ORIGINAL RESEARCH

Methylation analysis of Gasdermin E shows great promise as a biomarker for colorectal cancer

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Abstract
In addition to its implication in hereditary hearing loss, the Gasdermin E (GSDME) gene is also a tumor suppressor involved in cancer progression through programmed cell death. GSDME epigenetic silencing through methylation has been shown in some cancer types, but studies are yet to fully explore its diagnostic/prognostic potential in colorectal cancer on a large-scale. We used public data from The Cancer Genome Atlas (TCGA) to investigate differences in GSDME methylation and expression between colorectal cancer and normal colorectal tissue, and between left- and right-sided colorectal cancers in 432 samples. We also explored GSDME’s diagnostic capacity as a biomarker for colorectal cancer. We observed differential methylation in all 22 GSDME CpGs between tumor and normal tissues, and in 18 CpGs between the left- and right-sided groups. In the cancer tissue, putative promoter probes were hypermethylated and gene body probes were hypomethylated, while this pattern was inversed in normal tissues. Both putative promoter and gene body CpGs correlated well together but formed distinct methylation patterns with the putative promoter exhibiting the most pronounced methylation differences between tumor and normal tissues. Clinicopathological parameters, excluding age, did not show any effect on CpG methylation. Although the methylation of 5 distinct probes was a good predictor of gene expression, we could not identify an association between GSDME methylation and expression in general. Survival analysis showed no association between GSDME methylation and expression on 5-year patient survival. Through logistic regression, we identified a combination of 2 CpGs, that can discriminate between cancer and normal tissue with high accuracy (AUC = 0.95) irrespective of age and tumor stage. We also validated our model in 3 external methylation datasets, from the Gene Expression Omnibus database, and similar results were reached. Our results suggest that GSDME is a promising biomarker for the detection of colorectal cancer.

KEYWORDS
Biomarker, Colorectal cancer, Diagnosis, DNA methylation, GSDME
1 | INTRODUCTION

Colorectal cancer is the third most common cancer worldwide with 746,000 cases in men, 614,000 cases in women, and 694,000 deaths globally in 2012.\(^1\) Although incidence and mortality rates have been settling in several developed countries, the universal burden of colorectal cancer is projected to increase by more than 2.2 million cases and 1.1 million deaths by the year 2030.\(^1\) Its pathogenesis originates from epithelial cells lining the colon or rectum, which accumulate mutations in key cell signaling pathways such as Wnt signaling and, most commonly, in the APC tumor suppressor gene.\(^2\) Histologically, classical adenocarcinomas account for the majority of cases.\(^3\) More recently, varying disease characteristics have been described between left- and right-sided colorectal tumors.\(^4\) Epigenetic changes in cancer, namely DNA methylation, have attracted great attention, especially with the availability of high-throughput profiling methods. In colorectal cancer, normal methylation patterns are drastically reshaped and are characterized by widespread hypomethylation blocks.\(^5,6\) Hypermethylation has also been observed in specific CpG islands and contributes to tumor initiation and progression.\(^7,8\) Studies have already emphasized the substantial capacity of DNA methylation as biomarker for the early diagnosis of cancer.\(^9,10\)

The Gasdermin E (GSDME) gene was originally identified in our lab in 1998. Where gain of function mutations in the gene led to an autosomal dominant form of hearing loss. At the time of identification, its relation to other gasdermins had not yet been established, and the gene was initially named Deafness Autosomal Dominant 5 (DFNA5).\(^11\) only recently was it renamed GSDME. In addition to its role in hearing loss, \(GSDME\) is also a tumor suppressor gene capable of inducing programmed cell death as a result of a caspase-3 cleavage.\(^12\) Given its role in cancer, epigenetic silencing through methylation has been shown in 52% and 65% of primary gastric tumors\(^13\) and colorectal adenocarcinomas\(^14,15\) respectively. However, these studies were performed on a small number of tumors and only included a few CpGs from the putative promoter. More recently, \(GSDME\) putative promoter methylation has shown its potential as a biomarker for breast cancer.\(^16,17\)

To our knowledge, published large-scale studies are yet to thoroughly evaluate the potential of \(GSDME\) methylation and expression as markers in colorectal cancer. We postulate that the methylation of \(GSDME\) could serve as a worthy biomarker for the detection of colorectal adenocarcinomas. This study aimed to analyze \(GSDME\) methylation and expression in the largest colorectal cancer patient dataset to date \((N = 432)\) using publicly available data from The Cancer Genome Atlas (TCGA) in order to assess its diagnostic potential in colorectal cancer.

