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Decoding the Methylation Patterns of ABC Transporters in Colorectal Cancer

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Abstract: Colorectal cancer (CRC) is a significant global health concern, posing a major threat to morbidity and mortality rates. The molecular mechanisms that drive CRC, particularly the DNA methylation patterns in ATP-binding cassette (ABC) genes, have attracted considerable attention because of their potential roles in CRC drug resistance, initiation, and progression. This study aimed to investigate the methylation patterns of ABC genes in CRC using a high-throughput microarray technology. Microarray methylation data from CRC and adjacent normal tissues were subjected to preprocessing, differential methylation analysis, and correlation analysis using the MethSurv tool for a detailed study of

methylation patterns. The study revealed global hypomethylation of 43 ABC transporters and two pseudogenes in CRC compared to normal tissues. Receiver operating characteristic curve analysis identified 369 CpG sites as potential biomarkers for CRC diagnosis, with area under the curve values ranging from acceptable to outstanding. Survival analysis demonstrated the correlation between DNA methylation of individual CpG sites and patient survival probability in colon and rectal adenocarcinoma datasets from The Cancer Genome Atlas. The study provides insights into the epigenetic landscape of ABC genes in CRC, highlighting their potential roles in drug resistance, disease initiation, and progression. The findings offer opportunities for developing innovative therapeutic approaches and improving patient outcomes in the fight against CRC. Further research is needed to validate these results and investigate the functional implications of ABC gene methylation in CRC pathogenesis and treatment response.

Keywords: Colorectal cancer; DNA methylation; ABC transporters; Cancer biomarker; Precision medicine; Epigenetics; SDG 3 Good health and well-being

1. Introduction

Colorectal cancer (CRC) is a significant global health concern and is responsible for many cancer-related fatalities worldwide[1]. The pathogenesis of CRC is multifaceted, involving a multitude of genetic and epigenetic changes that are crucial to its onset, progression, and metastasis. Gaining a deeper understanding of these factors is vital for improving early detection and prognosis[2–6]. Among these factors, DNA methylation, a key epigenetic modification that regulates gene expression, has emerged as an important player in CRC development and progression[7–9].

ATP-binding cassette (ABC) genes encode a group of membrane transporters that can expel various chemotherapeutic agents from cells, thereby decreasing their intracellular concentration and reducing their therapeutic effectiveness[10]. This mechanism is a common cause of multidrug resistance (MDR) in cancer cells, particularly in CRC. MDR in CRC poses significant treatment challenges, limiting patient options and outcomes[11]. Understanding ABC genes is crucial because of their contribution to drug resistance[12]. Methylation often reduces ABC gene expression and function, emphasising the need to study ABC gene methylation in CRC. DNA methylation can affect the activity or stability of ABC genes, leading to their reduced expression or function[13]. Examining the methylation of ABC genes in CRC could aid in identifying novel biomarkers for predicting and monitoring MDR and chemoresistance in CRC patients. Additionally, this knowledge may facilitate the development of new therapeutic strategies aimed at targeting specific molecular pathways or restoring the tumour microenvironment.

Therefore, this study aimed to comprehensively investigate the methylation status of ABC transporter genes in CRC. We leveraged our existing publicly available dataset using high-throughput microarray technology to generate robust and extensive methylation profiles

of ABC genes. In addition, we employed various bioinformatics tools to analyse and interpret the data. Furthermore, we explored the potential correlation between these methylation patterns and clinical outcomes, such as disease progression and overall survival. Ultimately, our goal was to shed light on the role of ABC gene methylation in CRC chemoresistance and to identify potential therapeutic targets for overcoming drug resistance.

2. Materials and Methods

2.1. Data Retrieval of ABC Transporter Genes List

A comprehensive literature search was conducted to obtain the list of ATP-binding cassette (ABC) transporter genes[14,15]. All 49 known human ABC transporter genes were obtained and are divided into seven subfamilies based on their sequence homology and domain organisation. The gene list was used for subsequent analysis and served as the foundation for our study, ensuring that our analysis included the complete range of ABC transporter genes[14].

2.2. Human Methylation 450K Data Analysis

In-house methylation microarray data from fifty-four pairs of CRCs and the corresponding adjacent normal colon tissues (n = 108) were retrieved from the Gene Expression Omnibus (GEO) database under accession number GSE193535 and subjected to bioinformatics analysis. Quality control was performed on raw genomic data using Genome Studio version 2.0.4 (Illumina Inc., San Diego, California, USA)[16,17]. The ChAMP (Bioconductor package in R)[18] was used to further analyse IDAT files from 108 samples in a single analysis, and filters were applied to all probes by removing CpG sites with a detection p-value > 0.01. This includes the removal of probes located on sex chromosomes. The peak-based correction method (PBC)[19,20] was used for data normalisation prior to batch effect correction using ComBat[21]. The β-values were extracted and subjected to statistical analyses. The Limma (Bioconductor package in R)[22] was employed to identify differentially methylated CpG sites by applying false discovery rate (FDR) correction with a significance threshold of p-value < 0.01.

