Digital Transcript Profile Analysis With aRNA-LongSAGE Validates FERMT1 as a Potential Novel Prognostic Marker for Colon Cancer

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Grant support: This research was supported by the Key Basic Research Project of the Science and Technology Commission of Shanghai Municipality (05JC14029); the Program for Outstanding Medical Academic Leader of Shanghai Municipality (LJ06024); the National High-Technology Research and Development Program (“863” Program) of China (2007AA022003); and the Leading Project of the Science and Technology Commission of Shanghai Municipality (09411963500).
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Running title: LCM-LongSAGE analysis of colon cancer biomarkers

Nonstandard abbreviations

AHCY, adenosylhomocysteinase; FERMT1, fermitin family homolog 1; SAGE, serial analysis of gene expression; LongSAGE, long serial analysis of gene expression; LCM, laser capture microdissection; TMA, tissue microarrays; OS, overall survival; DFS, disease-free survival.
Translational Relevance

Upregulation of FERMT1 in colon carcinomas, compared to matched uninvolved epithelium was identified via laser capture microdissection–assisted specimen preparation and antisense RNA-long serial analysis of gene expression. High FERMT1 protein expression was found to be associated with lymph node metastasis, late AJCC stage, poor differentiation, positive vascular invasion, and poor patient survival. Therefore, FERMT1 may be used as a prognostic biomarker for identifying the poor prognosis subset of patients with colon cancer.
Abstract

**Purpose:** To use gene transcript profiling to identify cancer-associated gene expression.

**Experimental Design:** Methods included (1) marker discovery using laser capture microdissection (LCM)–assisted specimen preparation and antisense RNA-long serial analysis of gene expression (aRNA-LongSAGE) on matched colon cancer and uninvolved colon tissue specimens \(n = 5\). Candidate tumor-associated genes were selected by combining the LongSAGE libraries reported herein with our previous colon cancer LCM-microarray transcript profiling data; (2) marker selection and validation by real-time quantitative polymerase chain reaction (qPCR; \(n = 15\)) and immunohistochemistry \(n = 31\); and (3) independent validation on multiple tissue microarray \(n = 203\).

**Results:** Among 30 upregulated and 73 downregulated genes, upregulation of FERMT1, AHCY, SCRN1, and SAC3D1 expression and downregulation of IGJ and MALL expression in colon cancer were confirmed by qPCR. FERMT1 and AHCY protein expression was also upregulated in colon cancer compared with uninvolved colon mucosa, and FERMT1 expression showed upregulation in colon adenoma. Patients with moderate/strong tumor FERMT1 protein expression \(n = 122\) showed significantly poorer overall survival (OS) \(P = 0.011\) and disease-free survival (DFS) \(P = 0.005\) than patients with negative/weak tumor FERMT1 protein expression \(n = 81\). Multivariate Cox regression analysis showed that FERMT1 protein expression was also an independent prognostic factor for OS \(P = 0.018\) and DFS \(P = 0.009\). In addition, upregulated FERMT1 protein expression appeared to have some specificity among alimentary tract tumors.

**Conclusions:** FERMT1 is a novel prognostic factor for colon carcinoma.
**Key words**: colon carcinoma; FERMT1; laser capture microdissection; microarray; serial analysis of gene expression
Colorectal cancer is the fourth most common cancer in men and the third most common cancer in women worldwide. In China and other economically transitioning countries, colon cancer incidence rates have been rising rapidly, most likely owing to changes in lifestyle and nutritional habits (1). Laboratory investigations of differences in gene expression between normal cells and their corresponding carcinoma cells are crucial to understanding how oncogenes and tumor suppressor genes alter complex cellular functions and thus drive tumor progression (2). In colon cancer, a large number of disease biomarkers have been associated with clinical outcomes, including adenomatous polyposis coli (APC), beta-catenin, DNA mismatch repair genes, growth factors and their receptors, cell adhesion molecules, and more recently microRNAs and colon epithelial stem cell markers (3–9). Despite this rapidly accumulating knowledge, there remains a need for biomarkers of disease progression that can be utilized in a preventive strategy to stratify patients into appropriate screening, surveillance, treatment, and prevention programs.

At present, cDNA microarrays and serial analysis of gene expression (SAGE) are the most widely used techniques for determining gene expression levels and ratios in different disease states and in cells under different physiologic conditions (10). cDNA microarrays are used to measure the relative gene expression levels of thousands of known transcripts in different cell and tissue samples (11–14). SAGE is based on the high-throughput sequencing of concatemers of short sequence tags that originate from a known position within a transcript and therefore theoretically contain sufficient information for identification of a unique transcript. SAGE data and techniques have been utilized by a number of investigators to identify genes with differential regulation and potential contributions to colon cancer
progression (15–18). However, annotation of the short, 10-bp sequence tags may identify more than one transcript (19, 20). This lack of specificity can be overcome by using LongSAGE libraries that contain longer, 17-bp tags, thus generating more reliable mapping to Unigene clusters or the complete genome sequence (21–23).

