analyze the relationship between PEG3 methylation and RFS in colon cancer. Interestingly, the current study revealed that cg13960339 methylation level is positively associated with survival probability. This methylation site exists in the CpG island of chromosome19 and the distance to the transcription start site of PEG3 is 200 bp, suggesting this site might be an important transcriptional regulation position for gene expression. Methylation is strongly associated with negative regulation of mRNA expression, which was also shown in our analysis for the association between cg13960339 and PEG3 mRNA level. Additionally, the influence of these factors on RFS is also consistent with this phenomenon. The impact of PEG3 whole methylation density on the etiology and prognosis of colon cancer will be systematically analyzed in our future clinical studies.

Four microRNAs in the current study are reported to be diagnostic or prognostic biomarkers for colon cancer: hsa-miR-194-5p, hsa-miR-186-5p, hsa-miR-17-5p, hsa-miR-491-3p. Past studies have shown that miR-17- suppresses cell proliferation and invasion by increasing p53, decreasing p62 expression, or affecting cell cycle machinery, and might be a potential treatment target for adjuvant therapy in several cancers.[16,30,33–36] Additionally, a Spanish study of 110 patients undergoing surgery for colorectal cancer revealed that miR-17-5p had a trend to correlate with disease-free survival at early stages,[37] A meta-analysis revealed that the increased expression of miR-17 is associated with unfavorable prognoses for colon cancer. MiR-194-5p, miR-181a-5p, miR-494-3p and miR-491-3p are also reported as prognosis biomarkers in clear cell kidney carcinoma, ovarian cancer, lung (breast) cancer, and tongue cancer patients, respectively.[33,38–41] Tumor suppressive miR-296-3p and miR-186-5p also affect
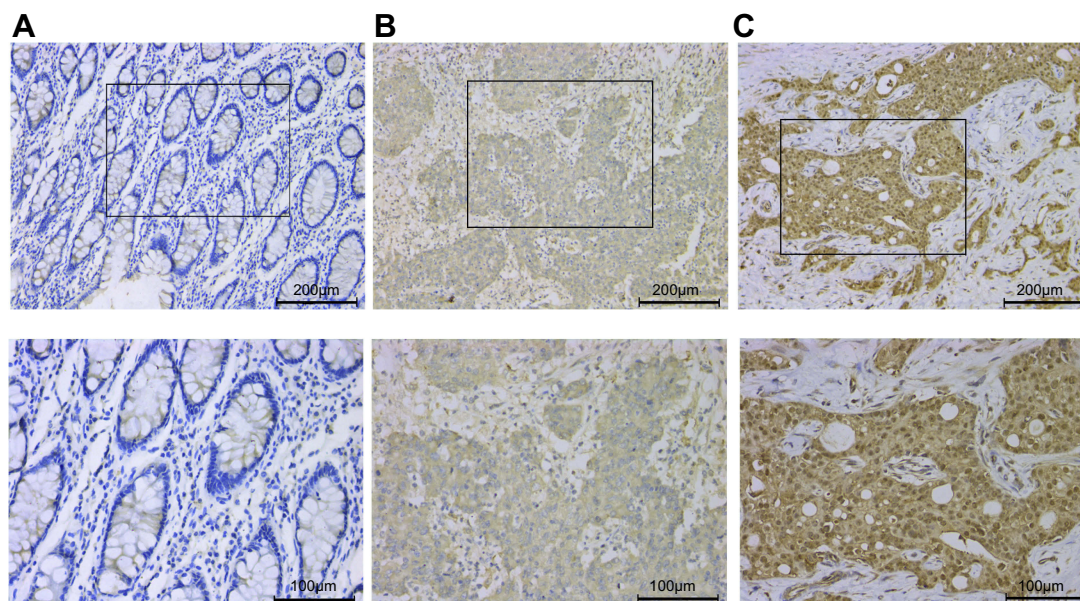
Figure 4 Representative immunohistochemical staining of PEG3 in colon cancer. (**A**) PEG3 negative expression. (**B**) PEG3 positive expression. (**C**) Positive control: immunohistochemical staining of PEG3 in breast cancer. Images in the lower panel were magnified from the black box area in the images in the upper panel.

tumorigenesis of non-small cell lung cancer and colorectal cancer cells.[42,43] Therefore, these microRNAs might be potential prognostic biomarkers of colon cancer and should be further investigated.

Conclusions

The current study revealed that the imprinted gene, PEG3, is a prognostic biomarker for colon cancer, and the potential mechanism might involve altered epigenetic regulation on different levels including microRNA and methylation of a CpG island for PEG3. Additionally, we speculate that the degradation and ectopic expression of PEG3 might be critical to the function it plays in the tumorigenesis of colon cancer.

Abbreviations

CI, Confidence interval; COAD, Colon Adenocarcinoma; GEO, Gene Expression Omnibus; GDSC, Genomics of Drug Sensitivity in Cancer; HR, Hazard ratio; MMR, Mismatch repair; PEG, Paternally expressed gene; RFS, Recurrence free survival; TCGA, The Cancer Genome Atlas; TNM, Tumor, node, metastasis; 5-fu, 5-fluorouracil.

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Disclosure

The authors declare no conflicts of interest in this work.

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