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Can Li Date

Cell-type-specific analysis in Alzheimer's disease

By

Can Li

Master of Science in Public Health

Biostatistics and Bioinformatics

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Hao Wu, PhD
Thesis Advisor

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Zhaohui Qin, PhD
Reader

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B.S.

The Ohio State University

2018

Thesis Committee Chair: Hao Wu, PhD

Reader: Zhaohui Qin, PhD

An abstract of
A thesis submitted to the Faculty of the
Rollins School of Public Health of Emory University
in partial fulfillment of the requirements for the degree of
Master of Science in Public Health
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2020

Abstract

Cell-type-specific analysis in Alzheimer's disease

By Can Li

Background:
Alzheimer's disease (AD) is one of the chronic neurodegenerative diseases which starts slowly but worsens over time. It has been found that distinct cell types in the brain play different roles in AD etiology and progression.

Methods and Materials:
We estimated cell-type-specific proportions in the brain applying reference-free DNA methylation mixture deconvolution (R/CRAN package RefFreeEWAS) and the newly developed TOAST (Tools for the Analysis of Heterogeneous Tissues, an R/Bioconductor package) statistical method. We detected cell-type specific differential signals between patients with Alzheimer's and normal controls. Differential methylation calling without consideration of cell mixtures was conducted to compare the results using Bioconductor package Minfi. We conducted the downstream analysis, including pathway/GO analysis and motif analysis using EnrichR

Results:
We assumed there are four brain cell types which are neuron, astrocyte, oligo, microglia during our analysis. We mainly focus on cell type 3 since both Reference-free DNA methylation mixture deconvolution and TOAST statistical method express the lowest average proportion on cell type 3. The average proportion of cell type 3 is 0.0809 (SD=0.0049) for AD patients and 0.0803 (SD=0.0042) for control groups resulted from the Reference-free DNA methylation mixture deconvolution. And the average proportion of cell type 3 generated from the TOAST statistical method is 0.0882 (SD=0.0043) for AD patients and 0.0803 (SD=0.0035) for control groups. The proportions of cell type 1 and 2 are highly negatively correlated to each other with correlation coefficients -0.94 indicating the possible cell type 1 and 2 might be neuron and oligo which are anti-correlated conducted from a recently published paper. The number of DMCs of cell type 3 identified by TOAST is less than that of RefFreeEWAS although it has the least proportions. The biological process regulation of adiponectin secretion has the highest combined score and the neurotransmitter receptor complex is the top cellular component GO term shown in the GO analysis.

Conclusion:
The study reveals that differential analysis could provide insights for diagnostic biomarkers and therapeutic targets and both RefFreeEWAS and TOAST approaches should be encouraged in the practice that future studies could address cell-type-specific idea in AD area.

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Introduction

Alzheimer’s disease (AD) is a progressive disease, starting with mild memory loss, which can lead to loss of ability to engage in conversation and respond to the environment, seriously impact daily activities. (Kukull, 2002) AD is one of the most prevalent forms of dementia which is a chronic neurodegenerative disease and is estimated to affect 36 million people worldwide. It accounts for 60% to 80% of cases of dementia. (Barnes, 2011) The incidence of AD, increasing exponentially, is not a normal part of aging and becomes a global burden and the majority of people with AD are 65 or older. (Qiu, 2009) While there are still approximately 200,000 Americans under the age of 65 develop younger-onset AD. (Alzheimer's Association, 2005) The most common early symptom of AD is short-term memory loss since AD changes the learning ability of the part of the brain typically. (Alzheimer's Association, 2005) In addition to memory problems, symptoms can be developed into problems with language, disorientation, changes in mood, personality or behavior. (Alzheimer's Association, 2005) The brain has 100 billion nerve cells(neurons) to connect with many others to form communication networks and store information through electrical and chemical signals. While AD disrupts the communication among nerve cells and characterized by developing amyloid plaque and neurofibrillary, or tau, tangles, and even the death of nerve cells. (National Institute on Aging, 2017)

The risk of AD is determined by genetics, lifestyle, environmental factors, as a combination among them to affect the brain over time. (Iatrou, 2017) Epigenetic mechanisms are acting as the mediation of genetic environment interactions and the factors to cause long-lasting gene changes including cognition, memory, and mood. Tremendous efforts have been spent to look for molecular mechanisms through analyzing the epigenetic changes at the genome level aimed to provide new perceptions into the relationship between brain epigenome and AD. (Hoffmann, 2017) Epigenetics alter gene expression and genomic functions, stable and heritable without changes in the DNA sequence. (Bollati, 2011) Epigenetic modification such as DNA methylation functions to modulate gene expression and associates with many key cellular processes and human diseases. (Anna, 2015) Therefore, people hope to identify epigenetic biomarkers and therapeutic targets to give early detection, diagnosis, prognosis, and prediction and identify methylation differences related to diseases. (Gao, 2018) Recently, abnormal DNA methylation is approved to be related to the pathogenesis of AD. (Jankowska, 2015)

Differential methylation (DM) analysis is an effective way to identify the modification of DNA methylation in brain regions through conducting statistical tests on CpG sites and identifying the ones associated with the disease. (Li, 2019) However, the brain has several distinct cell types with highly heterogeneous functions and various genomic profiles. To account for the cell type mixture, we conducted in-depth analysis of the DNA methylation of AD in brains using two newly developed statistical methods with hopes to detect novel biological signals and account for the cellular heterogeneity. We assumed there are four brain cell types which are neuron, astrocyte, oligo, microglia during our analysis. (Possel, n, d)

In recent years, the problem of solving cellular heterogeneity in DM and epigenome-wide association studies (EWAS) which can identify the association between epigenetic variation and a particular identifiable phenotype has attracted a lot of interest. (Rakyan, 2011) Several deconvolution methods for dealing with specific proportions of cell types in complex tissues have been proposed. Two deconvolution algorithms divided into "reference-free" and "reference-based" have been proposed, based on whether the use of DNA methylation reference profiles to conducting deconvolution. Although the reference-based method is considered to be more accurate and stable with given reference datasets, this design is laborious or expensive to collect and some solid tissues in which cell types may not be known. (Houseman, 2014) In addition, if the sample size of the reference data is small with limited clinical conditions, the reference-based method is unable to provide an accurate cell composition estimation with mixed tissues. (Li, 2019) Therefore, a RefFreeEWAS (EWAS using reference-free DNA Methylation Mixture Deconvolution) package was applied to infer cell-type-specific proportions and also compared with a newly developed statistical method TOAST (TOols for the Analysis of heterogeneouS Tissues) (Li, 2019)

After obtaining the estimated cell-type-specific proportions, cell-type specific differential methylation (csDM) or cell-type-specific differential expression(csDE) analysis can be also conducted using TOAST between patients with Alzheimer's and normal controls. TOAST improves reference-free cell composition estimation by cross-cell type differential analysis and provides great flexibility for detecting csDE/csDM by characterizing the data from mixed samples using the linear model in a rigorous statistical framework. The R package is available on GitHub (https://github.com/ziyili20/TOAST). The cell-type-specific proportions were conducted on the combination of AD patients and control groups. The csDM analysis is implemented of AD on four unknown types of brain cells in all samples. We also used the differential methylation calling without consideration of cell mixtures to compare the results using Bioconductor package Minfi. (Aryee, 2014) After that, we conducted the downstream analysis, including pathway/GO analysis and motif analysis using EnrichR to interpret the biological meaning of the results.(Kuleshov, 2016) As a follow-up, we hope to implement other methods to confirm my results and to detect specific brain cell types.

Methods

Description of subject and data

Our data set consists of the DNA methylation data of AD patients and controls which were generated on the samples collected from two prospective cohort studies at Rush University: The Religious Orders Study (ROS) and the Memory and Aging Project (MAP). (De Jager, 2014) These two cohort studies aimed to discover the difference of cognitive function in the aging brain. The dataset was obtained from De Jager et al. (2014) which studies a random selection of the older population with 734 subjects. The Infinium Human Methylation 450K BeadChip was used to measure the methylation profiles at 339,162 CpG sites and transformed into beta values. The final input data is beta value matrix with 339,162 rows (CpG sites) and 734 columns (subjects). The AD status for all subjects were determined by Braak stages that the subjects with Braak stage 0-3 were indicated as normal controls (368 subjects) and with Braak stage 4-6 were indicated as AD patients (366 subjects).

Proportions Estimation

Notations

Given DNA methylation profiles of ROS/MAP, R package RefFreeEWAS and TOAST were applied to solve for cell-type specific proportions in comparison. Since brain is complex tissue of cells derived from at least two cell types with potentially distinct DNA methylation profiles, reference-free deconvolution is the most suitable method to provide accurate cell composition estimation. Reference-free DNA methylation mixture deconvolution method assumes the decomposition with a variant of non-negative matrix factorization: $Y = M\Omega^T$ that Y, a m by n matrix that rows represent features and columns represent samples, denotes the observed DNA methylation. M is the Cell-type specific DNA methylation profiles from the mixed tissue which is the ROS/MAP data in this analysis. It consists of an unknown $m \times K$ matrix indicating m CpG-specific methylation for each of K cell types, i.e. four in this application. Ω denotes the unknown specimen-specific cell-type proportions consisted of unknown $n \times K$ matrix indicating the cell-type proportions for each subject. Ω is the interested variable in this analysis which entries lie between 0 and 1 and its rows sum to less than one. High values of the row-variance denoted as

$$Var(Y_{i\cdot}) = \Sigma_j \left(Y_{ij} - \overline{Y_{i\cdot}}\right)^2$$

which include the influences of within-cell type variances, cross-cell type variances and variation from the mixing proportions.

