Modern Pathology (2009) 22, 786–796

www.modernpathology.org

Transgelin as a suppressor is associated with poor prognosis in colorectal carcinoma patients

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We performed comparative proteomic analysis of colorectal cancer to investigate potential target proteins correlated with carcinogenesis and prognosis. Among them, transgelin, a 22 kDa protein also called SM22, was identified as a novel tumor suppressor protein, but little is known about this protein in tumors so far. A remarkable reduced expression of transgelin was found in colorectal cancer samples compared with normal colorectal mucosa. The effect of 5-aza-2′-deoxycytidine as a demethylation agent would obviously restore the original expression level of transgelin, implicating DNA hypermethylation of transgelin is important in the regulation of transgelin transcription in colorectal cancer. As a control, the investigation at cell line level confirms that transgelin protein comes from epithelium but not mesenchymal cells. Further, immunohistochemical staining for transgelin was performed on paraffin sections of 62 and 126 cases of normal colorectal mucosa and colorectal cancer specimens, respectively. As compared to normal colorectal tissue, we observed a significantly lower transgelin expression in colorectal cancer samples ($P<0.001$). Survival analysis demonstrated that patients without transgelin expression had shorter overall survival, whereas patients with transgelin expression had better survival ($P=0.006$). Multivariate analysis showed that negative transgelin expression was an independent prognostic indicator for patient's survival. Our results suggest that transgelin as a suppressor may serve as important biomarker of malignancy. Loss of transgelin involves gene promoter hypermethylation and is closely associated with poor overall survival in colorectal cancer patients.
Modern Pathology (2009) **22,** 786–796; doi:10.1038/modpathol.2009.29; published online 27 March 2009

Keywords: colorectal cancer; DNA methylation; poor prognosis; proteomics; transgelin

Colorectal cancer is the third most common malignancy in the world, and represents the main cause for cancer deaths in Europe and the USA.[1,2] In China, colorectal cancer occupies the fifth position in the mortalities caused by cancer, and its incidence still continues to increase.[2] Despite significant improvement in the treatment of colorectal cancer over the past decades, thanks to the introduction of new surgical techniques, improved radiotherapy techniques, and the use of chemotherapy, the overall survival rate of patients with colorectal cancer has not changed markedly.[3] One of the major factors for the poor outcome is lack of specific early diagnosis method. Although various genetic alterations have been identified, the precise mechanisms of colorectal cancer development still remain elusive.[4–6] Therefore, it is critical for us to advance in early diagnosis to increase the survival rate of patients with colorectal cancer. Identification of specific protein signatures associated with colorectal cancer development may provide novel biomarkers that allow more accurate prognostic information and help identify new molecular therapeutic targets, provide clues for understanding the molecular mechanisms governing colorectal cancer development as well as predicting the prognosis of colorectal cancer patients.

Among the currently available techniques, proteomic ones allow for identification of the

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Received 15 July 2008; revised and accepted 12 January 2009; published online 27 March 2009

protein changes caused by the disease process in a relatively high-throughput manner, because it permits an analysis of thousands of modified or unmodified proteins simultaneously. As a result, it is increasingly becoming popular in identifying biomarkers for cancer diagnosis, progression as well as therapeutic targets for improved treatment outcomes.[7] Currently, two-dimensional electrophoresis has become the most widely used method because of its simplicity, reliability, high information content and ready accessibility to researchers. Under optimal conditions, thousands of individual proteins can be resolved on a single two-dimensional electrophoresis gel, making this technology popular for studies of global proteome-scale differential expression despite a limited display of relatively abundant proteins. In our previous studies, we used phenotypic analyses and proteomic techniques to identify successfully protein changes between two colorectal cancer cell lines, SW480 and SW620, with different metastatic potentials.[8] However, as a marker filtered from cell lines *in vitro*, its clinical significance is limited, especially for early screening or diagnosis. In the present study, therefore, we performed comparative proteomic analysis to identify protein alterations during genesis of colorectal cancer at tissue level. To define further the importance of differentially expressed proteins in colorectal cancer progression, we substantiate our screening study by western blot and characterize their expressions by immunohistochemistry in a large series of colorectal cancer samples with a comprehensive set of clinicopathological and follow-up data.

In our study, we attempted to find differentially expressed proteins associated with colorectal cancer genesis. Among them, a remarkable decreased expression of transgelin, a 22 kDa protein also called SM22, WS3-10 and mouse p27, and found abundantly in smooth muscle cells,[9–11] was found in colorectal cancer specimens. Transgelin has been shown to bind to and colocalize with F-actin, indicating that it may be involved in cell differentiation by stabilizing the cytoskeleton through actin binding,[12,13] and diminished expression of this protein has been reported in a variety of cell types on transformation[14–16] as well as in several human cancers, including lung, renal, prostate and breast cancer.[14,17–19] According our current knowledge, there are few reports on its role in progression of colorectal cancer.

Materials and methods

Cell Lines

To confirm the origin of transgelin expression, the following human intestinal epithelial cell lines were analyzed for transgelin expression by immunoblotting: HT29, SW480, SW620 and Lovo (ATCC number: HTB-38, CCL-228, CCL-227 and CCL-229, respectively). In addition, a human colorectal cancer cell subline with unique liver metastatic potential, designated as SW480/M5, was established in our lab[20] and was also examined. Among them, HT29 and Lovo were used in the nucleotide analogue 5-aza-2′-deoxycytidine (5-AzaC) treatment assay. Cell lines were treated with 5-AzaC (Sigma-Aldrich) dissolved in RPMI 1640 just before use. Exponentially growing cells were cultured in RPMI 1640 medium containing 5 μmol/l 5-AzaC for 6 days. The medium was exchanged every other day with the same concentration of 5-AzaC. As a control, cell lines were cultured in normal medium, which was replaced every other day. All other cells were cultured in RPMI 1640 (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (Gibco BRL, Invitrogen, Paisley, UK) in a humidified atmosphere of 5% CO_2 at 37°C.

Tumor Samples

All cases were selected from the Nanfang Hospital tumor tissue bank. In total, 138 patients were involved in the study. In each case a diagnosis of primary colorectal cancer had been made, and the patients had undergone elective surgery for colorectal cancer, in Nanfang Hospital, between 2001 and 2004. The Nanfang Hospital tumor tissue bank is linked to a comprehensive set of clinicopathological data, including age, gender, size of primary tumor, degree of tumor differentiation, lymph node status and clinical stage. Supplementary Table 1 and present the data for patients included in the screening and validation study, respectively. Complete follow-up, ranging from 0 to 86 months, was available for all patients and the median patient survival was 56 months. At the time of censoring the data there had been 43 (34%) deaths in the patient group. The tumor samples were submitted to the Department of Pathology, Nanfang Hospital, Southern Medical University for diagnosis. The tumor excision specimens were fixed in formalin, representative blocks were embedded in wax and sections were stained with hematoxylin and eosin. Permission for this study was obtained from the ethics committee of Southern Medical University.

