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Methylation Profile of Cancer Testis Antigens in Colorectal Cancer

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ABSTRACT

Background: Cancer testis antigens (CTAs) are a class of immune-stimulating antigens often overexpressed in many types of cancers. The usage of the CTAs as immunotherapy targets have been widely investigated in different cancers including melanoma, hematological malignancies, and colorectal cancer. Studies have indicated that the epigenetic regulation of the CTAs such as the methylation status may affect the expression of the CTAs. However, the report on the methylation status of the CTAs is conflicting. The general methylation profile of the CTAs, especially in colorectal cancer, is still elusive.

Objective: To determine the methylation profile of the selected CTAs in our colorectal cancer patients.

Methods: A total of 54 pairs of colorectal cancer samples were subjected to DNA methylation profiling using the Infinium Human Methylation 450K bead chip.

Results: We found that most of the CTAs were hypomethylated, and CCNA1 and TMEM108 genes were among the few CTAs that were hypermethylated.

Conclusion: Overall, our brief report has managed to show the overall methylation profile in over the 200 CTAs in colorectal cancer and this could be used for further refining any immunotherapy targets.

Keywords: Tumor-associated Antigen, Cancer-testis Antigen, Epigenetics, Cancer vaccine, Immunotherapy

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INTRODUCTION

Immune-based therapies have been hailed as one of the most successful treatment routes in combating cancers. The basis of immune-based therapies depends on the expression of selective antigens that can be "flagged" as tumor markers and direct the immune response toward it. In general, two categories of antigens can be found in cancer, tumor-associated antigens, and tumor-specific antigens [1, 2]. Cancer testis antigens (CTA), or cancer germline antigens (CGA) are a type of tumor-associated antigens discovered a few decades ago. The first molecular evidence of the CTA, MZ2-E was discovered in 1991 in human melanoma samples, and was afterward termed MAGE-A1 [3].

Multiple studies have also characterized CT-X to be immunogenic, thus strengthening the confidence in utilizing these antigens as effective diagnostic markers and therapeutic tools [4, 5].

In colorectal cancer (CRC), there have been multiple discoveries of the potential CTAs that can be used as immunotherapeutic targets [6, 7]. Our previous work has identified PASD1 as a potential CTA for CRC patients [8]. Nevertheless, the expression of this particular CTA was inconsistent in our clinical samples [8]. Several studies have shown that the expression of the CTAs is epigenetically influenced. Multiple studies have also shown that the CTAs are known to be hypermethylated and by removing the methylation signatures, the recognition of these antigens is enhanced [9]. For instance, a study by Zhang et al. showed that the expression of the CT45 gene in ovarian cancer can be regulated through methylation [10]. An earlier study by Serrano et al. revealed the inverse correlation between the promoter methylation and MAGE-A1 expression. Not only that, but also the treatment with a demethylating agent, 5-aza-2'-deoxycytidine induced the expression of MAGE-A1 across cancer types [11]. Comparative DNA methylation analysis on normal and epithelial ovarian cancer (EOC) cells demonstrated a significantly higher level of NY-ESO-1 expression and reduced NY-ESO-1 promoter methylation. The results also remarkably revealed the introduction of DNA methyltransferase (DNMT) inhibitor, decitabine had induced the re-expression of NY-ESO-1 in non-expressing EOC samples [12].

In the light of these findings, the information on the regulation of DNA demethylation in cancer cells could be used as a strategy to enhance the expression of the CTA for a better immune detection. As such, in an effort to understand the correlation between the CTA expression patterns with DNA methylation, we seek to analyze the pattern of DNA methylation profile in CRC samples focusing on the Malaysian population. Previously, we have shown the difference in the methylation profile of open chromatin between CRC and non-CRC samples [13]. Using the same analysis, we repurposed the data to evaluate the methylation profile of the selected CTAs in this study.

MATERIALS AND METHODS

Clinical Specimens

A total of 54 pairs (n=108) of CRC and the corresponding adjacent normal tissues were collected from Hospital Tuanku Canselor Mukhriz (HCTM) and deposited at UMBI's Biobank. Written consent for biobanking was given by all the subjects. The IRB approval number for this study is UKM 1.5.3.5/244/UMBI-001-2014 as approved by the University of Kebangsaan Malaysia's Ethics Committee. The tissues were kept in liquid nitrogen. Afterward, the tissues were subjected to cryosectioning and H&E staining followed by verification by the pathologist. Then, the genomic DNA was extracted using the Allprep DNA/RNA/miRNA Universal Kit (Qiagen, Germany) according to the manual.

