Comparative assessment of DNA methylation patterns between reduced representation bisulfite sequencing and Sequenom EpiTyper methylation analysis

Aim: Validation of sequencing-based DNA methylation data is an important step for meaningful translation of findings. However, there has been limited assessment of different platforms to validate methylation data from next generation sequencing.

Methods: We performed a comparative methylation analysis between the genome-wide platform of reduced representation bisulfite sequencing with a targeted, Sequenom EpiTyper platform (four genes were analyzed in 15 cell lines covering 52 CpG sites). **Results:** We show that the accuracy of validation substantially improves if results from multiple and adjacent CpG sites are combined rather than at single CpG sites. We demonstrate increased read number improves accuracy of reduced representation bisulfite sequencing results. Further, by using series of replicates, we document variation in samples analyzed by Sequenom EpiTyper, indicating the importance of including replicates to increase precision. **Conclusion:** The results reveal potential sources of bias and provide a guideline for refining study design for DNA methylation analysis.

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Keywords: CpG site • DNA methylation • read number • replicate • RRBS • Sequenom EpiTyper • variation

The advent of next generation sequencing (NGS) technologies and the progressive increase in sequence throughput has allowed the quantification of DNA methylation on an unprecedented scale. Reduced representation bisulfite sequencing (RRBS) is a frequently used NGS-based method that allows genome-wide profiling of the DNA methylome. By enriching for CpG sites, RRBS covers 13.4% of CpG sites and includes information from 80% of CpG islands in the human genome [1], thereby providing a cost effective alternative to whole genome bisulfite sequencing.

NGS-based techniques, such as RRBS, are commonly used as a discovery platform to generate a list of regions showing significant methylation differences between experimental groups. The next essential step is to validate

these results by using a different technique either in the same samples (technical validation) or in independent cohorts (biological validation) [2]. There are several aspects in genome-wide studies that could lead to false identification of differential methylation, such as inadequate sequencing depth, the use of inappropriate statistical tests, overestimation of statistical significance from multiple testing and technical biases (e.g., PCR amplification bias). Thus an important question that arises in any large-scale DNA methylation analysis is whether the measured methylation values are accurate. Therefore, validation of results from genome-wide methylation profiles is of high importance for meaningful translation of the findings.

Several techniques allow locus-specific interrogation of methylation [3] including

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the Sequenom EpiTyper DNA methylation assay. Sequenom EpiTyper is a highly quantitative assay that combines base-specific enzymatic cleavage and MALDI-TOF (matrix-assisted laser desorption/ionization-time of flight) mass spectrometry to quantify individual CpG-site methylation [4]. Sequenom EpiTyper is commonly used as a validation platform for DNA methylation analyses due to its reputation of high precision [5–9].

In this study, we analyzed four genes from 15 RRBS libraries generated from human cell lines. We assessed the agreement between single CpG sites and larger regions in these genes (containing multiple CpG sites). We also assessed the level of variation at different read depth in RRBS data. Further, by performing several replicate experiments, we demonstrate technical variation in Sequenom EpiTyper methylation assays.

Results

Generating genome-wide & Sequenom EpiTyper methylation profiles for 15 cell lines

Genome-scale methylation profiles were obtained from 15 human cell lines by using RRBS. Four genes (*CBX8*, *HES5*, *POU3F2*, *EXOC3L2*, see **Tables 1 & 2**) were selected for comparison with the Sequenom EpiTyper assay. These regions were selected from an independent RRBS analysis [10] due to their good sequencing coverage (median read depth per CpG site was 38). In addition, these regions represented a range of methylation value (from 0 to 100% methylation) in the analyzed samples and therefore provide the opportunity for a comparative analysis at different methylation values. The analyzed Sequenom EpiTyper amplicon lengths varied from 62 to 454 bp. In total, 52 CpG sites were assessed by both techniques (i.e., RRBS and Sequenom EpiTyper) and the comparative data described in this article were based on these sites (**Supplementary Table 1**; the primer sequences are given in **Supplementary Table 2**).