Proteomic Analysis

Proteomic analysis, including two-dimensional gel electrophoresis, gel visualization and assessment, and mass spectrometry, was performed according to the protocol we have previously described.[8] Proteins were extracted from normal colorectal mucosa ($n = 12$) and primary colorectal cancer tissue ($n = 12$) samples. Tissue samples (50–100 mg) were crushed in liquid nitrogen and lysed in 1 ml lysis buffer consisting of 7 M urea, 2 M thiourea, 4% CHAPS, 65 mM DTT and 2% Pharmalyte (pH 3–10;

GE Healthcare, Piscataway, NJ, USA) by sonication on ice. The lysates were cleared by centrifugation at 12 000 r.p.m. for 1 h at 4°C. Subsequently, the protein concentration of the supernatants was determined by the modified Bradford method,[21] and aliquots of the protein samples were stored in −80°C. Before two-dimensional analysis, the protein samples were purified using 2D Clean-Up kit (GE Healthcare) according to the manufacturer's instructions. Differentially expressed proteins were identified using two-dimensional gel electrophoresis and mass spectrometry. Two-dimensional electrophoresis gel electrophoresis was performed using 3–10 p*I* Immobiline strips, with proteins being separated according to charge, and subsequently, molecular weight. The gels were then stained with silver staining to visualize proteins, and protein spots of interest were cut from the gels. Proteins were digested with trypsin, and peptide mass mapping was performed by matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF MS) using an ABI Voyager DE-STR mass spectrometer. To identify the original protein, the masses of the tryptic peptides were entered into MASCOT Database (http://www.matrixscience.com/search_form_select.html). The database search was restricted to human proteins, with no constraints on either the molecular weight or the isoelectric point of the protein.

Western Blot Analysis

Sample preparation for immunoblotting was carried out as previously described.[8] Briefly, proteins of tissue samples were obtained as mentioned above. Cells were washed twice with cold phosphate-buffered saline (PBS) and lysed on ice in RIPA buffer (1 × PBS, 1% NP-40, 0.1% sodium dodecyl sulfate (SDS), 5 mM EDTA, 0.5% sodium deoxycholate and 1 mM sodium orthovanadate) with protease inhibitors. Protein concentration was determined by the modified Bradford method.[21] Equal amounts of proteins were separated electrophoretically on 12% SDS–polyacrylamide gels and transferred onto polyvinylidene difluoride membranes (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The membrane was probed with an anti-transgelin rabbit polyclonal antibody (1:500; Abcam, Cambridge, UK). Expression of transgelin was determined with horseradish-peroxidase-conjugated anti-rabbit immunoglobulin G (1:20 000; Jingmei Biotech, China) and enhanced chemiluminescence (Pierce, Rockford, IL, USA). The immunoreactive bands were visualized on Kodak 2000 M camera system (Eastman Kodak, Rochester, NY, USA) according to the manufacturer's instructions. An anti-GAPDH goat polyclonal antibody (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used to confirm equal loading. The experiments were repeated three times.

Extraction of Total RNA and Semiquantitative RT-PCR

Total RNA was extracted using TRIzol solution (Invitrogen, USA) according to the manufacturer's protocol and RNase-free DNaseI was used to remove DNA contamination. Total RNA concentration and quantity were assessed by absorbency at 260 nm using a DNA/Protein Analyzer (DU 530; Beckman, USA). Reverse transcription (RT) was performed in a 20 μl reaction system with 2 μg total RNA treated by M-MLV Reverse Transcriptase to synthesize first-strand cDNA (Promega, USA) according to the manufacturer's recommendation, followed by cDNA amplification using the specific primer set for transgelin and *β*-actin as an internal control. The sequences of the sense and antisense primers were as follows: 5′-GCCAACAAGGGTCCTTCCTAT-3′ (F) and 5′-TAACTGATGATCTGCCGAGGT-3′ for transgelin; 5′-CCACACCTTCTACAATGAGC-3′ (F) and 5′-CGTAGCACAGCTTCTCCTTA-3′ (R) for *β*-actin, and the corresponding PCR products are 605 and 394 bp, respectively. Each PCR was generally performed in 30 thermal cycles and then the PCR products were observed by electrophoresis on 1% agarose gel and visualized after staining with ethidium bromide. To quantify the densities of the bands, the gray values were measured using the ImageQuant system (GE Healthcare). The values of transgelin were normalized by the corresponding values of *β*-actin.

Immunohistochemistry

Immunohistochemistry was carried out to study altered protein expression in 126 human colorectal cancer tissues. The corresponding antibody was selected based on its use and validation in previous study.[22] The procedures were performed similarly to previously described methods.[8] Briefly, 4 μm sections mounted on aminopropylethoxysilane slides and pretreated for immunohistochemistry were dewaxed in xylene and brought through graded ethanols to deionized distilled water. An antigen retrieval step was performed. Before staining for immunohistochemistry, the sections were incubated in a 750 W microwave oven for 15 min in 10 mM buffered citrate (pH 6.0) to complete antigen unmasking. Endogenous peroxidase was quenched by incubation of the sections in 0.1% sodium azide with 0.3% hydrogen peroxide for 10 min at room temperature. Nonspecific binding was blocked by incubation with nonimmune serum (1% bovine serum albumin for 15 min at room temperature). The sections were incubated for 1 h at room temperature with antibodies against transgelin at a dilution of 1:100. The following controls were performed: (1) omission of the primary antibody and (2) substitution of the primary antiserum with nonimmune serum diluted 1:500 in blocking buffer. No immunostaining was observed after any of the

control procedures. Bound antibodies were visualized using an EnVision kit (Dako Corporation, Carpinteria, CA, USA), which is based on EnVision polymer–peroxidase complex binding, according to the manufacturer's instructions. Mayer's hematoxylin was used for nuclear counterstaining. The sections were mounted with a synthetic medium. Two observers independently reviewed and assessed the cellular immunoreactivity in each section. Staining intensity was assessed as present or absent, not qualitatively. We used the same criteria as referred in the previous studies.[23–26] The cutoff value was set at 10%, and those cases with more than 10% of the area stained for transgelin were grouped as positive expression (present) and those with less than 10 as negative expression (absent). We chose this cutoff value of 10% positivity because this value showed the highest concordance between immunohistochemical detection of transgelin and different repeated measure. The small number of discrepancies ($<5\%$) was resolved by simultaneous re-evaluation.

Statistical Analysis

Quantitative values were expressed as means ± s.d. Student's *t*-test was used to compare mRNA and protein expression of transgelin in colorectal cancer tissue samples and cell lines. Categorical variables were enumeration data of counting the number of samples. Mann–Whitney *U*-test was used to analyze the relationship between transgelin expression and clinicopathological characteristics. Survival curves were plotted by the Kaplan–Meier method and compared by the log-rank test. The significance of various variables for survival was analyzed by the Cox proportional hazards model in the multivariate analysis. All statistical analyses were carried out using the SPSS 12.0 statistical software package. $P<0.05$ in all cases was considered statistically significant.