DNA Methylation Profiling

Methylation profiling was conducted using the Infinium Human Methylation 450K bead chip according to the protocol provided (Illumina, Inc.). Before the analysis, DNA was subjected to modification to convert all unmethylated cytosine to uracil using the EZ DNA methylation – Gold kit (Zymo Research, Inc.). Subsequently, the scanning of the bead chips was performed using the iScan scanner (Illumina, Inc.).

Data Analysis

Genome Studio software version 2.0.4 was used to do quality control once the raw IDAT data were exported from the scanner (Illumina Inc.). Using the ChAMP R program, we further examined the methylation data from the raw IDAT [14] and filters were

applied to all datasets where CpG sites that had detection P>0.01 in each probe were excluded from further analysis. Prior to batch effect correction using ComBat, the data were normalized using the Peak Based Correction (PBC) method [15, 16]. The retrieved values were subjected to statistical analysis. The t statistics in the limma Bioconductor package [17] were used to identify the differentially methylated CpG sites, and we employed the filtering property of an adjusted P value of 0.05 to find the significant probes. Then, using a value of |0.13|, we further ranked the hypermethylated and hypomethylated probes.

Microarray Validation

Since we were unable to perform functional validation, we utilized publicly

Table 1. Top 20 significantly differentially hypermethylated and hypomethylated probes

Gene	Probe	Delta Beta	Genomic Feature	CGI Feature
CCNA1	cg11513637	0.217	TSS1500	Shore
CCNA1	cg12417071	0.211	TSS1500	Shore
TMEM108	cg17385936	0.185	TSS200	Island
CCNA1	cg05089090	0.181	TSS1500	Shore
DMRT1	cg03168582	0.1784	1stExon	Island
CCNA1	cg08676975	0.175	TSS1500	Island
TMEM108	cg05075118	0.170	TSS200	Island
CCNA1	cg00282249	0.156	TSS1500	Shore
CCNA1	cg24334591	0.156	TSS1500	Shore
TMEM108	cg06288251	0.153	TSS200	Island
CCNA1	cg13711394	0.151	TSS1500	Shore
FBXO39	cg11871421	0.148	TSS200	Island
CTNNA2	cg20072442	0.145	Body	Island
TMEM108	cg02745211	0.141	TSS200	Island
CCNA1	cg18348647	0.141	1stExon	Island
TMEM108	cg18115215	0.137	TSS200	Island
TMEM108	cg09090724	0.137	5'UTR	Island
TMEM108	cg24198558	0.134	TSS200	Island
CCNA1	cg01040523	0.133	TSS1500	Shore
CCNA1	cg08215918	0.133	TSS1500	Shore
MAEL	cg18894878	-0.427	TSS1500	Island
IGF2BP3	cg18792116	-0.413	Body	Opensea
MAEL	cg11336590	-0.412	TSS200	Island
DPPA2	cg18992201	-0.409	TSS200	Opensea
DPPA2	cg11759378	-0.408	TSS200	Opensea
MAEL	cg27053975	-0.396	TSS1500	Island
TMEM108	cg18544974	-0.380	5'UTR	Opensea
IGSF11	cg23066675	-0.366	Body	Opensea
RNF17	cg10379992	-0.358	Body	Opensea
IGSF11	cg14841350	-0.347	Body	Shelf
CTNNA2	cg13784443	-0.344	Body	Opensea
DPPA2	cg18098079	-0.344	TSS200	Opensea
TEX14	cg20767977	-0.342	5'UTR	Shelf
CTNNA2	cg17733836	-0.335	3'UTR	Opensea
ODF1	cg10869265	-0.334	TSS1500	Opensea
TMEM108	cg03187125	-0.334	Body	Opensea
ODF1	cg25255602	-0.332	Body	Opensea
POTEE	cg22830663	-0.325	TSS200	Opensea
CEP55	cg25314624	-0.320	5'UTR	Shore
ELOVL4	cg05846476	-0.317	Body	Shore

available datasets that have conducted demethylation studies on CRC cell lines in the NCBI GEO database [18] with the accession number GSE79041. Further validation of the methylation profile was conducted using the TCGA dataset on the Wanderer platform [19].

