Promoter hypermethylation inactivate tumour suppressor *FAM134B* and is associated with poor prognosis in colorectal cancer

Running head: Prognostic significance of *FAM134B* methylation

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Abstract

The present study aims to examine promoter methylation status of *FAM134B* in a large cohort of patients with colorectal adenocarcinomas. The clinical significances and correlations of *FAM134B* promoter methylation with its expression are also analysed. Methylation-specific high-resolution melt-curve analysis followed by sequencing was used to identify *FAM134B* promoter methylation in colorectal adenomas (n=32), colorectal adenocarcinomas (n=164), matched adjacent non-neoplastic colorectal mucosae (n=83) and colon cancer cell lines (n=4). *FAM134B* expression was studied by real-time quantitative polymerase chain reaction, immunohistochemistry, and Western blots. *FAM134B* promoter methylation was more frequent in adenocarcinomas (52%; 85/164) when compared to that of adenomas (28%; 9/32) and non-neoplastic mucosae (35%; 29/83). Cancer cells exhibited higher methylation when compared to non-neoplastic cells. *FAM134B* promoter methylation was inversely correlated with low *FAM134B* copy number and mRNA/protein expressions, whereas in-vitro demethylation has restored *FAM134B* expression in colon cancer cells. *FAM134B* promoter methylation was associated with high histological grade ($p = 0.025$), presence of peri-neural infiltration ($p = 0.012$), lymphovascular invasion ($p = 0.021$), lymph node metastasis ($p = 0.0001$), distant metastasis ($p = 0.0001$) and advanced pathological stages ($p = 0.0001$). In addition, *FAM134B* promoter methylation correlated with cancer recurrence and poor survival rates of patients with colorectal adenocarcinomas. To conclude, *FAM134B* promoter methylation plays a key role in regulating FAM134B expression *in-vitro* and *in-vivo*, which in turn contributes to the prediction of the biological aggressiveness of colorectal adenocarcinomas. Furthermore, *FAM134B* methylation might act as a marker in predicting clinical prognosis in patients with colorectal adenocarcinomas.

Keyword: FAM134B, clinical, JK1, methylation, colorectal carcinoma

1. Introduction

DNA methylation is the most common epigenetic alterations involved in the pathogenesis of colorectal carcinoma [1-2]. Promoter hypermethylation mediated inactivation of tumour suppressor or cancer-related genes is one of the frequent genetic events in the pathogenesis of colorectal carcinomas [1, 3-4]. Hypermethylation mediated inactivation of tumour suppressor genes, including *APC, MGMT, VIM, CDKN2A, HML1*, and *SFRP* are associated with the pathogenesis of colorectal carcinoma [2, 5-7]. Hypermethylated guided silencing of these genes plays crucial roles in the early stages of the pathogenesis of colorectal carcinoma, namely in the progression of adenoma to carcinoma [2-3].

FAM134B (*Family with sequence similarity 134, member B*) is also called *JK1* or *RETREG1*. It is located at 5p15.1 and encodes an endoplasmic reticulum-receptor protein that regulates endoplasmic reticulum turnover by selective autophagy [8-10]. Loss of function mutations, absence and aberrant expression of *FAM134B* gene are associated with the pathogenesis of hereditary sensory and autonomic neuropathy type IIB, vascular dementia, allergic rhinitis and viral diseases [11-16]. In addition, genetic and epigenetic changes in *FAM134B* play an important role in the pathogenesis of breast, oesophageal and colorectal carcinomas [17-23]. Recent studies by our group have reported *FAM134B* mutations and its role in predicting the biological aggressiveness in patients with oesophageal and colorectal carcinomas [22-23].

Methylation mediated silencing of tumour suppressor has the potential to be used as a biomarker in colorectal adenocarcinomas [3]. Thus, screening of promoter methylation of *FAM134B* could be helpful to identify prognostic markers for colorectal adenocarcinoma. In the present study, we investigated the promoter methylation status of *FAM134B* in a large cohort of patients with colorectal adenocarcinoma. In addition, correlations of *FAM134B*

promoter methylation with clinicopathological parameters of patients with colorectal adenocarcinoma were investigated.

2. Materials and Methods

2.1 Patient's selection

Tissues samples were prospectively collected from the consecutive patients who had resection for a colorectal pre-invasive lesion (adenoma) and colorectal adenocarcinoma from hospitals in Queensland, Australia. Matched adjacent non-cancer mucoase (near the surgical resection margin) from the patient who underwent resection of colorectal carcinomas were also prospectively collected. All the collected tissues were snap frozen in liquid nitrogen and stored at -80°C until used for the present study. Patients were recruited with no selection bias. The tissue samples were excluded from the study if there are no adequate cancer cells after histological review. Ethical approval for this work has been obtained from the Griffith University Human Research Ethics Committee (GU Ref No: MSC/17/10/HREC). A schematic flow of the experimental design of the present study is presented in Fig. 1.

2.2 Clinicopathological parameters

In each case, the patient's resection specimen was fixed in formalin for pathological examination. Size (maximum dimension) and site of the cancers were recorded on macroscopic examination. Cancer located in the caecum, ascending colon and transverse colon were defined as proximal cancers whereas the cancers found in the region of descending colon, sigmoid colon and rectum defined as distal cancers.

The specimens were then dissected for selection of tissue blocks to be processed in paraffin. Histological sections from the paraffin-embedded formalin fixed tissues were cut and stained with haematoxylin and eosin for light microscopic examination. The pathological features of patients with colorectal adenocarcinoma were analysed by examining the histological sections and were reviewed and graded by a pathologist (AKL). The adenocarcinomas were graded into grade 1 (well differentiated), grade 2 (moderately

differentiated) and grade 3 (poorly-differentiated) according to the WHO classification [24]. The presence of lymphovascular invasion and peri-neural cancer infiltration in all the cancers were documented. The presence of co-existing adenomas or metachronous cancer were identified in every patient. The cancer tissues were investigated for microsatellite instability status (MLH1, PMS2, MSH2 and MSH6 by immunohistochemistry) according to the clinical guidelines. The carcinoma was staged according to the 8th edition of Cancer staging Manual of AJCC (American Joint Committee on Cancer) based on T, N and M staging [25].

After histological review, 164 patients (79 women; 85 men) with colorectal adenocarcinomas, 83 matched non-neoplastic mucosae and 32 adenomas were included in the present study (Fig. 1). Among the 164 adenocarcinomas studied, 19.5% (n = 32) had lymph node metastasis, whereas the other 81.5 % (n = 132) had no lymph node metastasis. There were 14.6 % (n = 24) stage I, 39.6 % (n = 65) stage II, 25.6 % (n = 42) stage III and 20.2 % (n = 33) stage IV carcinomas.

2.3 Clinical Management

Clinical management was performed by a pre-agreed standardized multi-disciplinary protocol. The use of post-operative adjuvant therapy was based on the pathological stage of cancer and the clinical status of the patient. The follow-up period was defined as the interval between the date of surgery for colorectal adenocarcinoma and the date of death or closing date of the study. The actuarial survival rate of the patients was calculated from the date of surgical resection of the colorectal adenocarcinomas to the date of death or last follow-up. Only cancer-related death was counted as the endpoint in the statistical analysis. Persistence or recurrence of the disease was also recorded.