Results

Differential Expression Proteins between Normal Mucosa and Colorectal Cancer Tissues

To determine a colorectal cancer genesis-specific protein expression pattern, comparative proteomic analysis of normal tissue ($n=12$) and primary colorectal cancer tissue ($n=12$) samples was performed. PDQuest software analysis revealed that the groups of colorectal cancer had average matching rates of 85.3%, and differentially expressed protein spots of 65.5 ± 16.3 (Figure 1). There were 22 differential spots that simultaneously existed in colorectal cancer groups compared with the normal tissue. All the protein spots of interest were successfully identified by MALDI-TOF MS and by subsequent comparative sequence search in the Mascot database. The MSDB identification number, the theoretical molecular mass, the theoretical p*I*, the sequence coverage and MASCOT score are presented in Table 1. Figure 2 shows the PMF of protein spots N9 representing transgelin.

Immunoblotting Confirmation of the Decreased Transgelin in Colorectal Cancer Samples

To confirm and extend the proteomic results, transgelin expression in 12 colorectal cancer tissue and paired normal colorectal mucosa was examined by western blot analysis (Figure 3). Equal protein loading was confirmed by parallel GAPDH immunoblotting, and signal quantification was performed by densitometric scanning. Despite interindividual variations in the extent of transgelin expression, this protein was found significantly downregulated in colorectal cancer specimens. The change pattern of transgelin expression was similar to that observed in the proteomic analysis.

Changes of Transgelin Expression in Colorectal Cancer Cell Lines after 5-AzaC Treatment

To investigate the expression levels of transgelin transcripts and protein in colorectal cancer cell lines under normal culture conditions, semiquantitative RT-PCR analysis and western blotting analysis were carried out in the following cell lines: HT29, SW480 (derived from primary lesion), Lovo, SW620 (derived from metastatic lymph node) and SW480/M5 (subline with unique liver metastatic potential). Among all five colorectal cancer cell lines, expression of transgelin mRNA was completely undetectable in HT29 cell line (Figure 4a). Immunoblotting analysis showed that positive immunoreactivity was only found in SW480, SW620 and SW480/M5, derived from the same patient, however, no positive signal was found in all the other cell lines tested (Figure 4b).

To determine whether transgelin expression was lost due to DNA methylation, we treated colorectal cancer cell lines with the demethylating agent 5-AzaC and then examined the level of mRNA by RT–PCR analysis and the level of protein by western blot analysis in treated cells compared with control cells. HT29 and Lovo cell lines, which were undetectable in protein level, were selected for analysis of DNA demethylation. As shown in Figure 4c and d, expression of transgelin mRNA and protein was restored after 6 days of treatment with 5-AzaC when compared with control HT29 cells, strongly suggesting that the mechanism for which transgelin decreased expression in colorectal cancer cells may involve DNA methylation. In contrast, however, the expression of transgelin mRNA and

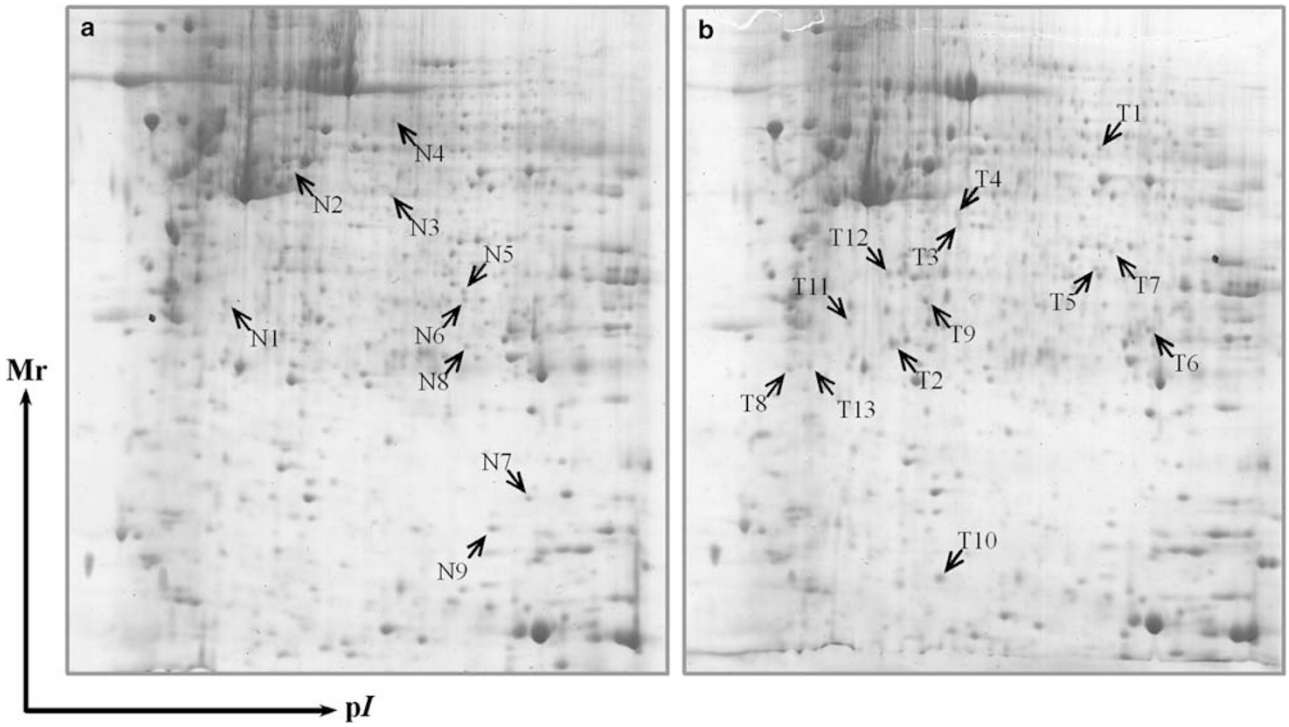

Figure 1 Representative two-dimensional gel of normal colorectal mucosa (**a**) and colorectal cancer tissue (**b**). All the 22 differentially expressed proteins were identified by MALDI-TOF MS (numbered arrows, for protein nomenclature see Table 1).

Table 1 The 22 characterized differentially expressed proteins among normal colorectal mucosa and colorectal cancer tissue