Agreement between RRBS & Sequenom EpiTyper assay increases when a contig of CpG sites is analyzed

First, we compared the methylation status of each commonly covered CpG site for the analyzed genes. In some closely adjacent CpG sites, the Sequenom EpiTyper platform is unable to resolve methylation values for individual sites and an aggregated methylation value is calculated (examples can be seen in the x-axis of **Figure 3**). To derive equivalent data for the comparison with RRBS, we took the mean methylation values of these aggregated CpG sites. The Bland-Altman analysis indicated that the standard deviations of the difference in these four genes were 0.18, 0.16, 0.21

and 0.054 for *CBX8*, *HES5*, *EXOC3L2* and *POU3F2*, respectively (**Table 1**). The Pearson's correlation coefficients (r) were 0.86, 0.82, 0.77 and 0.99, respectively. Next, we determined the extent to which a fragment-based analysis enhanced the agreement between methods compared with single CpG site comparison (**Supplementary Figures 1–4**). The standard deviations of the differences between the fragment quantification methods were 0.042, 0.085, 0.10 and 0.031, respectively. For all four genes, the correlation between measured methylation increased ($r = 0.99, 0.95, 0.96$ and 0.998 , respectively). Combining the data from the four genes yielded a correlation of 0.89 for all the 52 CpG sites (SD of difference = 0.16, see **Figure 1A & B**), and when the fragment methylation values were compared, the correlation was 0.98 (SD of the difference = 0.072, **Figure 1C & D**).

Relationship of sequenced read number & methylation differences

Next, we examined the size of the effect of increased read depth on agreement between these two techniques. We analyzed the difference between RRBS and Sequenom EpiTyper methylation values with incremental read depth (step size of 5; the distribution of the RRBS read depth is shown in **Supplementary Figure 5**) and found, as expected, that the difference decreases with increasing read depth (**Figure 2**). For RRBS read depths of ≥ 10 , the correlation coefficient for CpG sites with an RRBS read depth ≥ 10 was 0.908 (95% CI: 0.888–0.925) (see **Table 2**). At a read depth of ≥ 20 , the correlation coefficient was 0.918 (95% CI: 0.898–0.934), whereas at a read depth of ≥ 50 , the correlation coefficient was 0.960 (95% CI: 0.945–0.971) (**Table 2**).

Assessment of technical variation of the Sequenom EpiTyper platform

To assess variability of the Sequenom EpiTyper analysis process (PCR plus mass spectrometry), we used DNA from a normal melanocyte cell line (Hemn-LP). A single aliquot of DNA from the Hemn-LP cell line was bisulfite converted and used to generate the technical replicates for the subsequent experiments (described in **Figure 3**). We performed PCRs on replicate samples for three genes (*HES5*, *EXOC3L2*, *POU3F2*). In total, the length of the amplicons varied from 218 to 437 bp, and contained 81 CpG sites from which 32 quantifiable methylation values could be obtained (i.e., analyzable in Sequenom EpiTyper platform [**Supplementary Table 1**]). Of the 81 Sequenom EpiTyper CpG sites, 38 were also covered by RRBS. For *POU3F2* and *HES5*, there were seven replicate samples, whereas for *EXOC3L2*, there were four (**Figure 3A–C**). Sequenom

Table 1. Comparative validation analysis of reduced representation bisulfite sequencing versus Sequenom EpiTyper platform at single CpG sites and over an amplicon.

Nearest gene	Feature	CpG correlation (Sequenom EpiTyper-RRBS)	Bland-Altman analysis		
		r-value	Difference	SD of difference	Limits of agreement
<i>CBX8</i>	Promoter	0.859	-0.0110	0.179	-0.362–0.340
<i>HES5</i>	Promoter, CGI core	0.817	-0.014	0.160	-0.329–0.301
<i>EXOC3L2</i>	Promoter	0.770	-0.040	0.209	-0.451–0.370
<i>POU3F2</i>	Promoter, CGI core	0.992	-0.003	0.054	-0.110–0.103
All CpG sites	NA	0.896	-0.017	0.164	-0.338–0.304
		Common amplicon correlation (Sequenom EpiTyper-RRBS)	Bland-Altman analysis		
<i>CBX8</i>	Promoter	0.990	-0.012	0.042	-0.096–0.071
<i>HES5</i>	Promoter, CGI core	0.953	-0.023	0.085	-0.1907–0.1436
<i>EXOC3L2</i>	Promoter	0.959	-0.042	0.104	-0.246–0.162
<i>POU3F2</i>	Promoter, CGI core	0.998	-0.004	0.031	-0.066–0.058
All common amplicons	NA	0.982	-0.020	0.072	-0.161–0.120

RRBS: Reduced representation bisulfite sequencing; SD: Standard deviation.

EpiTyper methylation data for individual CpG sites were obtained for each of these replicates.