2.3. Receiver Operating Characteristic Curve Analysis

The diagnostic efficacy of the potential biomarker was assessed through receiver operating characteristic (ROC) curve analysis, generated using GraphPad Prism 8.0.2 (GraphPad Software Inc., Boston, Massachusetts, USA). The area under the ROC curve (AUC), constructed with a 95% confidence interval (CI), served as the accuracy criterion for evaluating potential biomarkers[23]. Methylation values from 369 probes, identified as potential biomarkers in CRC, were compared with their respective controls. The ideal diagnostic marker has an AUC value of 1. An AUC value ranging from 0.7 to 0.8 is deemed acceptable, 0.8 to 0.9 is considered excellent, and values above 0.9 are regarded as outstanding.

2.4. MethSurv Survival Analysis based on CpG Methylation Patterns

Survival analysis was performed using the MethSurv tool (https://biit.cs.ut.ee/methsurv/)[24], which requires individual CpG sites as input for each analysis. The Kaplan-Meier survival analyses were performed separately for both the Cancer Genome Atlas (TCGA) Colon Adenocarcinoma (COAD) and Rectal Adenocarcinoma (READ) datasets. A total of 369 CpG sites, along with CpG islands (CGIs) and genomic region data for individual CpG sites, were used as inputs for the analysis. The correlation between the DNA methylation of each CpG site and the probability of survival was assessed in these datasets.

3. Results

3.1. Human ABC Transporter Genes and Their Functions

Table 1 provides an overview of the 49 human ABC transporter genes, including their genomic locations, tissue-specific expressions, and known or putative functions based on previous studies[14,15]. These genes were categorised into seven distinct subfamilies, from subfamily A to G. Subfamily A, also known as ABC1, consists of twelve genes, most of which play crucial roles in lipid trafficking across various organs and cell types. Some ABC subfamily A (ABCA) proteins are among the longest ABC transporters, with a length of more than 2,100 amino acids (AA)[15]. The predicted ABCA13 protein, with 5,058 AA residues, is the longest known ABC protein[25]. Subfamily B is unique to mammals and comprises eleven genes. This subfamily includes four full transporters and seven half-transporters. Several members of this subfamily are known to confer MDR in cancer cells, hence its alternate name, the 'MDR family of ABC transporters'[14,15]. Subfamily C, on the other hand, also known as the multidrug resistance protein (MRP) subfamily, includes the cystic fibrosis transmembrane conductance regulator gene (*CFTR*, also known as *ABCC7*) and twelve other genes that encode transporters associated with multidrug resistance[14,15].

Subfamily D, also known as peroxisomal or ALD transporters, contains four genes encoding half-transporters. These subunits can form either homodimers or heterodimers to create a functional unit[14,15]. Subfamily E, represented by a single member, *ABCE1*, is an organic anion-binding protein (also known as OABP). *ABCE1* possesses an ATP-binding domain but lacks a transmembrane domain, suggesting that it likely does not function as a transporter[14,15]. Subfamily F (GGN20)[14,15], along with *ABCE1*, the three members of the ABCF subfamily, also have ATP-binding domains but lack transmembrane domains, making it unlikely that they function as transporters. Lastly, Subfamily G, also known as the ‘White’ subfamily, includes at least five genes that encode 'reverse half-transporters', which form the second half of a heterodimer[14,15].

Table 1. Overview of human ABC transporter genes: locations, expression, and functions [14, 15].

Sub-family	Gene	Chromosome location	AA	Expression	Function	General function
ABC1	*ABCA1*	9q3l.l	2261	Ubiquitous	Cholesterol efflux onto HDL	1. Lipid metabolism. 2. Transportation of cholesterol and certain fats
	ABCA2	9q34	2436	Brain	Drug resistance	
	ABCA3	16pl3.3	1704	Lung	Multidrug resistance	
	ABCA4	1p22	2273	Rod photoreceptors	N-retinylidene-phosphatidylethanolamine (PE) efflux	
	ABCA5	17q24.3	1642	Muscle, heart, testes	Urinary diagnostic marker for prostatic intraepithelial neoplasia (PIN)	
	ABCA6	17q24.3	1617	Liver	Multidrug resistance	
	ABCA7	19p13.3	2146	Spleen, thymus	Cholesterol efflux	
	ABCA8	17q24	1581	Ovary	Transports certain lipophilic drugs	
	ABCA9	17q24.2	1624	Heart	Potential role in monocyte differentiation and macrophage lipid homeostasis	
	ABCA10	l7q24	1543	Muscle, heart	Cholesterol-responsive gene	
	ABCA12	2q34	2595	Stomach	Has implications for prenatal diagnosis	
	ABCA13	7p12.3	5058	Low in all tissues	Inherited disorder affecting the pancreas	
MDR	*ABCB1*	7q21.1	1280	Adrenal, kidney, brain	Multidrug resistance	1. Multidrug resistance. 2. ATP-dependent drug efflux pumps for xenobiotic compounds.
	ABCB2 (TAPI)	6p21.3	808	All cells	Peptide transport	
	ABCB3 (TAP2)	6p21.3	703	All cells	Peptide transport	