2.4 Cell Culture

Three colon cancer cell lines (SW-480, SW-48 and HCT116) and one non-neoplastic colon epithelial cell line (FHC) were used in this study. All these cells were purchased from ATCC (American type culture collection) and maintained as previously described [26].

2.5 5-Aza-2′-deoxycytidine (5-Aza-CdR) treatment

5-Aza-CdR (Sigma-Aldrich Pty. Ltd. Sydney, VIC, Australia) was dissolved in 1% DMSO, stored at -20°C and used at the final concentration of 20 μM as previously published protocol with some modification [27]. FHC, SW-480, SW-48 and HCT116 cells were seeded in six wells plates and were exposed to 5-Aza-CdR for 48 and 72 hours respectively. DNA, RNA and protein were extracted for downstream analysis. Cells treated with 1% DMSO were used as solvent controls.

2.6 Extraction of DNA and RNA

Prospective fresh frozen tissues were sectioned by cryostat into 7 μm slices for RNA and DNA extractions. DNA and RNA were extracted and purified from the tissues and cells with Qiagen DNeasy Blood & Tissue kit and miRNeasy Mini kit (Qiagen Pty. Ltd., Hilden, Germany) using previously published protocol [28].

2.7 Bisulphite modification

Bisulphite conversion and purification of the genomic DNA was performed with MethylEasyXceed kit (Human Genetic Signatures Pty. Ltd., NSW, Australia) as recommended by the manufacturer. DNA quantification and purity was checked with Nanodrop Spectrophotometer (BioLab, Ipswich, MA, USA). Approximately 2 μg genomic DNA from each sample and cell line were the starting amount for the bisulphite treatment.

2.8 Methylation specific high-resolution melt (MS-HRM) curve analysis

Bisulphite treated genomic DNA from adenocarcinomas (n=164), matched non-neoplastic mucosea (n=83) and adenomas (n=32) were used for MS-HRM analysis to screen the methylation status. Methylation of *FAM134B* promoter sequence using methyl-specific primers was determined as previously described with some modification [29-30]. Briefly, MS-HRM curve analysis was performed by amplifying target sequences of *FAM134B* promoter region on the Rotor-Gene Q detection system (Qiagen) using the software - Rotor-Gene ScreenClust HRM Software. The polymerase chain reaction (PCR) reactions were amplified in a total reaction volume of 10 µl comprising 5 µl of 2Xsensimix HRM master mix, 1 µl of 40 ng/µl bisulphite converted genomic DNA, diethylpyrocarbonate (DEPC, RNase free) treated water 2 µl and 1 µl of each primer. The thermal cycling comprised 15 minutes at 95°C, followed by 50 cycles of 30 seconds at 95°C, 30 seconds at 61°C and 20 seconds at 72°C. Each PCR run included a no templates, a genomic unmethylated (negative) (Sigma) and a fully methylated (positive) (Thermo Fisher Scientific Australia Pty Ltd, Scoresby VIC, Australia) DNA as controls. The melt curve data were generated by increasing the temperature from 70°C to 95°C for all assays, with temperature increase rate of 0.05°C/seconds and recording fluorescence. The purity and size of the PCR product were checked in 1.5% agarose gel followed by electrophoretic separation (Supplementary data 1). All the primers used in this study are given in supplementary data 2.

2.9 Purification of MS-HRM PCR products and Sanger sequencing

FAM134B promoter methylations were further confirmed by Sanger sequencing followed by MS-HRM. Briefly, after MS-HRM curve analysis, methylated samples' PCR products were purified according to the manufacturer's protocols from the NucleoSpin® Gel and PCR Clean-up kit (Macherey- Nagel, Bethlehem, PA, USA). The purified DNA was

then mixed with methyl-specific primers. They were prepared for sequencing using Big Dye Terminator (BDT) chemistry Version 3.1 (Applied Biosystems, Foster City, CA, USA) under standardised cycling PCR conditions. The generated data were analysed at the Australian Genome Research Facility (AGRF) using a 3730xl Capillary sequencer (Applied Biosystems). Sequences were then analysed with Sequence Scanner-2 software (Applied Biosystems).

2.10 DNA copy number change and mRNA expression analysis

To study the correlation of *FAM134B* promoter methylation with DNA copy number changes in colorectal adenocarcinoma tissues (n=68), IQ5 multicolour real-time PCR detection system (Bio-Rad, Hercules, CA, USA) was used to perform RT-qPCR. The detail of the protocol was previously described [19]. In addition, the association of *FAM134B* promoter methylation with mRNA expression changes in colorectal cancer tissues (n=159) and cells (n=4) was studied. mRNA expression and copy number changes were obtained by our previously published data as acquired by quantitative reverse transcription polymerase chain reaction [17-19, 31]. Compared to the previous studies, the matched 83 cases were newly recruited for the current study. Copy number changes and mRNA expression, a fold change of >2 is used as copy number amplification/high mRNA expression, and a fold change ≤2 is used as copy number deletion/low FAM134B expression.

2.11 Immunohistochemistry

Immunohistochemical study was performed on paraffin blocks of colorectal adenocarcinomas (n = 79) to investigate the relationship between *FAM134B* promoter methylation and FAM134B protein expression. The tissue sections were then stained and analysed as published previously [17, 26]. To examine the relationship between *FAM134B*

promoter methylation and protein expression, data were used from our previous studies [17]. The protein expression was classified into four categories; “0” (0% to less than 10%), “+” (10% to <30%), “++” (30% to < 50%) and “+++” (>50%) according to the percentage of FAM134B protein staining. Tissues in the categories of “0” & “+” were classified as low whereas “++” & “+++” were considered as high expression.

2.12 Western blot analysis

To investigate the impact of *FAM134B* promoter methylation on protein expression in cells, total proteins were extracted from control and 5-Aza-CdR treated cells with lysis buffer (Bio-Rad, Gladesville, NSW, Australia). Afterwards, total protein (30 μg) was separated by 15% SDS-PAGE (Bio-Rad). The protein was transferred to nitrocellulose membranes (Bio-Rad). The membrane was then developed to detect protein bands according to the published protocol [22].

2.13 Statistical analysis

Comparisons between variable groups were analysed using the chi-square test, likelihood ratio and Fisher's exact test. All the data were entered into a computer database and the statistical analysis was executed using the Statistical Package for Social Sciences for Windows (version 24.0, IBM SPSS Inc., New York, NY, USA). Survival analysis was tested using Kaplan-Meier method. The significance level was taken at $p < 0.05$.

3. Results

3.1 *FAM134B* promoter hypermethylated in colorectal cancer tissues and cells

We have designed a pair of methyl-specific primers for *FAM134B* promoter region sequence (NM_019000; http://epd.vital-it.ch/search_EPDnew.php) using primer design tool (http://bisearch.enzim.hu/). Fig. 2A shows the schematic presentation of *FAM134B* promoter region with primer pair coverage. The levels of methylation in the cells and tissues samples were determined using unmethylated genomic DNA, 100% methylated genomic DNA and different dilutions of methylated DNA (Supplementary data 3). Sanger sequencing confirmed the results (Supplementary data 4). The pattern of *FAM134B* promoter methylation also exhibited high methylation levels in adenocarcinoma tissues and cells derived from aggressive colon cancers when compared to control tissues and non-neoplastic cells (Fig. 2B & 2C).

