Impact of LINE-1 hypomethylation on the clinicopathological and molecular features of colorectal cancer patients

Tai-Chuan Kuan, Pei-Ching Lin, Shung-Haur Yang, Chun-Chi Lin, Yuan-Tzu Lan, Hung-Hsin Lin, Wen-Yi Liang, Wei-Shone Chen, Jen-Kou Lin, Jeng-Kai Jiang, and Shih-Ching Chang

1 Division of Colon & Rectal Surgery, Department of Surgery, Taipei Veterans General Hospital, Taipei, Taiwan
2 Department of Surgery, Faculty of Medicine, School of Medicine, National Yang-Ming University, Taipei, Taiwan
3 Department of Clinical Pathology, Yang-Ming Branch, Taipei City Hospital, Taipei, Taiwan
4 Department of Health and Welfare, University of Taipei, Taipei, Taiwan
5 Department of Pathology, Taipei Veterans General Hospital, Taipei, Taiwan

☯ These authors contributed equally to this work.
* changsc@vghtpe.gov.tw (SCC); jkjiang@vghtpe.gov.tw (JKJ)

Abstract

Recent studies suggest that aberrant DNA methylation might occur early and commonly in colorectal tumorigenesis. In 111 normal subjects, the mean LINE-1 methylation level of peripheral blood was 81.0 ± 5.7%. Of 143 colorectal cancer (CRC) patients, the mean level of LINE-1 methylation was 60.5 ± 12.5%. We defined below 60% as cut-off value of LINE-1 hypomethylation, and 93 cases (65.0%) had LINE-1 hypomethylation in the tumor tissue. LINE-1 hypomethylation was not associated with any other clinical features. There was a trend that LINE-1 hypomethylation tumors were associated with advanced disease, but it did not reach statistical significance. There was no significant association between mutations of 12 genes, MSI-high, EMAST, and LINE-1 hypomethylation level. The median follow-up was 61.2 months. Five-year disease-free survival (DFS) and overall survival curves of patients with LINE-1 hypomethylation tumors were significantly lower than those of patients with normal LINE-1 methylation tumors (p = 0.032 and 0.001, respectively). Multivariate analysis showed that only TNM staging was an independent prognostic factor for CRC patients including DFS and overall survival (OS). LINE-1 did not impact patients’ outcomes in multivariate analysis including DFS and OS. In conclusion, LINE-1 hypomethylation is marginally related to advanced stage CRC and impacts patients’ outcomes in univariate analysis.

Introduction

Colorectal cancer (CRC) has become the most common cancer in Taiwan. More than 15,000 new diagnosed CRC cases were reported each year since 2013 [1]. As consistent with other models describing colorectal cancer originating from progressive accumulation of genetic and
epigenetic alterations [2–4], the molecular analysis in our previous studies[5–7] showed that CRCs had higher frequency of mutations in APC, TP53, and KRAS. These genomic alterations associating with chromosomal instability or aneuploidy were found in the majority of CRC cases [3,4,7]. In the screening of Lynch syndrome, analysis of microsatellite instability(MSI) and immunohistochemistry of mismatch-repair proteins showed that 10–15% CRC cases were MSI-high or had deficient MMR proteins.[8–11] Overall 2–3% CRC cases were found to have germline mutations in the mismatch repair system.(8,9,12) Hypermethylation of the MLH1 gene promoter resulting in silence of MMR proteins is another cause of MSI-high [12–14].

Recently, whole-genome methylation analyses of CRCs, precursor lesions, and normal colorectal mucosa provided evidence that aberrant DNA methylation might occur early in colorectal tumorigenesis and is a common event in CRC [15,16]. DNA methylation is known to add a methyl group to the fifth carbon atom of a cytosine ring at the “CG” dinucleotide sequence. Global DNA methylation occurs within highly repetitive DNA sequences, such as long interspersed nucleotide elements (LINE-1) and short repetitive sequences such as Alu repeats [17–18].

LINE-1 are retrotransposon elements composing about 17–18% of the human genome [19]. Line-1 might inactivate gene function through insertional mutagenesis or aberrant splicing or LINE-1-mediated insertions could result in disease through target-site deletions [20]. In normal tissue, LINE-1 is highly methylated and inactivated, but some of them still retaining the capacity to retrotranspose themselves to new genomic locations[21]. In cancer tissue, LINE-1 methylation is decreased [19–21]. Hypomethylation of Line-1 has been considered to be associated with increased retrotransposon activity[22], induced genomic instability and result in further progress of cancer formation [23–27]. Because Line-1 is highly abundant and randomly distributed throughout the genome, LINE-1 methylation could be used as a surrogate marker of global DNA methylation and is confirmed in several studies [18,23–30].

In this study, LINE-1 methylation levels in the peripheral blood were analyzed to understand the distribution of LINE-1 methylation level. In colorectal cancer tissue, cut-off value of LINE-1 methylation was determined and correlated with clinicopathological features and molecular alterations, including gene mutations, MSI, and elevated microsatellite alterations at selected tetranucleotides (EMAST).