2 | MATERIALS AND METHODS

2.1 Datasets and study population

The analyses presented in this manuscript were carried out on TCGA (colon and rectum adenocarcinoma) datasets that were downloaded from the GDC data portal website (https://portal.gdc.cancer.gov/) using an in-house developed Python script. The script automates the querying of TCGA in order to easily and quickly download the data. TCGA stores patient sample data under unique barcodes following a specific layout; these are used to access biological and clinical data in the database. First, all patient barcodes available for colorectal cancer were downloaded via the website. API URLs were generated using the downloaded barcodes in order to query the matching TCGA level 3 methylation 450k Illumina platform data, the RNAseq V2 gene expression data, and the Agilent 244K microarray expression data. Subsequently, the methylation and gene expression data was downloaded for each barcode (patient) and stored in separated JSON formatted files. The individual JSON files were then merged per data type (methylation, RNAseq expression and microarray expression), through Python’s dictionary functionality. This resulted in 3 data matrices, where sample data points (values: beta-value or normalized counts) were column-wise concatenated using the row name features (keys: methylation probe names or official gene symbols, 450K methylation or RNAseq V2 data respectively). The end result is a large table with probes row-wise and samples column-wise. The same principle was applied for downloading biospecimen and clinical data files. The biospecimen in the TCGA datasets were flash frozen/formalin-fixed paraffin-embedded, resection tissue samples, containing a minimum of 60% tumor nuclei and derived from primary, untreated colorectal tumor tissue. Using the in-house script, methylation (level 3) data were obtained from the portal for all 22 \(GSDME\) CpGs.

To determine the probes’ genomic location on the \(GSDME\) gene (gene body vs. putative gene promoter), we started from the most abundant \(GSDME\) RNAseq transcript (NM_004403) as the expression of the others was negligible. Additionally, NM_004403 is the only fully functional transcript of \(GSDME\), coding for the full-length protein of 496 amino acids. We annotated our transcript based on the GRCh37 Homo sapiens assembly which is also used by TCGA. To define the promoter and gene body regions of \(GSDME\) we used “Ensembl-Regulatory Build of \(GSDME\) gene” where the promoter region is indicated based on data of the ENCODE project (promoter: 24795602-24798199 and core promoter: 24797601-24796400). We considered the core promoter together with the flanking regions (24795602-24798199) as the putative promoter of \(GSDME\). Based on this annotation, 6 CpGs (CpG1-CpG6) are located in the gene body which
extends from exon 2 until exon 10. 15 (CpG7-CpG21) are located in the putative gene promoter which lies upstream of exon 2, while the last one (CpG22) is located in the upstream region, the details of which are described in Table 1. In this study however, we have considered CpG21 also a part of the upstream promoter region because its methylation pattern is clearly different from the other promoter CpGs and due to its very close proximity to the border of the GSDME promoter flanking region (24bp). Methylation is reported as β-value, which is the ratio of the methylated probe intensity over the sum of methylated and unmethylated probe intensities, ranging from 0 to 1. These values were obtained by TCGA using the Illumina Infinium HumanMethylation450 BeadChip microarrays (Illumina Inc, San Diego, California). RNA sequencing (RNAseq) and microarray expression datasets were obtained in a similar fashion. RNAseq expression values in TCGA were acquired using the IlluminaHiSeq platform (Illumina, San Diego, California), and the respective transcript abundances were quantified using the Expectation Maximization algorithm. The expression values are reported as log2 transformed value and the highest predicted transcript for GSDME in RNAseq was the most abundant (NM_004403), while the expression of the other transcripts was negligible. Microarray expression values were obtained in TCGA using the Agilent 244K Custom Gene Expression G4502A-07 microarrays (Agilent, Santa Clara, California) that contain 2 probes for GSDME (A_23_P82448 [36.3:chr.7:24705001-24705060] and A_23_P82449 [36.3:chr.7:24705092-24705151]), covering the 3 most abundant GSDME transcripts (NM_004403, NM_001127454.1, NM_001127453.1). Transcript NM_004403.2 was the most abundant, while the expression of the other transcripts was negligible and hence could not be included in the study. All microarray expression values are expressed as log2 transformed fold changes relative to the Universal Human Reference RNA (Stratagene).

Primary tumor samples for which clinical data was available were then split into 2 categories: “left-sided” and “right-sided,” based on the anatomical location of the neoplasm, with the splenic flexure acting as the demarcation line between the 2 categories. Inherently, samples taken from the caecum, ascending colon, hepatic flexure and transverse colon were part of the right-sided category, while samples from the splenic flexure, descending colon, sigmoid colon, rectosigmoid junction, and rectum comprised the left-sided category. This categorization is based on the pragmatic split between the embryological origins of the colorectal tissue such that the right part of the colon originates from the mid-gut, while the left part is derived from the hindgut. After data filtering and classification, several final datasets were available for the downstream analyses. Information about these datasets is found in Figure 1 and Supplement Table S1.