Model

TOAST statistical method was used to improve reference-free cell composition estimation by cross-cell type differential analysis. Based on the findings from recent published studies, good deconvolution is represented as low within-cell type variation but high cross-cell types variation. Therefore, TOAST introduces a method focus on data from mixed sample for cross-cell type differential analysis. First of all, the observe data are assumed as Y_m=$[Y_{m1}, Y_{m2}, \dots, Y_{mn}]^T, m = 1, \dots, m$. The proportions of the samples are following denoted as θ_s=$[\theta_{s1}, \theta_{s2}, \dots, Y_{sk}]^T$.

TOAST method conducted a linear model:

$$E(Y_m) = V\beta_m$$

Where

$$V = \begin{bmatrix} \theta_{11} & \theta_{12} & \cdots & \theta_{1K} \\ \theta_{21} & \theta_{22} & \cdots & \theta_{2K} \\ \vdots & \vdots & \vdots & \vdots \\ \theta_{N1} & \theta_{N2} & \dots & \theta_{NK} \end{bmatrix},$$

$$\beta_m = [\,\mu_{m1}\ \mu_{m2}\ \dots\ \mu_{mk}]^T.$$

μ_{mk} represents the mean level for the *m-th* feature in the *k-th* cell type.

This model is used to test the difference between cell type k versus other cell types under the hypothesis test that $H_0\colon \mu_{mk} - \frac{1}{K-1}\sum_{i \neq k} \mu_{mk} = 0,$ *k=1, … ,K* .which indicates the significant test results of features means the cell-type specific features. With this method, TOAST designs an iterative algorithm to improve feature selection in reference-free deconvolution.

Compare with reference-free DNA methylation mixture deconvolution, TOAST design a new algorithm for improve feature selection in RF deconvolution. M_0 is denoted as a list of initial features, choosing the top variable features from traditional methods. Y_{M_0} a sub-matrix of Y contains the corresponding rows of Y for feature in M_0. Then Y_{M_0} conducted RF deconvolution to estimate mixture proportions. The improved algorithm has been proved the improvements of proportion estimations that the RF deconvolution performs better with more accurate estimated results.

Cell-type specific DM (csDM) test using TOAST

With estimated mixture proportions of AD patients and controls together, we detect four cell-types specific DM signals using TOAST package. Let Z_i is a scaler or vector representing the subject's covariates (such as disease status, age, gender, etc.), TOAST assumes the pure cell-type profile X_{ik} for sample i and cell type k satisfies:

$$\mathrm{E}[\mathrm{X}_{ik}] = \mu_k + Z_i^T \beta_k$$

Where μ_k is the baseline DNA methylation profile of cell type *k,* and β_k are coefficients associated with covariate effect(s). Following the previous notation, we denote the estimated mixture proportions by W_{ik}. Although the cell-type specific pure profile X_{ik} is not directly observed, we can observe mixed signal Y_i for subject *i,* is weighted average of X_{ik} . Given the proportions of W_i, a linear model shows

$$\mathrm{E}[\mathrm{Y}_i; \theta_i] = \sum_k \theta_{ik}\, \mathrm{E}[\mathrm{X}_{ik}] = \sum_k (\theta_{ik}\, \mu_k + \theta_{ik} Z_i^T \beta_k).$$

In this model, Y_i, θ_{ik} and Z_i are observed (or estimated), and μ_k and β_k are unknown parameters to be solved. Since the observe data *Y* from a total number of *N* samples, linear model can be denoted as matrix form $Y = [\mathrm{Y}_1, \mathrm{Y}_2 \ldots \mathrm{Y}_\mathrm{N}]^\mathrm{T}$ and defined in a linear model: $E[Y]=W\beta$ where the design matrix W and the covariate vector β as

$$W = \begin{bmatrix} \theta_{11} & \theta_{12} & \cdots & \theta_{1K} & \theta_{11} \cdot Z_1^T & \theta_{12} \cdot Z_1^T & \cdots & \theta_{1K} \cdot Z_1^T \\ \theta_{21} & \theta_{22} & \cdots & \theta_{2K} & \theta_{21} \cdot Z_2^T & \theta_{22} \cdot Z_2^T & \cdots & \theta_{2K} \cdot Z_2^T \\ \vdots & \vdots & & \vdots & \vdots & \vdots & & \vdots \\ \theta_{N1} & \theta_{N1} & \cdots & \theta_{NK} & \theta_{N1} \cdot Z_N^T & \theta_{N1} \cdot Z_N^T & \cdots & \theta_{NK} \cdot Z_N^T \end{bmatrix}$$

$$\beta = \begin{bmatrix} \mu_1 \\ \mu_2 \\ \vdots \\ \mu_K \\ , \\ \beta_1 \\ \beta_2 \\ \vdots \\ \beta_K \end{bmatrix}$$

TOAST detects cell-type specific differential signals through testing linear combinations of the regression coefficients with estimated parameters. The ROS/MAP DNA methylation data were used to solve mixture proportion as inputs for TOAST and to detect four cell type-specific DM sites between AD patients and controls together. We explore correlations between proportions of each cell types and perform one-way ANOVA to evaluate the significance of the AD status in each cell type proportions. Five null hypotheses generated as : $\beta_1 = 0$ for cell type one-specific signals, $\beta_2 = 0$ for cell type two-specific signals, $\beta_3 = 0$ for cell type three-specific signals, $\beta_4 = 0$ for cell type four-specific signals and $\beta_1 = \beta_2 = \beta_3 = \beta_4 = 0$ for the joint signals from four cell types. Permutation test was conducted to test the differential expression of the joint signals analysis with deriving empirical p-values since we interested in the sharp null hypothesis that no signals difference between four cell types. Statistical results are corrected by multiple-testing by Benjamini-Hochberg False Discovery Rate (FDR). CpG sites with FDR less than 0.2 are considered as cell-type specific DM.

Then a flexible and comprehensive Bioconductor package for the analysis of Infinium DNA methylation microarrays called Minfi was used to detect DM of AD patients and control groups without considering the cell mixture. Each differentially methylated CpG (DMC) is matched with associated gene using Illumina Infinium methylation 450k methylation microarrays of human genome version 19 (hg19) using R/Cran package *IlluminaHumanMethylation450kanno.ilmn12.hg19*.

Pathway and Gene Ontologies Analysis

We use identified DMCs of Cell Type 3 in TOAST to conduct pathway analysis and gene ontology (GO) analyses using genes enrichment analysis tool *EnrichR* (McDermott et al. 2016) web server (http://amp.pharm.mssm.edu/Enrichr/). We set maximum entry as 1000 genes and use hg19 as reference gene sets libraries. The table generated consists of three enrichment scores to represent the significance of overlapping input DMCs list and gene sets libraries. The first one is the p-value which is computed from the Fisher exact test that is a proportion test assuming a binomial distribution and the probability of each gene within gene sets are independent. The second one is based on the z-score to assess the deviation from the expected rank derived from the Fisher exact test for many random gene sets. The third one is combined score, what we based on in this analysis, which is computed through taking log of the p-value from the Fisher exact test and multiply to the z-score from the expected rank. Kyoto Encyclopedia of Genes and Genomes (KEGG) cell signaling pathway database in 2019 was conducted to provide pathway analysis. For the GO Ontological analysis, we focus on the GO biological process 2018.

Results

Data Description

DNA methylation data of ROS/MAP include 339,162 CpGs for 734 subjects with 366 AD patients and 388 control groups. The reference-free deconvolution processes with AD patients and Control groups together.

Estimated cell Proportions

Top 10000 CpGs based on the coefficient of variation was selected as the most variant features among 339,162 CpGs. The cell-type-specific proportions for four unknown cell types in brains were measured applying reference-free DNA methylation mixture deconvolution (R/CRAN package RefFreeEWAS) and newly developed TOAST statistical method.

The boxplots of cell proportion for AD patients and control patients were generated under two methods with descriptive tables.

Reference-free DNA methylation mixture deconvolution (R/CRAN package RefFreeEWAS)

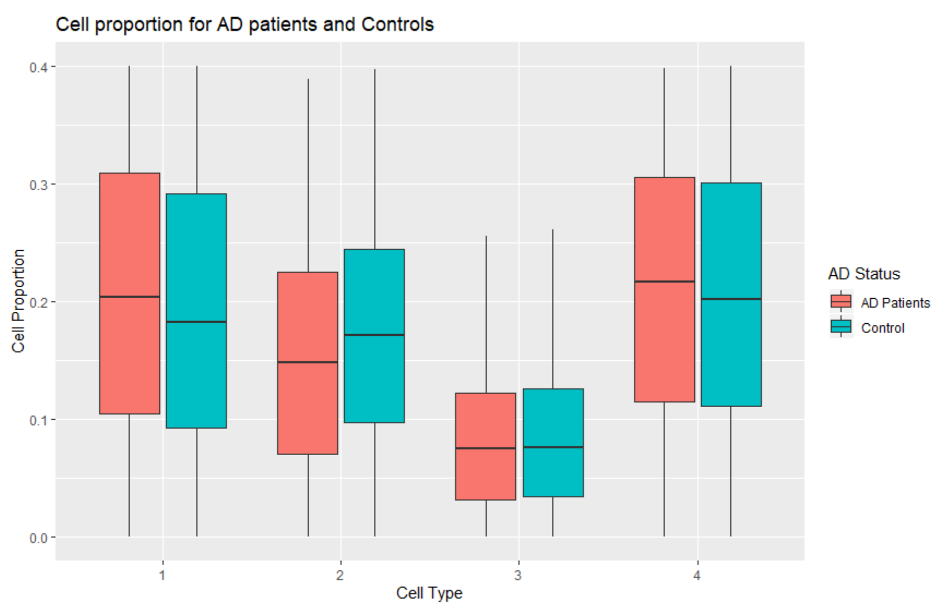

Figure 1: Cell Proportion for AD patients and Controls groups generated from Reference-free DNA methylation mixture deconvolution

Table1: The cell type, proportion and P-values of AD patients and Control groups by using Reference-free DNA methylation mixture deconvolution (R/CRAN package RefFreeEWAS) when K=4

Cell Type	AD Patients (Proportion ± SE)	Control groups (Proportion ± SE)	P-values
Cell Type 1	0.3117(0.0412)	0.2804(0.0388)	0.0345*
Cell Type 2	0.2982(0.0665)	0.3451(0.0689)	0.0148*
Cell Type 3	0.0809(0.0049)	0.0803(0.0042)	0.904
Cell Type 4	0.2934(0.0353)	0.2767(0.0347)	0.228

1. Abbreviations: AD=Alzheimer's Disease, SE= standard error.
2. A p-value less than 0.05 (typically ≤ 0.05) is statistically significant and labeled with *.
3. K means the number of the cell types.