Materials and methods
Clinical data
One hundred eleven healthy individuals, with informed consent, were enrolled from volunteer blood donors who had no history of malignant disease. DNA of peripheral blood from normal individuals was extracted and stored in Taipei Veterans General Hospital Biobank. One hundred forty-three samples were selected randomly from a prospective collected database consisting of 1505 patients with colorectal cancer who received surgery at the Taipei Veterans General Hospital between 2000 and 2010 [5–7]. This database excluded patients died of surgical complications, rectal cancer patients receiving preoperative chemoradiotherapy, and patients receiving emergency operations because of cancer complications. We prospectively collected data including age, sex, personal and family medical history, location of tumor, TNM stage, and other pathological prognostic features and follow-up condition. Colon length between the cecum and rectosigmoid colon was defined as the colon. The rectum was within 15 cm of the anal verge. After operation, patients were informed to be monitored every three months in the first two years and semiannually thereafter. Every clinical visits, patients received examinations physical, digital rectal examination, carcinoembryonic antigen and CA-199 analysis, chest radiography, abdominal sonogram. The computerized tomography was
arranged if any abnormal finding was found. Proton emission tomography or magnetic resonance imaging was arranged for patients with elevated levels of carcinoembryonic antigen but an uncertain site of tumor recurrence.

Source of samples
After approval by the Institutional Review Board of Taipei Veterans General Hospital (number 2013-11-013CCF), DNA of peripheral blood and samples of tumors were obtained from the Biobank. Tumor DNA was extracted using a QIAamp DNA Tissue Kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s recommendations. Quality and quantity of DNA were confirmed using a Nanodrop 1000 spectrophotometer (Thermo Scientific).

MassArray-based mutation characterization
The identification of 139 mutations in 12 genes detected by the MassDetect CRC panel (v2.0) was extracted from our previous studies [5,6]. In brief, polymerase chain reaction (PCR) and extension primers for the mutations were designed using MassArray Assay Design 3.1 software (Sequenom, San Diego, CA, USA). PCR products from the multiplexed reactions were spotted onto SpectroCHIP II arrays, and DNA fragments were resolved on a MassArray Analyzer 4 System (Sequenom). Each spectrum was then analyzed using Typer 4.0 software (Sequenom) to identify mutations. We defined a 5% abnormal signal as a putative mutation. Putative mutations were then filtered by manual review. Our previous study had verified the concordance between MassArray and Sanger sequencing up to 99%.[6]

MSI analysis
According to international criteria, five reference microsatellite markers were used to determine MSI: D5S345, D2S123, BAT25, BAT26, and D17S250. Primer sequences for these genes were obtained from GenBank (https://blast.ncbi.nlm.nih.gov/Blast.cgi). MSI detection was performed as previously described [7]. The specific microsatellite sequence was amplified with polymerase chain reactions (PCR). The PCR products were denatured and analyzed by 5% denaturing polyacrylamide gels using ABI-3730 analyzer (Applied Biosystems, CA, USA), and results were revealed using GenScan analysis software (Applied Biosystems, CA, USA). The samples with ≥2 MSI markers were defined as having high MSI, and those with 0–1 MSI markers were classified as microsatellite stable.

EMAST analysis
According to the definition by some studies [31,32], five tetranucleotide repeats markers were used to determine EMAST: D20S82, D20S85, D8S321, D9S242, and MYCL1. Primer sequences for these genes were obtained from GenBank (https://blast.ncbi.nlm.nih.gov/Blast.cgi). EMAST detection was performed as previously described [31,32] and the technical details were similar to those of MSI analysis. Samples with ≥2 tetranucleotide markers were defined as having EMAST, and those with 0–1 tetranucleotide markers were classified as without EMAST.

Methylation-Specific PCR for LINE-1
The methylation status of LINE-1 was examined using the EpiTect Methyl II PCR Array [33]. Briefly, input genomic DNA was aliquoted into four equal portions and subjected to mock, methylation-sensitive, methylation-dependent, and double restriction endonuclease digestion. After digestion, the enzymatic reactions were mixed directly with the quantitative PCR
(QPCR) master mix and were dispensed into a PCR array plate containing pre- aliquoted primer mixes. The sequences of the primers used for methylation-specific PCR were 185F: 5’-CATTGCCTCACCTGGG AAGC-3’ and 431R: 5’-CAGCCTC GTTGCCGCCTTG-3’. The assay amplified a region of LINE-1 element (position 305 to 331 in accession No. X58075) including 4 CpG sites.

Real-time PCR was conducted using the specified cycling conditions. Finally, the raw change in the threshold cycle number (ΔCt) was pasted into a data analysis spreadsheet, which automatically calculated the relative quantities of methylated and unmethylated DNA. The average of the relative amounts of C in the 4 CpG sites was used as overall LINE-1 methylation level in a given sample.

**Statistical analysis**

The statistical endpoint for disease-free survival (DFS) was defined to have disease since the date of diagnosis or even surgery. The overall survival (OS) was measured from the date of surgery or diagnosis to the date of death from any cause. Patients not known to have died were censored on the date of their last follow-up. The survival curves were plotted using Kaplan-Meier method and compared using the log-rank test. Cox regression univariate and multivariate analyses were performed to determine the impact of clinicopathological features on DFS and OS. The relationship between the genotype frequency and clinicopathological features were analyzed using the chi-square test and 2-tailed Fisher’s exact procedure. Numerical values were compared using Student’s t-test. Data were expressed as mean ± standard deviation. Statistical significance was defined as p < 0.05. Statistical analyses software was SPSS for Windows (version 16.0).