Cellular heterogeneity within patient samples very often presents a challenge to accurate tissue- or tumor-specific gene expression profiling. Gene expression measurements can become skewed owing to tissue heterogeneity, which in turn significantly confounds attempts at statistical analyses. This challenge can be overcome via the use of laser capture microdissection (LCM) to obtain purified cell populations from heterogeneous tissue, resulting in the derivation of precise information on the gene expression profiles of defined cell types (2, 24). Tissue microarrays (TMAs) can then be used to confirm gene expression in the clinical context.

In the present study, we first present a LCM-LongSAGE protocol for investigating global gene expression profiling of parenchymal cells in colon carcinoma. This protocol allowed for the rapid, quantitative measurement of genome-wide gene expression in specific cell populations. Differential regulation of a subset of genes was validated by quantitative real-time polymerase chain reaction (qPCR), immunohistochemistry, and clinical correlations with patient characteristics, tumor histopathology, and patient outcomes.

Materials and Methods

Construction of LongSAGE libraries. Use of amplified antisense RNA (aRNA) for transcript profile analysis of colon cancer cells and corresponding uninvolved colon
epithelium by LCM-microarray was described previously (25); this was used for the generation of LongSAGE libraries. aRNA was adjusted to a final volume of 10 μL with diethylpyrocarbonate-treated H₂O. A total of 2 μL of SAGE-random primer (5’-NNN NNN CAT G-3’; Eurofins MWG Operon, Ebersberg, Germany) and 1 μL dNTP mix (10 mM; Promega, Madison, WI, USA) were added to the aRNA, followed by 65°C incubation for 5 min and then frozen on dry ice. A total of 4 μL first strand buffer, 1 μL 0.1 M dithiothreitol, 1 μL RNaseOUT, and 1 μL SuperScript III RNase H⁻ reverse transcriptase were added to complete the reverse transcription reaction (Invitrogen, Breda, the Netherlands). The reaction was incubated for 5 min at 37°C, 60 min at 50°C, and 15 min at 70°C. RNase H (2 U/μL; USB Corp., Cleveland, OH, USA), was then added before a final incubation at 37°C for 20 min (2). LongSAGE libraries were then generated with an I-SAGE Long Kit according to the manufacturer’s instructions (Invitrogen).

A total of 2422 concatemer clones for colon cancer cells and 2600 concatemer clones for uninvolved colon epithelium were subjected to direct sequencing (ABI 3730 system, Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s instructions. All sequence files were processed with SAGE2000 software. Tags were matched to the human reliable tag database SAGEmap_Hs_NlaIII_17_best.gz (ftp://ftp.ncbi.nih.gov/pub/sage/mappings/).

**Comparison of gene expression profiles between two platforms.** Differentially expressed genes from the microarray platform were screened according to the protocol in the Affymetrix *Statistical Algorithms Descriptions Document*. The population of genes displaying overlapping differential expression as revealed by microarray and LongSAGE
analysis ($P < 0.05$) was selected with Microsoft Office Access 2003. Expression trends of these overlapping genes were compared between the two platforms with criteria for screening candidate tumor-associated genes for differential expression of a $P$ value $< 0.05$ in the LongSAGE libraries and a $\geq 4$-fold change in the cDNA microarray data.

**Validation by qPCR.** Matched colon cancer and uninvolved colon specimens ($N = 15$) were collected, snap frozen, and stored at $-80^\circ$C. Histopathology confirmed that each tumor sample contained at least 70% tumor cells and that the uninvolved colon samples contained $\geq 90\%$ epithelial cells. Total RNA was extracted with an RNEasy kit (Qiagen, Dusseldorf, Germany), and single-stranded cDNAs were synthesized with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). qPCR reactions were performed with SYBER Green PCR Master Mix (Applied Biosystems) and Mastercycler ep realplex (Eppendorf, Hamburg, Germany). Primers used for qPCR are listed in Supplemental Table 1. 18S rRNA was used as an internal control. Each qPCR reaction for each gene was performed in triplicate. Fold changes in expression were calculated and transformed to log2 with the following calculations: ratio $= 2^{\Delta\Delta C_t}$; log2 ratio $= \Delta\Delta C_t$.

**Tissue microarray for validation of protein expression for select genes.** A total of 203 radical colectomy specimens from 86 men and 117 women 22 to 95 years of age were obtained from the Department of Surgery, Shanghai Jiao Tong University Affiliated First People’s Hospital, and used for constructing the tissue microarray (TMA). The TMA included 24 stage I, 81 stage II, and 80 stage III, and 18 stage IV cancers; 99 well-differentiated, 74 moderately differentiated, and 30 poorly differentiated cancers; and 95 specimens with positive lymph node metastasis and 108 specimens with negative lymph node metastasis. All
tumors were adenocarcinomas and were graded according to World Health Organization
criteria (48). Tumor staging was conducted according to the American Joint Committee on
Cancer (AJCC) sixth edition cancer staging system (26). All patients with stage III or IV
disease were administered standard chemotherapeutic protocols with 5-fluorouracil after
operation at our institution. Clinical data were collected via medical record review. The final
patient follow-up date was June 29, 2008, and the median observation time for survivors was
61 months (range 9–89 months). All patients provided informed consent according to a
protocol approved by the Institutional Review Board of Shanghai First People’s Hospital.
None of the patients received any treatment for colon cancer prior to surgery. From each
colecotomy specimen, two cores each of primary cancer and uninvolved tissue adjacent to the
tumor at a distance of at least 2 mm from the tumor were arrayed. TMAs were created using a
Tissue Microarrayer (Beecher Instruments, Sun Prairie, WI, USA).