**Table 1** Table showing a simplified reference to the Illumina Infinium HumanMethylation450 probes, along with their genomic locations

| Probe abbreviation | Probe name     | Genomic coordinate | Location         |
|--------------------|----------------|--------------------|------------------|
| CpG1               | CpG17790129    | 24738572           | Gene body        |
| CpG2               | CpG14205998    | 24748668           | Gene body        |
| CpG3               | CpG04317854    | 24762562           | Gene body        |
| CpG4               | CpG12922093    | 24767644           | Gene body        |
| CpG5               | CpG17569154    | 24781545           | Gene body        |
| CpG6               | CpG19260663    | 24791121           | Gene body        |
| CpG7               | CpG09333471    | 24796355           | Putative promoter|
| CpG8               | CpG00473134    | 24796494           | Putative promoter|
| CpG9               | CpG03995857    | 24796553           | Putative promoter|
| CpG10              | CpG07320064    | 24796981           | Putative promoter|
| CpG11              | CpG07293520    | 24797192           | Putative promoter|
| CpG12              | CpG04770504    | 24797363           | Putative promoter|
| CpG13              | CpG24805239    | 24797486           | Putative promoter|
| CpG14              | CpG01733570    | 24797656           | Putative promoter|
| CpG15              | CpG25723149    | 24797680           | Putative promoter|
| CpG16              | CpG22804000    | 24797691           | Putative promoter|
| CpG17              | CpG07504598    | 24797786           | Putative promoter|
| CpG18              | CpG15037663    | 24797835           | Putative promoter|
| CpG19              | CpG19706795    | 24797839           | Putative promoter|
| CpG20              | CpG20764575    | 24797884           | Putative promoter|
| CpG21              | CpG06301139    | 24798175           | Upstream region  |
| CpG22              | CpG26712096    | 24798855           | Upstream region  |
2.2 | Statistical analyses

We designated the following clinicopathological parameters from the TCGA clinical patient data files with which to carry out association analyses: age at diagnosis, gender, pathological tumor stage (I-IV), anatomic neoplasm subdivision (left-sided or right-sided), and presence of colon polyps at procurement (Table 1). The GSDME sequence regions, methylation probe locations and chromatin states were explored using the UCSC genome browser.20 The statistical software R (version 3.4.1)21 was used to carry out all the statistical analyses. All reported p-values are 2-sided, and those less than or equal to 0.05 were considered statistically significant. To account for the nonindependence between measurements from the same individuals, a linear mixed model was fitted and included a random effect for sample barcodes. The significance of the fixed effects was then tested via the F-test with a Kenward-Roger correction for the number of degrees of freedom. Differences between groups were assessed through t-tests and linear regression models, while association between expression and CpG methylation was tested using Spearman's correlation and through linear regression models. In all regression models age was accounted for as a covariate, but it was excluded from the final model if its effect on the outcome was not significant.

5-year overall survival analysis was carried out by fitting Cox proportional hazard models to the methylation and expression datasets and including age as covariate. Additionally, stratified Cox models with separate baseline hazards for the 4 tumor stages were fitted. Censoring was carried out for individuals who died after the 5 years (1826 days) mark of the analysis and their respective follow-up time was set to 1826 days. Quantile-quantile plots showing the distribution of the 22 observed p-values as compared to the uniform distribution, which is expected in the absence of any true association signal, were generated.

To assess the viability of GSDME methylation and expression as a biomarker for colorectal cancer, binary logistic regression was fitted to predict tissue type (normal/tumor) based on methylation and expression values with age as covariate. Stepwise multiple regression analysis was carried out to determine the best combination of the 22 CpGs. The final model was chosen based on the best Akaike information criterion (AIC) values with the lowest number of predictors possible. The accuracy of the model predictions was assessed by plotting receiver operating characteristic (ROC) curves. A 10-fold cross validation of these results was then performed. Moreover, 3 additional Illumina 450K CpG methylation datasets were downloaded from the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/) (GEO accession numbers GSE77718, GSE42752, and GSE68060), and were used for the subsequent external validation (Figure 1). The final model was refit on each of the external datasets and the AUC was recalculated for the new predictions. The same methodology was also applied to RNAseq and microarray datasets to determine their predictive potential. A list of the R packages used in the analysis can be found on the last page of the Supplementary Material.