**Results and discussion**

In 111 normal subjects, LINE-1 methylation was analyzed in DNA of peripheral blood. As shown in Fig 1, the mean LINE-1 methylation level in the peripheral blood was 81.0 ± 5.7% (median 88%, range 35–100%). Of the normal subjects, 37.6% had < 80% methylation of LINE-1in the peripheral blood. CRC patients consisted of 93 men (65.0%) and 50 women (35.0%). The mean age at treatment was 79.6 ± 12.4 years (range: 38.8–85.2 years; median: 70.9 years). Ninety-eight (68.5%) and 45 (31.5%) cases were located in the colon and rectum, respectively. There were 33 (23.1%) stage I, 32 (22.4%) stage II, 34 (23.8%) stage III and 44 (30.8%) stage IV disease by tumor staging (TNM) respectively. Thirty-nine (27.3%), 10 (7.0%), and 11 (7.7%) tumors had lymphovascular invasion, mucinous component, and poor differentiation in histology, respectively. Twenty (14.0%) and 18 (12.6%) patients had cancers with MSI-high or EMAST, respectively.

Of the 143 CRC patients, the mean level of LINE-1 methylation was 60.5 ± 12.5% (median 65.2%, range 22.9–79.0%). According to previous studies [34,35], we defined below 60% as the threshold for LINE-1 hypomethylation, with 93 cases (65.0%) meeting this definition.

As shown in Table 1, tumors with the LINE-1 hypomethylation were not significantly associated with any other clinical features, including age, gender, and location. There was a trend that LINE-1 hypomethylation tumors associated with advanced disease, but it did not reach statistical significance (p = 0.093). The LINE-1 hypomethylation tumors had 21.5% and 36.6% stage III and stage IV disease, respectively. In contrast, the normal LINE-1 methylation tumors had 28% and 20% stage III and stage IV disease, respectively. The other pathological features, including lymphovascular invasion (LVI), mucinous histology, and poor differentiation were not significantly associated with LINE-1 hypomethylation tumors.
As shown in S1 Table, there was no significant association between mutations of 12 genes, MSI-high, EMAST, and tumor LINE-1 methylation level. Because of rarity in individual gene mutation, mutation in genes assuming to have function in similar pathway were organized together. However, we could not find any association between tumor LINE-1 methylation level and alterations of molecular pathways (Table 2).
The median follow-up was 61.2 months. There were 61 patients who developed metastatic disease, including liver (25), lung (17), peritoneal (12), and others (8). The five-year disease-free survival (DFS) curve of patients with LINE-1 hypomethylation tumors was 52%, significantly lower than that of normal LINE-1 methylation tumor patients (78%, p = 0.032; Fig 2A). In addition, the five-year overall survival (OS) curve of patients with LINE-1 hypomethylation tumors was 41%, significantly lower than that of normal LINE-1 methylation tumor patients (76%, p = 0.001; Fig 2B). The Cox regression model enrolling factors including TNM staging, LVI, mucinous histology, tumor differentiation and tumor LINE-1 hypomethylation (Tables 3 and 4) showed that only TNM staging was an independent prognostic factor for colorectal cancer patients including DFS (HR = 3.14, 95%; CI: 2.20–4.47) and OS (HR = 4.01, 95%; CI: 2.61–6.61).

This study provided three major contributions. First, the cut-off value of tumor LINE-1 methylation could be defined at 60%. Second, LINE-1 hypomethylation was not associated

| Table 1. Clinicopathological features of patients with hypo and normal methylation of Line-1 in colorectal cancer tissue. |
|-------------|------------------|------------------|------------------|
|             | <60% methylation | >60% methylation | p                |
| Age         | 70.76±12.7       | 68.5±12.3        | 0.542            |
| Gender(female) | 29(31.2)     | 21(42.0)         | 0.204            |
| Location    |                 |                 |                  |
| Colon       | 65(69.9)         | 33(66.0)         | 0.707            |
| Rectum      | 28(62.2)         | 17(34.0)         |                  |
| TNM stage   |                 |                 |                  |
| I           | 17(18.3)         | 16(32.0)         | 0.093            |
| II          | 22(23.7)         | 10(20.0)         |                  |
| III         | 20(21.5)         | 14(28.0)         |                  |
| IV          | 34(36.6)         | 10(20.0)         |                  |
| Lymphovascular Invasion(+) | 26(28.0) | 13(26.0) | 0.846 |
| Lymphovascular Invasion(-) | 67(72.0) | 37(74.0) | 0.329 |
| Differentiated poorly(+) | 9(9.7) | 2(4) | 0.093 |
| Differentiated poorly(-) | 84(93.5) | 48(96) | 0.093 |
| Mucinous histology(+) | 6(6.5) | 4(8.0) | 0.329 |
| Mucinous Histology(-) | 87(93.5) | 46(92.0) | 0.603 |

Hypomethylation defined as <60% methylation

https://doi.org/10.1371/journal.pone.0197681.t001

The median follow-up was 61.2 months. There were 61 patients who developed metastatic disease, including liver (25), lung (17), peritoneal (12), and others (8). The five-year disease-free survival (DFS) curve of patients with LINE-1 hypomethylation tumors was 52%, significantly lower than that of normal LINE-1 methylation tumor patients (78%, p = 0.032; Fig 2A). In addition, the five-year overall survival (OS) curve of patients with LINE-1 hypomethylation tumors was 41%, significantly lower than that of normal LINE-1 methylation tumor patients (76%, p = 0.001; Fig 2B). The Cox regression model enrolling factors including TNM staging, LVI, mucinous histology, tumor differentiation and tumor LINE-1 hypomethylation (Tables 3 and 4) showed that only TNM staging was an independent prognostic factor for colorectal cancer patients including DFS (HR = 3.14, 95%; CI: 2.20–4.47) and OS (HR = 4.01, 95%; CI: 2.61–6.61).

