Degree project

UNIVERSITY OF SKÖVDE

Identification of DNA Methylation Differences in Colon Mucosa Deriving from Healthy Controls and Patients with Colon Polyps

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Student: Ademola Aladesuru

Supervisor: Professor Yvonne Wettergren

Examiner: Professor Homa Tajsharghi

Abstract

Colorectal cancer develops through a multistep adenoma–carcinoma sequence driven by early genetic and epigenetic alterations. While advanced Colorectal cancer has been extensively characterized, less is known about molecular changes occurring in benign colorectal polyps. This study investigated early DNA methylation alterations and oncogenic mutation status in colorectal polyps compared with healthy colon mucosa to identify potential early biomarkers of colorectal tumorigenesis.

Colon mucosa samples were collected from 21 patients with colorectal polyps and 20 control individuals undergoing colonoscopy at Sahlgrenska University Hospital. DNA methylation levels of LINE-1, *MLH1, MGMT*, and *CDKN2A* (*p16INK4a*) were quantified using bisulfite pyrosequencing. *KRAS* and *BRAF* mutation status were assessed by digital polymerase chain reaction. Associations between methylation patterns, mutation status, and clinical variables were evaluated, and the diagnostic performance of LINE-1 methylation was assessed using receiver operating characteristic analysis.

Global LINE-1 methylation was significantly reduced in polyp-adjacent mucosa compared with control mucosa and demonstrated strong discriminatory power between groups (AUC = 0.863). In contrast, no aberrant hypermethylation of *MLH1* or *MGMT* was observed in polyp-adjacent mucosa, and *CDKN2A* (*p16INK4a*) methylation was significantly lower in polyps than in controls. *MLH1* methylation remained largely absent, consistent with the lack of *BRAF* mutations and microsatellite instability–associated features in this cohort. No activating *KRAS* or *BRAF* mutations were detected. The *CDKN2A* (*p16INK4a*) rs3814960 variant showed genotype-dependent methylation patterns but was not an independent predictor after adjustment for clinical variables.

These findings indicate that global DNA hypomethylation precedes tumor suppressor gene hypermethylation in early colorectal lesions and highlight LINE-1 hypomethylation as a promising early epigenetic biomarker in colorectal tumorigenesis.

Abbreviations

AUC – Area Under the Curve
Akt – Protein kinase B
BRAF – B-Raf proto-oncogene, serine/threonine kinase
CDKN2A – Cyclin-Dependent Kinase Inhibitor 2A
CI – Confidence Interval
CpG – Cytosine–phosphate–guanine dinucleotide
CIMP – CpG Island Methylator Phenotype
CRC – Colorectal Cancer
dPCR – Digital Polymerase Chain Reaction
DNA – Deoxyribonucleic Acid
DNMT – DNA Methyltransferase
EGFR – Epidermal Growth Factor Receptor
ERK – Extracellular Signal-Regulated Kinase
KRAS – Kirsten rat sarcoma viral oncogene homolog
LINE-1 – Long Interspersed Nuclear Element-1
MAFG – MAF bZIP Transcription Factor G
MAPK – Mitogen-Activated Protein Kinase
MGMT – O6-Methylguanine-DNA Methyltransferase
MLH1 – MutL Homolog 1
MMR – Mismatch Repair
MSI – Microsatellite Instability
PCR – Polymerase Chain Reaction
PI3K – Phosphoinositide 3-Kinase
ROC – Receiver Operating Characteristic
SD – Standard Deviation
SNP – Single Nucleotide Polymorphism

Popular Scientific Summary

Colorectal cancer is one of the most common cancers worldwide and often develops slowly over many years. In many cases, the disease begins as small growths in the intestine called polyps. Although most polyps are benign, some can gradually develop into cancer. Understanding the earliest molecular changes in these polyps is important for improving cancer prevention, early detection, and patient outcomes.

This study focused on changes in DNA methylation, a chemical modification that helps regulate how genes are switched on and off. Abnormal DNA methylation can disturb normal cell function and contribute to cancer development. Colon tissue samples were collected from individuals with colorectal polyps and from healthy control individuals undergoing colonoscopy. The study examined both global DNA methylation, using a marker called LINE-1, and gene-specific methylation in several important cancer-related genes.

The results showed that tissue from individuals with polyps had lower global DNA methylation compared with healthy tissue. This finding suggests that loss of DNA methylation is an early molecular change that may occur before cancer develops. In contrast, changes in specific tumor suppressor genes were limited or absent at this early stage, indicating that these gene alterations may happen later in disease progression. No common cancer-driving mutations were detected in the polyps, further supporting the idea that epigenetic changes occur early.

Overall, this research improves understanding of the earliest molecular events in colorectal disease. The findings suggest that global DNA methylation changes, such as LINE-1 hypomethylation, could potentially be used in the future as early markers to identify individuals at higher risk of developing colorectal cancer.

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1 Introduction

Colorectal cancer (CRC) is one of the most common cancers worldwide, ranking third in incidence and second in mortality, with rates approximately four times higher in developed compared with developing countries (Sung et al., 2021). Most CRC cases develop gradually from benign adenomatous polyps through the well-established adenoma–carcinoma sequence, driven by multiple genetic and epigenetic alterations (Nguyen et al., 2019). Colorectal polyps are classified histologically as neoplastic or non-neoplastic, and their malignant potential depends on various cellular factors, including polyp type and architectural features (Colucci et al., 2003). The adenoma–carcinoma sequence represents a molecular progression from normal mucosa to adenoma and eventually to carcinoma, involving pathways that deactivate tumor suppressor genes and activate oncogenes. Understanding the early molecular changes that occur in this sequence is therefore essential for improving early CRC diagnosis, prevention, and treatment.

Both genetic mutations and epigenetic modifications contribute significantly to the transition from colorectal polyps to CRC. Epigenetic alterations regulate gene expression without modifying the underlying DNA sequence and include DNA methylation, histone modifications, chromatin remodeling, and non-coding RNA-mediated regulation (Nina Hauptman et al., 2013; Debina Sarkar et al., 2015). Among these mechanisms, DNA methylation in CpG dinucleotides (CpG islands) plays a crucial role in maintaining genomic stability, controlling transcription, and regulating gene expression (Chen et al., 2020). Long interspersed nuclear elements (LINE-1), which constitute ~17% of the human genome, undergo epigenetic alterations that lead to global hypomethylation of retrotransposon repeats, contributing to genomic instability in CRC (Kuan et al., 2018). LINE-1 Hypomethylation also serves as an important prognostic biomarker in advanced CRC, influencing risk stratification and survival outcomes (Akbar Bagheri et al., 2025), highlighting its significance in both hereditary and sporadic disease contexts. At the same time, hypermethylation of CpG sites in promoter regions or first exons of tumor suppressor genes results in transcriptional silencing and directly contributes to oncogenic activation and tumor progression (Brenet et al., 2011).