The methylation values of each replicate sample at individual CpG sites are shown in [Figure 3D–F](#). We found that many of the CpG sites showed substantial variation. For example, CpG47 for *EXOC3L2* (SD = 0.20, coefficient of variation (CV) = 109%) and CpG11–13 for *POU3F2* (SD = 0.16 and CV =

59%, [Figure 3](#) and [Supplementary Data 1](#) contains detailed information for the individual CpG sites). We observed a general trend of higher variability (i.e., higher SD) if the methylation value is intermediate in the replicates ([Figure 4](#)). When the methylation values of the CpGs in each amplicon were averaged, the replicate samples showed better agreement: the SDs of the replicates for the amplicon were 0.05, 0.04

Table 2. Analysis of read depth and methylation correlation between reduced representation bisulfite sequencing and Sequenom EpiTyper.

Read cut off	Pearson co-efficient	Data points, n	95% CI
No cut off	0.896	383	0.875–0.914
5	0.895	378	0.873–0.913
10	0.908	356	0.888–0.925
15	0.912	329	0.892–0.928
20	0.918	295	0.898–0.934
25	0.930	262	0.912–0.945
30	0.932	238	0.913–0.946
35	0.942	208	0.925–0.956
40	0.953	188	0.938–0.964
45	0.959	159	0.945–0.970
50	0.960	147	0.945–0.971
55	0.967	125	0.953–0.976
60	0.971	111	0.958–0.980

Figure 1. Technical validation of all the analyzed CpG sites and common amplicon with reduced representation bisulfite sequencing and Sequenom EpiTyper MassARRAY techniques. (A) Dot plot showing correlation between DNA methylation as measured by RRBS (y-axis) and by Sequenom EpiTyper for the CpG site-based comparison; (B) the corresponding difference plot; (C) Dot plot showing correlation between DNA methylation for the fragment-based comparison; and (D) the corresponding difference plot. The solid red line in the difference plots shows the mean difference between Sequenom EpiTyper and RRBS. The top and bottom dotted black lines represent the 95% limits of agreement. RRBS: Reduced representation bisulfite sequencing.

Figure 2. Relationship between methylation difference and reduced representation bisulfite sequencing read depth in analysed CpG sites. Y-axis represents the methylation difference between Sequenom EpiTyper platform and reduced representation bisulfite sequencing (in a scale of -1–1) and x-axis shows the mapped read number of the corresponding CpG sites (combined data for four genes and each gene is highlighted with different color). The black lines indicate the 95% prediction interval at each read depth for the measured methylation difference, under the assumption that the measured difference is due to noise that scales as $1/\sqrt{\text{read depth}}$.

and 0.03 for *HES5*, *EXOC3L2* and *POU3F2*, respectively. The average CpG methylation of these seven replicates also showed good concordance with RRBS ($r = 0.908$, $p\text{-value} = 0.00002$ and SD of bias = 0.107, Supplementary Figure 6).

Discussion

Previous benchmark studies have compared the performance of several genome-wide methylation analysis platforms [11,12]. These analyses focused on genome-wide correlation, detection of differentially methylated regions and the effect of sequencing depth. Here, we present the use of a targeted approach using the quantitative Sequenom EpiTyper assay to compare the methylation of four genes that were first analyzed by using a genome-wide approach (RRBS). We have used a panel of 15 cell lines that includes primary and metastatic melanoma as well as normal melanocyte cell lines for comparison. Further, we have performed multiple repeats in our Sequenom EpiTyper methylation analysis to provide a measure of technical variation in this process. In general, we see significant concordance between RRBS and Sequenom EpiTyper

methylation patterns. Our observations are consistent with previous studies that demonstrated Sequenom EpiTyper platform to be a reliable targeted approach to validate genome-wide methylation data generated from RRBS [13–15].

By comparing RRBS and Sequenom EpiTyper methylation data, we demonstrate that methylation values between the two techniques are in better agreement if regions containing multiple CpG sites are compared instead of regions containing single CpG sites. There could be several reasons that might lead to increased variability when analyzing methylation data from single CpG sites. First, the technical platforms used to derive methylation calls (sequencing-based techniques and mass spectrometric-based techniques) have inherent differences in resolving methylation differences, which will affect the accuracy of methylation quantification. Second, a reduced number of reads covering a single site compared with a contig of sites can influence the methylation call. Finally, sampling-based variation could also contribute to the differences in methylation values between the platforms.

Figure 3. DNA methylation analysis of multiple technical replicates in Sequenom EpiTyper platform.

(A–C) Successful amplification of the technical replicates using EpiTyper primers for *HES5*, *EXOC3L2* and *POU3F2* (left to right). Ladder: 100 bp DNA ladder (Invitrogen). An additional replicate sample was prepared for each of these genes and is not included in this gel electrophoresis. (D–F) Methylation status of each CpG sites in the amplicon for replicate samples for *HES5* (7 replicates), *EXOC3L2* (4 replicates) and *POU3F2* (7 replicates) (left to right). For *HES5*, *EXOC3L2* and *POU3F2*, PCR gel of 6, 3 and 6 replicates is shown. Figure 3 shows examples of several adjacent CpG sites for which Sequenom EpiTyper was unable to resolve methylation values for individual sites (such as CpGs 4 and 5 and then 9 and 10 for the *HES5* gene amplicon, see x-axis).