	ABCB4	7q21.1	1279	Liver	Phosphatidylcholine (PC) transport	3. Broad substrate specificity.
	ABCB5	7p15.3	812	Ubiquitous	Melanogenesis	
	ABCB6	2q36	842	Mitochondria	Iron transport	
	ABCB7	Xq12-q13	753	Mitochondria	Fe/S cluster transport	
	ABCB8	7q36	718	Mitochondria	Intracellular peptide trafficking across membranes	
	ABCB9	12q24	766	Heart, brain	Located in lysosomes	
	ABCB10	1q42.13	738	Mitochondria	Export of peptides derived from proteolysis of inner-membrane proteins	
	ABCB11	2q24	1321	Liver	Bile salt transport	
MRP	*ABCC1*	16p13.1	1531	Lung, testes, peripheral blood mononuclear cell	Drug resistance	1. ATP-dependent efflux pumps. 2. Broad substrate specificity. 3. Transport endogenous and xenobiotic anionic substances.
	ABCC2	10q24	1545	Liver	Organic anion efflux	
	ABCC3	17q22	1527	Lung, intestine, liver	Drug resistance	
	ABCC4	13q32	1325	Prostate	Nucleoside transport	
	ABCC5	3q27	1437	Ubiquitous	Nucleoside transport	
	ABCC6	16p13.1	1503	Kidney, liver	Unknown	
	ABCC7 (CFTR)	7q31.2	1480	Exocrine tissues	Chloride ion channel	
	ABCC8	11p15.1	1581	Pancreas	Sulfonylurea receptor	
	ABCC9	12p12.1	1549	Heart, muscle	Drug-binding channel-modulating subunit of the extra-pancreatic ATP-sensitive potassium channels.	
	ABCC10	6p21.1	1464	Low in all tissues	Multidrug resistance	

	ABCC11	16q12.1	1382	Low in all tissues	Drug resistance in breast cancer	
	ABCC12	16q12.1	1359	Low in all tissues	Multidrug resistance	
	ABCC13	21q11.2	325	Biased expression in small intestine and duodenum	Encodes a polypeptide of unknown function	
ALD	*ABCD1*	Xq28	745	Peroxisomes	Very-long-chain fatty acid transport	Transport of very long-chain fatty acids into peroxisomes.
	ABCD2	12q11-q12	740	Peroxisomes	Major modifier locus for clinical diversity in X-linked ATP adenosine triphosphate	
	ABCD3	1p22-p21	659	Peroxisomes	Involved in the import of fatty acids and/or fatty acyl-coenzyme As into the peroxisome	
	ABCD4	14q24	606	Peroxisomes	Potentially modify the ATP adenosine triphosphate phenotype	
OABP	*ABCE1*	4q31	599	Ovary, testes, spleen	Oligoadenylate-binding protein	1. Blocking the activity of ribonuclease L. 2. Inhibit protein synthesis in the 2-5A/RNase L system. 3. Central pathway for viral interferon action.
GGN20	*ABCF1*	6p21.33	845	Ubiquitous	Susceptibility to autoimmune pancreatitis	Involvement in immune response, drug resistance and antiviral.
	ABCF2	7q36	634	Ubiquitous	Tumour suppression at metastatic sites and in the endocrine pathway for breast	

					cancer/drug resistance	
	ABCF3	3q27.1	709	Ubiquitous	Antiviral effect against flaviviruses	
White	*ABCG1*	21q22.3	678	Ubiquitous	Cholesterol transport	1. Xenobiotic transporters. 2. Multi-drug resistance. 3. Transport of various molecules, including lipids and sterols.
	ABCG2	4q22	655	Placenta, intestine	Toxicant efflux, drug resistance	
	ABCG4	q23.3	646	Liver	Functions as either a homodimer or as a heterodimer with another ABC subfamily protein such as *ABCG1*	
	ABCG5	2p2	65	Liver, intestine	Sterol transport	
	ABCG8	2p2	673	Liver, intestine	Sterol transport	

ABC - ATP-binding cassette, AA - Amino acid, HDL - High-density lipoprotein, MDR - Multidrug resistance, TAPI - Transporter associated with antigen processing I, TAP2 - Transporter associated with antigen processing II, PC – Phosphatidylcholine, Fe/S - Iron-sulfur, MRP - Multidrug resistance protein, CFTR - Cystic fibrosis transmembrane conductance regulator, OABP - Organic anion-binding protein

3.2. Mapping the Genomic Locations of Differentially Methylated ABC Genes

In this study, we analysed the differential methylation status of 54 CRC tissue samples and their corresponding adjacent normal samples, resulting in a total of 108 samples. Probe filtering was conducted to identify differentially methylated probes (DMPs) with an adjusted p-value of less than 0.01, and false discovery rate (FDR) correction was subsequently applied (Figure 1a), yielding 157,846 DMPs. To focus on ABC transporter genes, we implemented an additional filtering step to retain only the probes specific to these genes. This process resulted in a final selection of 369 probes for further analysis. We then categorised the selected probes as either hypermethylated or hypomethylated based on the positive β value difference ($\Delta\beta$) between CRC and adjacent normal tissues, resulting in the identification of 359 hypomethylated probes and 10 hypermethylated probes.