Aberrant hypermethylation of CpG islands in the promoter regions of tumor suppressor genes characterizes the CpG island methylator phenotype (CIMP), which is frequently observed in CRC (Parker et al., 2018; Mojarad et al., 2013). CIMP contributes to transcriptional silencing and plays a major role in the initiation and progression of adenomas toward carcinoma and has emerged as an early biomarker of CRC (Mun-Kar Ng et al., 2015; Wettergren et al., 2025). One of the key features associated with CIMP-positive CRC is hypermethylation-induced inactivation of the mismatch repair (MMR) gene *MLH1*. Loss of *MLH1* function leads to the accumulation of mutations in repetitive DNA sequences, resulting in microsatellite instability (MSI), a phenotype frequently observed in CRC (Bettington et al., 2013). Approximately 16% of CRCs are hypermutated, most of which display MSI caused by promoter hypermethylation of the *MLH1* gene (Michael T. Parsons et al., 2012; M. F. Kane et al., 1997), leading to sporadic MMR deficiency.

The *MGMT* gene (*O6-methylguanine-DNA methyltransferase*) encodes a DNA repair protein that plays a critical role in maintaining genomic stability by removing mutagenic O6-methylguanine adducts. Through this function, *MGMT* helps prevent replication and transcription errors that may otherwise lead to permanent mutations (Wettergren et al., 2025). Hypermethylation of *MGMT*, which results in transcriptional silencing of exon 1, has been associated with an increased frequency of G>A transition mutations in the *KRAS* gene (Zawlik et al., 2008; Nagasaka et al., 2008).

The *CDKN2A* gene is another extensively studied epigenetic biomarker in CRC. Its inactivation through promoter or exon hypermethylation disrupts the gene's ability to regulate the cell cycle, a defect widely reported across human malignancies (Herman et al., 1995; Zhao R et al., 2016). *CDKN2A* encodes two critical proteins: *p16INK4a* and *p14ARF*, which regulate cell cycle progression and apoptosis via both *p53*-dependent and *p53*-independent pathways. Silencing of *p14ARF* through promoter hypermethylation leads to the loss of *p53*-mediated apoptosis and has been linked to tumor development, including melanoma progression (Junichi Furuta et al., 2006). Aberrant methylation and transcriptional silence of *CDKN2A* result in uncontrolled cell division and proliferation, representing a key mechanism in CRC development (Ye et al., 2018).

Mutations in *BRAF and KRAS* represent two of the most common oncogenic events in CRC (Antonia R. Sepulveda et al., 2017). These mutations activate the MAPK signaling cascade, promoting sustained cellular proliferation. *BRAF* acts as a regulator of DNA methyltransferase (DNMT) gene expression, and the activating *BRAF* V600E mutation promotes hypermethylation of tumor suppressor genes by upregulating DNMT1 (Peng Hou et al., 2012). *BRAF* V600E driven signaling increases phosphorylated MAFG levels, strengthening MAFG–DNA interactions and driving aberrant CpG island hypermethylation (CIMP), including hypermethylation of *MLH1* that contributes to MSI (Minggang Fang et al., 2014). *KRAS* mutations typically arise at codons 12, 13, or 61, preventing GTP hydrolysis and resulting in constitutive activation of the *KRAS* protein (Ian A. Prior et al., 2012; Adrienne D. Cox et al., 2014). This persistent activation stimulates downstream MAPK and PI3K–AKT signaling pathways, promoting proliferation, survival, and metabolic reprogramming. In the adenoma–carcinoma sequence, *KRAS* represents an intermediate oncogenic event, while in the serrated pathway it defines a subset of traditional serrated adenomas (Fearon & Vogelstein, 1990; Bettington et al., 2017). Clinically, *KRAS* mutation status is important because it confers resistance to anti-EGFR therapies and is associated with a more aggressive tumor phenotype (Karapetis et al., 2008).

Although molecular features of advanced CRC have been well described, far less is known about early molecular alterations in benign polyps that may represent the initial stages of malignant transformation. Comparing methylation and mutation profiles between polyps mucosa and normal mucosa may provide insight into early carcinogenic events and support the identification of biomarkers for early detection. The present study, therefore, investigated epigenetic and genetic alterations in colonic mucosa 10cm away from colorectal polyps (polyps group) compared with healthy mucosa (control group) to identify early molecular changes of prognostic importance in colorectal tumorigenesis. DNA hypomethylation in LINE-1 and hypermethylation of *MLH1, MGMT,* and *p16INK4a (CDKN2A*) were quantified using pyrosequencing, and *KRAS* and *BRAF* mutation status in polyps mucosa was analyzed using digital polymerase chain reaction (dPCR). Associations between methylation levels, mutation status, and clinical variables (age, sex, colon side) were assessed. In addition, the diagnostic performance of methylation markers in distinguishing polyps from normal mucosa was evaluated. It was hypothesized that polyps would exhibit reduced LINE-1 methylation and increased methylation of *MLH1, MGMT,* and *p16INK4a*, that *KRAS* and *BRAF* mutations would be present in a subset of lesions, and that these molecular features would correlate with clinical characteristics and demonstrate potential as early diagnostic markers.

2 Patients and Methods

2.1 Study Subject and Sample Collection

Study subjects undergoing colonoscopy for various indications at Sahlgrenska University Hospital, Gothenburg, were included. The cohort consisted of twenty controls (11 females, 9 males) and twenty-one patients (10 females, 11 males) diagnosed with different types of colorectal polyps. Inclusion criteria for controls were age ≥18 years and a normal-appearing mucosa throughout the colon, with no evidence of polyps, inflammatory bowel disease, malignancy, or ischemic colitis. During colonoscopy, mucosal biopsies were obtained approximately 10 cm from the polyps in the sigmoid colon (left side) and from the mid-portion of the ascending colon (right side) using biopsy forceps. Samples were immediately stabilized in Allprotect Tissue Reagent (Qiagen) and stored at −80°C until further processing.

2.2 DNA Isolation

Genomic DNA was extracted from tissue samples using the Qiagen AllPrep DNA/RNA/Protein Kit (no. 80014) according to the manufacturer's instructions and stored at −20°C until analysis.

2.3 Bisulfite Conversion, PCR Amplification, and Methylation Analysis

Genomic DNA (200 ng/µL) from each sample was bisulfite-converted using the EpiTecht® Fast Bisulfite Kit (Qiagen, no. 181023354) according to the manufacturer's protocol. PCR amplification of bisulfite-converted DNA targeting *MLH1, MGMT, p16INK4a*, and LINE-1 was performed using the PyroMark Q24 CpG PCR Kit (Qiagen) on a Bio-Rad thermal cycler in a total reaction volume of 25 µL.