As expected, increased read depth in RRBS results in a better correlation with Sequenom EpiTyper methylation values. In fact, the improvement between RRBS and Sequenom EpiTyper methylation has a straightforward explanation: RRBS and Sequenom EpiTyper both give noisy measurements of the actual methylation, but, assuming independent reads, we expect the uncertainty in the RRBS measurement to have a component that scales as $1/\sqrt{\text{read depth}}$ (Figure 2). However, many CpG sites, in spite of the read depth being greater than ten, showed a large difference. It is possible that the mapping of reads results in erroneous methylation calls at some CpG sites. For mapping bisulfite sequenced reads, the mapping algorithms need to resolve C to T asymmetry [16] and can generate differences by mapping methylated reads more efficiently than unmethylated reads [17]. Therefore, inspection of read-mapping patterns might be an important step to consider before understanding sources of bias from RRBS experiments.

We detected notable variation in technical replicate samples in Sequenom EpiTyper assays for some CpG sites. This variability could be a result of the following: if adjacent CpGs cannot be resolved by Sequenom EpiTyper platform; if the CpGs under analysis are the first or last within an amplicon (these are more variable as previously reported by [4]); if the methylation value is intermediate (see Figure 4 and Supplementary Figure 7); and if suboptimal PCR performance biases the composition of the product under analysis. A previous study analyzed three genes in Sequenom EpiTyper platform and based on three PCR replicates they reported median standard errors of 0.02, 0.04 and 0.11 for these genes [4]. In our study, for the three analyzed genes we found the median standard error to be 0.007 (for *POU3F2*), 0.0205 (for *HES5*) and 0.0325 (for *EXOC3L2*) based on seven, seven and four replicates, respectively, see Supplementary Figure 7). These results are in agreement with previous reports of degree of variation in Sequenom EpiTyper platform. These results highlight the importance of including multiple replicates for validation to reduce sampling bias and PCR variation. An alternative strategy could be pooling multiple PCR replicates before profiling methylation patterns to reduce sampling noise. However, this needs to be tested in future studies.

DNA methylation at adjacent CpG sites is correlated [11,12]. Further, neighboring CpGs are likely to have similar experimental properties (such as similar read depth and similar PCR characteristics). Although methylation levels are generally similar in adjacent sites, there are exceptions. In our analysis, we observe substantial variation in specific CpG sites compared with their adjacent sites. For example, *EXOC3L2* CpGs 8,9 (aggregated sites, see Figure 3E), *HES5* CpG8 and *POU3F2* CpGs 11,12,13 (aggregated sites, see Figure 3F). Previously, we have demonstrated substantial methylation variation in adjacent CpG sites within single fragments in a relatively homogeneous cell population of neutrophils [18]. Mechanistic studies on adjacent DNA methylation dynamics are lacking and therefore firm conclusions cannot be drawn about the biological implications of adjacent CpG site methylation. However, it is plausible that some specific CpG sites in a contig might be under selective transcriptional control and therefore are necessary to alter binding of transcription factors, insulators or enhancer elements that are required to regulate transcription [19,20].

Although we analyzed sequencing-based RRBS data, the findings from this study could be utilized in other platforms for selection of CpG sites for validation. For example, Illumina's high density methylation array is currently the most widely used platform for profiling large number of samples due to the relative ease of extracting methylation calls. Almost half of

Figure 4. Relationship of standard deviation and methylation in Sequenom EpiTyper replicates.

X-axis: mean methylation of all the replicates in CpG sites; y-axis: corresponding standard deviation (SD).

the probes on 450 K CpG array were located around promoters and often one promoter contains multiple probes (average number of CpG probe/protein coding promoter is 9) [21]. The results from this work reveal potential sources of bias that can be used to refine experimental design for studies that are focused on validating methylation data generated from large-scale DNA methylation studies.