Based on the results shown on the Reference-free DNA methylation mixture deconvolution, the average proportion of the cell type 3 is 0.0809 (SD=0.0049) for AD patients and 0.0803 (SD=0.0042) for control groups, that expresses the lowest average proportion among four cell types. One-way ANOVA test was applied to compare the proportions of four cell types among AD patient and control groups. Considering the p-values of each cell type, cell types 1 and 2 show the p-value equals to 0.0345 and 0.0148, respectively less than 0.05. Therefore, indicating cell type 1 and cell type 2, there are significant differences in cell-type proportions between AD patients and control groups. The result shows non-significant p-values with 0.904 and 0.228 of cell types 3 and 4 respectively, indicating that there's no obvious cell proportion difference between AD patients and control groups.

TOAST Statistical method

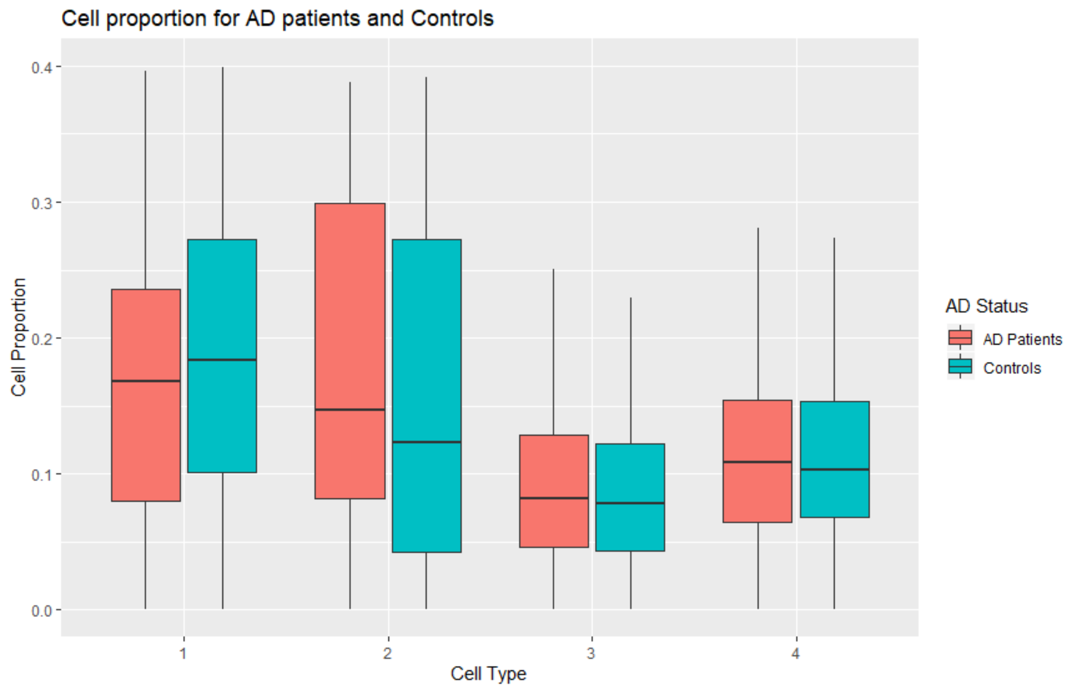

Figure 2: Cell Proportion for AD patients and Controls groups generated from TOAST statistical method

Table2: The cell type, proportion and P-values of AD patients and Control groups by using TOAST statistical method when K=4

Cell Type	AD Patients (Proportion ± SE)	Control groups (Proportion ± SE)	P-values
Cell Type 1	0.3058(0.0634)	0.3469(0.0650)	0.0284*
Cell Type 2	0.4752(0.0581)	0.4407(0.0612)	0.0557
Cell Type 3	0.0882(0.0043)	0.0830(0.0035)	0.264
Cell Type 4	0.1114(0.0054)	0.1081(0.0044)	0.531

1. Abbreviations: AD=Alzheimer's Disease, SE= standard error.
2. A p-value less than 0.05 (typically ≤ 0.05) is statistically significant and labeled with *.
3. K means the number of the cell types.

Based on the results shown on the statistical method TOAST, the average proportion of the cell type 3 is 0.0882 (SD=0.0043) for AD patients and 0.0803 (SD=0.0035) for control groups, that expresses the lowest average proportion among four cell types. One-way ANOVA test was applied to compare the proportions of four cell types among AD patient and control groups. Considering the p-values of each cell type, cell type 1 show the p-value equals to 0. 0284 less than 0.05 which means there is a significant difference in cell type proportion between AD patients and control groups. The result shows non-significant p-values with 0. 0557, 0. 264 and 0.531of cell types 2, 3 and 4 respectively, indicating that there's no obvious cell proportion difference between AD patients and control groups.

Compare the RefFreeEWAS method with TOAST, it can be shown that they both express cell type 3 and have the lowest average proportion among four cell types. Both of them given the result that cell type 1 has a significant difference in cell proportion between AD patients and control groups. While based on the boxplots, TOAST generated lower average proportion on cell type 4 than the results in RefFreeEWAS and TOAST also given an obvious higher average proportion on cell type 2 than those of in RefFreeEWAS method.

A correlation plot was generated to explore the correlation between each cell type proportions. Based on the plot, cell types 1 and 2 are highly negatively correlated to each other with correlation coefficients -0.84 from RefFreeEWAS and -0.94 from TOAST. From a recently published paper conducted that the neuron-enriched gene expression patterns are regionally anti-correlated with oligodendrocyte-enriched patterns. (Tan, 2013) Therefore, the opposite correlation between two cell types might be helpful in further analysis to find the specific brain cell types for cell types 1 and 2. In figure 3 and 4, the histograms indicate the distribution of each cell type proportion shown on the diagonal. On the bottom of the diagonal, the bivariate scatter plots with a fitted line are displayed shown the correlation between cell types.

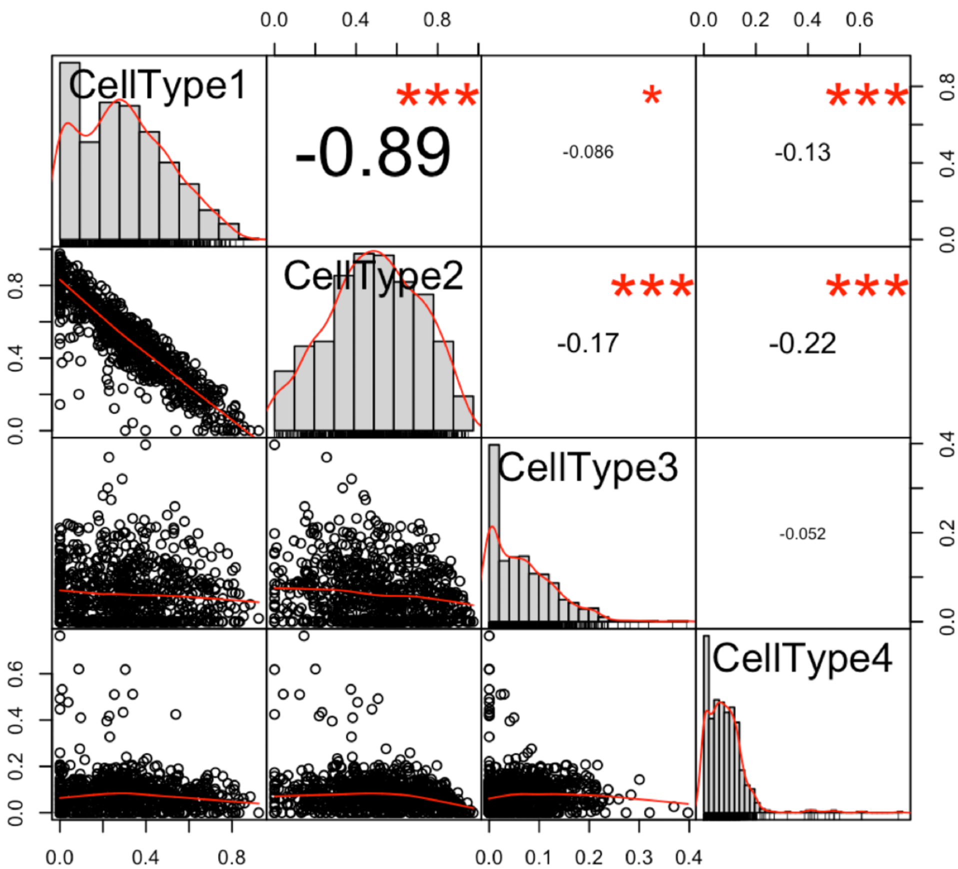

Figure 3: Correlation between each cell type proportion generated from RefFreeEWAS