This study provided three major contributions. First, the cut-off value of tumor LINE-1 methylation could be defined at 60%. Second, LINE-1 hypomethylation was not associated

| Table 2. Molecular alterations between patients with hypo and normal methylation of Line-1 in colorectal cancer tissue. |
|-------------|------------------|------------------|------------------|
| Alterations in Pathway | <60% methylation | >60% methylation | p value |
|              | Case no:93 (%)   | Case no:50 (%)   |       |
| APC- TP53- FBXW7 | 52 55.9 | 33 66.0 | 0.286 |
| KRAS- NRAS- HRAS | 38 40.9 | 23 46.0 | 0.597 |
| BRAF          | 10 10.8 | 5 10.0 | 1.000 |
| PI3KCA-PTEN- AKT1 | 8 8.8 | 4 8.0 | 1.000 |
| TGFB- SMAD4   | 5 5.4 | 3 6.0 | 1.000 |
| MSI-high      | 11 11.8 | 9 18.0 | 0.322 |
| EMAST         | 13 14.0 | 5 10.0 | 0.603 |

Hypomethylation defined as <60% methylation

https://doi.org/10.1371/journal.pone.0197681.t002
with mutation of the genes studied including MSI-high, and EMAST. Third, LINE-1 hypomethylation was insignificantly associated with advanced disease. Further, hypomethylation of LINE-1 in tumor tissue impacted patients’ outcomes including OS and DFS in univariate analysis but not in multivariate analysis.

Our series showed that LINE-1 methylation in normal subjects was near 81% in average. As shown in previous studies, the average LINE-1 methylation of other normal tissues was approximately 70–90%, including kidney, colon, stomach, and peripheral blood [35–37]. In our series, 37.6% of normal subjects had LINE-1 methylation lower than 80% in the peripheral blood. Previous studies demonstrated that LINE-1 hypomethylation was usually related to genomic instability and resulted in some neoplasms [28,38–41]. These groups of cases with LINE-1 lower than 80% deserved to be closely monitored for future disease development.

The LINE-1 methylation level in our CRC tumors was approximately 60% (median 65.2%, range 22.9–79.0%), similar to the large-scale study. In the Nurses’ Health Study and the Health Professionals Follow-Up Study, in 1121 CRC patients, tumor LINE-1 methylation level ranged from 23.1% to 93.1% with a mean of 62.7 ± 9.4% [42]. In another study of 217 CRC patients, tumor LINE-1 methylation level ranged from 24 to 68% with a mean of 54.3 ± 7.5% [13]. Therefore, the definition of less than 60% as the cut-off value of LINE-1 hypomethylation was reasonable.

---

**Table 3. Univariate and multivariate analysis for disease-free survival.**

|                      | Univariate analysis | Multivariate analysis |
|----------------------|---------------------|-----------------------|
|                      | HR      | 95% CI       | p         | HR      | 95% CI       | p         |
| TNM                  | 3.29    | 2.36–4.57    | <0.001    | 3.14    | 2.20–4.47    | <0.001    |
| Lymphovascular invasion | 2.67    | 1.62–4.39    | <0.001    | 1.99    | 1.18–3.38    | 0.010     |
| Mucinous Histology    | 2.51    | 1.14–5.53    | 0.023     | 1.92    | 0.84–4.40    | 0.119     |
| poor differentiation  | 1.03    | 0.37–2.77    | 0.99      | 1.21    | 0.43–3.43    | 0.707     |
| Line-1 hypomethylation | 1.86    | 1.07–3.25    | 0.027     | 1.50    | 0.87–2.75    | 0.132     |

HR: Hazard ratio; CI: confidence interval

---

https://doi.org/10.1371/journal.pone.0197681.t003

---

**Fig 2.** A) Green Line: 5-year disease-free survival of patients with normal LINE-1 methylation(76%) Blue Line: 5-year disease-free of patients with LINE-1 hypomethylation(41%, p = 0.001). B) Green Line: 5-year overall survival of patients with normal LINE-1 methylation(76%) Blue Line: 5-year overall survival of patients with LINE-1 hypomethylation(41%, p = 0.001).

https://doi.org/10.1371/journal.pone.0197681.g002
According to this definition, 65% of cases had LINE-1 hypomethylation. In contrast to previous studies showing that LINE-1 hypomethylation was associated with higher pN stage and metastatic disease, and inversely associated with poor tumor differentiation [43,44], our series demonstrated that other than a marginal association between tumor LINE-1 hypomethylation and advanced-stage disease (stages III and IV), tumor LINE-1 hypomethylation was not associated with any other clinicopathological features. Tumor LINE-1 hypomethylation associated with advanced-stage disease had been published in several studies but was not conclusive [44–47]. A study designed by Benard et al. demonstrated that LINE-1 methylation of normal tissues was approximately 90%, and 14.2% higher than those of tumor tissues on average [44]. As tumor node metastasis (TNM) stage increased, LINE-1 methylation decreased from 80% (stage I) to 65% (stage III) [44]. Sunami et al. suggested that genomic methylation level might decrease during CRC carcinogenesis and progression, because their data provided evidence of a linear correlation between tumor LINE-1 hypomethylation progression and TNM stage progression [45]. In contrast, Murata et al. [46] showed LINE-1 methylation levels of liver metastases were similar to those of primary tumors (69 ± 11.3%). In addition, a large population-based CRC study [47] including 869 cases demonstrated that LINE-1 methylation levels in tumors were not associated with tumor stage (stages I–IV). Our results showed LINE-1 hypomethylation affecting CRC patients’ outcomes in univariate analysis but not in multivariate analysis. A possible explanation is that TNM impacted patients’ outcomes in univariate and multivariate analysis and our LINE-1 hypomethylation was marginally associated with TNM stage.