Hypermethylation of *FAM134B* promoter in colorectal adenocarcinomas was more common when compared to that of adjacent non-neoplastic and adenoma tissue samples (Fig. 2D). Approximately, 52% (85/164) of the adenocarcinomas showed methylation, whereas only ~35% (29/83) of the non-neoplastic mucosae and ~28% (9/32) of the adenomas exhibited *FAM134B* promoter methylation (Supplementary data 5). *FAM134B* promoter methylation was detected in all colon cancer cells tested. The degree of methylation increased with the colon cancer cells derived from advanced pathological stages (stages III or IV) (Fig. 2E). SW48 and HCT116 cancer cells from patients with advanced stages colon cancers exhibited maximum (35.3%) methylation when compared to non-neoplastic colon epithelial cells (5.6%) (Fig. 2E).

Quantitative analysis of *FAM134B* methylation showed a high levels of methylation (median 44.11%, range 0.0-100%) in adenocarcinomas when compared to adenomas (median 7.64%, range 0-100%) and non-neoplastic mucosae (median 5.85%, range 0.0-30%) (p

<0.05) (Table 1). Collectively, these data indicated that *FAM134B* promoter remarkably hypermethylated in colorectal adenocarcinoma tissues and cells.

3.2 *FAM134B* epigenetically downregulated in colon cancer

DNA hypermethylation-mediated downregulation of *FAM134B* expression was investigated in cancer tissues and cell lines. Among the methylated cancer tissues, approximately 63% (20/32) shown *FAM134B* copy number deletion, whereas 39% (14/36) unmethylated cases showed *FAM134B* deletion (p = 0.04) (Fig. 3A). It is worth noting that, ~ 38% (12/32) of the methylated and 61% (22/36) of the unmethylated colorectal cancers showed amplification of *FAM134B* DNA (Fig. 3A). In addition, distribution of *FAM134B* promoter methylation in patients with *FAM134B* DNA copy number deletion was significantly higher when compared to those of *FAM134B* DNA copy number amplification (Fig. 3B).

A statistically significant inverse correlation of *FAM134B* promoter methylation and mRNA expression was noted (r = -0.268; p = 0.001, Fisher exact test). As shown in Fig. 3C, ~75% (62/83) methylated cancer samples had lower *FAM134B* mRNA expression, whereas *FAM134B* mRNA downregulation was only noted in 49% (37/76) of the unmethylated colorectal cancers (Fig. 3C-3D). Similarly, FAM134B protein expression inversely correlated with promoter methylation (r = -0.454; p = 001, Pearson test) (Fig. 3E-3F). Most of the methylated cancer tissue samples had low or absence of FAM134B protein expression (90% versus 53%) when compared to unmethylated samples (Fig. 3E-3H). Simultaneously, high FAM134B protein expression was noted in a higher number of unmethylated cancer tissues when matched to that of methylated tissues (10% versus 48%) (Fig. 3E-H). Patients with colorectal adenocarcinomas expressing low mRNA/proteins showed significantly higher *FAM134B* promoter methylation distribution in comparison to those expressing high

FAM134B mRNA/protein (Fig. 3D, 3F). A similar trend of association between methylation and *FAM134B* mRNA and protein expression changes were noted in cancer cells (Fig. 2E, Supplementary 6 and 7). No statistically significant relationship was found between *FAM134B* methylation, *FAM134B* mutations, microsatellite instability status and its target microRNA (miR-142-5p and miR-186-5p; n = 122) expressions [26]. Taken together, results from this study implied that DNA methylation was responsible for *FAM134B* downregulation in colorectal cancers and cells.

3.3 Demethylation by 5-Aza-CdR treatment restored *FAM134B* expression in cancer cells

Colon cancer cell cells treated with 5-Aza-CdR (an inhibitor of DNA methylation) shown complete demethylation of the *FAM134B* promoter sequence (Supplementary data 4). This demethylation significantly increased the expression of *FAM134B* mRNA and protein in all cancer cells when compared to the corresponding (methylated) control cells after 48 and 72 hours of treatment (Fig. 4 and Supplementary data 7). In 5-Aza-CdR untreated control cells, FAM134B expression was significantly difference in various types of colon cells; with the highest expression in FHC cells and lowest expression in HCT116 cells (Supplementary data 7).

3.4 *FAM134B* promoter methylation and its clinicopathological significances

The relationship between *FAM134B* promoter methylation and clinical-pathological characteristics is presented in Table 2. A significant correlation between methylation of cancer and high histological grade, the presence of peri-neural infiltration, lymphovascular invasion, advanced pathological (TNM) stages, cancer recurrence and use of post-operative chemotherapy were noted in the present study (Table 2). In addition, *FAM134B* promoter

methylation was also correlated with the presence of associated adenoma, metachronous colorectal cancer and cancers of other organs.

Poorly differentiated colorectal adenocarcinoma exhibited significantly higher *FAM134B* methylation (74% versus 48%) when compared to well or moderately differentiated colorectal adenocarcinomas (p = 0.025) (Table 2). Patients with adenocarcinoma having peri-neural infiltration showed high levels of *FAM134B* promoter methylation when compared to those without peri-neural infiltration (77% versus 48%; p = 0.012). Approximately, 68% (27/40) of adenocarcinomas with the presence of lymphovascular permeation in their colorectal cancer tissues have shown *FAM134B* promoter methylation, whereas only ~47% (58/124) of adenocarcinomas without lymphovascular permeation had shown *FAM134B* promoter methylation (p = 0.021). Among the 164 adenocarcinomas, those presented with associated adenoma(s) showed a higher frequency of *FAM134B* promoter methylation when compared to those without any associated adenoma in the colorectum (62% versus 44%; p = 0.028).

The majority of cancer patients with advanced T-stages (T3 & T4) showed *FAM134B* promoter methylation when compared to those with early T-stages (T1 & T2) (57% versus 27%; p = 0.009). In addition, compared to patients without any colorectal cancer metastasis, 75% and 88% patients with lymph node and distant metastasis, respectively showed *FAM134B* methylation, (p = 0.0001). Similarly, a significantly higher percentage of patients with advanced pathological stages (stage = III or IV) showed *FAM134B* methylation when compared to patients with early stages (stage = I or II) colorectal adenocarcinomas (76% versus 33%; p = 0.0001).

On the contrary, higher frequency of *FAM134B* promoter methylation was noted in patients with colorectal adenocarcinomas without having another neoplasm in the other parts of the body when compared to those having another neoplasm (36% versus 56%; p = 0.048).

In addition, patients with metachronous colorectal adenocarcinomas showed less frequency of *FAM134B* promoter methylation when compared to those without metachronous tumour in the colorectum (12% versus 54%, p = 0.029) (Table 2).