Protein index	*Theoretical Mr(kDa)/p*I	*Summary score*	*Protein coverage (%)*	*MSDB ID*	*Protein description*	*Protein level (tumor/ normal)*
N1	52200/5.35	101	30	Q5SP14_HUMAN	Heat shock 70 kDa protein 1B	↓
N2	48599/5.6	186	62	S37780	Keratin 20, type I-like, cytoskeletal	↓
N3	58339/7.95	117	39	KPYM_HUMAN	Pyruvate kinase, isozymes M1/M2	↓
N4	68354/5.67	146	45	E973181	α-Fetoprotein	↓
N5	53580/5.62	115	27	Q53HF2_HUMAN	Heat shock 70 kDa protein 8 isoform 2 variant	↓
N6	41973/6.69	101	44	1HJOA	Heat shock 70 kDa protein 42 kDa fragment	↓
N7	20146/6.76	127	70	CYHUAB	α-Crystallin chain B	↓
N8	68354/5.67	109	27	E973181	α-Fetoprotein	↓
N9	12312/6.95	73	84	Q59FA5_HUMAN	Transgelin variant	↓
T1	58339/7.95	205	50	KPYM_HUMAN	Pyruvate kinase, isozymes M1/M2	↑
T2	29678/5.5	139	65	Q6PJ43_HUMAN	ACTG1 protein	↑
T3	56577/8.54	71	32	FGHUB	Fibrinogen β-chain precursor	↑
T4	42568/5.72	76	40	A36898	Maspin	↑
T5	30185/6.11	118	42	S68234	Lasp-1 protein	↑
T6	28769/6.75	137	60	PGAM1_HUMAN	Phosphoglycerate mutase 1	↑
T7	30337/8.81	72	26	Q7KZ74_HUMAN	A+U-rich element RNA-binding factor	↑
T8	23108/5.21	82	37	1YER	Heat shock protein 90 residues 9–236	↑
T9	36393/5.63	137	49	ANXA3_HUMAN	Annexin A3	↑
T10	12770/5.55	71	80	CAA00999	Calgranulin B	↑
T11	28512/5.19	107	46	PSA3_HUMAN	Proteasome subunit-α type 3	↑
T12	48135/4.7	76	31	Q5JP53_HUMAN	Tubulin, β-polypeptide	↑
T13	20571/6.73	96	53	1CC0E	rho gdp dissociation inhibitor-α, chain E	↑

protein in Lovo cells was not restored after 6 days of treatment with 5-AzaC, indicating that transgelin expression might also be suppressed by unknown post-transcriptional regulation.

Immunohistochemical Analysis

Expression and subcellular localization of transgelin protein was determined by immunohistochemistry

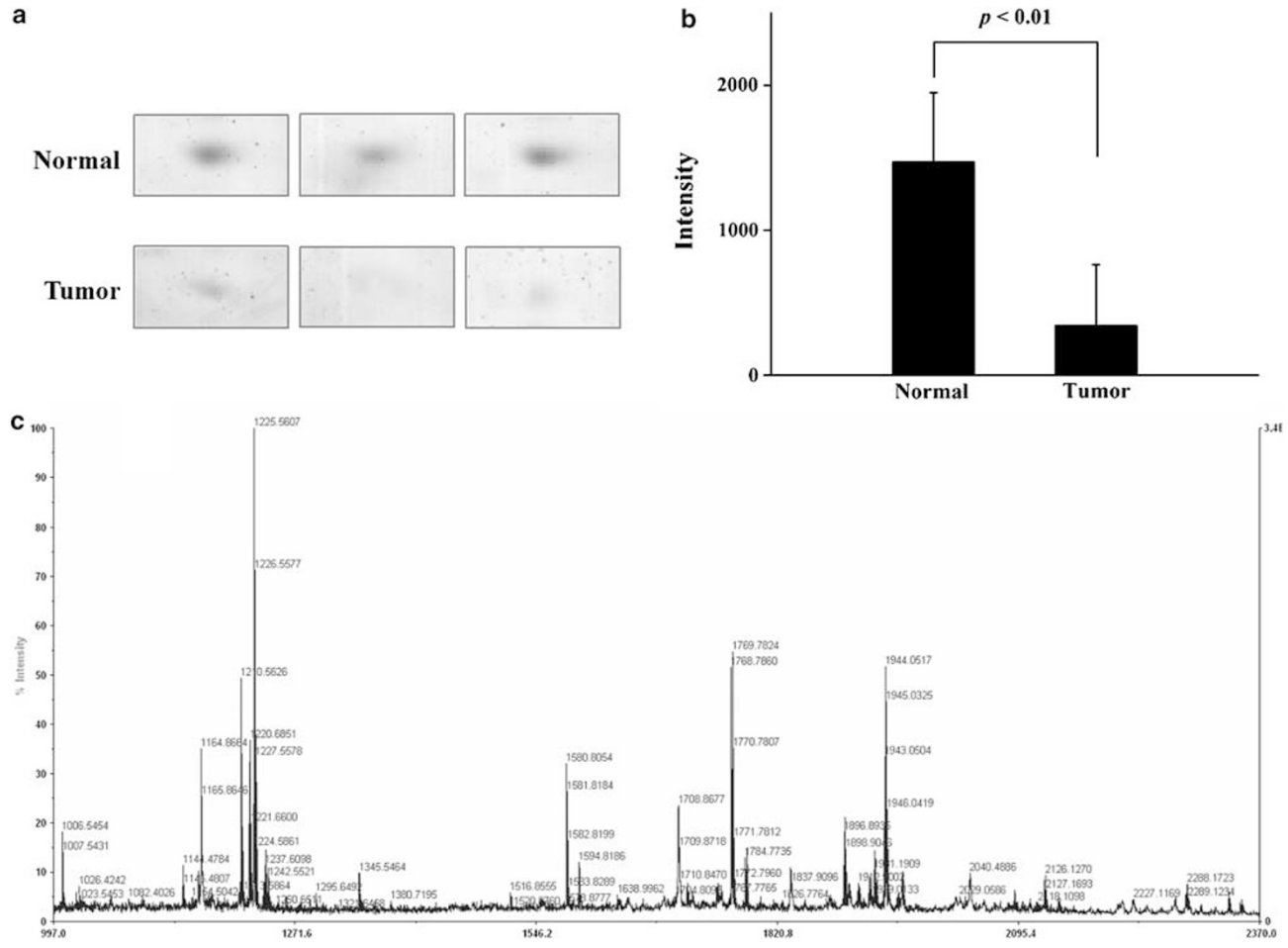

Figure 2 Identification of transgelin expression. (**a**) A section of each gel has been enlarged to show the downregulated progression of transgelin in normal colorectal mucosa and colorectal cancer. (**b**) The densitometric analysis of each protein was calculated from 12 different gels using PDQuest software. Each bar represents the mean ± s.d. of intensity, with significant difference between groups found by two-tailed Student's *t*-test. (**c**) MS identification of in-gel trypsin digests of the protein and analysis of the depicted peptide spectrum resulted in the identification of transgelin.

in 62 and 131 paraffin-embedded, archival normal colorectal mucosa and colorectal cancer tissues, respectively. Immunoreactivity to transgelin staining was observed in the cytoplasm region of benign and malignant epithelial cells (Figure 5). The positive signals were also frequently found in smooth muscle tissue and fibroblast of colorectal tissue. Transgelin was expressed in 84% (52 of 62) of normal colorectal epithelium tested. As compared to these normal colorectal tissues, we observed a significantly low positive rate of transgelin protein expression in 37% (47 of 126) of all colorectal cancer samples ($P<0.001$). The statistical evaluation of immunohistochemical checking, however, indicated no statistically significant relationship between transgelin expression and clinicopathological parameters derived from clinical materials, follow-up data and pathological findings (Table 2).

Survival Analysis

To investigate the prognostic value of transgelin expression, the association between transgelin expression and overall survival was initially evaluated using Kaplan–Meier survival curves with the log-rank test and then confirmed with univariate and multivariate Cox regression models. The overall survival rates were 57 and 81%, respectively, in patients with negative and positive transgelin expressions. There was a trend toward poorer survival for patients whose primary tumors showed negative transgelin immunoreactivity, compared with those patients whose primary tumors showed immunoreactivity (log rank 7.642, $P=0.006$; Figure 6). The mean survival in the poor survival group was 55.5 months (negative transgelin expression, $n=79$), whereas the mean survival in the good survival group was 72.5 months (positive transgelin expression, $n=47$).