DMPs were classified based on their location relative to CGIs, resulting in four distinct regions: CGIs, shores, shelves, and open-sea regions. Most hypomethylated probes were located in the open sea region, with 209 probes accounting for 58.2% of the total probes. The shore and shelf regions each contained 59 probes, representing 16.4% of the total in each region. The island region had the fewest hypomethylated probes, with 32 probes making up 8.9%. In contrast, the distribution of hypermethylated probes was slightly different. The open sea region still contained the majority, with six probes (60% of the total probes, whereas the

shore and shelf regions each contained only one probe (10% each). The island region had two probes, constituting 20% of the total. This distribution of DMPs provides valuable insights into the methylation patterns of ABC genes in CRC.

The distribution of hypomethylated probes across genomic regions showed that the majority (n = 249; 69.4%) were located in the body region, followed by the TSS1500 region (n = 42; 11.7%). The 3' UTR and 5' UTR regions had 33 (9.2%) and 30 (8.4%) probes, respectively. The 1st exon contained four probes (1.1%), and the TSS200 region contained the fewest probes (n = 1; 0.28%). In contrast, the distribution of the hypermethylated probes was less evenly spread. The majority were still found in the body region (n = 8; 80%), but only one probe each was identified in the 3'UTR and TSS1500 regions (10% each). No hypermethylated probes were detected in the 5'-UTR, TSS200, and 1st exon regions. The distribution is shown in Figure 1c.

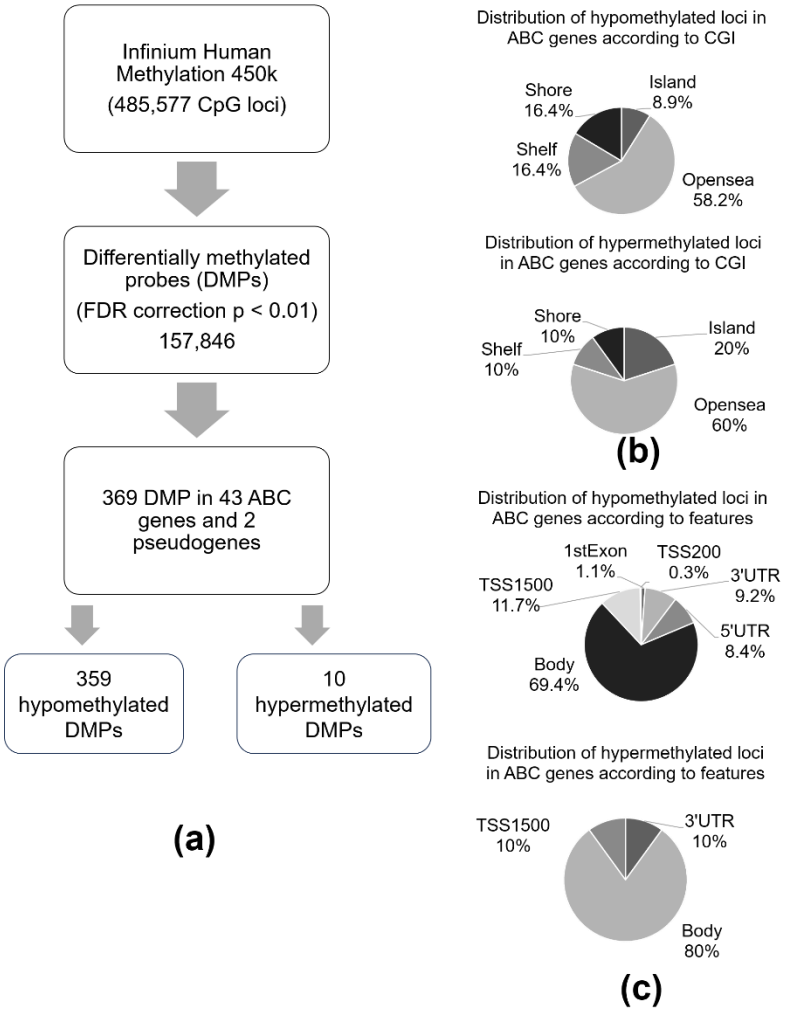

Figure 1. Identification and distribution of differentially methylated probes in ABC transporter genes. (a) Identification of DMPs in ABC transporter genes. (b) Distribution of DMPs relative to CGIs shows that most of the 359 hypomethylated and 10 hypermethylated probes are in the open sea region. (c) Distribution of probes across the genomic regions, with most probes found in the body region for both hypomethylated and hypermethylated probes.