3.5 *FAM134B* methylation is associated with poor patient survival

The overall median follow-up of patients with colorectal adenocarcinoma was 49 months and a significant correlation was noted between poor patient survival rates and advanced pathological stages of colorectal carcinomas (p=0.0001) (Fig. 5A). *FAM134B* promoter methylation was noted to be frequent among patients with cancer recurrence and use of post-operative chemotherapy (68% versus 42%; p = 0.001, 69.7% versus 40%; p = 0.0001, respectively) (Table 2). Patients with *FAM134B* promoter methylation had a significantly shorter survival time when compared to those with unmethylated colorectal carcinomas (102 months versus 127 months, p=0.009) (Fig. 5B). However, in multivariate analysis including other factors such as pathological stage, lymph node metastasis, and histological grade *etc.*, survival rates of the patients did not show statistical significance with *FAM134B* methylation (p > 0.05).

4. Discussion

The present study for the first time has demonstrated the biological and clinical significance of *FAM134B* promoter methylation in a large cohort of patients with colorectal adenocarcinomas. Increased frequency of *FAM134B* promoter methylation was noted in cancer tissues and cells when compared with control tissues and cells, respectively. *FAM134B* promoter methylation was significantly associated with loss or reduced *FAM134B* expression. Most importantly, *FAM134B* promoter methylation correlated with the adverse clinicopathological factors including high histological grade (grade 3), the presence of lymphovascular as well as perineural invasion and advanced stages of carcinomas. In addition, *FAM134B* promoter methylation associated with cancer recurrence or persistent use of post-operative chemotherapy and poor survival rates of patients with colorectal adenocarcinoma.

FAM134B acts as a tumour suppressor in colorectal adenocarcinoma and is frequently downregulated in cancer tissues and cells when compared to the non-neoplastic counter-parts, respectively [17-20, 22]. Promoter methylation mediated suppression or silencing of tumour suppressors in colorectal adenocarcinoma are common and involved in the adenoma-carcinoma sequence of carcinogenesis [2-3, 5-7]. *FAM134B* methylation was noted in adjacent non-neoplastic colonic mucosae to carcinoma as well as in adenomas. The methylation in these tissues was less frequent than in colorectal adenocarcinomas. Thus, it could be hypothesized that *FAM134B* methylation has potential implications in the pathogenesis of colorectal adenocarcinoma. However, the relatively high frequency of *FAM134B* promoter methylation was noted in matched non-neoplastic mucosae when compared to adenomas (35% versus 28%). This higher frequency of *FAM134B* promoter methylation in non-neoplastic mucosae might be due to the field effects of DNA methylation of cancer patients with colorectal carcinomas [32]. In addition, most of the adenoma samples

(22/32) used in the present study were of low-grade, which may be attributed to the lower frequency of *FAM134B* promoter methylation in adenoma samples in comparison to that of non-neoplastic mucosae. In addition, the majority of adenoma with high-grade dysplasia (7/10) used in the present study showed hypermethylation. Inverse correlation among *FAM134B* promoter methylation with copy number deletions, loss or reduced expression (both mRNA and protein) noted in the present study further endorse a tumour inhibitory properties of *FAM134B* in colorectal adenocarcinoma. Furthermore, demethylation induced upregulation of *FAM134B* expression in colon cancer cells imply that *FAM134B* maintain its tumour suppressor properties by being in demethylation state and methylation in its promoter region could have the potential to play roles in carcinogenesis in the colorectum.

In the present study, *FAM134B* promoter methylation was associated with the adverse clinical-pathological characteristics of patients with colorectal adenocarcinoma, which indicates the critical roles of *FAM134B* methylation in the progression of the disease. Gene methylations are reported to be significantly higher in poorly differentiated colorectal cancers when compared to well or moderately differentiated cancers [33-34]. Analogously, in the current study, patients with poorly differentiated colorectal adenocarcinomas showed a significant increase of *FAM134B* promoter methylation in comparison to those of well or moderately differentiated adenocarcinomas (74% versus 48%; $p=0.025$). This result further confirms the role of *FAM134B* promoter methylation and its associated genetic modulations in predicting biological and clinical aggressiveness in patients with colorectal adenocarcinomas.

Peri-neural infiltration is a pathological process characterised by cancer cells invasion to the nervous structures and distributed along the nerve sheaths [35]. The presence of peri-neural infiltration in cancer was associated with a higher rate of distant metastasis, cancer persistence or recurrence and poor survival rates of patients, thereby used as an independent

prognostic marker in cancers [36-37]. This process also involved in complex interactions of cancer cells with stromal and nerve cells [38]. Thus, the association of *FAM134B* promoter methylation with peri-neural invasions of patients with colorectal carcinomas suggested that *FAM134B* methylation could have the potential to induce peri-neural invasions in colorectal cancers, which in turn, could contribute its metastatic potential through complex stromal interactions between colon cancer cells and adjacent mesenchymal tissues.

Lymphovascular invasion in colorectal carcinoma is a high stage-dependent prognostic marker [39]. The presence of lymphovascular invasion has been associated with an increased risk of lymph node metastases and tumour invasion to the extramural veins, which in turn, has been associated with distant metastases [39]. Promoter hypermethylation of tumour suppressor gene *e.g. CDKN2A* is associated with the presence of lymphovascular invasion in patients with colorectal cancers [40]. Similarly, we have noted a significant correlation between *FAM134B* promoter methylation and lymphovascular invasion in colorectal adenocarcinoma ($p = 0.021$). Previous studies also reported the association of reduced *FAM134B* expression with the presence of lymphovascular invasion in the patients with colorectal adenocarcinomas [17]. Thus, from the consistent results, it can be hypothesized that methylation-mediated silencing of *FAM134B* could have the potential to permit cancer cells to invade local lymph nodes and veins, which in turn, could contribute to distant metastasis.

Adenoma in colorectum is a precursor lesion which can be transformed to colorectal adenocarcinoma if remain untreated [41]. The development of carcinoma from adenomatous lesions is referred to as the adenoma-carcinoma sequence in the pathogenesis of colorectal adenocarcinoma. In this study, *FAM134B* promoter methylation was noted to be frequent in patients who are reported with adenoma(s) along with an adenocarcinoma in their colorectum (62% versus 44%; $p = 0.028$). These results suggest a possible genetic correlation of

FAM134B methylation with the adenoma-carcinoma sequence in the colorectum. Furthermore, *FAM134B* methylation is less frequent in patients with metachronous colorectal adenocarcinomas (13%) and with other neoplasm(s) (36%). Presence of more than one primary colorectal adenocarcinoma detected consecutively in a single person after a set of time interval is known as metachronous colorectal carcinoma [42]. These results indicated that *FAM134B* methylation could have the potential to acts as a predictive marker for multiple cancers.

The pathological staging is assessed clinically by the joint assessment of tumour invasion (T), presences or absence of lymph node (N) and distant metastasis (M) of cancers [25]. *FAM134B* promoter methylation was correlated with higher T-stages ($p = 0.009$), presence of lymph node ($p = 0.0001$), distant metastasis ($p = 0.0001$) and advanced pathological stages ($p = 0.0001$) of patients with colorectal adenocarcinoma. *FAM134B* copy number deletion, reduced or loss of expression are associated with advanced cancer stages in colorectal adenocarcinoma [17, 19, 20]. Hypermethylation at promoter regions might be the causative events for downregulation of *FAM234B* in the majority of colorectal adenocarcinomas. In addition, we have noted mutations and microRNA (*miR-186-5p*) can induce reduced or loss of *FAM134B* expression and lead to the biological aggressiveness of colorectal adenocarcinomas [22, 26]. Thus, the concurrent genetic and epigenetic alterations impact confer loss or downregulation of *FAM134B* expression, which could contribute to the initiation, and progression of colorectal adenocarcinomas.