To identify the variables of potential prognostic significance in all the patients with colorectal cancer, univariate analysis of each variable was performed in relation to the survival time. The difference in predicting the prognosis was assessed by examining the hazard ratio and *P*-value for each variable. The relative importance of each variable was then determined by multivariate Cox proportional hazards model analysis. From univariate analysis, step-wise inclusion of variables in the model showed that the significant prognostic factors were transgelin expression, lymph node status and clinical stage (Table 3). Furthermore, to evaluate the potential of negative transgelin expression (negative *vs* positive) as an independent predictor for overall survival of colorectal cancer, multivariate Cox regression analysis was performed. Although positive lymph node status failed to demonstrate independence ($P = 0.218$; Table 3), only both clinical stage and negative transgelin expression might have a role to predict overall survival in colorectal cancer patients ($P < 0.001$ and 0.028, respectively; Table 3).

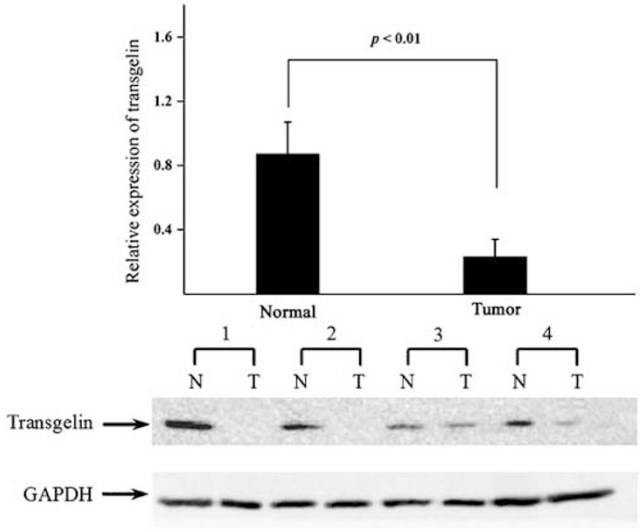

Figure 3 Expression of transgelin in normal colorectal mucosa and primary colorectal cancer samples. There are four representative samples in each group. Immunosignals were quantified by densitometric scanning. Transgelin expression in the individual tissue samples was calculated as transgelin expression relative to GAPDH expression. Data are means ± s.d. from three independent experiments. Immunoblotting analysis revealed the variation to be consistent with its appearance in the gel images (Figure 2).

Discussion

The initiation and progression of colorectal cancer involved multiple gene and protein alterations. Understanding the molecular basis of the disease is of great significance for its early detection and treatment. In the present study, comparative proteomic approach was applied to the identification of differential proteins between normal colorectal tissue and colorectal cancer tissue. There were 22 differential spots that simultaneously existed in

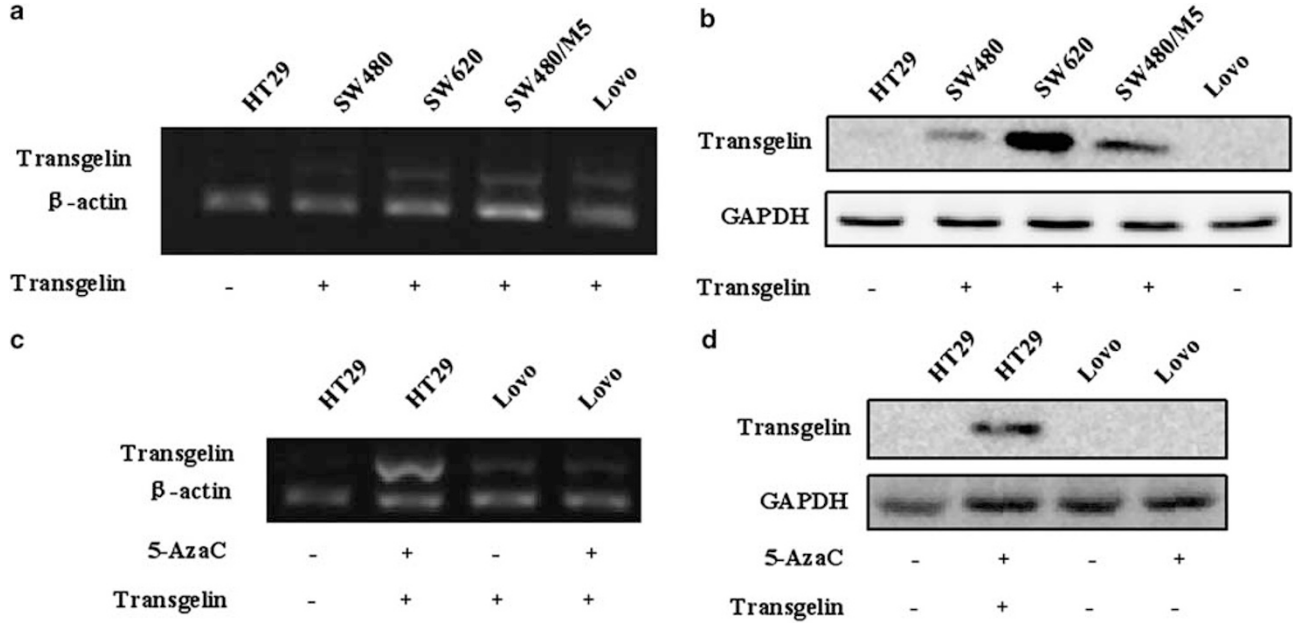

Figure 4 Expression and methylation status of transgelin in colorectal cancer cell lines. (**a**) Expression of transgelin mRNA using RT–PCR in colorectal cancer cell lines. *β*-Actin was used as a loading control. Among all five colorectal cancer cell lines, expression of transgelin mRNA was completely undetectable in HT29 cell line. (**b**) Corresponding expression of transgelin protein using western blot. GAPDH was used to confirm equal loading. Positive signals were only found in SW480, SW620 and SW480/M5, derived from the same patient, however, no positive signal was found in all the other cell lines tested. (**c**, **d**) Re-expression and demethylation of the transgelin gene in colorectal cancer cell lines treated with 5-AzaC. Expression of transgelin mRNA (**c**) and protein (**d**) was restored after 6 days of treatment when compared with control HT29 cells, but not for Lovo cells.