3.3. Analysis of Differentially Methylated ABC Transporter Genes

Probes with a positive Δβ value (tumour versus normal) were considered hypermethylated, whereas genes with a negative Δβ value were regarded as hypomethylated. Figure 2 illustrates the top ten ABC transporter genes with the highest number of differentially methylated loci.

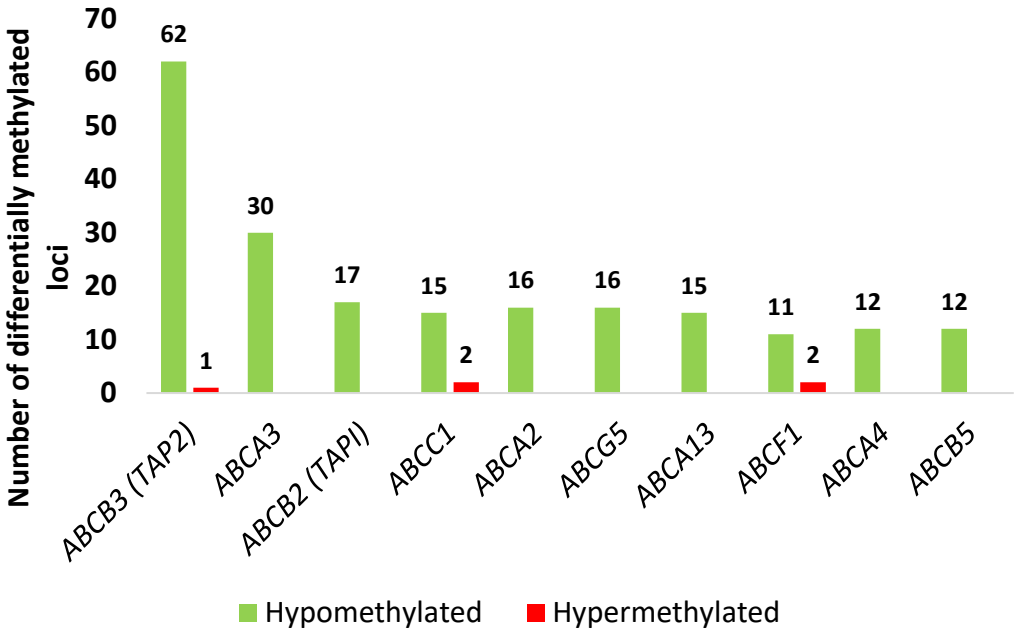

Figure 2. Top 10 ABC Transporter Genes with the Highest Number of Differentially Methylated Loci.

In this study, *ABCB3 (TAP2)* has 62 hypomethylated loci and one hypermethylated locus, resulting in a total of 63 differentially methylated loci. The substantial number of methylation changes in *ABCB3* (*TAP2*) suggests that its methylation status may influence CRC progression and outcomes.

Our study identified intriguing patterns in the methylation status of ABC transporter genes (Table 2). Generally, hypomethylated loci outnumbered hypermethylated loci across most genes, indicating a trend towards hypomethylation in our CRC samples. Detailed analysis revealed significant differential methylation in 43 ABC genes, of which 37 were hypomethylated. Notably, six genes exhibited a consistent hypomethylation trend despite having one or two hypermethylated probes, leading to their overall classification as hypomethylated.

In addition to the protein-coding genes, we identified two hypomethylated pseudogenes, *ABCC6P1* and *ABCA17P*. Although pseudogenes do not code for proteins, they can play regulatory roles, and their methylation status can affect gene expression. In contrast, six genes, *ABCCA10*, *ABCB6*, *ABCB7*, *ABCC6*, *ABCC7* (also known as *CFTR*), and *ABCD1*, showed no significant differential methylation in CRC. The relatively stable methylation status of these genes suggests that they may be less influenced by the factors driving differential methylation in other ABC transporter genes.

Table 2. Differential methylation patterns of ABC transporter genes in CRC.

ABC gene	No. of significantly differentially methylated probes	No. of hypomethylated probes	No. of hypermethylated probes
ABCB3 (TAP2)	63	62	1
ABCA3	30	30	-
ABCB2 (TAPI)	17	17	-
ABCC1	17	15	2
ABCA2	16	16	-
ABCG5	16	16	-
ABCA13	15	15	-
ABCF1	13	11	2
ABCA4	12	12	-
ABCB5	12	12	-
ABCC2	11	9	2
ABCA7	10	10	-
ABCB9	9	9	-
ABCC4	9	8	1
ABCA1	8	8	-
ABCB8	8	8	-
ABCC11	7	7	-
ABCC12	7	7	-
ABCC5	7	7	-
ABCA5	6	6	-
ABCC8	6	6	-
ABCG1	6	6	-
ABCA17P	5	4	1
ABCB1	5	5	-
ABCB11	5	5	-
ABCC9	5	5	-
ABCG8	5	5	-
ABCF3	4	3	1
ABCA12	3	3	-