Thermocycling conditions for *MLH1* and *p16INK4a* consisted of initial denaturation at 95°C for 15 minutes, followed by 45 cycles of 95°C for 20 seconds, 55°C for 20 seconds, and 72°C for 20 seconds, with a final extension at 72°C for 5 minutes. For *MGMT*, annealing was performed at 53°C under otherwise identical conditions. LINE-1 amplification included an initial denaturation at 95°C for 15 minutes, followed by 45 cycles of 94°C for 30 seconds, 50°C for 30 seconds, and 72°C for 30 seconds, with a final extension at 72°C for 10 minutes. PCR reaction compositions are detailed in Table 1.

Table 1. Component of PCR reaction

Component	Volume/Reaction	Final concentration
Template DNA	3 μL	20 ng bisulfite-converted DNA
PyroMark PCR Master Mix, 2x	12.5 μL	Contains HotStarTag DNA Polymerase, 1x PyroMark PCR Buffer, and dNTPs
CoralLoad Concentrate, 10x	2.5 μL	1x
Forward primer (10 mM)	0.5 μL	0.2 μM
Reverse primer (10 mM)	0.5 μl	0.2 μM
RNase-free water	6 μl	
Total Volume	25 μL	

Locus-specific methylation of *MLH1, MGMT,* and *p16INK4a* was quantified using PyroMark Q24 assays (Qiagen). The analyzed regions were *MLH1* −209 to −181 relative to the transcription start site, *MGMT* +17 to +39 in exon 1, and *p16INK4a* +68 to +120 in exon 1. Global LINE-1 methylation was assessed using the PyroMark Q24 CpG LINE-1 assay (Qiagen). Assay characteristics, including target sequences and CpG numbers, are summarized in Tables 2 and 3.

Table 2. PyroMark Q24 CpG assays used

Assay Names	Sequence to analyze	Numbers of CpGs
MLH1	YGGATAGYGATTTTTAAYGYGTAAGYGTATA	5
p16INK4a	TYGTTAAGTGTTYGGAGTTAATAGTATTTTTTTYGAGTATTYGT TTAYGGYGT	6
MGMT	YGTTTTGYGTTTYGAYGTTYGTAGGTTTT	5
LINE-1	TTYGTGGTGYGTYGTTT	3

Table 3. Methylation detection assay details

Gene	Assay	Cat No./ID.
MLH1	PyroMark Q24 CpG *MLH1* methylation detection assay	970022
MGMT	PyroMark Q24 CpG *MGMT* methylation detection assay	PM00039907
p16INK4a	PyroMark CpG Assay (200)	978746
LINE-1	PyroMark Q24 CpG LINE-1 methylation detection assay	970042

2.4 Pyrosequencing Procedure

For pyrosequencing, 40 µL PyroMark binding buffer (Qiagen) was mixed with 19 µL nuclease-free water and 1 µL streptavidin Sepharose beads. From this mixture, 60 µL was added to 20 µL of biotinylated PCR product in a PCR tube strip (Bio-Rad) and agitated at 1400 rpm for 10 minutes at room temperature. Each run included a negative control (nuclease-free water) and a positive control (EpiTect® Methylated Human DNA, bisulfite-converted; Qiagen). PyroMark Q24 cartridges were loaded with PyroMark Advanced Reagents and PyroMark Q24 Advanced CpG Reagents (Qiagen). Sequencing primers (0.375 µM) were prepared in 20 µL PyroMark Advanced Annealing Buffer and dispensed into the PyroMark Advanced Q24 Plate. Sample–primer annealing was performed using the PyroMark Vacuum Workstation followed by heating at 80°C for 5 minutes (*MLH1*) or 2.5 minutes (*p16INK4a, MGMT*, and LINE-1). Samples were then analyzed using the PyroMark Advanced Q24 instrument.

2.5 Quantification of DNA Methylation

Pyrosequencing data were analyzed using PyroMark Q24 software (Qiagen), with methylation percentages determined for each CpG site based on C/T ratios. Mean methylation levels were calculated across locus-specific CpG sites for *MLH1* (5 CpGs), *p16INK4a* (5 CpGs), *MGMT* (5 CpGs), and the global methylation level for LINE-1 (3 CpGs). For *p16INK4a*, the CpG site overlapping SNP *rs3814960* (C>T), located at the fifth CpG in the *p16INK4a* amplicon, was excluded from mean methylation calculations. The *T* allele eliminates this CpG site, so heterozygotes (*CT*) retain one CpG and homozygous *TT* lack that CpG on both alleles, resulting in reduced methylatable substrate and potentially lower measured methylation levels.

2.6 *KRAS* and *BRAS* Mutation Analysis

KRAS and *BRAF* mutation status was analyzed by digital PCR using the QIAcuity One system (Qiagen) according to the manufacturer's instructions. Each 40 µL reaction contained 10 µL QIAcuity Master Mix, 1.3 µL target dPCR LNA Mutation Assay-1 (FAM/HEX), 1 µL HaeIII restriction enzyme (TaKaRa), 25.2 µL RNase-free water, and 2.5 µL genomic DNA (20 ng/µL). Reactions were loaded onto a Nanoplate 26K (24-well; Qiagen). Negative (nuclease-free water) and positive (mutant DNA) controls were included in each run. PCR amplification consisted of an initial activation at 95°C for 2 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 30 seconds. Mutation calls were analyzed using QIAcuity Software Suite version 3.1.0.0_SOW-865-12 (Qiagen).

2.7 Statistical Analysis

Methylation data were analyzed using R version 4.5.0. Data are presented as mean ± standard deviation or median with 95% confidence intervals, as appropriate. Differences between polyp and control groups were assessed using Welch's t-test or the Wilcoxon test. Paired t-tests were applied for comparisons between left- and right-sided samples within the same individual. Associations between methylation levels and age were evaluated using Pearson and Spearman correlation analyses. Linear regression models were used to assess the effects of group (polyp vs control), age, and sex on methylation levels. Multiple testing across CpG sites was corrected using the Benjamini–Hochberg false discovery rate. A p-value <0.05 was considered statistically significant. Receiver operating characteristics (ROC) analysis was used to evaluate the ability of LINE-1 methylation to distinguish polyp from control mucosa, with an area under the curve (AUC) of 0.8 considered statistically significant.

For genotype analyses, genotype frequencies were calculated at the individual level (n = 41), while patient-level mean *p16INK4a* methylation values (the average of left and right samples) were used. Normality was assessed using Shapiro–Wilk tests; due to borderline non-normality in the *TT* genotype group, Kruskal–Wallis's testing with Benjamini–Hochberg-corrected Dunn post-hoc comparisons was applied. One-way ANOVA was also reported. Linear regression models, including genotype (*CC* reference), group, age, and sex were fitted, with robust MM-estimator regression used to reduce sensitivity to outliers.