3 | RESULTS

3.1 | GSDME methylation and expression in primary untreated colorectal adenocarcinomas and histologically normal colorectal tissue

Our results showed a significant methylation difference between primary tumor and normal colorectal tissue for
all 22 CpGs in the nonpaired samples ($P = 3.51E-24$ to $3.94E-2$) and in 19 of 22 CpGs in the paired samples ($P = 1.65E-16$ to $2.53E-2$) (Supplement Tables S2 and S3). For the significantly different CpGs located in the gene body, methylation levels in the normal tissue were higher than those in the tumor tissue, while the opposite holds true for CpGs located in the putative promoter region. The pattern switched again with 2 CpGs (CpG21 and CpG22), located upstream of the putative gene promoter region; these again showed increased methylation in the normal tissue as opposed to the tumor tissue (Figure 2A,B and Supplement Figure S1).

Two sources of GSDME expression were examined: RNAseq and microarray. The mean RNAseq expression for the normal tissues (5.80 95% CI: 3.31, 8.29) was slightly higher than that for the tumor tissues (5.45 95% CI: 2.68,

![Figure 2](image-url)

**FIGURE 2** GSDME methylation differences between representative CpGs in the different sample groups. (A, B) The 2 presented CpGs exhibit the most significant differences in methylation levels between normal tissues ($N = 43$) and colorectal adenocarcinomas ($N = 389$). The lines indicate the mean GSDME methylation for each group; for CpG03995857(CpG10), the mean methylation is 0.14 (95% CI: 0.05, 0.23) in the normal tissue and 0.50 (95% CI: 0.03, 0.97) in the tumor tissue, while for CpG12922093(CpG4) these values are at 0.67 (95% CI: 0.31, 1.03) and 0.84 (95% CI: 0.78, 0.91) respectively. (C, D) CpG25723149(CpG15) is representative of GSDME promoter CpGs, where significant differences in methylation levels between left-sided ($N = 202$) and right-sided ($N = 187$) adenocarcinomas were observed in 15 out of 16 CpGs located in the putative gene promoter. For CpG25723149(CpG15), the mean methylation is 0.57 (95% CI: 0.15, 0.98) in the left colon and 0.70 (95% CI: 0.40, 0.99) in the right colon, while for CpG04317854(CpG3) these values are at 0.78 (95% CI: 0.53, 1.03) and 0.80 (95% CI: 0.60, 1.01) respectively.
8.22), but these differences were not significant neither for the paired nor for the un-paired samples. The same held true for microarray data where no significant differences were observed between the normal and tumor tissues (means of $-3.18$, 95% CI: $-5.89$, $-1.38$ and $-0.46$, 95% CI: $-4.79$, $-1.38$ respectively). Additionally, we explored the correlation between the 2 sources of GSDME expression data in samples for which both microarray and RNAseq GSDME expression values were available. The 2 datasets were highly correlated with a Spearman’s coefficient of 0.89 for the whole datasets, 0.85 and 0.84 for the tumor tissues and normal tissues respectively, and 0.88 and 0.86 for the left-sided and right sided groups respectively, all of which having a $P < 2.2e-16$. With respect to the mentioned clinicopathological parameters, only age had a significant effect on the methylation of 14 out of 22 probes. These were CpGs 7-18, 20 and 22 (Supplement Table S7). The calculated regression slopes were very close to zero (0.0002-0.005) and as such the positive effect of age on probe methylation was somewhat minor; this is further elucidated by their weak Pearson’s correlation coefficients (Supplement Table S7). Both the analysis of variance (ANOVA) and Tuckey tests resulted in nonsignificant $P$-values, indicating that there is no significant difference in expression levels (microarray) between the different disease stages and that the observed variance is expression levels is due to intra-stage differences.

### 3.2 GSDME methylation and expression in left-sided and right-sided colorectal adenocarcinomas

With respect to left-sided and right-sided colorectal adenocarcinomas, our investigation showed a significant difference in methylation levels between the subgroups for 18 out of 22 CpGs ($P = 1.66E-13$ to $4.71E-2$) (Supplement Table S4). Interestingly, most significant differences were observed in the putative promoter region (CpG6-22), whereas only 2 CpGs in the gene body were significantly different in methylation between the 2 groups (CpG1, $P = 4.21E-2$ and CpG3, $P = 4.71E-2$) (Supplement Table S4). For the significant CpGs, the methylation levels in the left-sided subgroup were consistently lower than those in the right-sided group and followed the general trend of putative promoter CpGs in the normal colorectal tissue (Figure 2C.D and Supplement Table S4). No significant differences in GSDME expression between the 2 groups were found. The correlation between methylation and expression in the left-sided subgroup was 0.86 while in the right-sided subgroup it was 0.84.

### 3.3 GSDME methylation and genomic location

After plotting the average GSDME methylation per CpG versus the respective physical map position on chromosome...
7 (GRCh37), a clear trend in methylation was further elucidated (Figure 3). Methylation levels of the first 6 CpGs, located in the gene body, are higher in normal colorectal tissue as compared to tumor tissue. Conversely, the 14 following CpGs, located in the putative promoter region displayed a consistently lower methylation in the normal tissue as compared to tumor tissue. The inverse of this methylation pattern was seen for the last 2 CpGs, which are located upstream of the putative gene promoter region. As for the left-sided and right-sided groups, no difference can be seen in the methylation of gene body CpGs, in the putative promoter region the left-sided group shows lower methylation compared to its counterpart (Figure 3).