In this study, promoter methylation of *FAM134B* correlated with prognosis of patients with colorectal adenocarcinomas. Patients with *FAM134B* promoter methylation in their colorectal cancer tissues had a significantly poorer survival rates when compared to those having unmethylated *FAM134B* ($p=0.009$). In addition, we found that *FAM134B* methylation is more prevalent in patients with cancer recurrence or persistence ($p = 0.001$)

and have received post-operative chemotherapy ($p = 0.0001$). This association of *FAM134B* promoter methylation with poor patients' prognosis and cancer recurrence can be interrelated with its significant clinical correlations with tumour invasions and lymph node metastasis in colorectal adenocarcinomas. Taken together, *FAM134B* promoter methylation demonstrated to be a marker of aggressive biological behaviours and could be used as a potential prognostic marker for the patients with colorectal cancers.

In colorectal adenocarcinomas, there are different types genetic and epigenetic abnormalities, including chromosomal instability (CIN), microsatellite instability (MIN/MSI) and CpG island methylator phenotype (CIMP), which are associated with the various phenotypes of the disease [2]. The patients with colorectal adenocarcinomas having CIMP phenotype is associated with worse prognosis due to inactivation of various tumour suppressor genes. Thus, *FAM134B* methylation could represent a broader CIMP status. The poor prognosis of the patients with colorectal adenocarcinoma having *FAM134B* promotor methylation in this study may be related to this broader CIMP status.

The exact mechanism by which *FAM134B* mediated its tumour suppressive behaviours is yet to be identified. However, considering its endoplasmic reticulum-turnover function, it is assumed that absence or non-functional FAM134B protein could lead to generate endoplasmic reticulum-stress [10]. This endoplasmic reticulum-stress can activate unfolded protein response (UPR) in cancer cells, which in turn, allows cancer cells to adapt the tumour microenvironment and promote cancer growth and development [43-44]. Therefore, further studies of FAM134B protein are imperative to unveil its exact roles in cancer cell biology by identifying its cellular interacting partners.

In conclusion, we here report frequent *FAM134B* promoter methylation in colorectal adenocarcinomas. Its co-regulatory effects on FAM134B expressions in-vitro and colorectal cancer tissues indicate the significance of *FAM134B* promoter methylation in the genetic

landscaping of colorectal adenocarcinomas. In addition, the association of *FAM134B* methylation with adverse clinical and pathological factors in colorectal cancer as well as poor survival rates of patients with colorectal cancer implies the potential clinical applications of the gene in colorectal cancer.

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References

1. Hinoue T, Weisenberger DJ, Lange CP, et al. Genome-scale analysis of aberrant DNA methylation in colorectal cancer. Genome Res. 2012; 22: 271-282.
2. Gallois C, Laurent-Puig P, Taieb J. Methylator phenotype in colorectal cancer: a prognostic factor or not? Crit Rev Oncol Hematol. 2016; 99: 74-80.
3. Okugawa Y, Grady WM, Goel A. Epigenetic Alterations in Colorectal Cancer: Emerging Biomarkers. Gastroenterology 2015; 149: 1204-1225.e12.
4. Bird A. DNA methylation patterns and epigenetic memory. Genes Dev. 2002; 16: 6-21.
5. Veigl ML, Kasturi L, Olechnowicz J, et al. Biallelic inactivation of hMLH1 by epigenetic gene silencing, a novel mechanism causing human MSI cancers. Proc Natl Acad Sci USA. 1998; 95: 8698-8702.
6. Gonzalez-Zulueta M, Bender CM, Yang AS, et al. Methylation of the 5' CpG island of the p16/CDKN2 tumor suppressor gene in normal and transformed human tissues correlates with gene silencing. Cancer Res. 1995; 55: 4531-4535.
7. Suzuki H, Watkins DN, Jair KW, et al. Epigenetic inactivation of SFRP genes allows constitutive WNT signaling in colorectal cancer. Nat Genet. 2004; 36: 417-422.
8. Tang WK, Chui CH, Fatima S, et al. Oncogenic properties of a novel gene JK-1 located in chromosome 5p and its overexpression in human esophageal squamous cell carcinoma. Int J Mol Med. 2007; 19: 915-923.
9. Kurth I, Pamminger T, Hennings JC, et al. Mutations in FAM134B, encoding a newly identified Golgi protein, cause severe sensory and autonomic neuropathy. Nat Genet. 2009; 41: 1179-1181.
10. Khaminets A, Heinrich T, Mari M, et al. Regulation of endoplasmic reticulum turnover by selective autophagy. Nature 2015; 522: 354-358.

11. Kong M, Kim Y, Lee C. A strong synergistic epistasis between FAM134B and TNFRSF19 on the susceptibility to vascular dementia. Psychiatr Genet. 2011; 21: 37-41.
12. Melchiotti R, Puan KJ, Andiappan AK, et al. Genetic analysis of an allergic rhinitis cohort reveals an intercellular epistasis between FAM134B and CD39. BMC Med Genet. 2014; 15: 73.
13. Davidson G, Murphy S, Polke J, et al. Frequency of mutations in the genes associated with hereditary sensory and autonomic neuropathy in a UK cohort. J Neurol. 2012; 259:1673-1685.
14. Murphy SM, Davidson GL, Brandner S, Houlden H, Reilly MM. Mutation in FAM134B causing severe hereditary sensory neuropathy. J Neurol Neurosurg Psychiatry 2012; 83:119-120.
15. Ilgaz Aydinlar E, Rolfs A, Serteser M, Parman Y. Mutation in FAM134B causing hereditary sensory neuropathy with spasticity in a Turkish family. Muscle Nerve 2014; 49:774-775.
16. Chiramel AI, Dougherty JD, Nair V, Robertson SJ, Best SM. FAM134B, the selective autophagy receptor for endoplasmic reticulum turnover, inhibits replication of Ebola virus strains Makona and Mayinga. J Infect Dis. 2016; 214:s319-s325.
17. Kasem K, Gopalan V, Salajegheh A, Lu CT, Smith RA, Lam AK. The roles of JK-1 (FAM134B) expressions in colorectal cancer. Exp Cell Res. 2014; 326:166-173.
18. Kasem K, Sullivan E, Gopalan V, Salajegheh A, Smith RA, Lam AK. FAM134B (FAM134B) represses cell migration in colon cancer: a functional study of a novel gene. Exp Mol Pathol. 2014; 97:99-104.
19. Kasem K, Gopalan V, Salajegheh A, Lu CT, Smith RA, Lam AK. FAM134B (FAM134B) gene and colorectal cancer: a pilot study on the gene copy number

alterations and correlations with clinicopathological parameters. Exp Mol Pathol. 2014; 97: 31-36.