3 Results

3.1 Gene-Specific Methylation in Control Mucosa

The polyp group had a higher mean age (62.8 years; range 29–84) compared with controls (54.2 years; range 21–79), although this difference did not reach statistical significance (Welch's t-test p = 0.071). The polyp group included 21 individuals (10 females, 11 males), and the control group included 20 individuals (11 females, 9 males), yielding an overall female-to-male ratio of 21:20

In control mucosa, mean methylation levels were 2.86 for *p16INK4a*, 1.10 for *MLH1*, and 1.76 for *MGMT*. For *p16INK4a*, 200 CpG sites were analyzed, of which 23 sites (11.5%) exhibited methylation ≥5%. Elevated methylation was predominantly observed at the third CpG site, which consistently showed the highest methylation levels on both the left and right sides of the colon across all control samples.

MLH1 methylation levels across all 200 CpG sites ranged between 0–4%. For *MGMT*, methylation levels across 190 CpG sites were predominantly within the 0–4% range; however, 13 sites (6.84%) exceeded this threshold. Notably, five CpG sites (2.63%) on the left side of the colon in one control sample showed methylation levels of 5–7%, and another five CpG sites (2.63%) on the right side in a different control sample exhibited similarly elevated methylation. No significant differences in methylation levels were observed between left- and right-sided control mucosa for *p16INK4a*, *MLH1*, or *MGMT*.

3.2 Gene-Specific Methylation in Poly-Adjacent Mucosa

In mucosa sampled approximately 10 cm from polyps, mean methylation levels were 1.97 for *p16INK4a*, 0.85 for *MLH1*, and *1.63* for *MGMT*. Among 210 CpG sites analyzed for *p16INK4a*, only two sites (0.95%) displayed methylation ≥5%, both located on the left side of the colon in different cases. As observed in controls, the third CpG site showed the highest methylation levels on both sides of the colon across all samples.

MLH1 methylation levels across all 210 CpG sites ranged between 0–4%, with 67 sites (31.9%) showing no detectable methylation. For *MGMT*, 105 CpG sites were analyzed, with most exhibiting methylation between 0–4%. Elevated methylation was detected in a small number of sites, including five CpGs (4.76%) with methylation levels of 6–8% in one left-sided sample and two CpGs (1.90%) with methylation levels of 5–6% in another sample. No significant differences were observed between left- and right-sided methylation levels for *p16INK4a, MLH1,* or *MGMT* in polyp-adjacent mucosa, nor were differences observed in left–right asymmetry between control and polyp groups.

3.3 Global LINE-1 Methylation

Global LINE-1 methylation was significantly reduced in polyp mucosa (mean = 69.35%) compared with controls (mean = 76.67%) (Welch's t-test p = 2.66×10^{-10}; Wilcoxon p =

1.59×10^{-8}; Figure 1a). No significant differences were observed between right- and left-sided samples within or between groups.

LINE-1 methylation showed no significant correlation with age in either group (Pearson p = 0.29; Spearman p = 0.38) and did not differ between females and males (Welch p = 0.51; Wilcoxon p = 0.57). Multivariable regression analysis identified clinical group status as the only independent predictor of LINE-1 methylation; after adjustment for age, sex, and lesion side, polyp mucosa samples showed a marked reduction in LINE-1 methylation compared with controls ($\beta = -7.52$, $p = 9.38 \times 10^{-10}$; Figure 1b). ROC analysis demonstrated strong discriminatory power of LINE-1 methylation for distinguishing polyp mucosa from control mucosa (AUC = 0.863; Figure 1c). An optimal cut-off value of 73% yielded 81% sensitivity and 85% specificity.

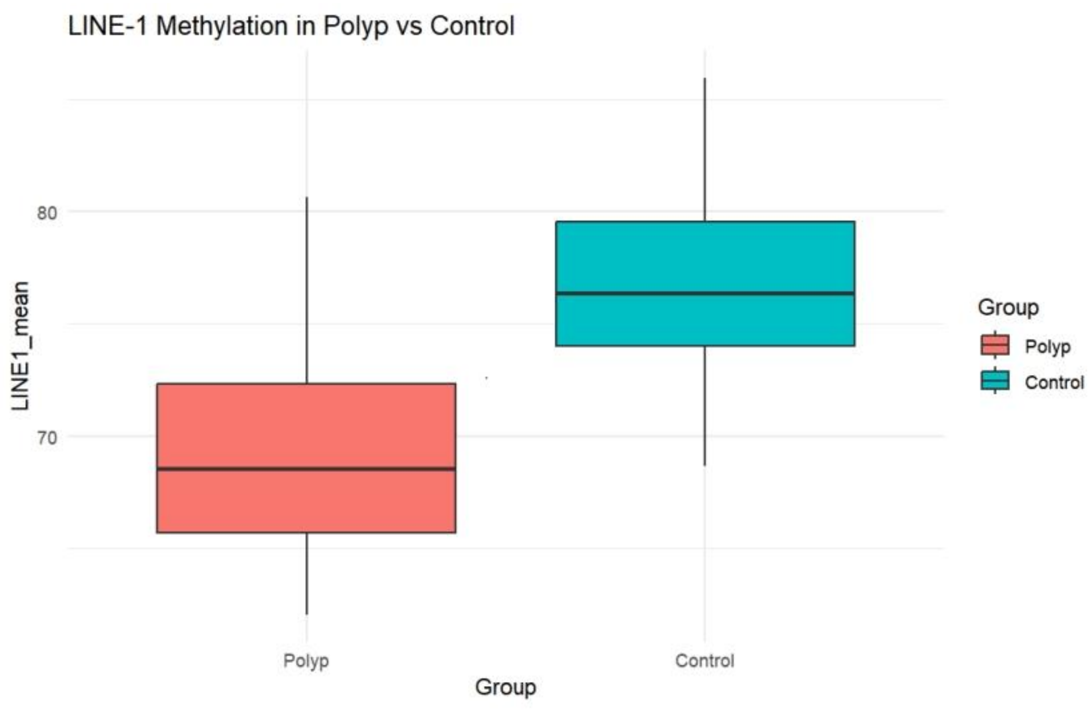

Figure 1a. LINE-1 methylation levels in controls and polyp patients Boxplot showing per-patient mean LINE-1 methylation levels in control subjects and patients with colorectal polyps. Boxes indicate the interquartile range (IQR), horizontal lines represent the median, and whiskers extend to 1.5×IQR. LINE-1 methylation was significantly lower in the mucosa of polyp patients compared with controls, consistent with global hypomethylation in premalignant tissue.