A correlation matrix for the methylation values of all 22 CpGs to investigate the association between the methylation of different regions in the GSDME gene, showed a block-like clustering comprising a smaller cluster made up of the 6 CpGs located in the gene body, and a larger cluster made up of the remaining 14 CpGs located in the putative gene promoter region (Figure 4). Additionally, the last 2 CpGs located upstream of the putative gene promoter region clustered together and had a pattern similar to the gene body cluster. In these clusters, the larger CpG group, pertaining to probes in the putative promoter region, had the largest positive pairwise correlation coefficients whereas the smaller group had lower positive coefficients, all of which having significant $P < 0.05$ (Figure 4). Moreover, an accumulation of methylation was observed in the promoter region of tumor tissues with a significant 32% increase over the normal tissues. When excluding CpGs 21 and 22, which are thought to be upstream of the putative promoter region and clearly follow the methylation patterns of gene body CpGs (Figure 4), a 43% increase in methylation is observed. With respect to the gene body, a 13% decrease in methylation is observed in the tumor tissues as opposed to the normal. CpG islands are normally larger than 200 bp in length with a GC content above 50%. Shore regions are located up to 2 kilo base pairs upstream or downstream from the CpG island, while shelves are regions 2 to 4 kilo base pairs away from the island.22 Based on the UCSC genome browser, a 946 bp CpG island was found to be part of the putative promoter, flanked by 2 enhancer regions (Figures 3 and 4). Moreover, high DNAse I activity is reported around the putative promoter region along with binding sites for E2F1 and PolR2A transcription factors.
3.4 | Association between GSDME methylation and expression

We calculated the Spearman correlation coefficient to study the association between GSDME methylation and expression in samples for which both methylation data and expression data were available (RNAseq dataset), but none of the calculated correlation coefficients were strong (Supplement Table S5). Regression analysis over the whole dataset resulted in significant P-values for CpGs 3, 6, 9, 20, and 22, this association however, was very weak indicated by the small exploratory variable slopes (Figure 5 and Supplement Table S5). Regression analysis on the grouped samples, showed that for the tumor samples about 40% of the variance in GSDME expression was attributable to variance in GSDME methylation ($R^2 = 0.38$, model $P = 2.20E-16$). Five CpGs (CpG3, CpG6, CpG9, CpG20, CpG22) showed significant association between methylation P-value and RNAseq expression. In the normal samples, a regression model could be fit, explaining around 60% of the variance in expression ($R^2 = 0.63$, model $P = 1.10E-2$). However, only one CpG (CpG20 $P = 3.50E-2$) showed a significant association with GSDME expression (Supplement Table S6). In the anatomical subgroups, around 40% of the variance could also be explained by the CpGs included in the models. In the left-sided group the methylation of only 2 CpGs (CpG9, CpG22) showed a significant association with GSDME expression, while in the right-sided group 4 CpGs (CpG6, CpG9, CpG20, CpG22) were significantly associated (Supplement Table S6). Overall, the regression analysis showed a heterogeneity in the effects of CpG methylation on expression. The coefficients were spread between positive and negative values with most of them clustering around zero, indicating minor effects between the variables (Figure 5). The results in both datasets are relatively disparate and hence the contribution of GSDME methylation to expression levels is still inconclusive, with no consistent association between the 2.

3.5 | Associations between GSDME methylation or expression and 5-year overall survival

The association between survival and methylation or expression was studied using Cox proportional-hazard models in patients for which follow-up data were available (N = 260). For the complete adenocarcinoma dataset, no significant association between methylation and 5-year survival could be found. For the left-sided and right-sided subgroups, a significant association was found only for 1 CpG (CpG22 $P = 1.60E-02$) and for 2 CpGs (CpG4 $P = 1.51E-2$, CpG21 $P = 3.13E-2$), respectively. By comparing the distribution of the P-values to the expected distribution under the null hypothesis of no association, no enrichment in low P-values was observed and hence CpG methylation does not seem to be a strong predictor of 5-year survival (Supplement Figures S2, S3 and Supplement Table S8). We repeated the same analysis for both RNAseq and microarray expression data, but again no clear association could be deduced. It is noteworthy that in all hazard proportion models, only age had a significant influence on survival (Supplement Figures S2, S3 and Supplement Table S8).