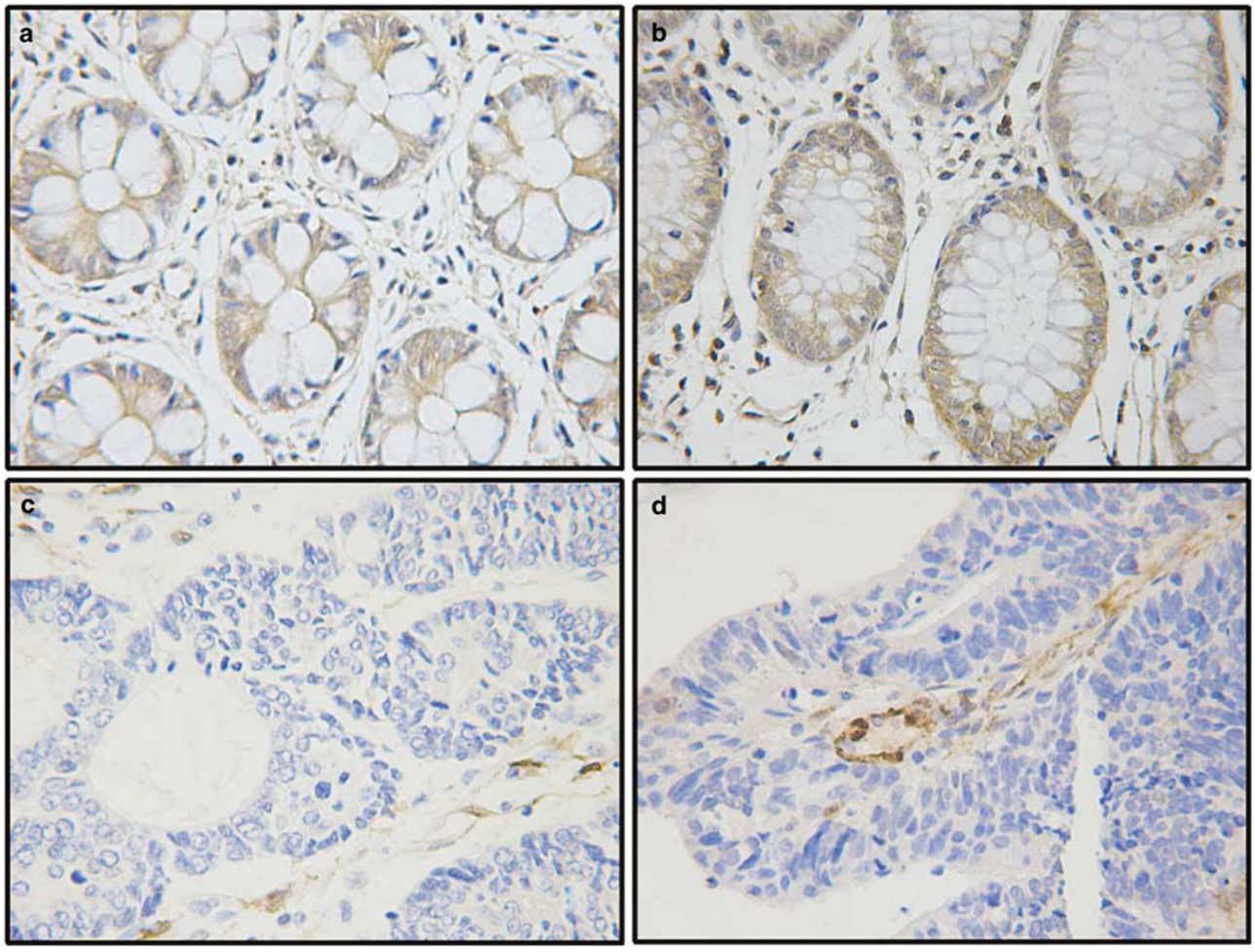

Figure 5 Representative immunohistochemical staining of transgelin in normal colorectal mucosa and colorectal adenocarcinoma (original magnification $\times 200$). Immunoreactivity to transgelin staining was localized to the cytoplasm region of benign and malignant epithelial cells. (**a**, **b**) Positive expression in normal colorectal mucosa; (**c**, **d**), negative expression in colorectal cancer tissue.

colorectal cancer groups compared with the normal tissue. To some extent, this result is consistent with the data reported by other groups, who listed several proteins involved in protein synthesis and folding (heat shock proteins), cellular reorganization and cytoskeleton (actin, tubulin), intracellular calcium-binding protein (calgranulin), and cell communication and signal transduction (annexin) in the proteomic profiles of colorectal cancer cell line and tissue.[27–30] However, there are still some different proteins between our data and other researchers' reports.[31,32] First, we consider that two-dimensional electrophoresis and MALDI-TOF MS-based PMF analysis for human tissue is more complex than that for cell line. Meanwhile, two-dimensional electrophoresis gels have extremely numerous biological information that is hard to be fully revealed by a single laboratory. A systemic collection and analysis for the sorted complementary data from various research groups will benefit the scientists in making global proteomic profiles of colorectal cancer. Moreover, the difference of races and region distributions, as well as the different methods of tissue collection and management, may contribute to the distinction among various laboratories. The methods used in this study of tissue washing and surface scraping from tissue are important for collecting pure tumor cell population free of contaminating serum proteins, red blood cells, connective tissues and necrotic tissue materials.[33] It was of clinical importance to identify the differential expression proteins that had potentially of being tumor markers and anticancer targets.

Among these candidate proteins, it was interesting that loss of transgelin expression was identified during genesis of colorectal cancer. Transgelin, also named SM22α, was first isolated from chicken gizzard,[34] as a transformation- and shape-change-sensitive actin-binding protein, the expression of which lost in virally transformed cells.[12,35] It was also originally described as predominantly expressed in smooth muscle cells to bind to actin, suggesting it is involved in cell differentiation and cytoskeletal rearrangement.[9–11] Yeo *et al*[36]

established an experimental model for colitic cancer, and using proteomic analysis to identify proteins involved in colitic cancer suggested that loss of transgelin could be a potential biomarker of repeated colitic-associated colon cancer. Recently, Shields *et al*[14] characterized transgelin as a gene, the expression of which was abolished by Ras, suggesting that loss of transgelin expression may contribute to transformation by oncogene Ras, and loss of transgelin expression might represent an early event for the tumor progression in breast and colon cancers. Functional studies revealed the identification of transgelin as a potential prostate cancer suppressor by inhibition of ARA54-enhanced AR transactivation and prostate cancer cell growth.[22] Similarly, our immunoblotting and immunohistochemical results confirmed the proteomic data that showed that transgelin was significantly decreased in colorectal cancer, compared with normal colorectal mucosa, suggesting its potential suppressor functions in colorectal cancer development. Taken together, these findings suggest that absence of transgelin may be a candidate biomarker of colorectal carcinogenesis, as well as being involved in the mechanism of colorectal cancer malignancy.