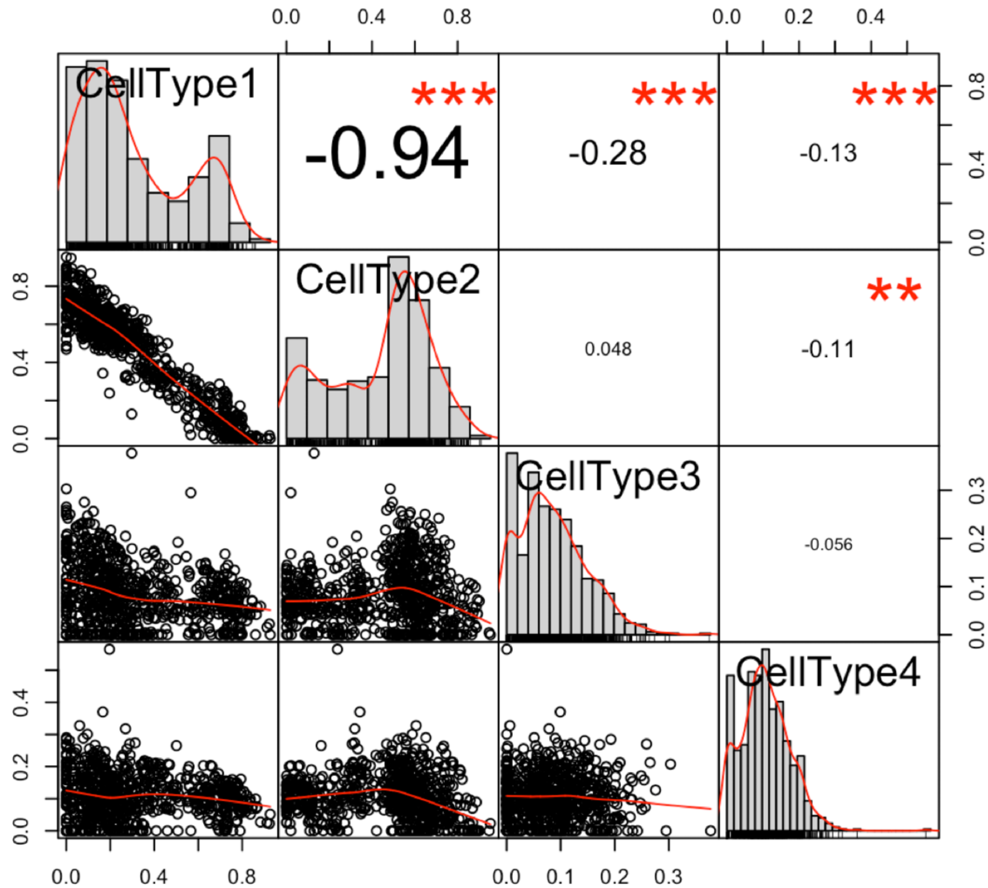

Figure 4: Correlation between each cell type proportion generated from TOAST

DMCs of four unknown cell types in brain, and joint signal analysis

For 339,162 input CpGs, we apply both RefFreeEWAS and TOAST methods to look for cell type-specific DM in AD patients and control groups.

Table 3: The number of DMCs of AD patients and control groups under each cell types and joint signal analysis generated by Reference-free DNA methylation mixture deconvolution (R/CRAN package RefFreeEWAS) and TOAST statistical method when K=4

	TOAST	RefFreeEWAS
Cell Types	**FDR (<0.2)**	**FDR (<0.2)**
Cell Type 1	1	0
Cell Type 2	1	0
Cell Type 3	501	1938
Cell Type 4	0	162
Joint Analysis	0	0

The results show that both TOAST and RefFreeEWAS methods generated the largest number of DMCs at cell type 3. RefFreeEWAS methods generate 1938 DMCs at cell type 3 and TOAST generate 501 DMCs which is less than the RefFreeEWAS method did. (Appendix 1&2) While the overlap DMCs at cell type 3 between the two methods is only 75 DMCs. (Appendix 3) There are no significant DMCs in the joint signal test ($\beta_1 = \beta_2 = \beta_3 = \beta_4 = 0$ for testing difference between AD and control from each cell types).

We also conduct DM calling without consideration of cell mixtures and found 6755 DMCs using Minfi. (Appendix 4) Compare DMCs generated from RefFreeEWAS method with Minfi, there are 121 overlapping DMCs on cell type 3 and 3 overlapping DMCs on cell type 4. (Appendix 5&6) Compare DMCs generated from TOAST with Minfi, there are 1 overlapping DMCs on cell type 2 and 18 overlapping DMCs on cell type 3. (Appendix 7&8)

Comparison with existing results

We compare results from TOAST of the cell type 3 with those from a published paper on ROS/MAP DNA methylation data (De Jager et al. 2014). For De Jager et al. study, the paper reported 71 associated CpGs from 415,848 input CpGs from ROS/MAP of 708 subjects. Out of their 71 identified DMCs, 64 are included in our input data, and among them, only 1 DMCs can match our results which is cg21644387 from De Jager.

Table 4: Overlap DMCs between DMCs generated from TOAST on cell type 3 and DMCs from De Jager.

Target ID	Chr.	Position	Gene
cg21644387	2	663024	TMEM18

Compare results from RefFreeEWAS of the cell type 3 with De Jager, there are 5 overlapping DMCs which are cg04157161, cg22962123, cg02308560, cg21806242 and cg15645660 with the following information from De Jager. (Table 7)

Table 5: Overlap DMCs between DMCs generated from RefFreeEWAS method on cell type 3 and DMCs from De Jager.

Target ID	Chr.	Position	Gene
cg04157161	17	7906847	GUCY2D, ALOX15B
cg22962123	7	27153605	HOXA1, HOTAIRM1, HOXA2, AK291164, HOXA3, AK311383, BC035889, HOXA4, LOC100133311, HOXA5, HOXA6, DQ655986, HOXA7, HOXA9, HOXA10-HOXA9
cg02308560	19	1071176	CNN2, ABCA7, HMHA1,

			POLR2E, GPX4, SBNO2
cg21806242	11	72532891	STARD10, MIR4692, ARAP1, ATG16L2, FCHSD2
cg15645660	1	55247356	HEATR8-TTC4, TTC4, PARS2, TTC22, C1orf177

Pathway Analysis and GO Ontologies Analysis

Since the cell type 3 given the enrichment of the DMCs in TOAST, we conduct KEGG pathway analysis for DMCs of the cell type 3 signals and GO Ontologies analysis using *EnrichR*. From the KEGG pathway, the enriched terms sorted by combined score ranking while there is no significant association between AD pathway and DMCs generated on cell type 3 (rank=187, Combined scores=0.49).

We conducted the GO biological process 2018 analysis on the enrichment of the DMCs on cell type 3 generated by the TOAST method. The biological process regulation of adiponectin secretion has the highest combined score.

Table 6: GO biological process 2018 of the corresponding genes

Name	P-value	Adjusted p-value	Odds Ratio	Combined Score	Genes
regulation of adiponectin secretion (GO:0070163)	0.00	0.20	27.47	249.24	C1QTNF3; RAB11FIP1; IL1B
positive regulation of heterotypic cell-cell adhesion (GO:0034116)	0.00	0.16	19.98	206.67	IL10; IL1B; GCNT2; CD44
negative regulation of membrane protein ectodomain proteolysis (GO:0051045)	0.00	0.14	23.55	200.78	IL10; TIMP2; TIMP3
adenylate cyclase-inhibiting G-protein coupled glutamate receptor signaling pathway (GO:0007196)	0.00	1.00	18.32	98.09	GRM4; GRM6
regulation of heterotypic cell-cell adhesion (GO:0034114)	0.00	0.21	11.57	92.49	IL10; IL1B; GCNT2; CD44

negative regulation of chemokine production (GO:0032682)	0.00	0.63	12.68	82.51	IL10; C1QTNF3; ELANE
regulation of monocyte chemotactic protein-1 production (GO:0071637)	0.01	1.00	15.70	78.99	C1QTNF3; IL1B
brown fat cell differentiation (GO:0050873)	0.01	1.00	15.70	78.99	PRDM16; METRNL
leukocyte aggregation (GO:0070486)	0.01	1.00	15.70	78.99	IL1B; CD44
regulation of calcidiol 1-monooxygenase activity (GO:0060558)	0.01	1.00	15.70	78.99	CYP27B1; IL1B

We followed up the GO Cellular Component 2018 analysis on the enrichment of the DMCs on cell type 3 generated by the TOAST method. Neurotransmitter receptor complex is top one cellular component with the highest combined score.

Table 7: GO Cellular Component 2018 of the corresponding genes

Name	**P-value**	**Adjusted p-value**	**Odds Ratio**	**Combined Score**	**Genes**
neurotransmitter receptor complex (GO:0098878)	0.00	0.52	13.74	92.80	DLG2; HTR3A; SHANK2
spindle pole centrosome (GO:0031616)	0.02	0.73	9.99	41.13	TBCCD1; DLGAP5
ionotropic glutamate receptor complex (GO:0008328)	0.01	0.80	5.64	29.45	GRIN2A; DLG2; CACNG3; SHANK2
MHC class II protein complex (GO:0042613)	0.03	0.89	7.85	28.64	HLA-DMB; HLA-DRA
contractile fiber (GO:0043292)	0.01	0.73	6.34	28.37	OBSCN; TWF2; LMOD3
myofibril (GO:0030016)	0.01	0.71	6.11	26.68	OBSCN; TWF2; LMOD3
specific granule lumen (GO:0035580)	0.01	0.60	4.43	23.15	GHDC; CTSZ; T IMP2; OLFM4; ELANE
mRNA cleavage and polyadenylation specificity factor complex (GO:0005847)	0.04	1.00	6.46	21.22	CPSF3; ZC3H3
platelet alpha granule membrane (GO:0031092)	0.04	1.00	6.46	21.22	ITGA2B; CD36
IPAF inflammasome complex (GO:0072557)	0.10	1.00	9.16	20.69	NLRC4

Discussion

There are certain limitations in this project such as the datasets don't show a significant difference in proportion between AD patients and control groups. The reason why the lowest proportion of cell type 3 but with the highest DMCs from the results of both RefFreeEWAS and TOAST is still unclear and needs further analysis. From the correlation plots, cell type 1 and cell type 2 shown a highly negative correlation and the possible brain cells of these two cell types would be neuron and oligodendrocyte from the former study. To detect specific brain cell types in this project, biomarker genes will be needed to classify the cells. Based on the limited number of DMCs generated by RefFreeEWAS and TOAST methods, the DNA methylation in the brain shows attenuation with a possible reason that the DNA methylation may be mediated by unknown cell types leading inaccurate results. We assumed K=4 that four types of brain cells in the application that may overestimate the heterogeneity of the brain cells that the glia differentiated from neuron while in the glia family, the astrocyte does not highly differentiate from other cell types such as oligo and microglia. The reference-free approach accounts for more cell types than reference-based approaches which focus on csDM analyses on two brain cell neuron and glia. RefFreeEWAS detected more DMCs than TOAST with the potential reason that TOAST focus more on data from the mixed sample for cross-cell type differential analysis.