3.6 | GSDME methylation and expression as potential detection biomarker for colorectal adenocarcinomas

In a logistic regression framework, we explored all combinations of the 22 CpGs that would yield discriminatory power...
Six CpGs had good predictive value in our models (CpG12, CpG14, CpG4, CpG17, CpG15, CpG2). In general, models with 2 CpGs led to a better prediction than those with only one. Their AUC values were in the range of 0.72-0.97 and 0.71-0.87, respectively (Figure 6, Table 1, and Supplement Figure S4). To analyze if the relation between CpG methylation and disease status (or tissue type) is homogeneous across tumor stages, we fitted logistic regression models. Tissue type was entered as dependent variable, and independent variables included CpG methylation, stage and the interaction between methylation and stage. The significance of this latter term (effect/interaction between methylation and diseases stage) tests the null hypothesis of homogeneity of the marker across the stages: in case the \( P \)-value of the interaction is significant, then the association between the CpG methylation and tissue type is not uniform across stages, in other terms, that CpG methylation is significantly different depending on disease stage. The significance of the interaction term was tested using a likelihood ratio test, comparing the fit of the model with both main effects and their interaction term, against the model with only the main effects of methylation and stage. None of the stages or interaction terms showed a significant outcome on tissue type prediction and hence methylation was not significantly altered by stage, reinforcing the marker's homogeneity across disease stages (Supplement Table S9).

For our final prediction model, CpG 12 located in the putative promoter region and CpG4 located in the gene body were chosen as predictors, resulting in a 0.95 (95% CI: 0.95-0.98) AUC value. A 10-fold cross-validation resulted in an AUC value of 0.95 (95% CI: 0.93-0.97, SE = 0.01). Sensitivities and specificities at the different cutoff values for the predicted probabilities are shown by means of an ROC plot (Figure 6). At a cutoff value of 0.72, a sensitivity of 93.3% and a specificity of 93.7% for detection of colorectal adenocarcinomas were reached without false positives, with an overall accuracy of 97.6%. As an external validation, we applied our trained model to 3 external CpG methylation datasets downloaded from the GEO database (Figure 1) using the same 2 CpGs as predictors. Sample tissue type was successfully predicted in all 3 datasets with AUC values comparable to that of the original TCGA dataset; GSE77718, GSE42752, and GSE68060 had AUCs of 0.97, 0.96 and 0.99 respectively. In all, the model exhibited a high predictive power and good generalizability across different datasets (Figure 6, Table 1).

We additionally investigated the potential of GSDME expression data as a biomarker. Using the same methodology, a ROC curve was constructed using RNAseq data for 453 tumor tissues and 41 normal tissues and microarray data for 221 tumor tissues and 20 normal tissues. The AUC values were 0.55 and 0.60 respectively, reflecting a low predictive power.

**4 | DISCUSSION**

Due to the rise of cost-effective genome-wide profiling methods such as the Illumina Infinium HumanMethylation450 and the more recent MethylationEPIC beadchips, several studies have emerged linking aberrant DNA methylation to the onset
and progression of colorectal tumors.\textsuperscript{23,24} Originally discovered in our lab as a gene responsible for autosomal hearing loss, \textit{GSDME} was also identified as a target of epigenetic silencing in several cancer types.\textsuperscript{13-17} In this analysis of TCGA methylation data, we provide evidence that \textit{GSDME} DNA methylation is a promising biomarker for the diagnosis of colorectal cancer.