Transgelin expression is thought to be regulated at the level of transcription.[13,37] Our results above showed that transgelin is downregulated at a high

Table 2 Correlation between the clinicopathological features and transgelin expression

Characteristics	*Transgelin (%)*		P-*value*
	Negative	*Positive*	
Gender			
Male	50 (63)	29 (37)	0.858
Female	29 (62)	18 (38)	
Age (years)			
<50	32 (70)	14 (30)	0.227
≥50	47 (59)	33 (41)	
Tumor location			
Proximal colon	33 (61)	21 (39)	0.332
Distal colon	12 (80)	3 (20)	
Rectum	34 (60)	23 (40)	
Tumor size (cm in diameter)			
<5	25 (61)	16 (39)	0.781
≥5	54 (64)	31 (36)	
Tumor differentiation			
Good	5 (50)	5 (50)	0.671
Moderate	55 (63)	32 (39)	
Poor	19 (66)	10 (34)	
Lymph node status			
Negative	48 (60)	32 (40)	0.409
Positive	31 (67)	15 (33)	
AJCC stage			
I	1 (33)	2 (67)	0.102
II	41 (59)	29 (41)	
III	22 (67)	11 (33)	
IV	15 (75)	5 (25)	

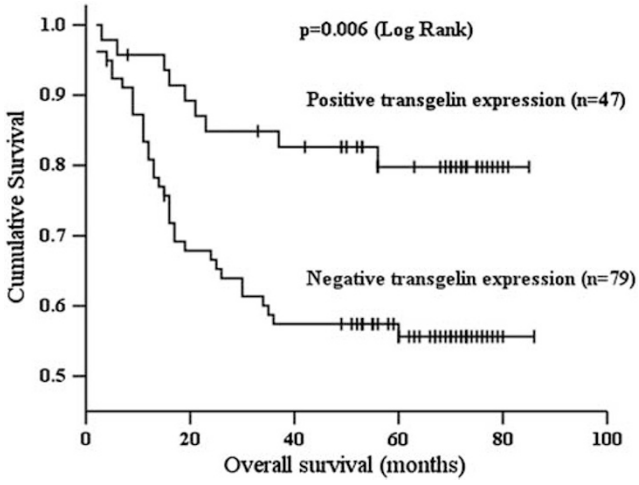

Figure 6 Kaplan–Meier curve for overall survival in patients with negative transgelin expression ($n=79$) *vs* positive expression ($n=47$) in the colorectal cancer. Poorer survival was seen in the patients whose tumors showed negative transgelin expression.

Table 3 Univariate and multivariate analyses of individual parameters for correlations with overall survival rate

Variables	*Univariate*			*Multivariate*		
	HR	*CI (95%)*	P-*value*	*HR*	*CI (95%)*	P-*value*
Gender	0.93	0.61–1.72	0.82			
Age	1.19	0.64–2.19	0.58			
Tumor location	0.99	0.72–1.35	0.92			
Tumor size	0.67	0.36–1.23	0.19			
Tumor differentiation	1.57	0.91–2.71	0.11			
AJCC stage	3.01	2.09–4.34	<0.001*	2.92	2.02–4.23	<0.001*
Transgelin expression	2.70	1.29–5.62	<0.001*	2.34	1.12–4.89	0.02*

Cox proportional hazards model.
*Statistically significant ($P<0.05$).
HR, hazard ratio; CI, confidence interval; AJCC, American Joint Committee on Cancer.

frequency in a variety of human tumor cell lines and patient-derived tumor tissue samples, suggesting that the mechanism by which transgelin is down-regulated is one that occurs frequently in carcinogenesis. One common mechanism for suppression of gene expression in human carcinomas involves DNA methylation of promoters of genes to block transcription.[38] For example, the expression of various tumor suppressor genes, including *BRCA1*, *E-cadherin*, *hMLH1*, *p16*, *VHL* and *Rb*, is frequently inhibited due to hypermethylation in human cancers. In addition,, promoter methylation has been described as a mechanism to repress the transcriptional activity of transgelin in smooth muscle cells.[39] Thus, we evaluated the possibility that the loss of transgelin expression was due to DNA methylation. Another study has showed that the promoter regions of transgelin were highly methylated in the hepatocellular carcinoma cell lines.[40] Our observation further confirmed that treatment with 5-AzaC restored expression of transgelin mRNA and protein in HT29 cell line, but not in Lovo cell, suggesting hypermethylation of transgelin is important in the regulation of transgelin transcription in colorectal cancer. In the mean time, however, the expression of transgelin mRNA and protein in Lovo cells was not restored after treatment with 5-AzaC, indicating that transgelin expression might also be suppressed by unknown post-transcriptional regulation.

Because transgelin is an actin-binding protein, we also examined the possible function of transgelin in tumor progression. Currently, however, little is known regarding transgelin function in tumor progression. To address this question, we used immunohistochemical assay to analyze the relationship between the expression of transgelin and clinical characteristics of the patients. The statistical evaluation, however, indicated no statistically significant relationship between transgelin expression and clinicopathological parameters. Nevertheless, a trend was identified between negative transgelin expression in colorectal cancer and worsening clinical prognosis. Furthermore, we have shown in univariate and multivariate analyses that negative expression of transgelin is a significant predictor of poor prognosis for colorectal cancer patients. As transgelin expression might be served as a new and independent predictor of overall patient survival, it may function as a new and independent predictor of prognosis for colorectal cancer patients as well. In combination with other biomarkers of colorectal cancer, transgelin expression status may be useful to stratify patients for novel therapeutic strategies, such as adjuvant chemotherapy, radiosensitization or the establishment of rational treatment selection criteria for patients. However, these findings still need to be replicated, and further investigation in another patient population is required to verify these hypotheses.

In conclusion, two-dimensional electrophoresis-based proteomics is an efficient tool for biomarker distinction in colorectal cancer. The study revealed a series of protein alterations during colorectal cancer genesis. Among the candidate differential proteins, absence of transgelin, which was confirmed by immunoblotting and immunohistochemical assays, may serve as a useful molecular marker for colorectal cancer development. DNA hypermethylation of transgelin was important in the regulation of transgelin transcription in colorectal cancer. Further, diminished transgelin expression was also found as a significant prognostic marker of poor survival in colorectal cancer patients. Further studies are needed to clarify the mechanism by which transgelin is involved in the development and progression of colorectal cancer.

Acknowledgement

This work was financially supported by Key Science and Technology Research Program of Guangdong Province (No. 2003A308401), National Natural Science Foundation of China (Nos. 30670967 and 30770977) and Natural Science Foundation of Guangdong Province (No. 5200512).

References

1 Tan S, Seow TK, Liang RC, *et al.* Proteome analysis of butyrate-treated human colon cancer cells (HT-29). Int J Cancer 2002;98:523–531.
2 You WC, Jin F, Devesa S, *et al.* Rapid increase in colorectal cancer rates in urban Shanghai, 1972–97, in relation to dietary changes. J Cancer Epidemiol Prev 2002;7:143–146.
3 Li S, Wang J, Lu Y, *et al.* Screening and early diagnosis of colorectal cancer in China: a 12 year retrospect (1994–2006). J Cancer Res Clin Oncol 2007;133: 679–686.
4 Bellacosa A. Genetic hits and mutation rate in colorectal tumorigenesis: versatility of Knudson's theory and implications for cancer prevention. Genes Chromosomes Cancer 2003;38:382–388.
5 Rajagopalan H, Nowak MA, Vogelstein B, *et al.* The significance of unstable chromosomes in colorectal cancer. Nat Rev Cancer 2003;3:695–701.
6 Redston M. Carcinogenesis in the GI tract: from morphology to genetics and back again. Mod Pathol 2001;14:236–245.
7 Celis JE, Celis P, Ostergaard M, *et al.* Proteomics and immunohistochemistry define some of the steps involved in the squamous differentiation of the bladder transitional epithelium: a novel strategy for identifying metaplastic lesions. Cancer Res 1999;59:3003–3009.
8 Zhao L, Liu L, Wang S, *et al.* Differential proteomic analysis of human colorectal carcinoma cell lines metastasis-associated proteins. J Cancer Res Clin Oncol 2007;133:771–782.
9 Lees-Miller JP, Heeley DH, Smillie LB, *et al.* Isolation and characterization of an abundant and novel 22-kDa protein (SM22) from chicken gizzard smooth muscle. J Biol Chem 1987;262:2988–2993.