After pathway analysis using the enrichment DMCs in cell type 3 generated from the TOAST method, we clarify the enrichment of disease-related pathways and cell processes in corresponding gene groups. However, Alzheimer's disease is not an enrichment term with a very small combined score. GO enrichment analysis provides the biological processes information and also the cellular component. The genes C1QTNF3, RAB11FIP1 and IL1B are ranked top on the regulation of adiponectin secretion biological processes in GO enrichment analysis. And the genes DLG2, HTR3A, and SHANK2 indicate the neurotransmitter receptor complex in the cellular component during the analysis. This project provides the study on epigenetic modification in AD by estimating the cell composition and conducting cell-type-specific DM analysis. Since brain tissue has many different cell types, which exhibit highly heterogeneous functions, it is necessary to identify changes in specific cell types involved in the pathogenesis and progression of the disease at different levels and roles.

Conclusion

According to our study, cell-type-specific DM analysis provides a new version to study epigenetic modification in AD through conducting a statistical test on CpG sites to identify potential disease. Several natural extensions would be looking forward to identifying epigenetic biomarkers and therapeutic targets to validate new treatments on AD.

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Appendix

Appendix 1: Top 100 DMCs defined at lowest 100 FDR for Cell Type 3 generated by RefFreeEWAS method

CpGs	beta	beta_var	mu	effect_size	f_statistics	p_value	fdr
cg05929755	-0.1463018	5.83E-04	0.77799395	-0.2075665	36.6974073	2.21E-09	7.51E-04
cg22777249	-0.1683412	9.14E-04	0.75547722	-0.2507665	30.9998691	3.63E-08	0.00435673
cg16186938	-0.213742	0.00151205	0.37110844	-0.8089015	30.2143507	5.36E-08	0.00435673
cg21921016	-0.1085983	3.94E-04	0.78911051	-0.1477906	29.9479156	6.12E-08	0.00435673
cg07623567	-0.1154369	4.56E-04	0.8077396	-0.1539115	29.2336993	8.71E-08	0.00435673
cg12616935	-0.0937486	3.01E-04	0.71285711	-0.1407673	29.1947782	8.88E-08	0.00435673
cg01231381	-0.1027783	3.62E-04	0.8567129	-0.1276236	29.1701242	8.99E-08	0.00435673
cg20240091	-0.073896	1.97E-04	0.83271862	-0.092861	27.7084207	1.86E-07	0.00695393
cg16350446	-0.1112975	4.53E-04	0.79857192	-0.1498102	27.3349511	2.24E-07	0.00695393
cg14138235	-0.0840136	2.59E-04	0.89465147	-0.098533	27.2003983	2.39E-07	0.00695393
cg17706411	-0.0793617	0.0002319	0.83631175	-0.0996217	27.1594076	2.44E-07	0.00695393
cg27338202	-0.0879929	2.85E-04	0.85163283	-0.1089511	27.1462418	2.46E-07	0.00695393
cg19863740	-0.1332481	6.73E-04	0.77793502	-0.1873275	26.3776906	3.61E-07	0.00929578
cg11380128	-0.103034	4.04E-04	0.65818401	-0.1698362	26.2558922	3.84E-07	0.00929578
cg12002745	-0.1003094	3.88E-04	0.77142773	-0.1390727	25.965011	4.44E-07	0.00956458
cg17471062	-0.1099461	4.66E-04	0.63309428	-0.1901783	25.9317561	4.51E-07	0.00956458
cg02258534	-0.0912237	3.25E-04	0.83744834	-0.1152052	25.6178767	5.28E-07	0.01053267
cg18720803	-0.0989348	3.84E-04	0.73730177	-0.1438353	25.4824793	5.65E-07	0.01064516
cg07092805	-0.0976773	3.87E-04	0.8356627	-0.1241412	24.633624	8.65E-07	0.0153733
cg26957187	-0.1317257	7.08E-04	0.74537519	-0.1938533	24.4969114	9.26E-07	0.0153733
cg25095518	-0.093442	3.58E-04	0.7399703	-0.1347884	24.3612805	9.91E-07	0.0153733
cg14016236	-0.1608819	0.00106591	0.7448123	-0.2421566	24.2826106	1.03E-06	0.0153733
cg09809672	-0.0940245	3.64E-04	0.91907629	-0.1078183	24.2608914	1.04E-06	0.0153733
cg17636309	0.12461323	6.45E-04	0.72802855	0.15767137	24.0930647	1.13E-06	0.016029
cg17785515	-0.0692483	2.00E-04	0.81606307	-0.0886164	23.9625381	1.21E-06	0.01643133
cg26889879	-0.0765394	2.45E-04	0.85658299	-0.0935332	23.8778999	1.26E-06	0.01648627
cg04157161	0.24202467	0.00246504	0.03717216	1.53001509	23.7626975	1.34E-06	0.01682272
cg23997365	-0.0625651	1.66E-04	0.85212281	-0.0762208	23.6214189	1.44E-06	0.017417
cg20924136	-0.0865565	3.19E-04	0.81805139	-0.1117185	23.4828317	1.54E-06	0.01744912
cg01234251	-0.115262	5.66E-04	0.85786424	-0.1440356	23.4806914	1.54E-06	0.01744912
cg00280235	-0.1920428	0.00158321	0.46057391	-0.5267905	23.2947114	1.69E-06	0.01854417
cg27658026	-0.0886959	3.39E-04	0.80086561	-0.1172423	23.175281	1.80E-06	0.01888543
cg26336265	-0.0771545	2.58E-04	0.89345351	-0.0902522	23.0555158	1.91E-06	0.01888543
cg09796170	-0.102284	4.55E-04	0.83845728	-0.1299149	22.9970154	1.97E-06	0.01888543

cg06942814	0.2548858	0.00283204	-0.0060037	2.09887553	22.9399271	2.03E-06	0.01888543
cg09889997	-0.0972064	4.12E-04	0.90123528	-0.1140075	22.9253505	2.04E-06	0.01888543
cg21543346	-0.0928429	3.76E-04	0.89249906	-0.1097333	22.9074773	2.06E-06	0.01888543
cg03684062	-0.1690763	0.00125309	0.77196669	-0.2459547	22.8131111	2.16E-06	0.01928478
cg11772020	-0.1051437	4.87E-04	0.65056498	-0.1758277	22.7130612	2.27E-06	0.01976307
cg02097616	-0.1301155	7.48E-04	0.80264871	-0.176406	22.6348197	2.36E-06	0.01992696
cg19856263	-0.0662377	1.94E-04	0.86857371	-0.0792835	22.5975988	2.41E-06	0.01992696
cg13266773	-0.1356056	8.20E-04	0.7191861	-0.2081809	22.4356058	2.61E-06	0.02069199
cg18900271	-0.1140559	5.81E-04	0.85000899	-0.1438319	22.3877965	2.68E-06	0.02069199
cg11328127	-0.0990378	4.39E-04	0.78862601	-0.1339966	22.3637062	2.71E-06	0.02069199
cg02879960	-0.1169589	6.12E-04	0.74531919	-0.1702855	22.3386282	2.75E-06	0.02069199
cg25228737	-0.0757416	2.59E-04	0.89559362	-0.0883055	22.1346781	3.04E-06	0.02155624
cg04781580	-0.0937265	3.97E-04	0.86012097	-0.1152482	22.1196278	3.07E-06	0.02155624
cg02965078	-0.1187415	6.39E-04	0.73736011	-0.1751377	22.0534881	3.17E-06	0.02155624
cg24411075	-0.0744754	2.52E-04	0.82401469	-0.0946589	22.0012097	3.26E-06	0.02155624
cg11794384	-0.097315	4.30E-04	0.78385609	-0.1323656	22.001143	3.26E-06	0.02155624
cg25313447	-0.101233	4.67E-04	0.84383348	-0.1276233	21.9607039	3.32E-06	0.02155624
cg15911153	0.21664957	0.00213983	0.0870901	1.1086646	21.9349308	3.37E-06	0.02155624
cg21520613	-0.0717477	2.35E-04	0.88128554	-0.0848672	21.9339346	3.37E-06	0.02155624
cg14050954	-0.100813	4.65E-04	0.77855771	-0.1384506	21.8795589	3.46E-06	0.021747
cg06389019	-0.1326777	8.10E-04	0.65472978	-0.2254923	21.7427362	3.71E-06	0.022882
cg08747557	-0.094765	4.14E-04	0.78643601	-0.1282248	21.6702361	3.85E-06	0.02331331
cg10548978	-0.085672	3.40E-04	0.84555526	-0.1067272	21.6089926	3.97E-06	0.02362551
cg24964103	-0.0801999	2.99E-04	0.84239764	-0.0999627	21.4992363	4.20E-06	0.02454503
cg22998020	-0.0889099	3.71E-04	0.82604423	-0.1137553	21.3141085	4.61E-06	0.02650135
cg14878852	-0.0705091	2.35E-04	0.80963041	-0.0910528	21.1686919	4.96E-06	0.02779146
cg24499605	-0.106393	5.35E-04	0.82685647	-0.137519	21.1497038	5.01E-06	0.02779146
cg16860686	-0.1286245	7.84E-04	0.46605834	-0.3201637	21.1034151	5.13E-06	0.02779146
cg01951274	-0.088468	3.71E-04	0.76570666	-0.1226215	21.0699172	5.22E-06	0.02779146
cg00078456	-0.0901124	3.86E-04	0.87595629	-0.1084516	21.0598723	5.24E-06	0.02779146
cg01816936	-0.1297733	8.03E-04	0.86421075	-0.1623538	20.9812312	5.46E-06	0.0284774
cg15048480	-0.060937	1.77E-04	0.87473948	-0.072177	20.9242161	5.62E-06	0.02886897
cg07060794	-0.1037478	5.16E-04	0.83067158	-0.1332153	20.8500394	5.83E-06	0.02952884
cg25693349	-0.0658868	2.09E-04	0.85019084	-0.0806204	20.793941	6.00E-06	0.02993485
cg07814707	-0.0860839	3.57E-04	0.77002998	-0.1184117	20.7581184	6.11E-06	0.02999699
cg14945086	-0.1813645	0.00158653	0.75094858	-0.2746839	20.7327498	6.19E-06	0.02999699
cg07251887	-0.1313929	8.35E-04	0.76835698	-0.1869934	20.6782353	6.36E-06	0.02999876
cg08530760	-0.0615479	1.83E-04	0.86390849	-0.0738752	20.6771473	6.37E-06	0.02999876
cg19403909	-0.165252	0.00133101	0.33427452	-0.656678	20.5169466	6.91E-06	0.03209585