Additional TMAs were purchased from Outdo Biotech (Shanghai, China). The
standard tissue section OD-CT-DgCol03-001 TMA included 31 matched colon carcinoma and
uninvolved colon tissues, and the OD-CT-DgCol01-001 TMA included 22 colon carcinoma
and 20 colon adenoma specimens. The OD-CT-Dg03-001 TMA included 5 pairs of cancer
tissue and corresponding uninvolved tissue from liver carcinoma, esophageal carcinoma,
gastric carcinoma, colon carcinoma, and rectal carcinoma, and six paired samples of
pancreatic carcinoma and corresponding uninvolved pancreas tissue. A flow chart of the
screening strategy is presented in Supplemental Fig. 1.

**Immunohistochemistry.** Fermitin family member 1 (FERMT1) protein expression
was detected on the OD-CT-DgCol03-001, OD-CT-DgCol01-001, and OD-CT-Dg03-001
TMAAs and our 203-case colon cancer TMA with a polyclonal rabbit antibody (U1610-01; United States Biological, Swampscott, MA, USA; diluted 1:1000). This antibody showed a single protein band by Western blot at this dilution (Supplemental Fig. 2).

Adenosylhomocysteinase (AHCY) protein expression was detected on OD-CT-DgCol03-001 with a polyclonal rabbit antibody (10757-2-AP; Proteintech Group, Chicago, IL, USA; diluted 1:100). A goat anti-rabbit IgG (Dako, Carpinteria, CA, USA) was used as a secondary antibody. Tissue sections were counterstained with Mayer hematoxylin. Positive staining was scored by two independent investigators without knowledge of patient outcomes (double-blinded; HT [coauthor] and HH [Dept. of Pathology, Shanghai First People’s Hospital]) then classified into four groups: negative, weakly positive, moderately positive, and strongly positive according to percentage of positive cells and staining intensity. A mean value of the two independent scores was calculated for each sample. When different values were reported for a given sample, the samples were re-evaluated, and if they were still different, the investigators discussed until a unanimous agreement was reached.

**Statistical analysis.** Continuous variables are presented as the mean ± standard deviation (SD). Categorical variables are expressed as the number (n) and percentage and were compared with the Fisher exact test (27). FERMT1 and AHCY staining for the 31 matched uninvolved and carcinoma tissues were compared with the signed-rank test (28). Overall survival (OS) and disease-free survival (DFS) curves were calculated with the Kaplan-Meier method with log-rank test (29). To investigate independent risk factors for death and lymph node metastasis, Cox proportional hazard models were used (30) and expressed as the hazard ratio (HR) with 95% confidence intervals (CI). Significant factors in
the univariate Cox proportional hazard models were selected for the final multivariate Cox proportional hazard model with the forward conditional method (31). All statistical analyses were set with a significance level of 0.05 and were performed with SPSS 15.0 statistical software (SPSS Inc., Chicago, IL, USA).

Results

**LongSAGE data libraries.** Pure cell populations were collected from 203 paired colon carcinoma and uninvolved mucosa specimens by LCM and were used to create a LongSAGE library. A total of 100,666 tags were generated including 45,560 unique tags (Supplemental Table 2). The duplication ditag proportions were 6.47 in the uninvolved colon LongSAGE library and 6.15 in the colon carcinoma LongSAGE library. These tags were divided into four groups according to their abundance in the libraries. The 848 transcripts with $P$ value < 0.05 were screened for differential gene expression between uninvolved colon and tumor tissues.

When the results from LCM-LongSAGE and TMAs were compared, 404 genes showed overlapping differential transcripts in the two platforms. Among these transcripts, the expression trend of 388 transcripts representing 326 unique genes was identical between the two platforms. A total of 30 upregulated genes and 73 downregulated genes met our criteria for screening candidate tumor-associated genes. The top 20 upregulated and downregulated genes are presented in Supplemental Tables 3 and 4, respectively. Eight of these upregulated genes, including FERMT1 and AHCY (32–36), have been previously reported as being upregulated in colon cancer, whereas 12 represented newly identified upregulated genes.
Likewise, differential regulation of eight of the downregulated genes has been previously reported in colon cancer.

**qPCR for validation of altered gene expression.** qPCR was performed to validate the expression of six differentially expressed genes, four with significantly upregulated expression, and two with significantly downregulated expression. Our rationale for focusing on these genes was based on fold difference in expression, the potential function of these genes in tumor development, and the fact that there have been few or no detailed studies on these genes. Upregulation of FREMT1, AHCY, SCRN1, and SAC3D1 expression and downregulation of immunoglobulin J (IgJ) and MAL-like protein (MALL) expression in colon carcinomas were confirmed by qPCR (Supplemental Fig. 3).