RESULTS

Most of the Detected CTAs Were Hypomethylated

For this study, we identified the methylation profile of the 215 selected CTAs related to CRC, and the list of these CTAs was obtained from the Cancer Immune Atlas [20]. We profiled the methylation status of the 215 CTAs in 108 samples consisting of 54 CRC tissue with the paired adjacent normal tissue, and found 90 hypermethylated and 413 hypomethylated probes between CRC and normal tissues after filtering for substantially methylated probes using FDR 0.05. There were 203 differentially methylated probes, including 192 hypomethylated and 11 hypermethylated probes, once we further filtered the probes based on the delta beta value of |0.13|. The CCNA1 gene's cg11513637 probe had the highest level of hypermethylation, whereas the MAEL gene's cg18894878 probe had the lowest level of hypomethylation, as shown in Table 1. According to Figure 1A, the body and opensea sections contained the majority of the probes that had differential methylation.The same pattern was also seen in the hypomethylated probes. Nevertheless, since there were only 11 hypermethylated probes, the only genomic regions that were affected were the 1st exon, TSS1500, and TSS200. Whereas for the CGI features, the probes were only located at the island and shore region, as illustrated in Figure 1C.

TMEM108 is Hypermethylated and MAEL Is Hypomethylated in CRC

Our research revealed that most areas of TMEM108 were hypermethylated, particularly the promoter region. 14 of the 26 TMEM108-targeting probes had significantly varied methylation patterns. While the remaining 11 probes were hypomethylated, three of the probes were hypermethylated. The TSS200-island region contained all of the hypermethylated probes, whereas the Body-opensea region was mainly affected by the hypomethylated probes. To further validate the methylation profile of TMEM108, we used the TCGA dataset in the Wanderer platform. The most hypermethylated probe, cg17385936, was indeed significantly hypermethylated in CRC tissues, as shown in Figure 1B. We also analyzed the general expression of TMEM108 using the TCGA cohort, as shown in Figure 2A, the expression of TMEM108 was downregulated in CRC tissues. Accordingly, the Spearman correlation between the methylation level and the mRNA expression was indeed

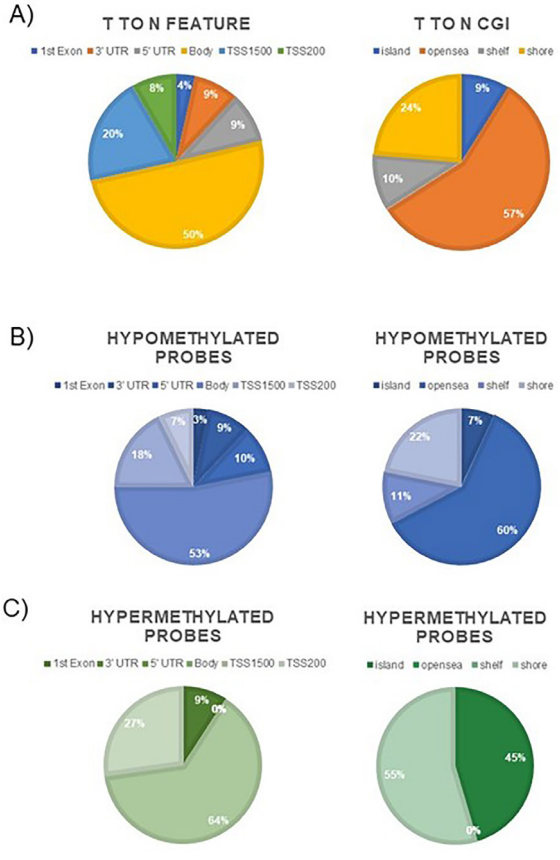

Figure 1. A) Distribution of all differentially methylated probes (left: Genomic Feature, right: CGI feature). B) Distribution of hypomethylated probes (left: Genomic Feature, right: CGI feature) C) Distribution of hypermethylated probes (left: Genomic Feature, right: CGI feature).