Genome-wide DNA hypomethylation has been associated with genomic and chromosomal instability (CIN) [48–50]. Further, LINE-1 hypomethylation was found to be associated with p53 mutation [13] and activation of proto-oncogenes including MET [51], but inversely correlated with MSI [42,52]. However, our series did not find any association between LINE-1 hypomethylation, mutations of 12 genes studied, and MSI-high. EMAST is a phenomenon of tetranucleotide instability. Our series did not find any association between LINE-1 methylation and EMAST. Until now, there have been no reports mentioning LINE-1 methylation and EMAST.

Although this study collected several types of molecular alterations and had LINE-1 data of normal subjects, its limitation was a sample size inadequate to achieve statistical significance. With the development of next-generation sequencing (NGS), this type of study (especially mutational analysis) should take advantage of NGS to detect the whole length of targeted genes.

**Conclusion**

Our study provided evidence that first a mean methylation level of 80% was found in normal subjects, and that this could indicate a potential threshold for pathologic activity. Second, CRC

---

Table 4. Univariate and multivariate analysis for overall survival.

|                      | Univariate analysis |           | Multivariate analysis |           |
|----------------------|---------------------|-----------|-----------------------|-----------|
|                      | HR                  | 95% CI    | p                     | HR        | 95% CI    | p         |
| TNM                  | 4.26                | 2.79–6.48 | <0.001                | 4.01      | 2.61–6.61 | <0.001    |
| Lymphovascular invasion | 2.21                | 1.30–3.73 | 0.003                 | 1.20      | 0.70–2.05 | 0.503     |
| Mucinous Histology   | 1.82                | 0.78–4.26 | 0.165                 | 2.10      | 0.51–8.19 | 0.307     |
| poor differentiation | 2.29                | 0.56–9.43 | 0.249                 | 1.44      | 0.61–3.43 | 0.409     |
| Line-1 hypomethylation | 1.80                | 1.01–3.28 | 0.049                 | 1.20      | 0.65–2.23 | 0.555     |

HR: Hazard ratio; CI: confidence interval

[https://doi.org/10.1371/journal.pone.0197681.t004](https://doi.org/10.1371/journal.pone.0197681.t004)
patients’ clinicopathological features, DFS, and OS had no association with tissue LINE-1 hypomethylation in multivariate analysis. Only TNM staging was significantly associated with patients’ outcome. Tumor LINE-1 hypomethylation was marginally associated with advanced stages of CRC. The molecular alterations including specific gene mutations, MSI and EMAST LINE-1 were not associated with tumor LINE-1 hypomethylation.

**Supporting information**

S1 Table. Molecular alterations of hypo and normal methylation of Line-1 and abbreviations. (XLSX)

S1 Dataset. The detailed information of 143 CRC patients including clinicopathological features and molecular alterations. (XLSX)

**Acknowledgments**

The authors acknowledge the Clinical and Industrial Genomic Application Development Service Center of National Core Facility Program for Biotechnology, Taiwan (MOST 106-2319-B-010-001) for sequencing.

**Author Contributions**

**Conceptualization:** Pei-Ching Lin, Jeng-Kai Jiang, Shih-Ching Chang.

**Data curation:** Shung-Haur Yang, Chun-Chi Lin, Yuan-Tzu Lan, Wei-Shone Chen, Jeng-Kai Jiang.

**Formal analysis:** Pei-Ching Lin, Shih-Ching Chang.

**Funding acquisition:** Shung-Haur Yang, Hung-Hsin Lin, Shih-Ching Chang.

**Investigation:** Yuan-Tzu Lan, Hung-Hsin Lin, Wei-Shone Chen, Shih-Ching Chang.

**Methodology:** Hung-Hsin Lin, Shih-Ching Chang.

**Project administration:** Jeng-Kai Jiang, Shih-Ching Chang.

**Resources:** Wei-Shone Chen, Jen-Kou Lin.

**Supervision:** Jen-Kou Lin, Jeng-Kai Jiang, Shih-Ching Chang.

**Validation:** Chun-Chi Lin, Jen-Kou Lin, Shih-Ching Chang.

**Visualization:** Wen-Yi Liang.

**Writing – original draft:** Tai-Chuan Kuan.

**Writing – review & editing:** Shih-Ching Chang.

**References**

1. The Department of Health. Healthy statistics: Cancer Registry Annual Report in Taiwan Area. Taiwan, R.O.C.: The Department of Health, the Executive Yuan; 2013.