ABCA8	3	3	-
ABCA9	3	3	-
ABCB4	3	3	-
ABCC10	3	3	-
ABCC13	3	3	-
ABCC3	3	3	-
ABCE1	3	3	-
ABCB10	2	2	-
ABCG4	2	2	-
ABCA6	1	1	-
ABCC6P1	1	1	-
ABCD2	1	1	-
ABCD3	1	1	-
ABCD4	1	1	-
ABCF2	1	1	-
ABCG2	1	1	-

ABC - ATP-binding cassette

3.4. Exploring the Correlation between DNA Methylation Patterns of ABC Transporter Gene Probes and Survival Rates in CRC Patients

Out of the 369 DMPs, 10 exhibited a significant association with survival in either TCGA COAD or TCGA READ datasets (Table 3). Nevertheless, none of the DMPs demonstrated a significant association with survival across both datasets.

Table 3. Significantly associated DMPs for survival across both TCGA COAD and TCGA READ datasets.

ABC genes	**Probes ID**	**Delta-beta value**	**LR test P-value**	
			COAD	READ
ABCA13	cg0337204	-0.260761646	0.017	ns
ABCB1	cg22899422	-0.248964354	0.05	ns
ABCC12	cg26758427	-0.228402759	0.029	ns
ABCC12	cg07861968	-0.225775438	0.0024	ns
ABCA3	cg02715407	-0.211525860	ns	0.025
ABCA3	cg05814755	-0.200106537	ns	0.00075
ABCC12	cg07878951	-0.233117456	ns	0.012

ABCC12	cg07861968	-0.225775438	ns	0.012
ABCC9	cg20025970	-0.305320541	ns	0.046
ABCG4	cg17168875	-0.269257273	ns	0.035

ABC - ATP-binding cassette, COAD - Colon adenocarcinoma, ns – Not significant, READ - Rectal adenocarcinoma

4. Discussions

To date, most studies on DNA methylation of ABC transporters have focused on CRC lines[26,27] and a model of an MDR cell line[28]. Although numerous studies have explored DNA methylation of ABC transporters in various types of cancers, including bladder[29], brain[30], breast[31], oesophageal[32], gastric[33], liver[34], leukaemia[35], lung[36], myeloma[37], ovarian[38], prostate[39], and renal[40] cancers, there remains a notable gap in the literature specifically addressing CRC[13]. This underrepresentation suggests that understanding the DNA methylation of ABC transporters concerning CRC is limited and could be significantly improved. Addressing this gap could provide valuable insights into CRC, including its pathogenesis, progression, and treatment[11,41,42]. Future research should, therefore, focus on investigating the role of ABC transporters in CRC, examining the impact of DNA methylation status on the disease, and exploring their potential as therapeutic targets. Such research could bridge this gap and make substantial contributions to the field of CRC research.

The role of specific ABC transporters, such as *ABCB1*[43], *ABCC1*[44,45] and *ABCG2*[46,47], in MDR is well documented. However, the mechanisms by which these transporters influence cancer cell proliferation, differentiation, migration, and invasion are poorly understood and remain a subject of ongoing debate among stakeholders[48].

The ABCA subfamily is the largest among the ABC transporter families and is primarily involved in lipid transport, including cholesterol and phospholipids[25,49]. Although the precise mechanisms by which *ABCA* genes contribute to cancer progression and drug resistance remain unclear, it is generally reported that *ABCA* gene expression correlates with tumour progression and resistance to therapy[50,51]. This correlation is likely linked to their role in lipid homeostasis[52–54]. Moreover, elevated expression of *ABCA* transporter genes is associated with drug resistance and poor patient outcomes across various cancers[55]. In our study, we observed significant hypomethylation of *ABCA1-9* and *ABCA12/13/17P*, except for *ABCA10*. This hypomethylation suggests that these genes might be overexpressed, potentially contributing to cancer progression through mechanisms such as drug resistance or abnormal lipid metabolism. Although the documentation on the hypomethylation of *ABCA1*, *ABCA2*, and *ABCA5* in CRC is limited, their overexpression has been associated with worse CRC prognosis and tumour differentiation in other studies[50,56].