Materials & methods

Cell line experiments

In total, 15 cell lines were used in this article. These cell lines were obtained from America Type Culture Collection (VA, USA). These cell lines are: WM-115 (ATCC® CRL-1675™), WM266-4 (ATCC CRL-1676™), Hs688(A).T (ATCC CRL-7425™) and Hs688(B).T ATCC CRL-7426™). These cell lines were grown and maintained in Minimum Essential Media and supplemented with 1% penicillin-streptomycin (Gibco, NY, USA) and 10% fetal bovine serum (Moregate biotech, Australia & New Zealand) under standard conditions (5% CO₂, 37°C, humidified atmosphere). Three normal melanocyte cell lines (Hemn-LP, HemA-LP and Mel-ST) were obtained from Robert Weinberg, MIT, USA. Eight additional cell lines were used for preparing the RRBS libraries. For these cell lines (WM793, WM853-2, WM278, WM39, WM1341D, and WM15552C, WM75, WM373), DNA material was obtained from The Wistar Institute (PA, USA).

RRBS library preparation & sequencing

RRBS libraries were prepared according to previously published protocols [22]. Briefly, genomic DNA was digested with the MspI enzyme followed by end-repair and ligation of sequencing adaptors. The fragments were then size-selected (40–220 bp) and bisulfite converted prior to a PCR amplification step. The quality of the libraries was checked using a bioanalyzer, and four libraries (per flow cell lane) were sequenced on an Illumina HiSeq2500 machine (100 bp, single-ended run).

RRBS data analysis

Processing of the reduced representation bisulfite sequencing data was analyzed using our in-house bioinformatics pipeline [23]. Analysis of mapped RRBS data and extraction of CpG methylation was performed using DMAP (differential methylation analysis package) tools [24].

Sequenom EpiTyper DNA methylation assay

The primers used for analyzing four genes described

here were designed using the EpiTyper assay designer [25]. The sequences of the primers are given in **Supplementary Table 2**. Briefly, 1 µg of genomic DNA was bisulfite converted using the EZ DNA methylation kit (Zymo Research) using the ‘alternative cycle protocol’ as stated by the manufacturer. PCR was performed with the following cycling protocol: 15 min at 94°C, 45 cycles of (20 s at 94°C, 30 s at optimal annealing temperature, 1 min at 72°C), 3 min at 72°C for extension and hold at 4°C. The mass spectrometry-based Sequenom EpiTyper assay was performed using the manufacturers’ protocol on one 384-well plate (Sequenom Facility, University of Auckland). During the mass spectrometry run, the Sequenom EpiTyper assay generates spectrum quality data for each CpG site that were analyzed [26]. Our spectrum quality data were of acceptable quality to be used for the subsequent analysis. The spectrum characteristics of the assay were analyzed at the facility and the methylation values for each CpG site in the designed amplicons were obtained. Analysis of methylation data was performed using excel and R statistical software (Version 3.02).

Code availability

Standard R commands were used to perform statistical tests and generate the figures. The codes are available from the authors on request (aniruddha.chatterjee@otago.ac.nz). In addition, data analysis of single genes was performed using Prism Graphpad software.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/full/10.2217/epi-2016-0176

Author contributions

A Chatterjee, E Macaulay, MR Eccles and IM Morison conceptually designed the project. A Chatterjee analyzed the data and wrote the first draft of the manuscript together with E Macaulay. A Ahn performed the EpiTyper PCR experiments. JL Ludgate prepared multiple RRBS libraries. PA Stockwell developed the computational pipeline for data analysis and performed alignments and helped in data analysis. RJ Weeks helped in the design of the EpiTyper assays and interpretation of data. MF Parry contributed to the statistical analysis and integration of the data. IM Knarston and TJ Foster contributed to the Sequenom EpiTyper PCR experiments and data analysis, respectively. All authors read, contributed and approved the final manuscript.

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terials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The experiments and analysis described here were carried out in accordance with the Health and Disability Ethics Committee of New Zealand protocol LRS/11/02/002/AM04.

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Summary points

- Validating this next generation sequencing (NGS)-based DNA methylation data is an important step for meaningful translation of findings. However, there has been limited assessment of different platforms to validate methylation data from NGS.
- We provide a comparative assessment of DNA methylation patterns between a NGS-based, genome-wide platform (reduced representation bisulfite sequencing) with a targeted, mass spectrometry based platform (Sequenom EpiTyper). We compared the DNA methylation patterns of four genes in 15 cell lines (covering 52 CpG sites).
- We show that the accuracy of validation of DNA methylation profiles between the two methods substantially improves if results from multiple and adjacent CpG sites are combined (i.e., investigating a region) rather than at single CpG sites.
- Increased read number improves accuracy of reduced representation bisulfite sequencing results and we provide estimates of variation at different read number cut offs.
- By using series of replicates, we demonstrate variation within technical replicate samples analyzed by Sequenom EpiTyper, which indicates that the use of replicate samples will enhance precision.
- This work reveals potential sources of bias that can be used to refine experimental design for studies that are focused on validating methylation data generated from large-scale DNA methylation studies.

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