**Immunohistochemical staining of matched cancer and uninvolved colon tissues for AHCY and FERMT1 protein expression.** Among FERMT1, AHCY, secernin-1 (SCRN1), and SAC3 domain-containing protein 1 (SAC3D1), there were no commercially available antibodies for SAC3D1, and antibody for SCRN1 resulted in ambiguous immunohistochemical staining. Antibodies for FERMT1 and AHCY were found to be useful for titration and staining, but preliminary results indicated that AHCY protein expression was not a significant prognostic factor for tumor outcome. Therefore, we focused on FERMT1. For further confirmation of altered gene expression leading to changes in protein levels, AHCY and FERMT1 protein expression was detected on a commercial TMA containing 31 matched colon carcinoma and uninvolved colon tissues. Semi-quantitative immunohistochemical results are presented in Fig. 1A. Significantly stronger AHCY and FERMT1 staining was observed in carcinomas compared to uninvolved colon tissue ($P <$...
Among the 31 matched tissues, 30 (96.8%) showed stronger FERMT1 staining in carcinoma tissues than in uninvolved colon tissues, and no matched tissue showed stronger FERMT1 staining in uninvolved colon tissues than in carcinoma tissues. Similar results were found for AHCY staining, with 24 (77.4%) matched tissues showing stronger AHCY staining in carcinoma tissues than in uninvolved colon tissues, and again no matched uninvolved tissues showed stronger AHCY staining than in respective carcinoma tissues (Table 1).

FERMT1 protein expression detected in multiple TMAs. Differential expression of FERMT1 in colon cancer has been shown at the mRNA level (32), but changes in FERMT1 protein expression in colon cancer have not been reported. To explore changes in FERMT1 protein expression during the progression of colon cancer, FERMT1 protein was detected on a TMA that included 22 colon carcinoma and 20 colon adenoma specimens. Strong or moderate FERMT1 protein–positive staining was detected in 72.7% (16/22) of the colon carcinomas and 15% (3/20) of the colon adenomas ($P = 0.0002$, Fig. 1B). Thus, FERMT1 may be progressively upregulated during colon cancer development.

Immunohistochemical detection of FERMT1 protein expression was also performed on a TMA comprised of multiple types of alimentary tumors (Fig. 1C–N). All esophageal carcinomas and corresponding uninvolved esophageal tissues showed weak/negative FERMT1 protein expression. Likewise, similar moderate FERMT1 protein expression was detected in two pairs of matched liver carcinoma and uninvolved liver tissue, but FERMT1 protein was downregulated in the other three cases of liver carcinoma. FERMT1 protein was also downregulated in three cases of pancreatic carcinoma and had similar expression between carcinoma and uninvolved tissue in the other three cases. Weak FERMT1 protein
staining was detected in all of the uninvolved gastric tissue and two gastric carcinoma specimens, whereas three gastric carcinoma tissues showed moderate FERMT1 protein staining. In contrast, FERMT1 protein was upregulated in all of the colon and rectal carcinomas on the TMA. Thus, upregulated FERMT1 protein expression appeared to support the tissue-specific nature of FERMT1 expression.

Clinicopathologic significance of FERMT1 and AHCY protein expression in colon cancer. The clinicopathologic significance of FERMT1 and AHCY is summarized in Table 2. Overexpression of FERMT1 was significantly associated with lymph node metastasis ($P = 0.006$), AJCC stage ($P = 0.012$), tumor differentiation ($P = 0.036$), and vascular invasion ($P = 0.049$). Interestingly, AJCC stage III and moderately differentiated tumors showed the highest FERMT1 expression. Increased AHCY protein expression was also associated with advanced AJCC stage ($P = 0.021$), but unlike FERMT1, AHCY was associated with advanced tumor T stage ($P = 0.002$) and metastasis ($P = 0.004$).

Patient overall and disease-free survival. Given that eight patients with stage IV disease underwent noncurative surgery, a total of 195 patients were included in the following survival analyses to avoid the possible confounding effects of unresectable metastatic tumors. Results of the OS analyses are presented in Fig. 2 and Table 3. Patients with negative/weak and moderate/strong tumor FERMT1 protein expression had a 5-year survival rate of 77.6% and 60.8%, respectively, with significantly worse OS for patients with moderate/strong FERMT1 expression ($P = 0.011$). However, there was no significant difference between the 2 AHCY protein expression groups in OS.

T stage, AJCC stage, differentiation, and FERMT1 expression showed significant
effects on OS in multivariate Cox models and were included into the final multivariate Cox
proportional hazard model. As seen in Table 3, the risk of death for patients with T3 stage
disease was significantly lower than patients with T4 stage disease (HR [95% CI]: 0.38 [0.21,
0.67]). The risk of death for those with AJCC stage IV was significantly higher than those
with AJCC stage I (HR [95% CI]: 14.68 [1.33, 161.83]), and the risk of death for those with
moderate or poor tumor differentiation was significantly higher than for patients with
well-differentiated tumors (HR [95% CI]: 1.81 [1.01, 3.24] for moderate differentiation and
3.05 [1.52, 6.14] for poor differentiation). Finally, the risk of death for patients with
moderate/strong tumor FERMT1 expression was significantly higher than for patients with
negative/weak FERMT1 expression (HR [95% CI]: 1.92 [1.12, 3.30]).