cg00422624	-0.0702428	2.41E-04	0.89200969	-0.0819743	20.4893159	7.01E-06	0.03210971
cg12036735	-0.099416	4.85E-04	0.81785584	-0.129423	20.3765929	7.42E-06	0.03354933
cg03557950	-0.0642252	2.03E-04	0.84731484	-0.0787844	20.2900712	7.75E-06	0.03417814
cg21829239	-0.092415	4.22E-04	0.78384405	-0.1252853	20.2534455	7.90E-06	0.03417814
cg07243366	-0.140643	9.77E-04	0.66342043	-0.2371324	20.2432811	7.94E-06	0.03417814
cg19048532	0.23469166	0.00272438	0.08354948	1.16822869	20.2175084	8.04E-06	0.03417814
cg03409108	-0.0905196	4.05E-04	0.67991487	-0.142628	20.2131211	8.06E-06	0.03417814
cg25143652	-0.1177383	6.87E-04	0.75506709	-0.1691162	20.1742019	8.22E-06	0.03434958
cg15031685	-0.0867616	3.74E-04	0.76499476	-0.1202327	20.1199561	8.45E-06	0.03434958
cg03463994	-0.1073418	5.73E-04	0.81633216	-0.1407463	20.1035768	8.52E-06	0.03434958
cg15596913	-0.0558203	1.55E-04	0.83417621	-0.0692331	20.0892024	8.59E-06	0.03434958
cg24254196	-0.0826777	3.40E-04	0.8756159	-0.099101	20.0840945	8.61E-06	0.03434958
cg02555923	-0.122869	7.54E-04	0.81366286	-0.16334	20.0349084	8.83E-06	0.0348105
cg05106502	-0.0838506	3.54E-04	0.80450516	-0.1099564	19.8547876	9.67E-06	0.03771424
cg25844471	-0.1527387	0.00118073	0.83468357	-0.2014187	19.7582724	1.02E-05	0.03780236
cg18885125	-0.0747756	2.83E-04	0.80838025	-0.0969862	19.7545217	1.02E-05	0.03780236
cg05862438	-0.0789603	3.16E-04	0.81522888	-0.1017859	19.7242848	1.03E-05	0.03780236
cg07059469	-0.1094629	6.08E-04	0.66784567	-0.1785359	19.722045	1.04E-05	0.03780236
cg19607229	-0.0865507	3.81E-04	0.85432351	-0.1067146	19.6825299	1.06E-05	0.03780236
cg06758644	-0.0996613	5.05E-04	0.77921871	-0.1366369	19.6630648	1.07E-05	0.03780236
cg03186440	-0.1069414	5.82E-04	0.67787213	-0.1712702	19.6476878	1.08E-05	0.03780236
cg06011086	-0.1004126	5.13E-04	0.77634763	-0.1382824	19.6353605	1.08E-05	0.03780236
cg15829665	-0.1032676	5.44E-04	0.83499461	-0.1318264	19.5952046	1.10E-05	0.03780236
cg11525052	-0.1009547	5.20E-04	0.84023541	-0.1278299	19.5937223	1.11E-05	0.03780236
cg16942327	-0.0864429	3.82E-04	0.80807729	-0.1130185	19.5731407	1.12E-05	0.03780236
cg12787209	-0.0821845	3.45E-04	0.73388385	-0.118628	19.5670268	1.12E-05	0.03780236
cg05283597	-0.0871727	3.88E-04	0.80851503	-0.1139618	19.5643951	1.12E-05	0.03780236

Appendix 2: Top 100 DMCs defined at lowest 100 FDR for Cell Type 3 generated by RefFreeEWAS method

CpGs	beta	beta_var	mu	effect_size	f_statistics	p_value	fdr
cg07174627	0.02161298	1.76E-05	0.12567784	0.15835502	26.5992005	3.23E-07	0.07734656
cg04001090	0.02300005	2.04E-05	0.14902737	0.14327805	25.9101859	4.56E-07	0.07734656
cg11142333	0.02066705	1.79E-05	0.92714485	0.02204536	23.8153586	1.30E-06	0.11616838
cg11503710	0.02389047	2.53E-05	0.89828696	0.02624657	22.5890551	2.42E-06	0.11616838
cg07202981	0.04083999	7.48E-05	0.16170165	0.22424565	22.3120366	2.78E-06	0.11616838
cg09096234	0.02370158	2.52E-05	0.92313352	0.02534971	22.2493883	2.87E-06	0.11616838
cg23250910	0.02673554	3.23E-05	0.12404785	0.1945597	22.1198179	3.07E-06	0.11616838
cg16189217	0.03913417	6.94E-05	0.88294803	0.04336123	22.076217	3.13E-06	0.11616838
cg14453509	0.03270709	4.88E-05	0.25235836	0.12171804	21.9384163	3.36E-06	0.11616838
cg16658579	0.02435646	2.74E-05	0.91018902	0.02640647	21.6581818	3.87E-06	0.11616838
cg09431416	-0.0305494	4.34E-05	0.10889385	-0.326316	21.4979346	4.20E-06	0.11616838
cg26438665	0.05229318	1.28E-04	0.86887246	0.05842689	21.4396405	4.33E-06	0.11616838
cg16237262	0.02420046	2.74E-05	0.90195293	0.02647598	21.3826806	4.45E-06	0.11616838
cg20305610	0.02009378	1.96E-05	0.15414001	0.12238358	20.6153563	6.57E-06	0.15919705
cg03653601	0.03093112	4.69E-05	0.88845973	0.03421867	20.3838149	7.39E-06	0.15946096
cg20733250	0.02642014	3.44E-05	0.9098468	0.02862244	20.264845	7.85E-06	0.15946096
cg03469057	0.01749861	1.54E-05	0.93305076	0.01857996	19.8954663	9.48E-06	0.15946096
cg17007693	0.02221914	2.48E-05	0.92971611	0.02361664	19.878018	9.56E-06	0.15946096
cg06927343	0.02221993	2.49E-05	0.93094072	0.02358676	19.8498155	9.70E-06	0.15946096
cg00959883	0.03249926	5.33E-05	0.87160166	0.0366044	19.8038473	9.93E-06	0.15946096
cg17439660	0.0360619	6.57E-05	0.20471654	0.16189588	19.7903155	1.00E-05	0.15946096
cg03557950	0.0179819	1.65E-05	0.9327861	0.01909358	19.6463964	1.08E-05	0.15946096
cg01154241	0.02302545	2.71E-05	0.20406906	0.10680609	19.5836861	1.11E-05	0.15946096
cg02205181	-0.0424521	9.22E-05	0.19880653	-0.2390584	19.5526577	1.13E-05	0.15946096
cg13325346	-0.0462601	1.10E-04	0.30146092	-0.1662055	19.4362908	1.20E-05	0.16243827
cg00155609	0.01927032	1.92E-05	0.13005939	0.13794609	19.3351525	1.26E-05	0.16445789
cg09353251	0.02407713	3.02E-05	0.88329173	0.0268919	19.2029978	1.35E-05	0.16680417
cg10355997	0.01746462	1.59E-05	0.1098609	0.14726488	19.1621386	1.38E-05	0.16680417
cg05951763	0.02181333	2.49E-05	0.9144091	0.02357393	19.0798446	1.44E-05	0.16714407
cg04561804	-0.0616076	2.00E-04	0.46790452	-0.1409461	18.9784575	1.51E-05	0.16714407
cg19834711	0.01585707	1.33E-05	0.9172401	0.01713966	18.8801992	1.59E-05	0.16714407
cg02984092	0.044279	1.04E-04	0.8571299	0.05035886	18.8529115	1.61E-05	0.16714407
cg08140542	0.01566003	1.31E-05	0.91769204	0.01692021	18.7427486	1.71E-05	0.16714407
cg04452534	0.0407007	8.87E-05	0.86815792	0.0458079	18.6749274	1.77E-05	0.16714407
cg23380672	0.05497318	1.64E-04	0.53385241	0.09793222	18.4757293	1.96E-05	0.16714407