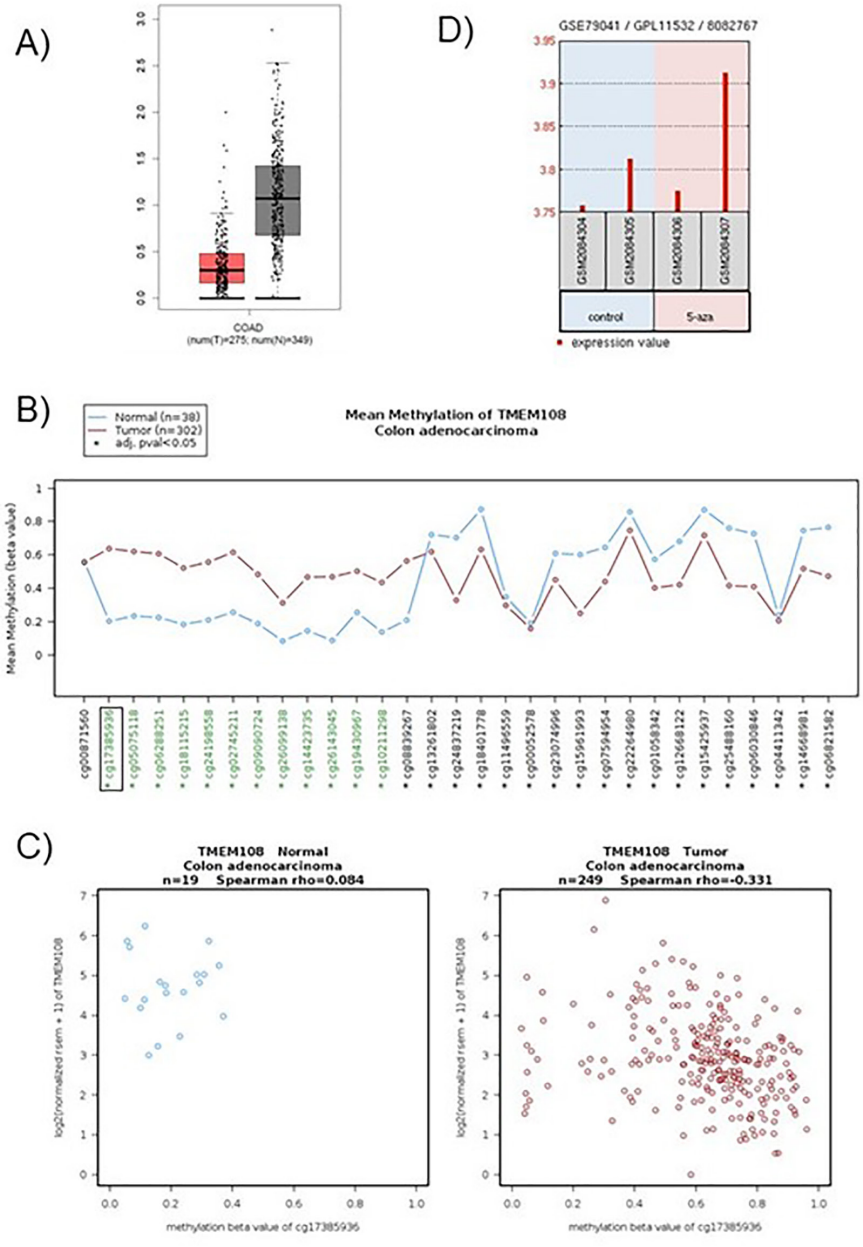

Figure 2. A) Gene expression of TMEM108 of CRC tumor tissues and normal colonic tissues in the TCGA dataset. Data obtained from GEPIA2 [34]. B) Overall profile of methylation probes covering the TMEM108 gene in the TCGA cohort. Data obtained from TCGA Wanderer[19]. C) Spearman correlation between the gene expression and methylation value of the cg17385936 probe in normal colon tissues and CRC tissues. Data obtained from TCGA Wanderer [19]. D) Gene expression of TMEM108 in HCT116 cells upon treatment with 5-azacytidine. Data analyzed from GEO2R using the GSE79041 dataset.

negative, -0.228. Since we were unable to perform validation using cancer cell lines, we resorted to public datasets that utilized colorectal cancer cell lines treated with demethylating agents. Based on the methylation profile, the MAEL gene was one of the most hypomethylated genes. All of the significantly methylated probes targeting MAEL were hypomethylated. Nevertheless, when we looked at the methylation profile in the TCGA cohort, the MAEL gene had a heterogeneous profile of methylation as compared with the normal tissues, which were significantly hypermethylated (Figure 3A). In the HCT116 cell line, upon treatment with 5-azacytidine, the expression of the MAEL gene was among the most significantly upregulated (Figure 3C).