The results of the DFS analyses are presented in Fig. 3 and Table 3. Patients with
negative/weak and moderate/strong tumor FERMT1 protein expression had 5-year DFS rates
of 69.8% and 54.7% respectively. Kaplan-Meier survival curves showed that patients with
moderate/strong tumor FERMT1 protein expression had significantly worse DFS than
patients with negative/weak tumor FERMT1 protein expression ($P = 0.013$).

Similar to the Cox models for OS, T stage, AJCC stage, differentiation, and FERMT1
expression showed independent effects on the risk of disease and were included into the final
multivariate Cox proportional hazard model. As seen from the table, the risk of recurrent
disease for patients with T3 stage tumors was significantly lower than for patients with T4
stage tumors (HR [95% CI]: 0.42 [0.24, 0.71]). The risk of recurrent disease for patients with
AJCC stage IV tumors was significantly higher than for patients with AJCC stage I tumors
(HR [95% CI]: 9.06 [1.01, 81.34]); the risk of disease for patients with moderate or poor
tumor differentiation was significantly higher than for patients with well differentiated tumors (HR [95% CI]: 1.87 [1.11, 3.16] for moderate differentiation and 2.56 [1.27, 5.17] for poor differentiation). Finally, the risk of recurrent disease for patients with moderate/strong FERMT1 expression were significantly higher than for patients with negative/weak FERMT1 expression (HR [95% CI]: 1.99 [1.19, 3.35]).

Discussion

In the present study, LCM–assisted tissue specimen preparation was combined with cDNA microarray and LongSAGE analysis for the rapid identification and validation of tumor-associated genes in colon cancer. Combined analyses revealed significant upregulation of 30 genes and downregulation of 73 genes in colon carcinoma. Upregulation of FERMT1, AHCY, SCRN1, and SAC3D1 expression and downregulation of IGJ and MALL expression in colon cancer were confirmed by real-time PCR. Upregulated FERMT1 protein expression in colon carcinoma was associated with significantly poorer DFS and OS and was confirmed as an independent prognostic factor for DFS.

The FERMT1 protein is expressed predominantly in skin, intestine, and kidney. It localizes to cell junctions and regulates integrin function to facilitate the linkage of cell adhesion structures to the actin cytoskeleton (37). As a crucial connector between cytoskeletal structures and the extracellular matrix, FERMT1 may also be involved in the assembly and stabilization of actin filaments and likely plays a role in modulating cell adhesion, morphology, and motility (37). FERMT1 mutations causes Kindler syndrome in humans, an autosomal recessive form of genodermatosis (38). Interestingly, FERMT1 may
also have roles in neonatal intestinal development and maintenance of epithelial intestinal barrier function (39).

FERMT1 has also been suggested to have a role in human cancer following a report of significant upregulation of FERMT1 mRNA in lung (60-fold) and colon (6-fold) carcinomas as identified by microarray analysis and confirmed by quantitative RT-PCR (32). In that report, FERMT1 was overexpressed in 70% of the colon carcinomas and 60% of the lung carcinomas tested. In the present study, 66.7% of colon carcinomas showed an increase in FERMT1 expression by 3- to 28-fold, with a mean fold increase of 7.9-fold.

Transforming growth factor-β1 (TGF-β1) contributes to tumor invasion and cancer progression by increasing the motility of tumor cells (40–42). Interestingly, FERMT1 is regulated by TGF-β1, and a TGF-β1–induced increase in FERMT1 expression results in increased cell spreading correlated with epithelial to mesenchymal cell transition, an important step in carcinogenesis (43). It has also been reported that FERMT1 protein is predictive of breast cancer lung metastasis (44). In the present study, the majority of uninvolved colon tissues and colon adenoma tissues were negative for FERMT1 expression, whereas moderate or strong FERMT1 protein expression was detected in the majority of colon carcinomas. High FERMT1 protein expression was also associated with aggressive tumor phenotypes, including positive lymph node metastasis, late AJCC stage, poor differentiation, positive vascular invasion, and poor patient survival. Interestingly, upregulated FERMT1 protein expression showed some specificity amongst alimentary tract tumors and may be a useful prognostic biomarker in colon cancer. Although a definitive mechanistic role for FERMT1 in colon cancer progression will need to be confirmed by
additional experimentation with techniques such as RNA interference in model systems, existing data suggest a strong biologic basis for the association reported herein.

AHCY catalyzes the reversible hydrolysis of S-adenosylhomocysteine to adenosine and L-homocysteine. Thus, it regulates the intracellular S-adenosylhomocysteine concentration, which is thought to be important for transmethylation reactions (GeneCards v.3. http://www.genecards.org/cgi-bin/carddisp.pl?gene=AHCY&search=AHCY ).