cg13775005	-0.0353465	6.77E-05	0.13875498	-0.2919224	18.4436912	1.99E-05	0.16714407
cg02389292	0.02186543	2.60E-05	0.15081347	0.13518361	18.3821974	2.05E-05	0.16714407
cg03408271	0.02905428	4.59E-05	0.28813628	0.09599533	18.3748598	2.06E-05	0.16714407
cg00557354	0.02536402	3.51E-05	0.13096729	0.17656906	18.3451156	2.09E-05	0.16714407
cg02147126	0.02508671	3.46E-05	0.90491603	0.02734368	18.2078377	2.24E-05	0.16714407
cg23188547	0.02019327	2.24E-05	0.12951967	0.14463404	18.1812675	2.27E-05	0.16714407
cg11974710	-0.0402925	8.95E-05	0.23686039	-0.1859247	18.1371538	2.33E-05	0.16714407
cg25195960	0.01893906	1.98E-05	0.1590816	0.11236387	18.089188	2.38E-05	0.16714407
cg02202589	0.01910423	2.02E-05	0.9325161	0.02027903	18.0889066	2.38E-05	0.16714407
cg22050705	-0.0350039	6.78E-05	0.24339608	-0.1549569	18.0795006	2.40E-05	0.16714407
cg01862641	0.05625015	1.75E-04	0.42469505	0.12422184	18.075965	2.40E-05	0.16714407
cg26119330	0.01781358	1.76E-05	0.93327591	0.01890672	18.0716063	2.41E-05	0.16714407
cg04572898	0.0268639	4.00E-05	0.14296487	0.17176757	18.0306779	2.46E-05	0.16714407
cg17735631	0.01839706	1.88E-05	0.93664458	0.01945044	18.0294219	2.46E-05	0.16714407
cg08159743	0.02754514	4.21E-05	0.92033194	0.02948828	18.0244185	2.46E-05	0.16714407
cg14692768	-0.045117	1.14E-04	0.31989861	-0.1517353	17.9184181	2.60E-05	0.17145486
cg06977900	0.01498265	1.25E-05	0.93219007	0.01594439	17.8975208	2.63E-05	0.17145486
cg15937634	-0.0257273	3.71E-05	0.10299309	-0.2854481	17.8610885	2.68E-05	0.17145486
cg20147920	0.04107093	9.54E-05	0.88139939	0.04553647	17.6788186	2.94E-05	0.17762896
cg00480429	0.01847555	1.93E-05	0.93463119	0.01957428	17.6744968	2.95E-05	0.17762896
cg22123784	0.03186761	5.80E-05	0.88996619	0.03517784	17.5062297	3.21E-05	0.17762896
cg23431851	-0.0258804	3.83E-05	0.18972883	-0.1463916	17.4849138	3.25E-05	0.17762896
cg18840832	-0.0418668	1.00E-04	0.1880016	-0.2505973	17.4650708	3.28E-05	0.17762896
cg23689219	0.02111245	2.55E-05	0.9223155	0.02263167	17.457697	3.30E-05	0.17762896
cg02877269	0.02104391	2.54E-05	0.91827295	0.02265721	17.4500021	3.31E-05	0.17762896
cg26248552	-0.0364368	7.61E-05	0.24258228	-0.1624003	17.4455177	3.32E-05	0.17762896
cg22694931	0.01744741	1.75E-05	0.92503257	0.01868519	17.4297041	3.34E-05	0.17762896
cg23002708	0.01929693	2.14E-05	0.9242231	0.02066336	17.4181082	3.36E-05	0.17762896
cg23851026	0.01979062	2.26E-05	0.92084288	0.02126336	17.3583171	3.47E-05	0.17762896
cg10916459	0.01931279	2.15E-05	0.89241547	0.02140937	17.3515391	3.48E-05	0.17762896
cg12488005	0.01597951	1.48E-05	0.93689938	0.01691152	17.2976583	3.58E-05	0.17762896
cg20052751	0.01931643	2.16E-05	0.13256758	0.13581522	17.2647511	3.64E-05	0.17762896
cg26289450	-0.0455373	1.20E-04	0.24386738	-0.205959	17.2490475	3.67E-05	0.17762896
cg03808001	0.03190991	5.90E-05	0.88821911	0.03529178	17.246522	3.67E-05	0.17762896
cg03345925	0.02242948	2.92E-05	0.93756201	0.02364042	17.2176346	3.73E-05	0.17762896
cg16461729	0.01825611	1.94E-05	0.14047107	0.12203353	17.2049739	3.75E-05	0.17762896
cg16117757	0.0265084	4.09E-05	0.9158103	0.02853236	17.1955379	3.77E-05	0.17762896
cg07215975	0.0188441	2.07E-05	0.94116547	0.01982364	17.1304118	3.90E-05	0.17900601
cg00284511	0.01594263	1.49E-05	0.94268147	0.0167702	17.0710708	4.02E-05	0.17900601

cg14138235	0.01893034	2.10E-05	0.92391782	0.02028143	17.0581665	4.05E-05	0.17900601
cg03604573	0.01583878	1.47E-05	0.10326481	0.14245533	17.052466	4.06E-05	0.17900601
cg00078456	0.02306822	3.12E-05	0.92089731	0.02473985	17.0472317	4.07E-05	0.17900601
cg16421621	0.01556516	1.42E-05	0.92772171	0.01663825	17.0249014	4.12E-05	0.17900601
cg24403959	0.02229881	2.93E-05	0.1642313	0.12714516	16.9536735	4.27E-05	0.17954669
cg18454045	0.02222529	2.92E-05	0.89709783	0.02447152	16.9435526	4.29E-05	0.17954669
cg22025064	0.02548673	3.84E-05	0.7884031	0.03181283	16.9308782	4.32E-05	0.17954669
cg04324999	-0.0406999	9.79E-05	0.2740969	-0.1603955	16.9218627	4.34E-05	0.17954669
cg06710735	0.02284425	3.11E-05	0.9244425	0.02440978	16.7955285	4.63E-05	0.18019205
cg19737540	0.03560797	7.56E-05	0.86998249	0.04010871	16.7710206	4.69E-05	0.18019205
cg16333262	0.03489976	7.26E-05	0.87896383	0.03893265	16.7705424	4.69E-05	0.18019205
cg18033671	0.01902868	2.16E-05	0.92715484	0.02031526	16.7542022	4.73E-05	0.18019205
cg08304525	0.01754178	1.84E-05	0.9356415	0.01857428	16.7500556	4.74E-05	0.18019205
cg23389776	0.017426	1.82E-05	0.87398405	0.01974176	16.7293699	4.79E-05	0.18019205
cg23426002	0.01997076	2.38E-05	0.92685046	0.02131725	16.7289916	4.79E-05	0.18019205
cg11142013	0.01590007	1.51E-05	0.90385174	0.01743808	16.7017418	4.86E-05	0.18019205
cg17729694	0.01477212	1.31E-05	0.92876319	0.01577966	16.6777211	4.92E-05	0.18019205
cg05401312	0.01970514	2.33E-05	0.9199216	0.02119347	16.6663196	4.95E-05	0.18019205
cg27128984	0.02225455	2.97E-05	0.14490634	0.14262664	16.6560957	4.98E-05	0.18019205
cg02865149	0.02143912	2.76E-05	0.13469311	0.14743638	16.6497128	4.99E-05	0.18019205
cg20240091	0.01628251	1.60E-05	0.92571056	0.01743586	16.6169611	5.08E-05	0.18075359
cg16942327	0.02265271	3.09E-05	0.91760009	0.0243859	16.602824	5.12E-05	0.18075359
cg02879960	0.02864182	4.96E-05	0.86629003	0.03252494	16.5474445	5.26E-05	0.18313254
cg17034181	0.02435813	3.59E-05	0.32005856	0.07331538	16.5205284	5.34E-05	0.18313254
cg00382463	-0.0409347	1.02E-04	0.25439072	-0.174992	16.5003754	5.39E-05	0.18313254
cg21293902	0.01870735	2.12E-05	0.93729295	0.0197617	16.496621	5.40E-05	0.18313254

Appendix 3: Overlap DMCs at cell type 3 in two methods.

CpGs
cg14138235
cg26957187
cg09809672
cg09889997
cg21543346
cg18900271
cg02879960
cg25228737
cg04781580
cg25313447
cg24499605
cg01816936
cg07060794
cg15829665
cg16942327
cg05283597
cg00903584
cg00476955
cg00153919
cg12299361
cg11442608
cg00255726
cg16611352
cg03103850
cg07420362
cg27641961
cg05102794
cg19692322
cg08237401
cg26749306
cg22514173
cg13882285
cg08474901
cg13798621
cg11241278
cg03429643

cg00980649
cg18657389
cg22322277
cg02370707
cg24248680
cg08616585
cg14145338
cg20038477
cg11733245
cg24055461
cg13069237
cg19677267
cg03699843
cg19819912
cg27573991
cg19223119
cg25032968
cg03062454
cg13494191
cg00235484
cg01787084
cg04685387
cg07749597
cg08688659
cg05767404
cg13385220
cg19532942
cg03039701
cg13185177
cg18148349
cg21750887
cg18545991
cg09096234
cg09620718
cg17852032
cg12405265
cg23166250
cg14083015
cg25314284

Appendix 4: Top 100 DMCs defined at lowest 100 q-value generated without consideration of cell mixtures.