2. Grady WM, Carethers JM. Genomic and epigenetic instability in colorectal cancer pathogenesis. Gastroenterology. 2008; 135(4):1079–99. Epub 2008/09/09. https://doi.org/10.1053/j.gastro.2008.07.076 PMID: 18773902

3. Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. Cell. 1990; 61(5):759–67. Epub 1990/06/01. PMID: 2188735
4. Vogelstein B, Fearon ER, Hamilton SR, Kern SE, Preisinger AC, Leppert M, et al. Genetic alterations during colorectal-tumor development. N Engl J Med. 1988; 319(9):525–32. Epub 1988/09/01. https://doi.org/10.1056/NEJM198809011980901 PMID: 2841597

5. Lan YT, Lin JK, Lin CH, Yang SH, Lin CC, Wang HS, et al. Mutations in the RAS and PI3K pathways are associated with metastatic location in colorectal cancers. J Surg Oncol. 2015; 111(7):905–10. Epub 2015/04/30. https://doi.org/10.1002/jso.23895 PMID: 25920435

6. Chang SC, Lin PC, Lin JK, Lin CH, Yang SH, Liang WY, et al. Mutation Spectra of Common Cancer-Associated Genes in Different Phenotypes of Colorectal Carcinoma Without Distant Metastasis. Ann Surg Oncol. 2016; 23(3):849–55. Epub 2015/10/17. https://doi.org/10.1245/s10434-015-4899-z PMID: 26471487

7. Lin JK, Chang SC, Yang YC, Li AF. Loss of heterozygosity and DNA aneuploidy in colorectal adenocarcinoma. Ann Surg Oncol. 2003; 10(9):1086–94. Epub 2003/11/05. PMID: 14597448

8. Cunningham JM, Kim CY, Christensen ER, Tester DJ, Parc Y, Burgart LJ, et al. The frequency of hereditary defective mismatch repair in a prospective series of unselected colorectal carcinomas. Am J Hum Genet. 2001; 69(4):780–90. Epub 2001/08/29. https://doi.org/10.1086/323658 PMID: 11524701

9. Markowitz SD, Bertagnolli MM. Molecular origins of cancer: Molecular basis of colorectal cancer. N Engl J Med. 2009; 361(25):2449–60. Epub 2009/12/19. https://doi.org/10.1056/NEJMr0804588 PMID: 20018966

10. Sinicrope FA, Sargent DJ. Molecular pathways: microsatellite instability in colorectal cancer: prognostic, predictive, and therapeutic implications. Clin Cancer Res. 2012; 18(6):1506–12. Epub 2012/02/04. https://doi.org/10.1158/1078-0432.CCR-11-1469 PMID: 22302899

11. Poullogiannis G, Frayling IM, Arends MJ. DNA mismatch repair deficiency in sporadic colorectal cancer and Lynch syndrome. Histopathology. 2010; 56(2):167–79. Epub 2010/01/28. https://doi.org/10.1111/j.1365-2559.2009.03392.x PMID: 20102395

12. Chang SC, Lin PC, Yang SH, Wang HS, Liang WY, Lin JK. Taiwan hospital-based detection of Lynch syndrome distinguishes 2 types of microsatellite instabilities in colorectal cancers. Surgery. 2010; 147(5):720–8. Epub 2010/01/05. https://doi.org/10.1016/j.surg.2009.09.069 PMID: 2004164

13. Sahnane N, Magnoli F, Bernasconi B, Tibiletti MG, Romualdi C, Pedroni M, et al. Aberrant DNA methylation profiles of inherited and sporadic colorectal cancer. Clin Epigenetics. 2015; 7:131. Epub 2015/12/24. https://doi.org/10.1186/s13148-015-0165-9 PMID: 26697123

14. Levine AJ, Phipps Al, Baron JA, Buchanan DD, Ahnen DJ, Cohen SA, et al. Clinicopathologic Risk Factor Distributions for MLH1 Promoter Region Methylation in CIMP-Positive Tumors. Cancer Epidemiol Biomarkers Prev. 2016; 25(1):68–75. Epub 2015/10/30. https://doi.org/10.1158/1055-9965.EPI-15-0935 PMID: 26512054

15. Belshaw NJ, Pal N, Tapp HS, Dainty JR, Lewis MP, Williams MR, et al. Patterns of DNA methylation in individual colonic crypts reveal aging and cancer-related field defects in the morphologically normal mucosa. Carcinogenesis. 2010; 31(6):1158–63. Epub 2010/04/17. https://doi.org/10.1093/carcin/bgp077 PMID: 20395289

16. Cryns VV, O’Reilly A, Finnegan TM, Stewart L, Warrack NJ, et al. Metastatic potential in colorectal cancer: microsatellite instability and the expression of proteins associated with DNA mismatch repair. Clin Cancer Res. 2002; 8(8):2695–704. Epub 2002/09/04. https://doi.org/10.1158/1078-0432.CCR-01-0340 PMID: 12233190

17. Yang AS, Estacio MR, Doshi K, Kondo Y, Tajara EH, Issa JP. A simple method for estimating global DNA methylation using bisulfite PCR of repetitive DNA elements. Nucleic Acids Res. 2004; 32(3):e38. Epub 2004/02/20. https://doi.org/10.1093/nar/gnh032 PMID: 14973332

18. Ardeljan D, Taylor MS, Ting DT, Burns KH. The Human Long Interspersed Element-1 Retrotransposon: An Emerging Biomarker of Neoplasia. Clin Chem. 2017; 63(4):816–22. Epub 2017/02/12. https://doi.org/10.1373/clinchem.2016.257444 PMID: 28186229

19. Kazazian HH Jr. Genetics. L1 retrotransposons shape the mammalian genome. Science. 2000; 289(5482):1152–3. Epub 2000/09/02. PMID: 10970230

20. Hancks DC, Kazazian HH Jr. Roles for retrotransposon insertions in human disease. Mobile DNA. 2016; 7:9. Epub 2016/05/10. https://doi.org/10.1186/s13100-016-0066-9 PMID: 27158268