20. Islam F, Gopalan V, Wahab R, Smith RA, Qiao B, Lam AK. Stage dependent expression and tumor suppressive function of FAM134B (JK1) in colon cancer. Mol Carcinog. 2017; 56: 238-249.
21. Haque MH, Gopalan V, Chan KW, Shiddiky MJ, Smith RA, Lam AK. Identification of novel FAM134B (JK1) mutations in oesophageal squamous cell carcinoma. Sci Rep. 2016; 6: 29173.
22. Islam F, Gopalan V, Wahab R, et al. Novel FAM134B mutations and their clinicopathological significance in colorectal cancer. Hum Genet. 2017; 136: 321-337.
23. Dai X, Hua T, Hong T. Integrated diagnostic network construction reveals a 4-gene panel and 5 cancer hallmarks driving breast cancer heterogeneity. Sci Rep. 2017; 7: 6827.
24. Hamilton SR, Bosman FT, Boffetta P, et al. Carcinoma of the colon and rectum. In: Bosman FT, Carneiro F, Hruban RH, Theise ND (eds) WHO classification of tumors of the digestive system. IARC Press, Lyon, 4th edition, pp 134–146. 2010.
25. Milburn Jessup J, Goldberg RM, Asare EA, Benson III AB, et al. Colon and rectum. In AJCC Cancer staging Manual, Amin MB, Edge S, Greene F, Byrd DR, Brookland RK, Washington MK, Gershenwald JE, Compton CC, Hess KR, Sullivan DC, Jessup JM, Brierley JD, Gaspar LE, Schilsky RL, Balch CM, Winchester DP, Asare EA, Madera M, Gress DM, Meyer LR. (Eds.) eighth edition, Springer 2017; Chapter 20, p251-274. 2017.
26. Islam F, Gopalan V, Vider J, et al. MicroRNA-186-5p overexpression modulates colon cancer growth by repressing the expression of the FAM134B tumour inhibitor. Exp Cell Res. 2017; 357: 260-270.

27. Zhou D, Tang W, Su G, Cai M, An HX, Zhang Y. PCDH18 is frequently inactivated by promoter methylation in colorectal cancer. Sci Rep. 2017; 7: 2819.

28. Islam F, Gopalan V, Law S, Tang JC, Chan KW, Lam AK. MiR-498 in esophageal squamous cell carcinoma: clinicopathological impacts and functional interactions. Hum Pathol. 2017; 62: 141-151.

29. Wojdacz TK, Dobrovic A, Hansen LL. Methylation-sensitive high-resolution melting. Nat Protoc. 2008; 3: 1903-1908.

30. Haque MH, Gopalan V, Islam MN, et al. Quantification of gene-specific DNA methylation in oesophageal cancer via electrochemistry. Anal Chim Acta. 2017; 976: 84-93.

31. Gopalan V, Islam F, Pillai S, et al. Overexpression of microRNA-1288 in oesophageal squamous cell carcinoma. Exp Cell Res. 2016; 348: 146-154.

32. Giovannucci E, Ogino S. DNA methylation, field effects, and colorectal cancer. J Natl Cancer Inst. 2005; 97: 1317.

33. Issa JP. CpG island methylator phenotype in cancer. Nat Rev Cancer 2004; 4: 988-993.

34. van Rijnsoever M, Grieu F, Elsaleh H, Joseph D, Iacopetta B. Characterisation of colorectal cancers showing hypermethylation at multiple CpG islands. Gut 2002; 51: 797-802.

35. Batsakis JG. Nerves and neurotropic carcinomas. Ann Otol Rhinol Laryngol. 1985; 94: 426-427.

36. Krasna MJ, Flancbaum L, Cody RP, Shneibaum S, Ben Ari G. Vascular and neural invasion in colorectal carcinoma. Incidence and prognostic significance. Cancer 1988; 61: 1018-1023.

37. Fleming M, Ravula S, Tatishchev SF, Wang HL. Colorectal carcinoma: Pathologic aspects. J Gastrointest Oncol. 2012; 3: 153-173.

38. Liebig C, Ayala G, Wilks JA, Berger DH, Albo D. Perineural invasion in cancer: a review of the literature. Cancer 2009; 115: 3379-3391.

39. Harris EI, Lewin DN, Wang HL, et al. Lymphovascular invasion in colorectal cancer: an interobserver variability study. Am J Surg Pathol. 2008; 32: 1816-1821.

40. Xing X, Cai W, Shi H, et al. The prognostic value of CDKN2A hypermethylation in colorectal cancer: a meta-analysis. Br J Cancer. 2013; 108: 2542-2548.

41. Hardcastle JD, Armitage NC. Early diagnosis of colorectal cancer: a review. J R Soc Med. 1984; 77: 673-676.

42. Lam AK, Gopalan V, Carmichael R, et al. Metachronous carcinomas in colorectum and its clinicopathological significance. Int J Colorectal Dis. 2012; 27: 1303-1310.

43. Rao RV, Niazi K, Mollahan P, et al. Coupling endoplasmic reticulum stress to the cell-death program: a novel HSP90-independent role for the small chaperone protein p23. Cell Death Differ. 2006; 13: 415-425.

44. Yadav RK, Chae S-W, Kim H-R, Chae HJ. Endoplasmic Reticulum Stress and Cancer. Journal of Cancer Prevention 2014; 19: 75-88.

Figure legends

Fig.1 Schematic presentation of the current study design. Patients with colorectal adenocarcinomas underwent surgical resection in the present study.

Fig.2 *FAM134B* promoter structure and methylation status. A) Schematic representation of *FAM134B* promoter structure, CpG sites (short vertical lines) and primers for MS-HRM. The curved arrow indicates the transcriptional start site. **B)** Schematic presentation of *FAM134B* promoter methylation level in representative tissue samples. **C)** Level of methylation in four cell lines tested in this study. Remarkable differences of methylation level were noted among adenocarcinomas, adenomas and adjacent non-neoplastic colorectal tissues and cells. The degree of methylation significantly higher in adenocarcinomas and in more aggressive cancer cells when compared to adenomas or non-neoplastic counters parts. The closed circle represents methylated CpG whereas open circle represents unmethylated CpG sites. **D)** Percentages of methylation of a *FAM134B* promoter in adenocarcinomas, adenomas and adjacent non-neoplastic mucosae. Significant differences in methylation frequencies were noted among adenocarcinomas, adenomas and adjacent non-neoplastic mucosae ($p<0.05$). The methylation cut-off for scoring of tissue samples as methylation positive (hypermethylation) was used >10%, whereas ≤ 10% was used methylation negative (non-hypermethylated). **E)** Methylation status of *FAM134B* in the non-neoplastic colon (FHC) and colon cancer (SW480, SW48 and HCT116) cells. Extensive hypermethylation was noted in cancer cells derived from patients with colon cancer of advanced stages (SW48 and HCT116) in comparison to that of early-stage colon cancer (SW480) and non-neoplastic FHC cells.