Our study also revealed significant hypomethylation in the ABCB subfamily. Notably, *ABCB1* (*MDR1*)[57], which is widely recognised for its role in chemoresistance,

exhibits hypomethylation, indicating potential upregulation that could contribute to the reduced efficacy of chemotherapy in CRC patients. Previous research supports that knockout of the *ABCB1* gene by CRISPR/Cas9 increased the chemosensitivity of *ABCB1* overexpressed MDR HCT8/VCR cells to doxorubicin by increasing the intracellular accumulation of doxorubicin[58]. Similarly, Lei *et al.* found that *ABCB1* knockout could reverse 3H-paclitaxel resistance in SW620/Ad300 CRC cells by decreasing drug efflux[59]. Additionally, knockout of the redox-inducible antioxidant protein RING-box 2 (RBX2) using CRISPR/Cas9 has been reported to enhance the sensitivity of HCT116 and SW480 cell lines to paclitaxel, possibly by deactivating mTOR/S6 kinase 1 (S6K1)[60].

The observed hypomethylation of *ABCB2* and *ABCB3*, which are involved in peptide transport and antigen presentation, could influence the tumour microenvironment and immune response, potentially impacting immunotherapy outcomes. Future studies should investigate whether hypomethylation of *ABCB2* and *ABCB3* affects the immunogenicity of CRC tumours and contributes to immune evasion.

Furthermore, *ABCB4* and *ABCB5* are noteworthy for their roles in phosphatidylcholine transport and chemoresistance, respectively. *ABCB5*, in particular, has been implicated in the resistance of melanoma cells to chemotherapy[61] and has been shown to confer similar resistance in CRC[62]. The hypomethylation of *ABCB5* observed in our study suggests potential upregulation, which could contribute to the chemoresistant phenotype in CRC. The role of *ABCB4* in liver health, specifically in the prevention of cholestasis, also makes it a candidate for further investigation in the context of CRC metastasis to the liver, a common site of CRC spread. *ABCB4* is significantly downregulated in CRC tumour tissues compared to normal tissues, and lower *ABCB4* expression predicts shorter recurrence-free survival and overall survival in CRC patients[63]. This indicates that *ABCB4* may function as a tumour suppressor gene. While hypermethylation of the *ABCB4* promoter has been linked to its silencing in other cancers, such as lung and breast cancer[63,64], direct evidence of *ABCB4* hypomethylation in CRC remains limited.

The hypomethylation observed in other *ABCB* genes, such as *ABCB8*, *ABCB9*, *ABCB10*, and *ABCB11*, indicates broader epigenetic deregulation within this subfamily, which may have far-reaching implications for cellular processes, including mitochondrial function and intracellular trafficking. Significant hypomethylation was also observed across *ABCC*, *ABCD*, *ABCE*, and *ABCF* gene subfamilies in CRC tissues, indicating potential epigenetic deregulation that could contribute to disease progression and treatment resistance. The ABCC genes, well-known for their role in MDR, likely exhibit upregulation that enhances drug efflux and reduces chemotherapy effectiveness. Upregulation of *ABCC1* and *ABCC2* in CRC has been previously reported and corroborated[65].

Hypomethylation in the ABCD subfamily, which is involved in metabolic processes, may alter cellular metabolism, while the hypomethylation of *ABCE1* could interfere with apoptosis and promote tumour survival. *ABCE1* is significantly upregulated in CRC tumour tissues compared to controls, a finding that highlights its relevance as a potential target for

therapy, as confirmed by other independent studies[65]. Additionally, the hypomethylation observed in ABCF genes, which play roles in protein synthesis and immune response, might contribute to immune evasion and tumour microenvironment alterations that further complicate the treatment landscape in CRC.

The ABCG gene subfamily, particularly *ABCG2*, has been extensively studied in CRC. In our study, the significant hypomethylation observed in these genes suggests potential upregulation. Notably, upregulation of the ABCG2 protein has been reported in CRC[66]. A recent clinicopathological study further demonstrated that the *ABCG2* gene and its protein expression are higher in CRC than in non-carcinoma tissues. Moreover, high ABCG2 expression in CRC patients was associated with lower histological differentiation, increased lymph node metastasis, and shorter 5-year survival, indicating that *ABCG2* overexpression is associated with poorer prognosis[67]. While other ABCG members may also play roles in drug transport and resistance mechanisms, their specific contributions to CRC are less well characterized than those of *ABCG2*.

ABCC6P1, a pseudogene, is essentially a nonfunctional copy of a gene. It is closely related to the *ABCC6* gene, which plays a significant role in the transport of drugs out of cells. This function is particularly relevant in the context of cancer, as it can contribute to drug resistance in cancer cells. The *ABCC6P1* pseudogene is located on chromosome 16 and is coexpressed with *ABCC6* in various human tissues. This finding suggests a potentially functional relationship between the two. Interestingly, a study has indicated that overexpression of *ABCC6P1* can increase the expression of *ABCC6*, which, in turn, can enhance drug resistance in breast cancer cells[68]. This finding underscores the potential significance of *ABCC6P1* in cancer research and treatment. Nevertheless, the exact role and mechanism of *ABCC6P1* in human health and disease remains unclear. In the present study, we found that *ABCC6* was not differentially methylated, suggesting that other factors may also play a role in regulating the activity of this gene.