Upregulation of AHCY gene expression in colorectal carcinoma compared to uninvolved colon mucosa has been reported in three independent studies of transcript profile analysis with cDNA microarrays (33–36). However, validation of differential AHCY protein expression in colon cancer has not been reported. In the present study, we report the first confirmation of elevated AHCY mRNA and protein expression in colon cancer compared to uninvolved colon tissue. Mechanistic studies of AHCY contributions to colon cancer development remain a topic for future investigations.

Comparison of research results obtained with microarrays and SAGE is very complicated owing to the challenges associated with selecting differentially expressed genes from microarray and SAGE data. Fold change and P values are two commonly used criteria for selecting differentially expressed genes. Use of these two ranking criteria often produces different lists of differentially expressed genes (45, 46). In the present study, 9.4% (404/4300) of the differentially expressed transcripts identified in the microarray data and 47.6% (404/848) of the differentially expressed transcripts in the SAGE data were identical. These data indicate that the intraplatform correlation was modest. Most of the overlapping transcripts (388/404, 96.0%) between the two platforms showed identical gene expression
trends, demonstrating the reliability of LongSAGE data. Among the top 20 upregulated genes and downregulated genes represented by the overlapping transcripts, the differential expression of 40.0% (16/40) of the genes have been confirmed in other reports. The present study validated the differential expression of six of these genes by qPCR.

It has been reported that antibody concentration can affect the apparent relation between biomarker expression and outcome (47). A potential limitation of the present study is that although we performed antibody titration for each antibody used for immunohistochemistry at the beginning of the study, and a single FERMT1 protein band was obtained by Western blot at an antibody dilution of 1:1000 (the same dilution as that used for immunohistochemistry), the fact that we used a single antibody dilution for immunohistochemistry may have led to results that may have differed from those had we used multiple dilutions. Further studies will clarify this issue.

In conclusion, we present the first report using LCM-LongSAGE for quantitative measurement of genome-wide gene expression in paired colon carcinomas and uninvolved colon mucosa. Gene profiling results were verified via evaluation of differential protein expression in more than 200 patients, together with histopathologic and clinical correlation with different stages of disease and patient outcomes. This new, comprehensive approach to the accurate and rapid identification and validation of tumor-associated genes in colon cancer may serve as a model for future investigations. Identification and validation of upregulated FERMT1 protein expression as a molecular event in late-stage tumor progression demonstrated that FERMT1 is a new prognostic biomarker in colon cancer for predicting patient outcomes.
Disclosure of Potential Conflicts of Interest:

None to declare.

Acknowledgments

The authors wish to thank Dr. H Hu, Dept. of Pathology, Shanghai First People’s Hospital, for review of stained samples.

This research was supported by the Key Basic Research Project of the Science and Technology Commission of Shanghai Municipality (05JC14029); the Program for Outstanding Medical Academic Leader of Shanghai Municipality (LJ06024); the National High-Technology Research and Development Program (“863” Program) of China (2007AA022003); and the Leading Project of the Science and Technology Commission of Shanghai Municipality (09411963500).
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Figure Legends

Fig. 1. Immunohistochemical validation of AHCY and FERMT1 protein expression in multiple tissue microarrays. (A) Immunohistochemical staining of 31 matched colon carcinoma and uninvolved colon tissue specimens was performed with specific antibodies against AHCY and FERMT1. AHCY and FERMT1 protein expression were confirmed to be upregulated in colon cancer compared to uninvolved colon mucosa (P <0.001). (B) Immunohistochemical staining of 22 colon carcinomas and 20 colon adenomas was performed with a FERMT1-specific antibody. FERMT1 protein expression was upregulated in colon cancer compared to colon adenoma (P = 0.0002). Representative photomicrographs of FERMT1 protein expression in colon cancer (C) and uninvolved colon mucosa (D). Representative photomicrographs of AHCY protein expression in colon cancer (E) and uninvolved colon mucosa (F). Representative photomicrographs of FERMT1 protein expression in liver cancer (G) and uninvolved liver tissue (H). Representative photomicrographs of FERMT1 protein expression in pancreatic cancer (I) and uninvolved pancreas tissue (J). Representative photomicrographs of FERMT1 protein expression in esophageal cancer (K) and uninvolved esophageal tissue (L). Representative photomicrographs of FERMT1 protein expression in gastric cancer (M) and uninvolved gastric tissue (N).

Fig. 2. Kaplan-Meier survival curves for overall survival (OS) as determined by expression of FERMT1 and AHCY. (A) Patients with moderate/strong FERMT1 expression (n = 122) showed significantly poorer OS (P = 0.011) than patients with negative/weak FERMT1
expression \((n = 81)\). \((B)\) No significant difference in OS was detected based on AHCY expression \((P = 0.082)\).