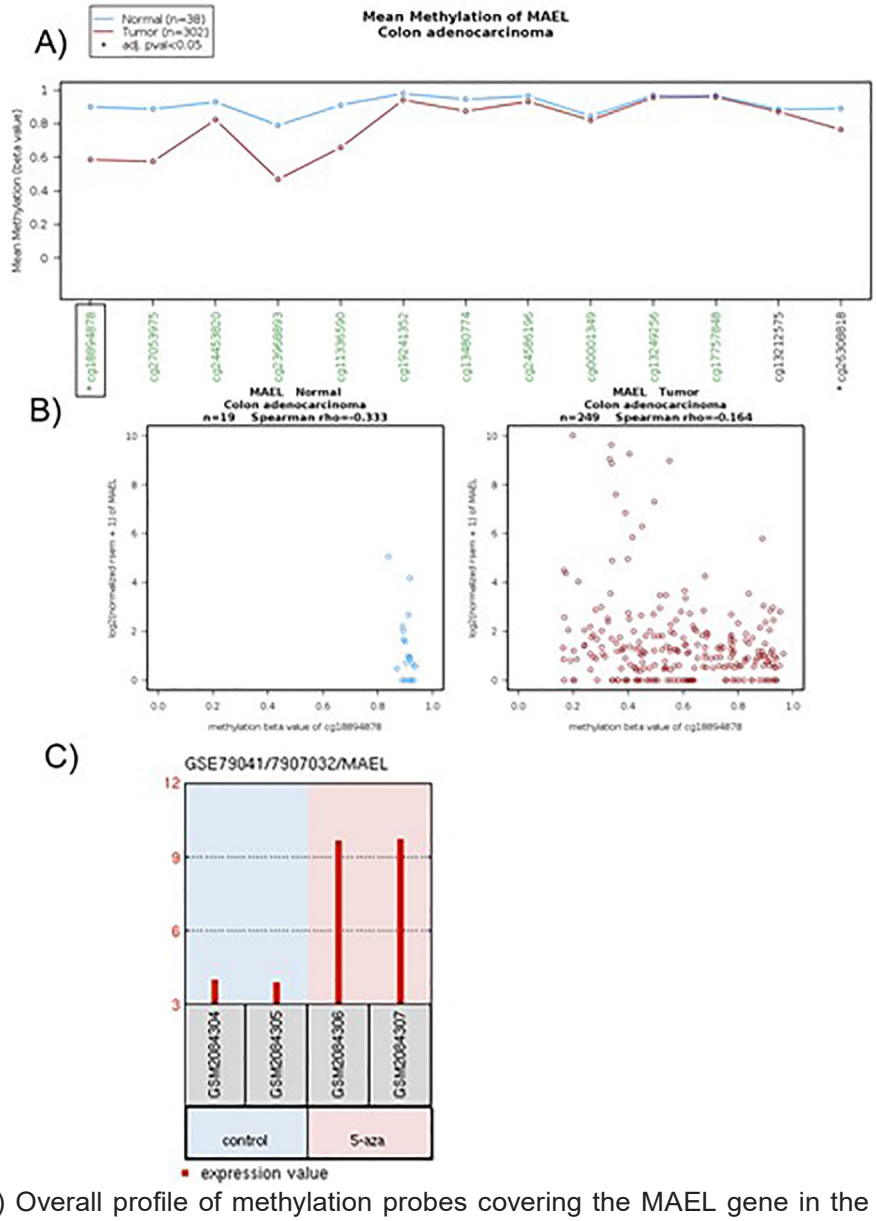

Figure 3. A) Overall profile of methylation probes covering the MAEL gene in the TCGA cohort. Data obtained from TCGA Wanderer[19]. B) Spearman correlation between the gene expression and methylation value of the cg18894878 probe in normal colon tissues and CRC tissues. Data obtained from TCGA Wanderer [19]. C) Gene expression of MAEL in HCT116 cells upon treatment with 5-azacytidine. Data analyzed from GEO2R using the GSE79041 dataset.