ABCA17P is another pseudogene that was differentially methylated in our study. It is the human orthologue of the rodent testis-specific ABC transporter Abca17, which is involved in lipid transport and spermatogenesis[69]. *ABCA17P* shares a 5’ end with another ABC transporter gene, *ABCA3*, which causes lung surfactant deficiency and cystic fibrosis 2. Both genes are in the same genomic region and have evolved through gene duplication. *ABCA17P* and *ABCA3* form a complex of overlapping genes that may have important implications for the functional analysis of the disease gene *ABCA3*[69]. We observed overall hypomethylation of *ABCA17P* and *ABCA3* in CRC tissues compared to normal tissues. However, although these changes in methylation patterns have been observed, it is unclear how they might contribute to the development or progression of CRC. It is possible that these changes could influence the behaviour of cancer cells, but more research is needed to understand their exact role.

The colon, divided into the right-sided (ascending) and left-sided (descending and sigmoid) regions, along with the rectum, exhibits significant functional and biological

differences, including distinct methylation patterns[70]. For example, the gene *EVL* shows a decrease in promoter methylation from 15% in the rectum (left side) to 5% in the ascending colon (right side), indicating a methylation gradient across these regions[71]. This tissue-specific regulation is also observed in cancers like TCGA COAD and READ. Methylation patterns in genes such as *ABCA3*, *ABCC12*, and *ABCG4* differ between COAD and READ, with *ABCA3* hypomethylated in READ but not COAD, and *ABCC12* showing distinct patterns between the two, suggesting varying roles in cancer progression. These differences may stem from diverse environmental[72,73], genetic, and epigenetic factors, emphasizing the need for tailored treatments based on tumor location within the colon or rectum.

Although this study provides valuable insights into the role of ABC genes in CRC, it is imperative to acknowledge its limitations for a comprehensive understanding of the context and implications of the findings. First, it was predicated on a relatively small cohort of 54 CRC tissue samples. Although this sample size is adequate for exploratory investigations, it may not accurately represent the broader demographics of individuals with CRC. Consequently, extrapolating these findings should be cautiously approached until corroborated in larger, more diverse cohorts. This would facilitate more robust results and a better understanding of the generalisability of the findings. Future research should include larger and more diverse sample sizes to validate these findings.

Second, although the study successfully identified differentially methylated ABC genes associated with CRC, marking a significant advancement in understanding the molecular mechanisms underlying this disease, it does not provide functional or clinical validation of these genes. This implies that while these genes exhibit differential methylation in CRC, the impact of this methylation on gene function or the clinical outcome of the disease remains elusive. To address this gap, experimental studies using in vitro or in vivo models and clinical studies in patients are needed to ascertain the biological significance of these findings and their potential diagnostic or therapeutic implications. Future studies should aim to functionally validate these genes to better understand their roles in CRC.

Finally, the study did not account for inter-individual variability in DNA methylation patterns. Myriad factors, including age, lifestyle factors like diet and exercise, and genetic background, can influence DNA methylation. These factors can induce significant variations in DNA methylation patterns among individuals, which could potentially influence the results of this study. Future studies should consider these factors and include them as covariates in their analyses to provide a more comprehensive understanding of the role of ABC genes in CRC.

Understanding these limitations can guide future studies and ultimately contribute to our knowledge of CRC and its underlying molecular mechanisms. Future research could include expanding the sample size, performing functional and clinical validation of the identified genes, accounting for inter-individual variability in DNA methylation patterns, and exploring the impact of environmental factors on the methylation of ABC genes. These efforts could significantly enhance our understanding of the role of ABC genes in CRC and

potentially lead to the development of novel diagnostic and therapeutic strategies in the foreseeable future.

5. Conclusion

The ABC transporter superfamily, a group of transmembrane proteins, plays a crucial role in maintaining cellular homeostasis by actively transporting endogenous and exogenous substances across biological membranes. In the context of cancer, one of the major challenges in chemotherapy is the development of multidrug resistance (MDR), often driven by increased efflux of chemotherapeutic agents—a process heavily mediated by ABC transporters. Our study provides significant insights into the DNA methylation patterns of ABC transporter genes in colorectal cancer (CRC), revealing widespread hypomethylation in 43 ABC transporters, which may contribute to tumor progression and drug resistance.

Furthermore, our analysis uncovered distinct methylation patterns between colon and rectal adenocarcinomas, highlighting region-specific regulatory mechanisms that differentiate these cancer types at the molecular level. Specific CpG sites within ABC genes were found to be associated with patient survival, underscoring their potential as prognostic biomarkers. These findings not only enhance our understanding of the epigenetic landscape of ABC transporters in CRC but also point toward their role in influencing chemotherapy resistance.

By identifying these epigenetic alterations, this study lays the groundwork for future therapeutic strategies that target ABC gene methylation, potentially offering novel approaches to overcoming MDR in CRC. Further validation in larger cohorts and functional studies will be crucial to fully understand the implications of these findings and their potential to improve treatment outcomes in CRC patients.

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