10 Nishida W, Kitami Y, Hiwada K. cDNA cloning and mRNA expression of calponin and SM22 in rat aorta smooth muscle cells. Gene 1993;130:297–302.
11 Shanahan CM, Weissberg PL, Metcalfe JC. Isolation of gene markers of differentiated and proliferating vascular smooth muscle cells. Circ Res 1993;73:193–204.
12 Shapland C, Hsuan JJ, Totty NF, *et al.* Purification and properties of transgelin: a transformation and shape change sensitive actin-gelling protein. J Cell Biol 1993;121:1065–1073.
13 Shapland C, Lowings P, Lawson D. Identification of new actin-associated polypeptides that are modified by viral transformation and changes in cell shape. J Cell Biol 1988;107:153–161.
14 Shields JM, Rogers-Graham K, Der CJ. Loss of transgelin in breast and colon tumors and in RIE-1 cells by Ras deregulation of gene expression through Raf-independent pathways. J Biol Chem 2002;277:9790–9799.
15 Genini M, Schwalbe P, Scholl FA, *et al.* Isolation of genes differentially expressed in human primary myoblasts and embryonal rhabdomyosarcoma. Int J Cancer 1996;66:571–577.
16 Zuber J, Tchernitsa OI, Hinzmann B, *et al.* A genome-wide survey of RAS transformation targets. Nat Genet 2000;24:144–152.
17 Klade CS, Voss T, Krystek E, *et al.* Identification of tumor antigens in renal cell carcinoma by serological proteome analysis. Proteomics 2001;1:890–898.
18 Li LS, Kim H, Rhee H, *et al.* Proteomic analysis distinguishes basaloid carcinoma as a distinct subtype of nonsmall cell lung carcinoma. Proteomics 2004;4:3394–3400.
19 Shi YY, Wang HC, Yin YH, *et al.* Identification and analysis of tumor-associated antigens in hepatocellular carcinoma. Br J Cancer 2005;92:929–934.
20 Zhang YF, Liu L, Ding YQ. Isolation and characterization of human colorectal cancer cell subline with unique metastatic potential in the liver. J South Med Univ 2007;27:126–130.
21 Ramagli LS. Quantifying protein in 2-D PAGE solubilization buffers. Methods Mol Biol 1999;112:99–103.
22 Yang Z, Chang YJ, Miyamoto H, *et al.* Transgelin functions as a suppressor via inhibition of ARA54-enhanced androgen receptor transactivation and prostate cancer cell growth. Mol Endocrinol 2007;21:343–358.
23 Koukourakis MI, Giatromanolaki A, Simopoulos C, *et al.* Lactate dehydrogenase 5 (LDH5) relates to up-regulated hypoxia inducible factor pathway and metastasis in colorectal cancer. Clin Exp Metastasis 2005;22:25–30.
24 Galizia G, Lieto E, Ferraraccio F, *et al.* Determination of molecular marker expression can predict clinical outcome in colon carcinomas. Clin Cancer Res 2004;10:3490–3499.
25 Resnick MB, Routhier J, Konkin T, *et al.* Epidermal growth factor receptor, c-MET, beta-catenin, and p53 expression as prognostic indicators in stage II colon cancer: a tissue microarray study. Clin Cancer Res 2004;10:3069–3075.
26 Khorana AA, Ryan CK, Cox C, *et al.* Vascular endothelial growth factor, CD68, and epidermal growth factor receptor expression and survival in patients with stage II and stage III colon carcinoma: a role for the host response in prognosis. Cancer 2003;97:960–968.
27 Pei H, Zhu H, Zeng S, *et al.* Proteome analysis and tissue microarray for profiling protein markers associated with lymph node metastasis in colorectal cancer. J Proteome Res 2007;6:2495–2501.
28 Stulik J, Kovarova H, Macela A, *et al.* Overexpression of calcium-binding protein calgranulin B in colonic mucosal diseases. Clin Chim Acta 1997;265:41–55.
29 Roblick UJ, Hirschberg D, Habermann JK, *et al.* Sequential proteome alterations during genesis and progression of colon cancer. Cell Mol Life Sci 2004;61:1246–1255.
30 Friedman DB, Hill S, Keller JW, *et al.* Proteome analysis of human colon cancer by two-dimensional difference gel electrophoresis and mass spectrometry. Proteomics 2004;4:793–811.
31 Zhang M, Volpert O, Shi YH, *et al.* Maspin is an angiogenesis inhibitor. Nat Med 2000;6:196–199.
32 Chen ZM, Wang HL. Alteration of cytokeratin 7 and cytokeratin 20 expression profile is uniquely associated with tumorigenesis of primary adenocarcinoma of the small intestine. Am J Surg Pathol 2004;28:1352–1359.
33 Alaiya AA, Roblick UJ, Franzen B, *et al.* Protein expression profiling in human lung, breast, bladder, renal, colorectal and ovarian cancers. J Chromatogr B Analyt Technol Biomed Life Sci 2003;787:207–222.
34 Lees-Miller JP, Heeley DH, Smillie LB. An abundant and novel protein of 22 kDa (SM22) is widely distributed in smooth muscles. Purification from bovine aorta. Biochem J 1987;244:705–709.
35 Lawson D, Harrison M, Shapland C. Fibroblast transgelin and smooth muscle SM22alpha are the same protein, the expression of which is down-regulated in many cell lines. Cell Motil Cytoskeleton 1997;38:250–257.
36 Yeo M, Kim DK, Park HJ, *et al.* Loss of transgelin in repeated bouts of ulcerative colitis-induced colon carcinogenesis. Proteomics 2006;6:1158–1165.
37 Prinjha RK, Shapland CE, Hsuan JJ, *et al.* Cloning and sequencing of cDNAs encoding the actin cross-linking protein transgelin defines a new family of actin-associated proteins. Cell Motil Cytoskeleton 1994;28:243–255.
38 Jones PA. Altering gene expression with 5-azacytidine. Cell 1985;40:485–486.
39 Yamamura H, Masuda H, Ikeda W, *et al.* Structure and expression of the human SM22alpha gene, assignment of the gene to chromosome 11, and repression of the promoter activity by cytosine DNA methylation. J Biochem 1997;122:157–167.
40 Hirasawa Y, Arai M, Imazeki F, *et al.* Methylation status of genes upregulated by demethylating agent 5-aza-2′-deoxycytidine in hepatocellular carcinoma. Oncology 2006;71:77–85.

Supplementary Information accompanies the paper on Modern Pathology website (http://www.nature.com/modpathol)