21. Beck CR, Collier P, Macfarlane C, Malig M, Kidd JM, Eichler EE, et al. LINE-1 retrotransposition activity in human genomes. Cell. 2010; 141(7):1159–70. Epub 2010/07/07. https://doi.org/10.1016/j.cell.2010.05.021 PMID: 20602998

22. Tubio JMC, Li Y, Ju YS, Martincorena I, Cooke SL, Tojo M, et al. Mobile DNA in cancer. Extensive transcription of nonrepetitive DNA mediated by L1 retrotransposition in cancer genomes. Science. 2014; 345(6196):1251343. Epub 2014/08/02. https://doi.org/10.1126/science.1251343 PMID: 25082706

23. Barchitta M, Quattrocchi A, Mauger A, Vinciguerra M, Agodi A. LINE-1 hypomethylation in blood and tissue samples as an epigenetic marker for cancer risk: a systematic review and meta-analysis. PLoS
LINE-1 and colorectal cancer

One. 2014; 9(10):e109478. Epub 2014/10/03. https://doi.org/10.1371/journal.pone.0109478 PMID: 25275447

24. Jeyapalan JN, Doctor GT, Jones TA, Alberman SN, Tep A, Haria CM, et al. DNA methylation analysis of paediatric low-grade astrocytomas identifies a tumour-specific hypomethylation signature in pilocytic astrocytomas. Acta neuropathologica communications. 2016; 4(1):54. Epub 2016/05/28. https://doi.org/10.1186/s40478-016-0123-6 PMID: 27229157

25. Kiss NB, Kogner P, Johnsen JI, Martinsson T, Larsson C, Geli J. Quantitative global and gene-specific promoter methylation in relation to biological properties of neuroblastomas. BMC Med Genet. 2012; 13:83. Epub 2012/09/19. https://doi.org/10.1186/1471-2350-13-83 PMID: 22984959

26. Symer DE, Connelly C, Szak ST, Caputo EM, Cost GJ, Parmigiani G, et al. Human L1 Retrotransposition Is Associated with Genetic Instability In Vivo. Cell. 2002; 110(3):327–38. https://doi.org/10.1016/S0092-8674(02)00839-5 PMID: 12176320

27. Gilbert N, Lutz-Prigge S, Moran JV. Genomic deletions created upon LINE-1 retrotransposition. Cell. 27.

28. Harada K, Baba Y, Ishimoto T, Chikamoto A, Kosumi K, Hayashi H, et al. LINE-1 methylation level and patient prognosis in a database of 208 hepatocellular carcinomas. Ann Surg Oncol. 2015; 22(4):1280–7. Epub 2014/10/17. https://doi.org/10.1245/s10434-014-4134-3 PMID: 25319577

29. Ikeda K, Shiraishi K, Eguchi A, Shibata H, Yoshimoto K, Mori T, et al. Long interspersed nucleotide element 1 hypomethylation is associated with poor prognosis of lung adenocarcinoma. Ann Thorac Surg. 2013; 96(5):1790–4. Epub 2013/09/04. https://doi.org/10.1016/j.athoracsur.2013.06.035 PMID: 23998411

30. Joyce BT, Gao T, Zheng Y, Liu L, Zhang W, Dai Q, et al. Prospective changes in global DNA methylation and cancer incidence and mortality. Br J Cancer. 2016; 115(4):465–72. Epub 2016/06/29. https://doi.org/10.1038/bjc.2016.205 PMID: 27351216

31. Venderbosch S, van Lent-van Vliet S, de Haan AF, Ligtenberg MJ, Goossens M, Punt CJ, et al. EMAST promoter methylation in relation to biological properties of neuroblastomas. BMC Med Genet. 2012; 9:128. Epub 2012/03/10. https://doi.org/10.1186/1471-2350-9-128 PMID: 22355437

32. Lee SY, Chung H, Devaraj B, Iwaizumi M, Han HS, Hwang DY, et al. Microsatellite alterations at selected tetranucleotide repeats are associated with morphologies of colorectal neoplasias. Gastroenterology. 2010; 139(5):1519–25. Epub 2010/08/17. https://doi.org/10.1053/j.gastro.2010.08.001 PMID: 20708618

33. Goel A, Arnold CN, Niedzwiecki D, Carethers JM, Dowell JM, Wasserman L, et al. Frequent inactivation of PTEN by promoter hypermethylation in microsatellite instability-high sporadic colorectal cancers. Cancer Res. 2004; 64(9):3014–21. Epub 2004/05/06. PMID: 15126336

34. de Sa Pereira BM, Montalvao-de-Azevedo R, Faria PA, de Paula Silva N, Nicolau-Neto P, Maschietto M, et al. Association between long interspersed nucleotide element-1 methylation levels and relapse in Wilms tumors. Clin Epigenetics. 2017; 9:128. Epub 2017/12/20. https://doi.org/10.1007/s13148-017-0431-6 PMID: 29255437

35. Kupcinskas J, Steponaitiene R, Langner C, Smalylte G, Skiecevieciene J, Kupcinskas L, et al. LINE-1 hypomethylation is not a common event in preneoplastic stages of gastric carcinogenesis. Sci Rep. 2017; 7(1):4828. Epub 2017/07/08. https://doi.org/10.1038/s41598-017-05143-3 PMID: 28684753

36. Barchitta M, Quattrocci A, Mauger A, Canto C, La Rosa N, Cantarella MA, et al. LINE-1 hypermethylation in white blood cell DNA is associated with high-grade cervical intraepithelial neoplasia. BMC Cancer. 2017; 17(1):601. Epub 2017/09/01. https://doi.org/10.1186/s12885-017-3582-0 PMID: 28854904