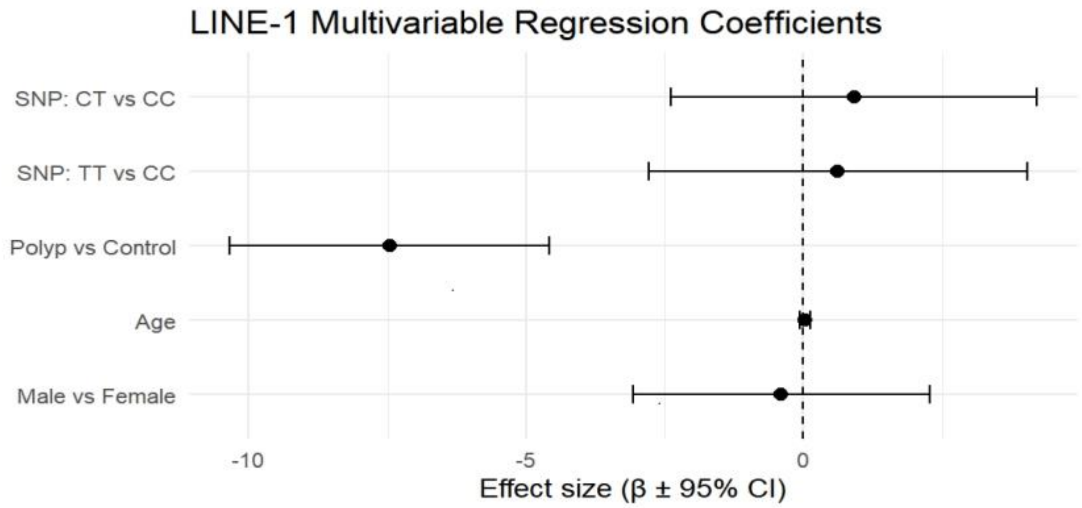

Figure 1b. Multivariable linear regression of factors associated with LINE-1 methylation

Forest plot of regression coefficients from a multivariable linear model assessing the association between LINE-1 methylation and clinical variables (group, age, gender, and lesion side). Points represent estimated regression coefficients and horizontal lines indicate 95% confidence intervals. Polyp status was the only variable significantly associated with LINE-1 methylation, demonstrating that LINE-1 hypomethylation is primarily driven by disease status rather than demographic or anatomical factors.

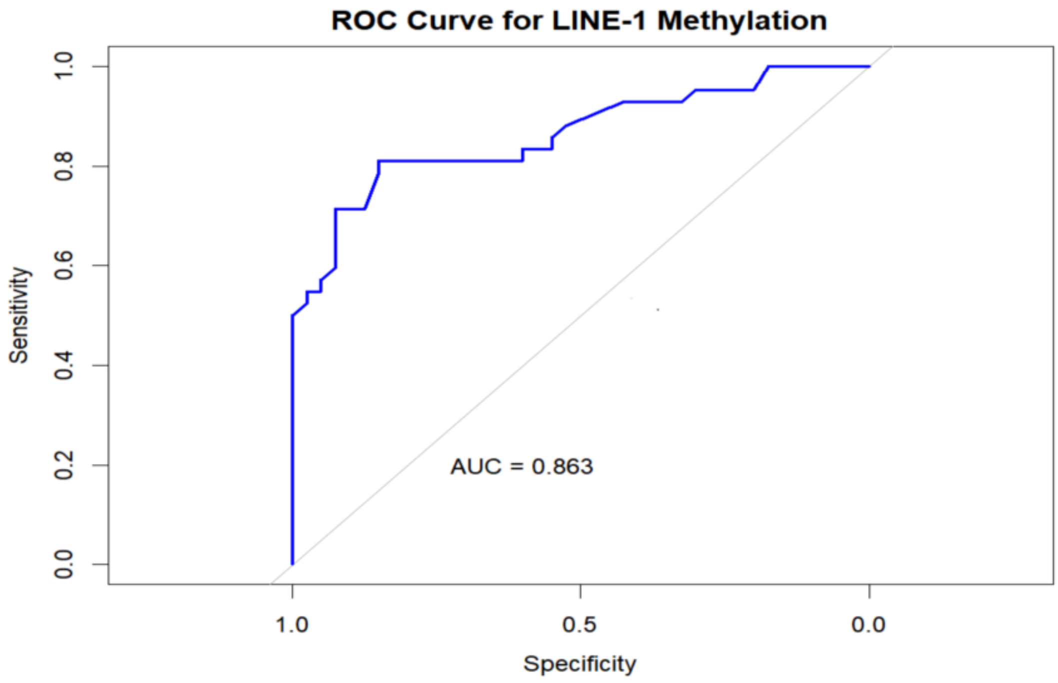

Figure 1c. Receiver operating characteristic (ROC) curve for LINE-1 methylation
Receiver operating characteristic (ROC) curve evaluating the performance of LINE-1 methylation levels in distinguishing polyp patients from controls. The area under the curve (AUC) indicates good discriminatory ability, supporting the potential of LINE-1 hypomethylation as a marker of early colorectal neoplastic change.

3.4 *P16INK4a* Methylation

p16INK4a methylation was significantly lower in polyp mucosa samples (mean = 1.97) compared with controls (mean = 2.86; Welch's t-test $p = 9.6 \times 10^{-13}$; Figure 2). No significant differences were observed in left–right methylation asymmetry between groups or within either group. *p16INK4a* methylation showed a weak negative association with age (Pearson $r = -0.263$, $p = 0.017$), although this relationship was not significant using Spearman correlation ($\rho = -0.179$, $p = 0.108$). No significant differences were observed between males and females.

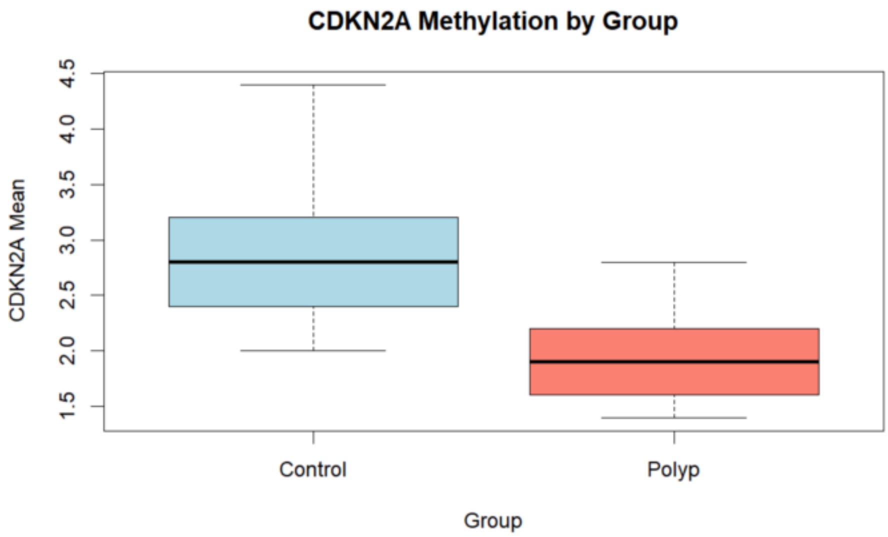

Figure 2. *p16INK4a (CDKN2A)* methylation by group
Distribution of mean *p16INK4a* methylation levels in control mucosa and polyp tissue. Boxplots show the median and interquartile range, with whiskers representing the data range excluding outliers. *p16INK4a* methylation was significantly lower in polyp mucosa samples compared with controls, indicating disease-associated promoter hypomethylation.