CpGs	intercept	f	pval	qval
cg05066959	0.76036119	71.7341942	1.34E-16	3.72E-11
cg03169557	1.16924804	68.7646999	5.31E-16	7.35E-11
cg26102082	1.14170563	65.7245187	2.18E-15	2.01E-10
cg22883290	1.14111998	63.1648882	7.19E-15	4.98E-10
cg11823178	0.95797137	62.1400243	1.16E-14	6.43E-10
cg25018458	1.135116	61.3575209	1.67E-14	7.73E-10
cg13076843	0.83317312	60.093504	3.03E-14	1.20E-09
cg05810363	1.02065989	58.969209	5.14E-14	1.78E-09
cg23968456	1.16964579	56.8128413	1.42E-13	4.37E-09
cg12163800	0.91641289	53.9983547	5.37E-13	1.49E-08
cg19803550	1.14662602	53.4901888	6.84E-13	1.72E-08
cg12309456	1.01446987	52.6225561	1.03E-12	2.38E-08
cg16588649	0.95798283	52.1808396	1.27E-12	2.71E-08
cg22639325	1.15593989	51.1537157	2.07E-12	4.10E-08
cg17693222	0.79520598	47.8439016	1.01E-11	1.86E-07
cg06436667	1.22155839	47.0909522	1.45E-11	2.50E-07
cg25285237	0.89036337	46.6224678	1.81E-11	2.95E-07
cg10120897	0.90724417	45.8320823	2.64E-11	4.07E-07
cg12305431	-0.5307214	45.182388	3.61E-11	5.27E-07
cg13327545	-0.399089	44.6360161	4.70E-11	6.51E-07
cg22373622	0.93565327	44.1289507	6.00E-11	7.91E-07
cg22962123	-0.530836	43.8223287	6.95E-11	8.41E-07
cg21775279	1.02860944	43.8129812	6.99E-11	8.41E-07
cg04027736	-0.7389092	43.5361865	7.98E-11	9.21E-07
cg07207652	-0.676042	43.4520119	8.31E-11	9.21E-07
cg02798280	-0.4329402	42.5882054	1.26E-10	1.34E-06
cg16406967	-0.6447337	42.1787227	1.54E-10	1.58E-06
cg20535966	1.11009278	42.0599381	1.63E-10	1.61E-06
cg09221482	0.65885695	41.7301797	1.91E-10	1.77E-06
cg20618448	1.05893867	41.721337	1.92E-10	1.77E-06
cg07104958	-0.2251312	41.3027857	2.35E-10	2.10E-06
cg03776506	1.0615208	41.1776367	2.50E-10	2.16E-06
cg19240213	-0.6612199	41.0755493	2.62E-10	2.20E-06
cg21806242	-0.5858235	40.8441211	2.93E-10	2.39E-06
cg07012687	0.67025544	40.7766018	3.03E-10	2.40E-06

cg25594100	0.56627369	40.4202981	3.60E-10	2.77E-06
cg05845757	0.40277369	40.3653098	3.70E-10	2.77E-06
cg01217984	-0.4303959	39.9682479	4.49E-10	3.26E-06
cg14830003	-0.5561937	39.9192142	4.59E-10	3.26E-06
cg19153828	-0.8195715	39.7948728	4.88E-10	3.38E-06
cg01463828	-0.4866292	39.7461814	5.00E-10	3.38E-06
cg04157161	-0.4669351	39.5822106	5.41E-10	3.57E-06
cg20457732	-1.0251614	39.4339301	5.82E-10	3.73E-06
cg15821544	0.83610923	39.3957932	5.92E-10	3.73E-06
cg19940077	0.44883952	39.0857864	6.89E-10	4.24E-06
cg24524285	-0.8194689	38.8905898	7.57E-10	4.56E-06
cg05800416	-0.7738871	38.6618128	8.47E-10	4.98E-06
cg03760191	0.6567395	38.6240153	8.62E-10	4.98E-06
cg18680977	-0.5478798	38.4869631	9.22E-10	5.21E-06
cg07679492	0.84864164	38.4278863	9.49E-10	5.26E-06
cg15755240	0.98067185	38.3709779	9.75E-10	5.30E-06
cg17636309	0.92555282	38.1160248	1.10E-09	5.88E-06
cg23279355	-0.7772917	38.0362475	1.15E-09	6.00E-06
cg12561474	1.16445301	37.9946688	1.17E-09	6.01E-06
cg15374751	-0.8262674	37.2088598	1.72E-09	8.65E-06
cg26600753	0.89155446	37.1060144	1.81E-09	8.80E-06
cg20056593	1.10993222	37.0712928	1.84E-09	8.80E-06
cg05118960	-0.1739906	37.0648741	1.84E-09	8.80E-06
cg07883124	0.39959471	37.0189466	1.89E-09	8.85E-06
cg02317313	-0.6855155	36.8760225	2.02E-09	9.33E-06
cg22245494	1.13857542	36.8403631	2.06E-09	9.34E-06
cg00228891	-0.2725399	36.5817386	2.33E-09	1.03E-05
cg11532431	-1.0099219	36.5710179	2.35E-09	1.03E-05
cg08202399	-0.7704307	36.471786	2.46E-09	1.07E-05
cg07923390	0.85514085	36.3366094	2.63E-09	1.12E-05
cg24379915	0.73137433	35.9627172	3.16E-09	1.33E-05
cg15974867	0.78438924	35.9138765	3.24E-09	1.34E-05
cg06804344	0.69454848	35.7260652	3.55E-09	1.44E-05
cg23669081	-0.9513941	35.4731963	4.01E-09	1.61E-05
cg10895952	0.27784158	35.3056016	4.36E-09	1.72E-05
cg01964852	-0.8341977	35.2389181	4.50E-09	1.76E-05
cg22904711	-0.7784089	35.1759618	4.64E-09	1.79E-05
cg06902698	-0.7712576	34.9107323	5.29E-09	2.01E-05
cg26247646	0.87258023	34.8156408	5.54E-09	2.07E-05

cg11652496	0.62938917	34.7759928	5.65E-09	2.09E-05
cg11666770	0.49512177	34.6440831	6.03E-09	2.20E-05
cg19138865	0.57965719	34.3677306	6.90E-09	2.48E-05
cg13154057	1.00429006	34.32644	7.04E-09	2.50E-05
cg11236550	-0.6860774	34.2244732	7.40E-09	2.60E-05
cg25749512	-0.0369449	34.0716339	7.98E-09	2.75E-05
cg02803819	-0.7988239	34.0574665	8.04E-09	2.75E-05
cg05851442	-0.3070148	33.8926201	8.71E-09	2.94E-05
cg24550149	-0.6773301	33.7006239	9.58E-09	3.20E-05
cg24402332	0.86819011	33.5655849	1.02E-08	3.37E-05
cg04972348	0.27027851	33.4229035	1.10E-08	3.58E-05
cg06381803	-0.7527142	33.3560706	1.13E-08	3.65E-05
cg17104258	-0.9071068	33.2240916	1.21E-08	3.85E-05
cg23995914	-0.2431463	33.1390137	1.26E-08	3.97E-05
cg06768599	-0.2272962	33.0213331	1.34E-08	4.16E-05
cg11724984	0.55979098	32.9970132	1.35E-08	4.17E-05
cg00979931	-0.4651405	32.9392184	1.39E-08	4.24E-05
cg26587870	-0.2537288	32.9118954	1.41E-08	4.25E-05
cg16672810	0.6946911	32.7286054	1.54E-08	4.58E-05
cg04717895	-0.5351243	32.7169888	1.55E-08	4.58E-05
cg17474422	0.38204212	32.6514465	1.60E-08	4.60E-05
cg07061298	-0.6151077	32.6511812	1.60E-08	4.60E-05
cg18102633	0.82168182	32.6435956	1.61E-08	4.60E-05
cg00921266	-0.8342793	32.5896718	1.65E-08	4.64E-05
cg09825979	-0.5978436	32.573364	1.67E-08	4.64E-05
cg17804348	0.38302948	32.5560612	1.68E-08	4.64E-05

Appendix 5: Overlap DMCs without considering cell mixture and DMCs generated on cell type 3 by RefFreeEWAS method.

CpGs
cg22962123
cg07207652
cg02798280
cg19240213
cg21806242
cg01217984
cg14830003
cg01463828
cg04157161
cg24524285
cg17636309
cg00228891
cg05851442
cg06381803
cg06768599
cg00979931
cg26587870
cg00921266
cg15911153
cg07522913
cg22090150
cg19048532
cg01301319
cg09490371
cg20591728
cg03503871
cg15645660
cg02308560
cg04917446
cg00452882
cg08101036
cg19875532
cg08441803
cg01287088
cg08056778

cg27317046
cg01478234
cg19827875
cg25595793
cg22595230
cg04874795
cg05398321
cg08027484
cg18383668
cg06942814
cg05258935
cg11629889
cg08230957
cg09482050
cg24670552
cg27624319
cg09595050
cg07184237
cg09830866
cg07060794
cg24636368
cg21080562
cg09482093
cg02879960
cg04345034
cg04301614
cg15967278
cg14496753
cg22660933
cg09809672
cg16308533
cg08203284
cg21757872
cg17606183
cg10380221
cg14083015
cg25143652
cg14166284
cg15488009

cg00994629
cg20839206
cg23377551
cg25167447
cg09889997
cg18180783
cg24466241
cg09475796
cg07136133
cg07450210
cg01016592
cg16450309
cg11442608
cg26619671
cg27616541
cg20273670
cg04798016
cg25730428
cg24026619
cg04533276
cg25551168
cg10359157
cg13185177
cg24114014
cg06721393
cg04279801
cg25382900
cg12461930
cg05767404
cg11067407
cg03684062
cg27573991
cg23588605
cg26881102
cg20781880
cg17757980
cg08688659
cg07420362
cg19863740

cg04781580
cg07749597
cg04005059
cg02158978
cg26053066
cg07871971
cg03240301
cg07623567

Appendix 6: Overlap DMCs without considering cell mixture and DMCs generated on cell type 4 by RefFreeEWAS method.

CpGs
cg02879960
cg00959883
cg26417346

Appendix 7: Overlap DMCs without considering cell mixture and DMCs generated on cell type 2 by TOAST method

CpGs
cg08101036

Appendix 8: Overlap DMCs without considering cell mixture and DMCs generated on cell type 3 by TOAST method.

CpGs
cg23392845
cg07060794
cg02879960
cg09809672
cg14083015
cg09889997
cg23652026
cg17696044
cg11442608
cg04147621
cg13185177
cg05767404
cg27573991
cg08688659
cg07420362
cg04781580
cg07749597
cg23201812