DISCUSSION

The CTAs are a class of tumor-associated antigens that have the potential to elicit an immune response and be used for immune-based therapies. Nevertheless, the challenge with the CTAs is that the expression is irregular and this may be caused by epigenetic modifications [5]. Several studies have shown that methylation may affect the expression of the selected CTAs [21-23], and therefore we postulate that most CTAs will be hypermethylated. Nevertheless, from our CRC data, we found that the majority of probes targeting our selected CTAs were hypomethylated. This shows, that while in fact, methylation may be a factor in repressing gene expression of certain CTAs, there could be other factors regulating the expression as well. For instance, in a recent study by Jakobsen et al. the authors discovered that the VCX2 gene could be activated by treatment with DNA methyltransferase and histone deacetylase inhibitors [24]. In a different

study by Song et al. the expression of the CTA HCA587/MAGE-C2 could be regulated by the binding of microRNAs such as miR-874 [25]. A study by Kutlitn et al. showed that the transcriptional regulation of the CTAs in CRC can be influenced by copy number variations, the presence of miRNAs, and the expression of DNA methyltransferase genes [26].

From our data, the probe targeting the CCNA1 gene was the most hypermethylated. This was in agreement with several other studies that have shown hypermethylation of the CCNA1 gene in correlation with several types of cancer [27-29]. For instance, a study by Zuo et al. demonstrated that the promoter of the CCNA1 is methylated in cervical cancer patients which could serve as a biomarker [28]. In head and neck squamous cancers, the same observation was seen where the CCNA1 gene was also hypermethylated [27]. With regards to CRC, there has been no report on the methylation status of the CCNA1, however, it has been shown that the expression of the CCNA1 may be linked with enriched copy number alterations [30].

The second most hypermethylated gene from our results was the TMEM108 gene. TMEM108 is a gene encoding for a transmembrane protein involved in several neurological disorders [31]. Nevertheless, its function in cancer, or CRC particularly is not well established yet. Here, it was shown that the expression of TMEM108 was indeed lower in CRC tissues as evidenced by the TCGA dataset. Furthermore, our results indicated that only three probes were hypermethylated, and the expression of the most hypermethylated probe was in concordance with the TCGA dataset. This specific probe has not been reported elsewhere to be involved in cancer or CRC. The presence of hypermethylation was further evidenced by the treatment of a demethylating agent in CRC cell lines, where the expression of TMEM108 was further elevated. This shows that methylation is indeed a factor that regulates the expression of TMEM108 in CRC. Nevertheless, more study is needed to further understand the transcriptional regulation of TMEM108.

One of the most hypomethylated probes targeted the MAEL gene in the island region. Although the methylation pattern was similar to the TCGA dataset, the expression of the MAEL gene was higher in normal samples, albeit not significantly. Of note, the hypomethylated probe has not been reported elsewhere either. Interestingly, as shown in Figure 3, in cell lines treated with 5-azacytidine, the expression of MAEL was higher compared with the untreated, suggesting that methylation repressed the expression. It has been shown that in hypospermatogenesis, the MAEL gene was hypermethylated instead [32]. An earlier study by Xiao et al. indicated that treatment with 5-azacytidine increases the expression of the MAEL gene, confirming the role of DNA methylation in the regulation of this gene [33].

Overall, our brief report has shown that while methylation could regulate the expression of the CTAs in CRC, further in-depth analysis is needed to determine the role of methylation. Our data has also shown that methylation only affected the selected CTAs and this should be further validated by other studies as well.

AUTHORS' CONTRIBUTION

MI, RB, SNN, NSAM performed the experiments, NSAM, MI, RB NA performed the data analysis, IMR provided clinical interpretation of samples, NA, NAARB, SNN drafted the manuscript, RJ NSAM provided critical input on the manuscript.

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