37. Michels KB, Harris HR, Barault L. Birthweight, maternal weight trajectories and global DNA methylation of LINE-1 repetitive elements. PLoS One. 2011; 6(9):e25254. Epub 2011/09/07. https://doi.org/10.1371/journal.pone.0025254 PMID: 21980406

38. Woo HD, Kim J. Global DNA hypomethylation in peripheral blood leukocytes as a biomarker for cancer risk: a meta-analysis. PLoS One. 2012; 7(4):e34615. Epub 2012/04/18. https://doi.org/10.1371/journal.pone.0034615 PMID: 22509334

39. Kitkumthorn N, Tuangsintanakul T, Rattanatanyong P, Tiwawei D, Muthrangura A. LINE-1 methylation in the peripheral blood mononuclear cells of cancer patients. Clin Chim Acta. 2012; 413(9–10):869–74. Epub 2012/02/14. https://doi.org/10.1016/j.cca.2012.01.024 PMID: 22326975

40. Liu J, Hesson LB, Meagher AP, Bourke MJ, Hawkins NJ, Rand KN, et al. Relative distribution of folate species is associated with global DNA methylation in human colorectal mucosa. Cancer Prev Res (Phila). 2012; 5(7):921–9. Epub 2012/05/23. https://doi.org/10.1158/1940-6207.capr-11-0577 PMID: 22609762

41. Xu X, Gammon MD, Hernandez-Vargas H, Herceg Z, Wetmur JG, Teitelbaum SL, et al. DNA methylation in peripheral blood measured by LUMA is measured with breast cancer in a population-based
42. Inamura K, Yamauchi M, Nishihara R, Lochhead P, Qian ZR, Kuchiba A, et al. Tumor LINE-1 methylation level and microsatellite instability in relation to colorectal cancer prognosis. J Natl Cancer Inst. 2014; 106(9). Epub 2014/09/06. https://doi.org/10.1093/jnci/dju195 PMID: 25190725

43. Mima K, Nowak JA, Qian ZR, Cao Y, Song M, Masugi Y, et al. Tumor LINE-1 methylation level and colorectal cancer location in relation to patient survival. Oncotarget. 2016; 7(34):5509–109. Epub 2016/07/09. https://doi.org/10.18632/oncotarget.10398 PMID: 27391152

44. Benard A, van de Velde CJ, Lessard L, Putter H, Takeshima L, Kuppen PJ, et al. Epigenetic status of LINE-1 predicts clinical outcome in early-stage rectal cancer. Br J Cancer. 2013; 109(12):3073–83. Epub 2013/11/14. https://doi.org/10.1038/bjc.2013.654 PMID: 24220694

45. Sunami E, de Maat M, Vu A, Turner RR, Hoon DS. LINE-1 hypomethylation during primary colon cancer progression. PLoS One. 2011; 6(4):e18884. Epub 2011/05/03. https://doi.org/10.1371/journal.pone.0018884 PMID: 21533144

46. Murata A, Baba Y, Watanabe M, Shigaki H, Miyake K, Ishimoto T, et al. Methylation levels of LINE-1 in primary lesion and matched metastatic lesions of colorectal cancer. Br J Cancer. 2013; 109(2):408–15. Epub 2013/06/15. https://doi.org/10.1038/bjc.2013.289 PMID: 23764749

47. Ogino S, Kawasaki T, Nosho K, Ohnishi M, Suemoto Y, Kirkner GJ, et al. LINE-1 hypomethylation is inversely associated with microsatellite instability and CpG island methylator phenotype in colorectal cancer. Int J Cancer. 2008; 122(12):2767–73. Epub 2008/03/28. https://doi.org/10.1002/ijc.23470 PMID: 18366060

48. Gasior SL, Wakeman TP, Xu B, Deininger PL. The human LINE-1 retrotransposon creates DNA double-strand breaks. J Mol Biol. 2006; 357(5):1383–93. Epub 2006/02/24. https://doi.org/10.1016/j.jmb.2006.01.089 PMID: 16490214

49. Gaudet F, Hodgson JG, Eden A, Jackson-Grusby L, Dausman J, Gray JW, et al. Induction of Tumors in Mice by Genomic Hypomethylation. Science. 2003; 300(5618):489–92. https://doi.org/10.1126/science.1083558 PMID: 12702876

50. Rodriguez J, Frigola J, Vendrell E, Risques RA, Fraga MF, Morales C, et al. Chromosomal instability correlates with genome-wide DNA demethylation in human primary colorectal cancers. Cancer Res. 2006; 66(17):8462–9468. Epub 2006/09/05. https://doi.org/10.1158/0008-5472.CAN-06-0293 PMID: 16951157

51. Hur K, Cejas P, Feliu J, Moreno-Rubio J, Burgos E, Boland CR, et al. Hypomethylation of long interspersed nuclear element-1 (LINE-1) leads to activation of proto-oncogenes in human colorectal cancer metastasis. Gut. 2014; 63(4):635–46. Epub 2013/05/25. https://doi.org/10.1136/gutjnl-2012-304219 PMID: 23704319

52. Estecio MR, Gharibyan V, Shen L, Ibrahim AE, Doshi K, He R, et al. LINE-1 hypomethylation in cancer is highly variable and inversely correlated with microsatellite instability. PLoS One. 2007; 2(5):e399. Epub 2007/05/04. https://doi.org/10.1371/journal.pone.0000399 PMID: 17476321