3.5 *MLH1* Methylation

MLH1 methylation levels were lower in polyp mucosa samples (mean = 0.843) than in controls (mean = 1.105). This difference was not significant using Welch's t-test (p = 0.08) but reached significance using the Wilcoxon test (p = 0.032), with higher methylation observed in controls. No significant differences were detected between left- and right-sided samples or between sexes, and no associations with age were identified.

3.6 *MGMT* Methylation

MGMT methylation did not differ significantly between control and polyp groups (t-test p = 0.74; Wilcoxon p = 0.74), although mean levels were slightly higher in controls. No significant left–right asymmetry was observed. *MGMT* methylation showed a weak, non-significant positive correlation with age. Females (mean = 2.135) exhibited higher *MGMT* methylation than males (mean = 1.250) overall (t-test p = 0.016; Wilcoxon p = 0.0097); however, after stratification by group, this difference was no longer statistically significant in polyp mucosa samples. In controls, females (mean = 2.17) showed higher *MGMT* methylation than males (mean = 1.31), reaching significance by Wilcoxon testing (p = 0.049).

3.7 Distribution of *P16INK4a* rs3814960 Variants

Pyrogram analysis identified the *rs3814960* C>T SNP at the fifth CpG site within the *p16INK4a* region (Figure 3a). This CpG was excluded from mean methylation calculations due to the loss of the CpG site in the T allele. Genotype distribution did not differ significantly between control and polyp groups (χ^2 p = 0.175; Fisher's exact p = 0.188). The frequency distribution of rs3814960 (C>T) genotypes differed between the polyp and control groups and is summarized in Table 4a.

p16INK4a methylation showed genotype dependence, with *TT* carriers exhibiting the lowest mean methylation, *CT* carriers the highest, and *CC* carriers intermediate levels (Table 4b). Global genotype effects showed a non-significant trend (Kruskal–Wallis p = 0.083). *CT* vs *TT* indicated the strongest comparison (unadjusted $p = 0.032$, adjusted $p = 0.097$), while *CC* vs *TT* (adj $p = 0.165$) and *CC* vs *CT* (adj $p = 0.666$) were not significant (Figure 3b). Effect size for the genotype effect was moderate (eta2[H] = 0.0785). In multivariable regression, genotype was not an independent predictor of *p16INK4a* methylation, whereas clinical group status was strongly associated with reduced methylation in polyp mucosa samples (Table 4c). Genotype was not associated with LINE-1, *MLH1,* or *MGMT* methylation after adjustment for age, sex, and group.

Table 4a. Distribution of rs3814960 (C>T) genotypes in polyp patients and controls.
Genotype frequencies (*CC, CT, TT*) are shown as absolute numbers and percentages for the polyp (n = 21) and control (n = 20) groups.

Group	CC	CT	TT
Polyps, n (%)	5 (23.8)	6 (28.6)	10 (47.6)
Control, n (%)	7 (35)	9 (45)	4 (20)

Table 4b. *p16INK4a* methylation differences by SNP genotype

Genotype	n	Mean methylation	SD	Polyps	Control	Male	Female	Median	Interpretation
CC	12	2.49	0.64	5	7	6	6	2.45	Intermediate methylation
CT	15	2.58	0.56	6	9	7	8	2.40	Highest methylation
TT	14	2.14	0.48	10	4	7	7	2.00	Lowest methylation

Table 4c. *p16INK4a* methylation (patient mean) predicted by SNP genotype, Group, Age, Gender, and Side.

Predictor	**Effect**	**p-value**	**Interpretation**
Group (Polyp)	–0.85	p <0.001	Strongly lower CDKN2A methylation in polyps
SNP (CT vs CC)	+0.07	0.61	No effect
SNP (TT vs CC)	–0.09	0.54	No effect
Age	~0	0.64	No effect
Gender	+0.17	0.15	Not effect
Side (Left vs Right)	~0	>0.60	No effect