Differential methylation was observed across \textit{GSDME} (Figure 2A,B and Supplement Figure S1, Supplement Tables S2 and S3). The putative promoter region of healthy tissues was hypomethylated as compared to the gene body, whereas the opposite was observed in tumor tissues. Our findings conform to the global genomic methylation paradigm of CpGs in cancer,\textsuperscript{24} with \textit{GSDME}’s methylation patterns following those of other known tumor suppressor genes in colorectal cancer.\textsuperscript{13,14,24} The methylation status of probes in the putative promoter region were highly correlated, as was the case with those in the gene body region, but not between these 2 distinct regions, and a clear positional segregation between them was observed. On average, the largest and most significant differences in methylation were observed in putative promoter CpGs as compared to the gene body. This clear difference between the 2 regions is further illustrated through the bimodal clustering of methylation correlation coefficients in the matrix (Figure 4). CpGs 1-6, which are located in the gene body are highly correlated together, as are CpGs 7-19, which are located in the putative gene promoter. CpGs 21 and 22, which are thought to be located upstream of the putative promoter, correlate strongly with CpGs 1-6, and follow their methylation patterns. The methylation levels of the 2 CpG clusters seems to act conversely; levels of putative promoter CpGs seemed to increase simultaneously in tumor tissues, while that of gene body CpGs decreased in a similar fashion. The opposite was true when considering normal tissues. To date the impact of CpGs located outside of promoter regions, such as intragenic CpGs, is yet to be fully understood as they do not seem to directly regulate gene expression. Nevertheless, a few studies have already hinted at their potential role in modulating alternative promoters\textsuperscript{25} or in long-range regulation.\textsuperscript{26} More recently, Neri et al\textsuperscript{27}, proposed intragenic DNA methylation as a safeguard against spurious transcription initiation. Its absence in cancerous cells could affect biological processes and favour neoplastic predisposition. Despite the scarcity of studies on \textit{GSDME} methylation, our findings are still in agreement with those reported for colorectal\textsuperscript{14,15} and breast cancer,\textsuperscript{16,17} albeit their analyses involved TaqMan-MSP and pyrosequencing respectively, as opposed to the Illumina 450K beadchip data that was used here. Our results show that methylation alterations occur not only within the strict putative gene promoter but also in the mentioned shore regions (Figures 3, 4 and Supplement Tables S2 and S3), which is in agreement with the original findings of Irizarry et al that outlined the involvement of CpG island shores in colon cancer.\textsuperscript{28} The observed higher methylation in the gene body of normal cells is hypothesized to act as a protective strategy against unwanted intragenic transcription from highly active genes and the possible production of aberrant gene products. Such methylation patterns are hypothesized to protect the gene body from faulty RNA polymerase II entry and cryptic transcription initiation. Evidently, tumors are characterized by global DNA hypomethylation, especially in intragenic regions, a fact that hints at a functional epigenetic crosstalk between DNA methylation, RNA polymerase II and gene expression.\textsuperscript{27} Taken together, we believe that our findings are in line with the global CpG methylation paradigm of tumor suppressors in cancer.\textsuperscript{29} Clinical parameters did not have any significant outcome on methylation, with the exception of age, which showed a minor positive effect on 14 out of the 16 CpGs in the putative promoter region, for one of the CpGs upstream of that region a minor negative effect was observed (Supplement Table S7).

We investigated the association between \textit{GSDME} methylation and expression through correlation tests and linear regression, but no significant correlation between them, for both tumor and normal tissues could be observed. Some association between methylation and expression was found in 5 distinct probes across the tested groups (Figure 5 and Supplement Tables S5 and S6), of which CpGs 20 and 22 recurred in 3 out of the 4 groups followed by CpG9 in 2 groups. The effect of methylation on expression seemed low and disparate, even for the significant probes, with the exception of CpG20, whose methylation showed a fivefold positive effect on RNAseq expression. Due to the discrepancy in the results, we could not establish a conclusive relationship between \textit{GSDME} methylation and its expression, which is somewhat discordant with the general dogma of gene expression in cancer and in particular with the findings of Akino et al that showed a significant downregulation in colorectal cancer tissues as compared to normal tissue.\textsuperscript{13} Although a strictly linear relationship between \textit{GSDME} methylation and expression is not anticipated, we would expect that increased methylation leads to decreased gene expression in cancer. The discrepancy in our results and in conflicting findings,\textsuperscript{13,30} make it hard to clearly define the effect of \textit{GSDME} methylation on its expression, particularly in light of recent studies suggesting that the relation between the 2 could be more complex.\textsuperscript{31} One conflicting factor could be miRNAs (mir-3p and mir26b-5p) that are known to modulate \textit{GSDME} expression,\textsuperscript{32} and another could be copy number variations (CNVs) that play an important role in complex phenotypes such as cancer.\textsuperscript{33} Moreover, the location of CpG methylation (in or near the island) is crucial to its influence on gene expression such that CpGs located outside the islands have a bigger impact on gene expression than those located within.\textsuperscript{34} A novel finding of this study is the differential methylation between left-sided and right sided tumors. Several studies
have outlined the differences between the 2 groups, attributing higher incidence but better prognosis to left-sided tumors versus right-sided ones; however, differential methylation patterns have not yet been widely considered. Our findings are in line with various communications that have reported a significant association between hypermethylated CpGs and right-sided tumor locations. This same rationale could also apply to the worse disease progression and prognosis of the right-sided group where we report higher GSDME methylation. GSDME could be one of several tumor suppressor genes that are differentially activated between left- and right-sided colorectal cancers leading to differences in prognosis and survival. To date, there is no clear explanation for the observed methylation differences. Considering that diet type is one of the main risk factors of colorectal cancer, we speculate that the concentration gradient of substances or gut microbiota across different parts of the colon, might be one of the causes of such methylation patterns. Another could be the divergent embryological origins of the colon and their inherent cellular differences. Tumor location has a prognostic value in CRC as it provides information about the overall cancer outcome, independent of treatment received. Its predictive value on the other hand, provides information on the likelihood of response to a given therapy, and therefore helps to optimize treatment decisions. In addition to microsatellite instability, chromosome instability, invasive polymicrobial biofilms and gene mutations (KRAS, BRAF, PIK3CA, NRAS, and TPS3), the difference in GSDME methylation (amongst other genes) could reflect a different disease etiology and outcome, but to clarify this, more research is necessary.