**Fig. 3.** Kaplan-Meier survival curves for disease-free survival (DFS) as determined by expression of FERMT1 and AHCY. \((A)\) Patients with moderate/strong FERMT1 expression \((n = 122)\) showed significantly poorer DFS \((P = 0.013)\) than patients with negative/weak FERMT1 expression \((n = 81)\). \((B)\) No significant difference in DFS was detected based on AHCY expression \((P = 0.397)\).
Table 1. FERMT1 and AHCY immunohistochemical staining for protein expression in matched colon cancer and uninvolved colon tissues

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<tr>
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<td>6 (19.4%)</td>
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<td>27 (87.1%)</td>
<td>1 (3.2%)</td>
<td>3 (9.7%)</td>
<td>0 (0%)</td>
<td>31 (100%)</td>
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<tr>
<td>AHCY</td>
<td>Negative</td>
<td>6 (19.4%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>6 (19.4%)</td>
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<td>Moderate</td>
<td>2 (6.5%)</td>
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<td>0 (0%)</td>
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<td>8 (25.8%)</td>
</tr>
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<td>Strong</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
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<tr>
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<td>Total</td>
<td>24 (77.4%)</td>
<td>7 (22.6%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>31 (100%)</td>
</tr>
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†Significant difference in protein expression in matched colon cancer and uninvolved colon tissues.
Table 2. Association between clinicopathologic features and FERMT1 or AHCY protein expression

<p>|                | Total (n = 203) | FERMT1 Expression | AHCY Expression | p value | | Total (n = 203) | FERMT1 Expression | AHCY Expression | p value |
|----------------|-----------------|-------------------|-----------------|---------|----------------|-----------------|-----------------|---------|
|                | (n = 81) | (n = 122) | P value | (n = 134) | (n = 69) | P value | (n = 134) | (n = 69) | P value |
| Age            |              |                  |                 |          |              |                  |                 |          |        |
| &lt;65 years      | 81 (39.9%) | 38 (46.9%) | 43 (35.2%) | 0.109   | 56 (41.8%) | 25 (36.2%) | 0.455 |
| ≥65 years      | 122 (60.1%) | 43 (53.1%) | 79 (64.8%) |          | 78 (58.2%) | 44 (63.8%) |        |
| Gender         |              |                  |                 |          |              |                  |                 |          |        |
| Male           | 86 (42.4%) | 33 (40.7%) | 53 (43.4%) | 0.772   | 51 (38.1%) | 35 (50.7%) | 0.100 |
| Female         | 117 (57.6%) | 48 (59.3%) | 69 (56.6%) |          | 83 (61.9%) | 34 (49.3%) |        |
| Tumor location |              |                  |                 |          |              |                  |                 |          |        |
| Right          | 84 (41.4%) | 32 (39.5%) | 52 (42.6%) | 0.509   | 58 (43.3%) | 26 (37.7%) | 0.606 |
| Left           | 100 (49.3%) | 39 (48.1%) | 61 (50.0%) |          | 65 (48.5%) | 35 (50.7%) |        |
| T stage        |              |                  |                 |          |              |                  |                 |          |        |
| T1             | 8 (3.9%)   | 4 (4.9%)    | 4 (3.3%)    | 0.321   | 6 (4.5%)    | 2 (2.9%)    | 0.002† |
| T2             | 23 (11.3%) | 12 (14.8%) | 11 (9.0%)  |          | 16 (11.9%) | 7 (10.1%)  |        |
| T3             | 76 (37.4%) | 25 (30.9%) | 51 (41.8%) |          | 61 (45.5%) | 15 (21.7%) |        |
| T4             | 96 (47.3%) | 40 (49.4%) | 56 (45.9%) |          | 51 (38.1%) | 45 (65.2%) |        |</p>
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<th>N1</th>
<th>N stage N0 108 (53.2%)</th>
<th>N1 95 (46.8%)</th>
<th>M0 185 (91.1%)</th>
<th>M1 18 (8.9%)</th>
<th>AJCC stage I 24 (11.8%)</th>
<th>II 81 (39.9%)</th>
<th>III 80 (39.4%)</th>
<th>IV 18 (8.9%)</th>
<th>Differentiation Well 99 (48.8%)</th>
<th>Moderate 74 (36.5%)</th>
<th>Poor 30 (14.8%)</th>
<th>Vascular Absent 189 (93.1%)</th>
<th>Present 14 (6.9%)</th>
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<td>28 (34.6%)</td>
<td>67 (54.9%)</td>
<td>9 (11.1%)</td>
<td>72 (88.9%)</td>
<td>9 (7.4%)</td>
<td>13 (16.0%)</td>
<td>38 (46.9%)</td>
<td>21 (25.9%)</td>
<td>9 (11.1%)</td>
<td>46 (56.8%)</td>
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<td>67 (54.9%)</td>
<td>113 (92.6%)</td>
<td>9 (7.4%)</td>
<td>113 (92.6%)</td>
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<td>11 (9.0%)</td>
<td>43 (35.2%)</td>
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<td>9 (7.4%)</td>
<td>53 (43.4%)</td>
<td>53 (43.4%)</td>
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<td>110 (90.2%)</td>
<td>12 (9.8%)</td>
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<td>57 (42.5%)</td>
<td>128 (95.5%)</td>
<td>6 (4.5%)</td>
<td>128 (95.5%)</td>
<td>6 (4.5%)</td>
<td>16 (11.9%)</td>
<td>59 (44.0%)</td>
<td>53 (39.6%)</td>
<td>6 (4.5%)</td>
<td>64 (47.8%)</td>
<td>48 (35.8%)</td>
<td>22 (16.4%)</td>
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<td>31 (44.9%)</td>
<td>38 (55.1%)</td>
<td>57 (82.6%)</td>
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<td>57 (82.6%)</td>
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<td>8 (11.6%)</td>
<td>22 (31.9%)</td>
<td>27 (39.1%)</td>
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<td>35 (50.7%)</td>
<td>26 (37.7%)</td>
<td>8 (11.6%)</td>
<td>64 (92.8%)</td>
<td>5 (7.2%)</td>
</tr>
</tbody>
</table>