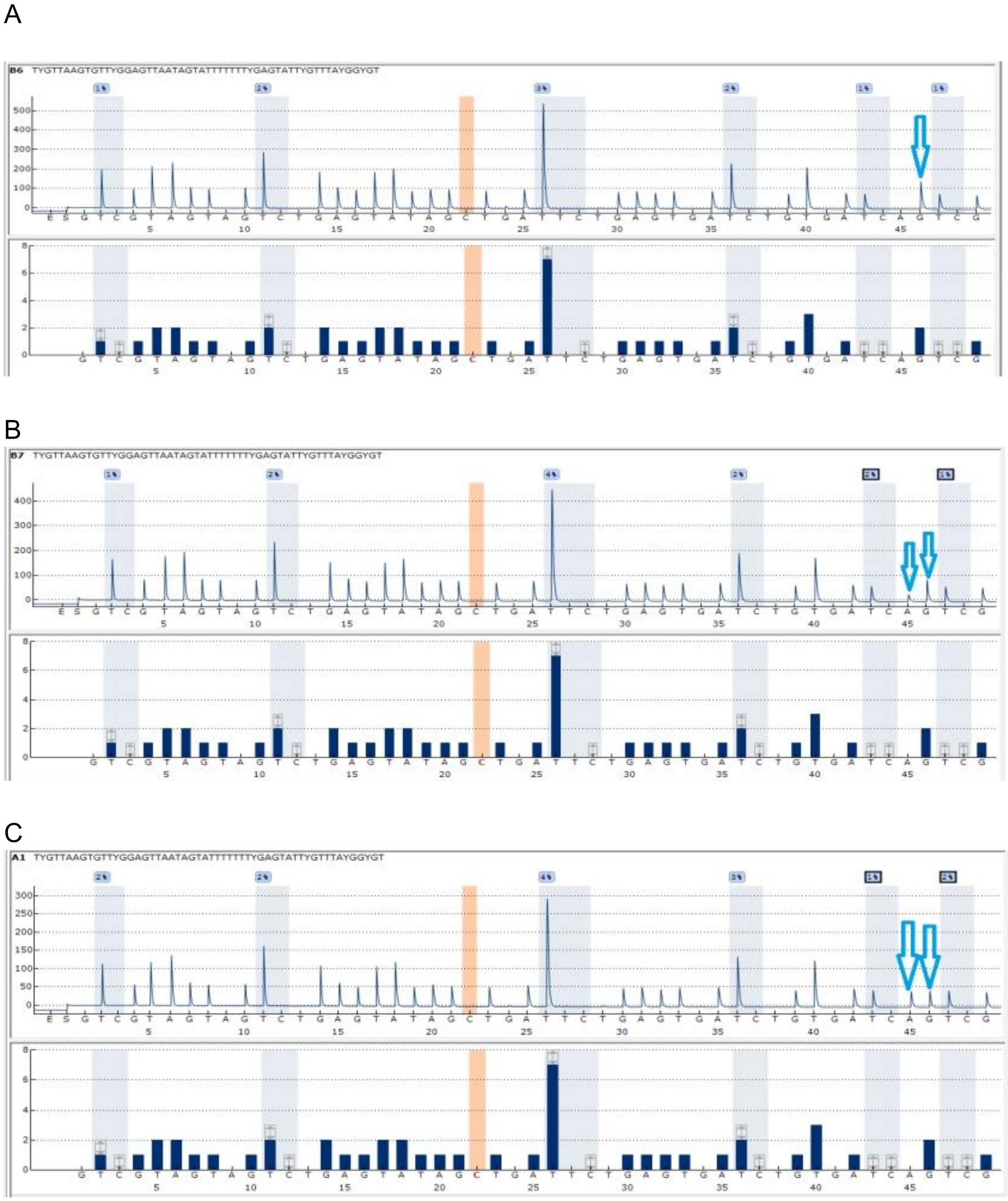

Figure 3a. Pyrograms illustrating the *p16INK4a* rs3814960 (*C*>*T*) SNP
Representative pyrograms generated using the PyroMark Q24 Advanced system showing the three rs3814960 genotypes in the *p16INK4a* locus.
Top (A):*CC* genotype, characterized by a double G peak indicating an intact CpG site.
Middle (B): *CT* genotype, showing a reduced A peak together with a higher G peak, reflecting heterozygosity at the SNP position.
Bottom (C): *TT* genotype, displaying comparable A and G peak heights, consistent with loss of the CpG site at this position. The SNP overlaps the fifth CpG site of the *p16INK4a* assay and was therefore excluded from methylation quantification to avoid technical bias.

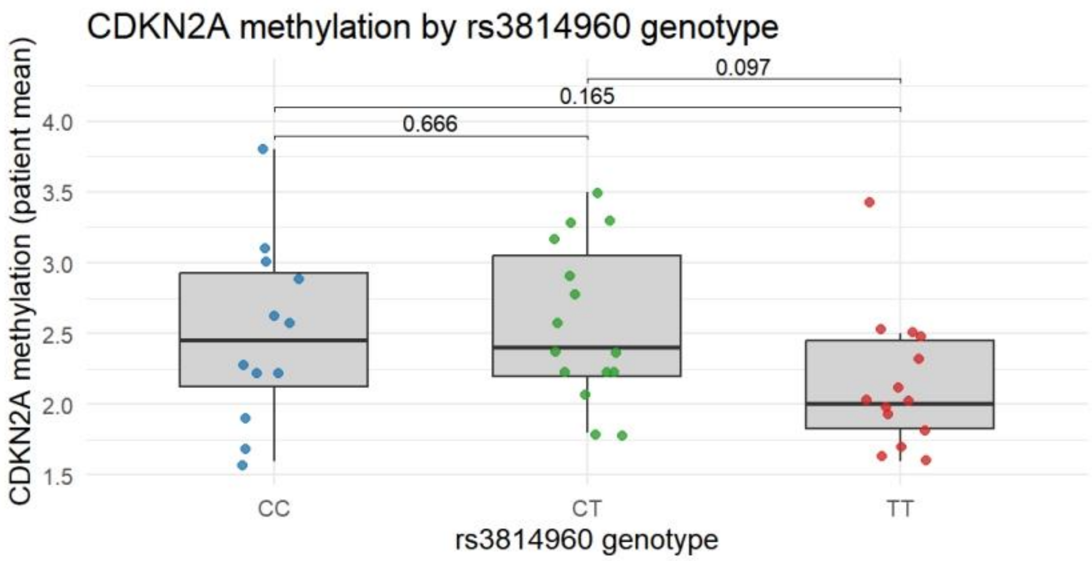

Figure 3b. *p16INK4a* (*CDKN2A*) methylation stratified by rs3814960 genotype
Comparison of mean *p16INK4a* promoter methylation levels across rs3814960 genotypes (*CC, CT, TT*). Each point represents a per-patient mean value, with group distributions shown as boxplots. Pairwise comparisons were assessed using non-parametric tests with multiple-testing correction; adjusted p-values are indicated. No statistically significant differences were observed after correction.

3.8 *KRAS* and *BRAS* Mutation Status

Digital PCR analysis detected no *KRAS* or *BRAF* mutations in polyp mucosa samples from either side of the colon (range 0.00–0.03%). One polyp case showed a low-level *KRAS* G>A signal above background (right: 0.09%; left: 0.11%), which remained below 1% and was considered non-significant.

4 Discussion

4.1 Global LINE-1 Hypomethylation as an Early Epigenetic Alteration in Colorectal Polyps

This study demonstrated significant global hypomethylation of LINE-1 in polyp-adjacent mucosa compared with control mucosa. Receiver operating characteristic (ROC) analysis showed good discriminatory performance (AUC = 0.863), supporting the potential utility of LINE-1 hypomethylation as an early marker of colorectal neoplastic change. The finding that polyp status was the only clinical variable independently associated with

LINE-1 methylation further suggests that this epigenetic alteration is driven primarily by disease-related processes rather than demographic or anatomical factors. These observations are consistent with previous reports linking reduced LINE-1 methylation to colorectal cancer progression and adverse outcomes (Kuan et al.; Bagheri et al.), supporting the relevance of LINE-1 hypomethylation as an early prognostic indicator in colorectal tumorigenesis.

4.2 Gene-Specific Methylation patterns in Polyps-Adjacent and Control Mucosa

Previous work by Wettergren et al. demonstrated that hypermethylation of tumor suppressor genes such as *MLH1, MGMT,* and *p16INK4a* can be detected not only in colorectal tumors but also in non-cancerous mucosa, suggesting potential utility as early risk markers. In contrast, the present study did not detect aberrant hypermethylation of these tumor suppressor genes in mucosa adjacent to polyps. However, the presence of significant LINE-1 hypomethylation indicates early genomic instability, which may precede gene-specific hypermethylation events. These findings support a model in which global hypomethylation occurs early in colorectal tumorigenesis, with tumor suppressor gene silencing emerging at later stages, warranting longitudinal genomic studies in polyp cohorts.