With respect to colorectal cancer screening, several tests have been devised, including the fecal immunochemical test (FIT) and the fecal DNA test. Moreover, genes with aberrant methylation patterns have been reported as biomarkers, for example, the silencing of VIM through promoter methylation has been exploited clinically as a tumor determinant as part of the Cologuard kit. More recently, SEPT9 promoter hypermethylation was approved for use in colorectal cancer screening as part of 3 commercially available kits (Epi proColon, ColoVantage, and RealTime mS9). Despite their near perfect specificity and superior performance over FIT, such methods still compromise on sensitivity and some even show significant variability based on disease stage; hence the need for new and improved biomarkers remains. In our case, the identified CpGs performed well in tumor classification with both a high sensitivity and specificity. The external validation, confirms the model’s validity and its generalizability over external cohorts and colorectal cancer tissue types (Figure 6, Table 1). Normally, the level of aberrant DNA methylation of genes is higher in later stages as compared to early stages in most cancers. This makes it difficult to identify genes that can be used in a diagnostic setting as their methylation status is highly dependent on disease progression and is not always detected in early stages where the need for diagnosis is paramount. In GSDME’s case, our prediction model metrics were unaffected by age and disease stage (Supplement Table S9). This makes GSDME an excellent candidate gene for early diagnosis irrespective of CRC stage as its aberrant methylation status can be ubiquitously used for predictions across disease progression. Although most combinations of 2 probes yielded good prediction results (Supplement Figure S4), the final step-wise binary logistic regression model included CpGs 2, 4, 12-17 and 21. These probes had the highest contribution in accurately predicting tissue type based on methylation values. CpGs 4 and 12 yielded the best AUC, thus their importance in the biomarker context. Given the strong correlation between probes in the different regions, pairwise combinations using any of the mentioned probes still yielded a high prediction AUC. Overall, differences in methylation were more prominent in the putative promoter region as compared to the gene body, with CpG9 and CpG4 having the highest mean differences respectively, and more of the putative promoter probes contributing to the prediction model. Moreover, the largest differences in methylation were observed in CpGs 9, 10, 12, and 13, all of which exhibited the highest tissue prediction capacities in both the single and pairwise models (Supplement Table S10 and Supplement Figure S4). CpG13 exhibited the most variation across all our analyses and is possibly located within a region of high epigenetic activity. CpG12 is the most important clinically as it yielded the best ROC curve and had the biggest contribution to tissue type prediction.

Our findings regarding GSDME methylation can be translated into a noninvasive biomarker assay through digital-droplet PCR (dd-PCR) for example. To that end, the next step would be investigating GSDME methylation in collected tissue biopsies from a cohort of colorectal cancer patients and healthy individuals. This would then be followed by testing in circulating tumor DNA extracted from a similar cohort using blood-based liquid biopsies. Evidently, such an assay would still need to undergo clinical trials and be compared to existing early detection kits before making its way to the clinic. To date, a handful of “proof of concept” studies have emerged, outlining the basis of such methods in breast and colorectal cancers. Given that colorectal cancer initially develops from colon polyps, and that epigenetic modifications are believed to be precursors of cancer formation, another step would be testing GSDME methylation in polyps to assess its potential as an early biomarker for colorectal cancer. Considering the models’ robustness and the low number of predictor probes needed, the answer might ultimately lie in the use of GSDME methylation in liquid biopsies as a sensitive, minimally-invasive and cost-effective detection method for colorectal cancer.

The exceptional in silico performance of the thoroughly identified CpG dinucleotides in a large patient cohort, makes
this study a stepping stone towards developing a biomarker assay for the detection of colorectal cancer, in the context of liquid biopsy-based dd-PCR. With respect to left-sided versus right-sided colorectal cancer, the underlying causes of nonadherence and their effects on treatment outcome warrant further investigation. In this context, exploring the differences in methylation and expression between additional clinicopathological parameters, such as neoadjuvant therapy status or microsatellite instability would be beneficial to understanding their effects on tumor sidedness. One limitation of this study is the lack of clinically collected tissue methylation data; hence the next step would be the validation of in silico data using tissue biopsies.

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**How to cite this article:** Ibrahim J, Op de Beeck K, Fransen E, et al. Methylation analysis of *Gasdermin E* shows great promise as a biomarker for colorectal cancer. *Cancer Med*. 2019;8:2133–2145. [https://doi.org/10.1002/cam4.2103](https://doi.org/10.1002/cam4.2103)