† Significant associations between two categorical variables.
AJCC: American Joint Committee on Cancer.
Table 3. Univariate and multivariate Cox proportional hazard models for overall survival and disease-free survival

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<th>Overall Survival</th>
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<th>Disease-Free Survival</th>
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<td>Multivariate</td>
<td>Univariate</td>
<td>Multivariate</td>
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<tr>
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<td>HR (95% CI)</td>
<td>P value</td>
<td>HR (95% CI)</td>
<td>P value</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥65 years</td>
<td>0.96 (0.61, 1.53)</td>
<td>0.875</td>
<td>0.98 (0.62, 1.55)</td>
<td>0.938</td>
</tr>
<tr>
<td>&lt;65 years</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>0.74 (0.46, 1.20)</td>
<td>0.223</td>
<td>0.88 (0.56, 1.38)</td>
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<tr>
<td>Female</td>
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<td>–</td>
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<td>Tumor location</td>
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<td>–</td>
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<tr>
<td>Transverse</td>
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<tr>
<td>Left</td>
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<td>1.12 (0.70, 1.79)</td>
<td>0.631</td>
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<tr>
<td>T1</td>
<td>0.36 (0.09, 1.46)</td>
<td>0.152</td>
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<td>0.11 (0.03, 0.44)</td>
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<td>0.16 (0.05, 0.52)</td>
<td>0.002 *</td>
</tr>
<tr>
<td>T3</td>
<td>0.34 (0.20, 0.58)</td>
<td>&lt;0.001 *</td>
<td>0.42 (0.26, 0.70)</td>
<td>0.001 *</td>
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<td>N stage</td>
<td>M stage</td>
<td>AJCC stage</td>
<td>Differentiation</td>
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<td>---------</td>
<td>------------</td>
<td>----------------</td>
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<tr>
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<td>–</td>
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<td>–</td>
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<td>14.74 (8.15, 26.67)</td>
<td>2.08 (0.47, 9.21)</td>
<td>2.37 (1.34, 4.18)</td>
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<td>2.08 (0.47, 9.21)</td>
<td>9.51 (2.29, 39.47)</td>
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<tr>
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<td>0.58 (0.05, 6.51)</td>
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<td>AJCC stage</td>
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<td>1.91 (0.18, 9.91)</td>
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<td>0.58 (0.05, 6.51)</td>
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<td>II</td>
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<td>IV</td>
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<tr>
<td>Differentiation</td>
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<td>0.58 (0.05, 6.51)</td>
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<td>Well</td>
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<td>0.58 (0.05, 6.51)</td>
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<tr>
<td>Moderate</td>
<td>2.37 (1.34, 4.18)</td>
<td>1.81 (1.01, 3.24)</td>
<td>0.048*</td>
<td>2.26 (1.35, 3.79)</td>
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<td>Poor</td>
<td>7.50 (4.11, 13.68)</td>
<td>&lt;0.001*</td>
<td>0.002*</td>
<td>4.87 (2.64, 8.97)</td>
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<td>Vascular invasion</td>
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<td>3.05 (1.52, 6.14)</td>
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<td>3.05 (1.52, 6.14)</td>
<td>0.002*</td>
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<td>Present</td>
<td>4.68 (2.55, 8.59)</td>
<td>&lt;0.001*</td>
<td>4.12 (2.16, 7.86)</td>
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<tr>
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<td>4.12 (2.16, 7.86)</td>
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</table>
Weak
Moderate/
Strong 1.91 (1.15, 3.17) 0.013* 1.92 (1.12, 3.30) 0.018* 1.83 (1.12, 2.98) 0.015* 1.99 (1.19, 3.35) 0.009*
Negative/
AHCY
Weak –
Moderate/
Strong 1.50 (0.94, 2.39) 0.085 1.22 (0.77, 1.94) 0.401

CI: confidence interval; HR: hazard ratio; AJCC: American Joint Committee on Cancer.

*P < 0.05 indicates that the 95% CI of HR did not include 1.
Digital Transcript Profile Analysis With aRNA-LongSAGE Validates FERMT1 as a Potential Novel Prognostic Marker for Colon Cancer

Junwei Fan, Dongwang Yan, Mujian Teng, et al.

Clin Cancer Res  Published OnlineFirst January 10, 2011.

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