Fig.3 FAM134B epigenetically silenced in colorectal cancer. A) *FAM134B* promoter methylation was associated with copy number deletion, whereas unmethylation of *FAM134B* correlated with gene copy number amplification (p = 0.04). **B)** Distribution of FAM134B promoter methylation in DNA copy number deletion and amplification of patients with colorectal adenocarcinomas. **C)** Inverse correlation between methylation and mRNA expression was noted in adenocarcinomas (*r* = -0.268; p = 0.001, Fisher exact test)**.** *FAM134B* promoter methylation induced loss or reduced FAM134B mRNA expression in colorectal adenocarcinomas. **D)** Distribution of *FAM134B* promoter methylation in high and low *FAM134B* mRNA expression of patients with colorectal adenocarcinomas. **E)** Similarly, a significant reduction or loss of FAM134B protein expression was noted in methylated colorectal adenocarcinomas when compared to those of unmethylated colorectal adenocarcinomas (*r* = -0.454; p = 001, Pearson test). **F)** Distribution of FAM134B promoter methylation in low and high FAM134B protein expressing of patients with colorectal adenocarcinomas. **G)** FAM134B protein staining by immunohistochemistry in hypermethylated adenocarcinoma showing low protein expression (3, 3'-diaminobenzidine; DAB × 25)**.** The nuclei of many carcinoma cells are not stained up by DAB and appear blue on counterstain (arrows) **H)** Adenocarcinoma with *FAM134B* promoter unmethylation showing high protein expression (DAB × 25). The nuclei of the nearly all the carcinoma cells are strongly stained up by DAB and appear brown (arrows).

Fig.4 Demethylation induced restore of FAM134B expression in cells. Treatment of cells with demethylating agent 5-Aza-CdR induced FAM134B re-expression in cancer cells. Demethylation induced almost similar expression of FAM134B in all tested cells irrespective of cancer or non-neoplastic cells. *FAM134B* mRNA expression increased remarkably in

cancer cells at 48 (A) and 72 (B) hours of 5-Aza-CdR treatment. Similarly, protein expression also increased in all three cancer cells following 48 (C) and 72 (D) hours of treatment.

Fig.5 *FAM134B* methylation correlated with poor survival of patients with colorectal cancer. Survival rates of patients with colorectal cancers could be stratified by *FAM134B* promoter methylation or unmethylation. Patients with adenocarcinoma having methylated *FAM134B* promoter had significantly shorter survival time when compared to those without *FAM134B* promoter methylation (102 months versus 127 months) ($p = 0.009$).

Table 1. *FAM134B* promoter methylation status in adenocarcinoma, adenoma and matched non-neoplastic tissues

Tissue Samples	Number	Methylation status (%)
Adenocarcinoma	164	44.11 (0.0-100)
Matched Non-neoplastic	83	5.85 (0.0-30)
Adenoma	32	7.64 (0-100)

Table 2. Association of *FAM134B* promoter methylation with clinicopathological factors of patients with colorectal adenocarcinoma

Variables	Number	Unmethylated	Methylated	P values
Total patients	164	79 (48.2%)	85 (51.8%)	-
Age				
≥60	43 (26.2%)	20 (46.5%)	23 (53.5%)	0.860
<61	121 (73.8%)	59 (48.8%)	62 (51.2%)	
Sex				
Female	79 (48.2%)	35 (44.3%)	44 (55.7%)	0.353
Male	85 (51.8%)	44 (51.8%)	41 (48.2%)	
Site				
Proximal colon	126 (76.8%)	58 (46.0%)	68 (54.0%)	0.357
Distal colorectum	38 (23.2%)	21 (55.3%)	17 (44.7%)	
Size (mm)				
≤40	83 (50.6%)	46 (55.4%)	37 (44.6%)	0.063
>40	81 (49.4%)	33 (40.7%)	48 (59.3%)	
Grade				
Well or moderate	141 (86.0%)	73 (51.8%)	68 (48.2%)	**0.025**
Poor	23 (14.0%)	6 (26.0%)	17 (74.0%)	
Lymphovascular invasion				
Presence	40 (24.4%)	13 (32.5%)	27 (67.5%)	**0.021**
Absence	124 (75.6%)	66 (53.2%)	58 (46.8%)	
Peri-neural infiltration				
Presence	22 (13.4%)	5 (22.7%)	17 (77.3%)	**0.012**
Absence	142 (86.6%)	74 (52.1%)	68 (47.9%)	
Associated adenoma				
Presence	73 (44.5%)	28 (38.4%)	45 (61.6%)	**0.028**
Absence	91 (55.5%)	51 (56.0%)	40 (44.0%)	

Associated cancer in other organs				
Presence	31 (18.9%)	20 (64.5%)	11 (35.5%)	**0.048**
Absence	133 (81.1%)	59 (44.4%)	74 (55.6%)	
Metachronous colorectal cancer				
Presence	8 (4.9%)	7 (87.5%)	1 (12.5%)	**0.029**
Absence	156 (95.1%)	72 (46.2%)	84 (53.8%)	
***FAM134B* mutation[δ]**				
Positive	41 (50.0%)	17 (41.5%)	24 (58.5%)	0.412
Negative	41 (50%.0)	19 (46.3%)	22 (53.7%)	
MSI*				
Positive	22 (20.2%)	13 (59.0%)	9 (40.0%)	0.244
Negative	87 (79.8%)	39 (44.8%)	48 (55.2%)	
T-stage				
I & II	26 (15.9%)	19 (73.1%)	7 (26.9%)	**0.009**
III & IV	138 (84.2%)	60 (43.5%)	78 (56.5%)	
Lymph node metastasis (N)				
Presence	71 (43.3%)	18 (25.4%)	53 (74.6%)	**0.0001**
Absence	93 (56.7%)	61 (65.6%)	32 (34.4%)	
Distant metastasis (M)				
Presence	32 (19.5%)	4 (12.5%)	28 (87.5%)	**0.0001**
Absence	132 (80.5%)	75 (56.8%)	57 (43.2%)	
Overall stage				
I & II	89 (54.3%)	61 (68.5%)	28 (32.5%)	**0.0001**
III & IV	75 (45.7%)	18 (24.0%)	57 (76.0%)	
Post-operative chemotherapy				
Positive	66 (40.2%)	20 (30.3%)	46 (69.7%)	**0.0001**
Negative	98 (59.8%)	59 (60.2%)	39 (39.8%)	
Cancer recurrence				
Positive	63 (38.4%)	20 (31.7%)	43 (68.3%)	**0.001**
Negative	101 (61.6%)	59 (58.4%)	42 (41.6%)	

*Microsatellite instability (MSI) was done in 109 cases; [δ]*FAM134B* mutation was performed in 82 cases by Sanger sequencing.