MGMT methylation did not differ significantly between polyp-adjacent and control mucosa overall. However, higher *MGMT* methylation levels were observed in females compared with males, particularly in the control group. Although this sex-associated difference did not remain significant after stratification within the polyp group, the trend toward higher *MGMT* methylation in female polyp samples suggests a possible disease-related effect rather than a purely biological sex difference. This interpretation is supported by previous findings in glioblastoma, where higher *MGMT* promoter methylation in females was associated with improved survival outcomes (Barnett et al.). It is important to note that the *MGMT* dataset in this study was smaller than that for other genes due to technical limitations related to reagent availability, which may have reduced statistical power. A complete *MGMT* dataset could potentially have strengthened these observations.

p16INK4a methylation levels were significantly lower in polyp-adjacent mucosa compared with controls mucosa. This observation is consistent with findings by Bihl et al**.**, who reported that p16INK4a methylation correlates positively with molecular and clinicopathological features of more advanced colorectal disease, including right-sided tumor location, mucinous histology, higher tumor grade, microsatellite instability (MSI), *BRAF* mutation, and *KRAS* mutation in the MSI setting. These associations suggest that *p16INK4a* promoter methylation is more strongly linked to specific tumor subtypes and later molecular events rather than early lesion formation. This interpretation is further supported by Shima et al**.**, who concluded that *p16INK4a* promoter methylation or loss of expression in colorectal cancer is not independently associated with patient prognosis. Another plausible explanation is the higher frequency of the rs3814960 *TT* genotype in the polyp group, as *TT* carriers exhibited the lowest mean *p16INK4a* methylation levels.

Although the control group in the present study was carefully defined as normal-appearing mucosa throughout the colon, without evidence of polyps, inflammatory bowel disease, malignancy, or ischemic colitis, the precise epigenetic state of histologically normal mucosa cannot be fully determined. The observation that control mucosa exhibited slightly higher methylation across tumor suppressor genes compared with polyp-adjacent mucosa is therefore noteworthy. While some studies recommend the use of adjacent non-neoplastic mucosa as a reference (Bihl et al., 2012), other reports suggest that truly normal control mucosa may provide a more appropriate baseline, as adjacent tissue can already harbor aberrant methylation changes. These considerations highlight the complexity of selecting appropriate reference tissue and may partly explain the lower *p16INK4a* methylation observed in polyp-adjacent mucosa. Within the analyzed *p16INK4a* amplicon, the third CpG site located near the CpG island boundary consistently showed higher methylation across all samples, including controls. This pattern is consistent with prior genome-wide studies demonstrating that differential methylation often occurs in CpG island shores rather than core promoter regions (Irizarry et al.; Doi et al.), and that regional methylation tends to be coordinated across neighboring CpG sites (Hansen et al.). *MLH1* methylation did not differ significantly between groups, and the prevalence of unmethylated CpGs suggests that mismatch repair gene hypermethylation may occur later than *p16INK4a* and *MGMT* alterations during colorectal carcinogenesis. *MLH1* methylation is typically associated with CRC molecular subtypes characterized by CIMP positivity, microsatellite instability, and *BRAF* mutation; however, aberrant *MLH1* methylation and *BRAF* mutations were absent in the present study, which may further explain the high prevalence of unmethylated CpG sites observed in *MLH1* within polyp-adjacent mucosa.

4.3 Influence of rs3814960 Genotype on *P16INK4a* Methylation

The rs3814960 polymorphism is a benign intronic variant that abolishes a CpG site in the *p16INK4a* region and is characterized by population-specific allele frequencies. In this cohort, genotype distribution showed a higher frequency of the *TT* genotype among polyp patients compared with controls. Although *p16INK4a* methylation demonstrated a genotype-dependent pattern, with *TT* carriers showing the lowest methylation and *CT* carriers showing the highest, the global genotype effect did not reach statistical significance, and genotype was not an independent predictor after adjustment for group, age, and sex. These findings align with previous reports indicating no strong association between rs3814960 and *p16INK4a* methylation or expression (Wettergren et al.). Li et al. reported that individuals carrying the *TC/CC* genotypes of rs3814960 were associated with decreased overall survival in patients with esophageal squamous cell carcinoma in a Chinese population, whereas the *TT* genotype was associated with improved patient outcomes. Although these findings originate from a different cancer type and population, they support the biological plausibility that rs3814960 genotype may influence disease behavior and clinical outcome rather than acting as a direct determinant of levels DNA methylation.

Differences in allele frequencies between this cohort (*C*=47.6%; *T*=52.4%) and previously studied Chinese populations (*C*=58.3%; *T*=41.7%; Li et al.) likely reflects differences in population genetics, cancer type, and disease stage rather than inconsistent biological effects of the variant. Comparison with a colorectal cancer cohort study by Wettergren et al. further suggested enrichment of the *T* allele in disease states. In both studies, individuals with colorectal pathology (polyps or carcinoma) showed a higher frequency of the *T* allele compared with controls, while the *C* allele was more prevalent among controls, although these observations require cautious interpretation due to limited sample size.

Across LINE-1, *MLH1*, and *MGMT*, no associations with rs3814960 genotype were observed, indicating that this variant does not exert a broad regulatory effect on DNA methylation. Any potential influence appears to be localized to the *p16INK4a* region and modest in magnitude. Larger cohorts will be required to determine whether this SNP contributes meaningfully to colorectal cancer risk or progression. Together, these observations suggest that rs3814960 may exert its influence primarily through disease prognosis or progression-related mechanisms, rather than through broad regulation of DNA methylation.

4.4 Absence of KRAS and BRAS Mutations in Early Lesions

KRAS and *BRAF* hotspot screening revealed no activating mutations in the polyp cohort, because no activating *KRAS* or *BRAF* mutations were detected, genotype–epigenotype comparisons involving these oncogenes could not be performed in this cohort. The absence of these canonical oncogenic alterations suggests that the epigenetic changes observed, particularly LINE-1 hypomethylation, may precede *KRAS* and *BRAF* mutations in early colorectal lesion development. This is consistent with prior studies reporting relatively low frequencies of *BRAF* mutations and associations between *KRAS* mutations and more advanced disease stages (Kakinya et al).

4.5 Ethical Considerations and Societal Relevance

All participants provided written informed consent, and the study was approved by the Regional Ethical Review Board in Gothenburg (no. 987–17). Ethical principles including participant confidentiality, transparency, and data integrity were upheld throughout the study. Sampling of mucosa 10 cm from polyps enabled assessment of early epigenetic alterations relevant to cancer risk without confounding by overt tumor tissue. The findings provide insight into early molecular events in colorectal pathology and may contribute to the development of improved diagnostic and prognostic tools, ultimately supporting risk-adapted prevention strategies and improved patient outcomes.

4.6 Conclusion, Clinical Implications, and Future Directions

This study demonstrates that pyrosequencing enables reliable quantification of very low DNA methylation levels across individual CpG sites. Global LINE-1 hypomethylation emerged as a robust early epigenetic alteration in polyp-adjacent mucosa, while tumor suppressor gene methylation changes were limited. The observed associations involving *p16INK4a* methylation, *MGMT* sex differences, and the rs3814960 variant highlight biologically plausible trends that merit further investigation in larger cohorts. Longitudinal tissue and liquid biopsy studies may provide valuable insight into the temporal evolution of methylation changes preceding the adenoma–carcinoma sequence and support the identification of early biomarkers for colorectal cancer risk.

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