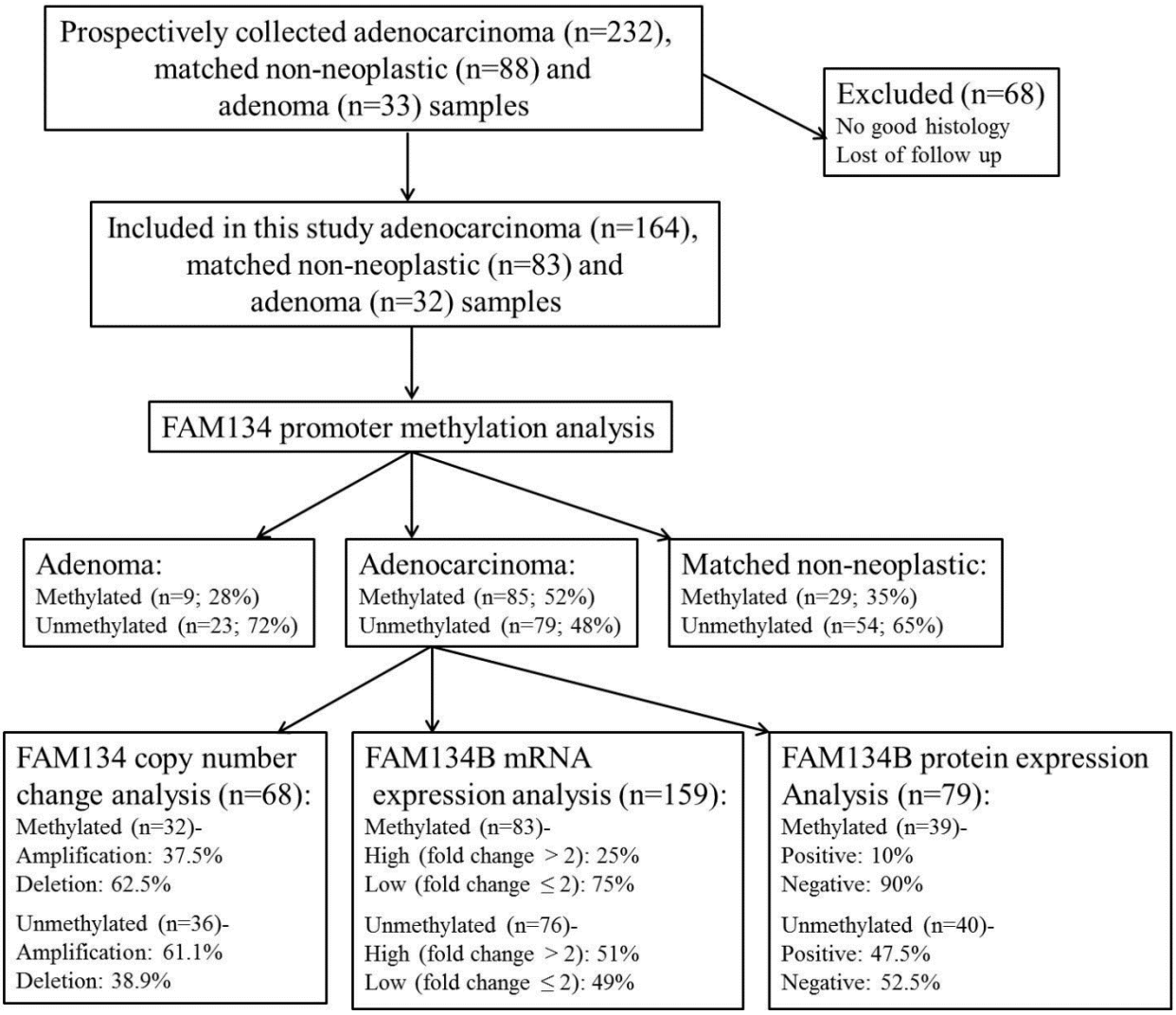
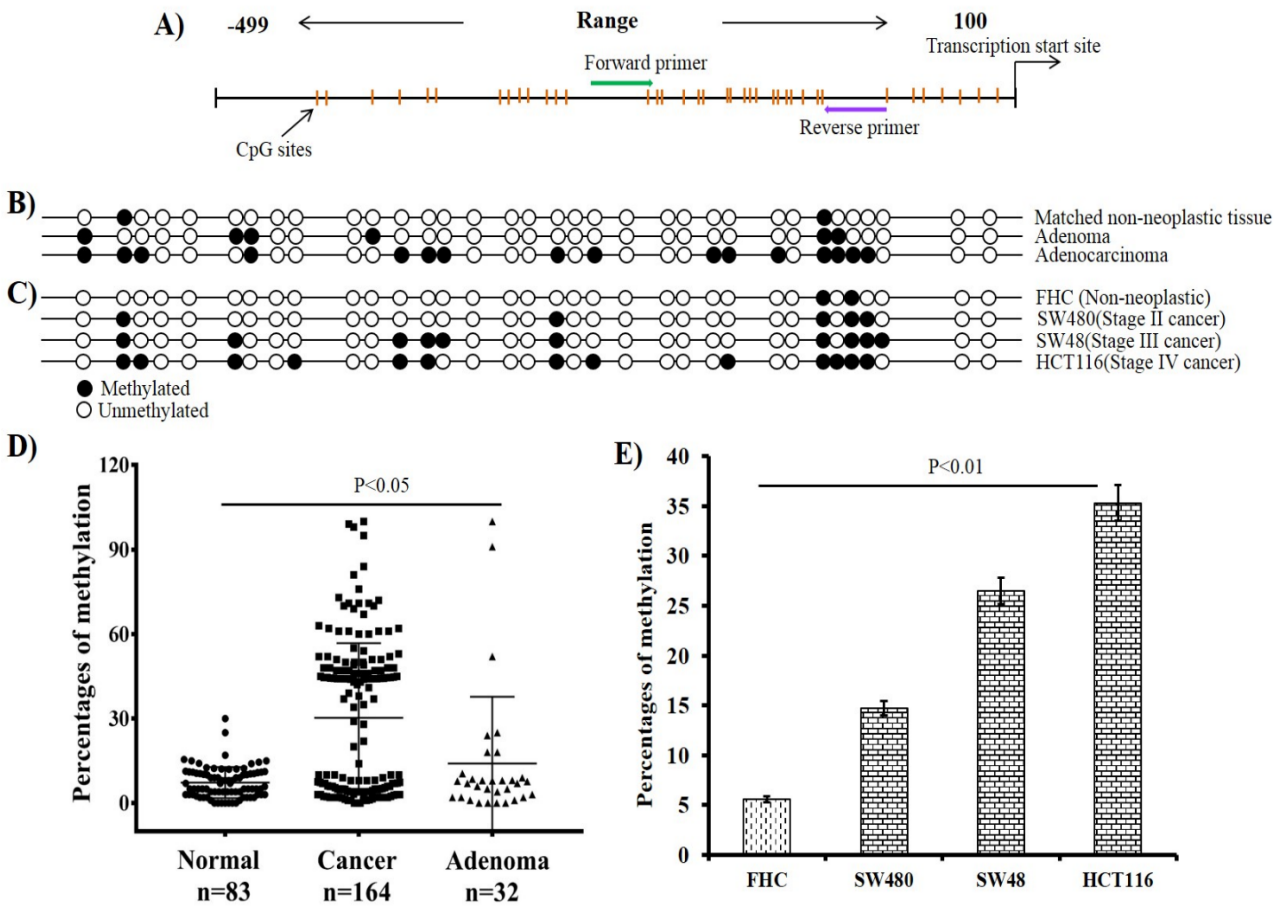


FIGURE 1 Schematic presentation of the current study design. Patients with colorectal adenocarcinomas underwent surgical resection in the present study



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