

METHOD

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DiffVar: a new method for detecting differential variability with application to methylation in cancer and aging

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Abstract

Methylation of DNA is known to be essential to development and dramatically altered in cancers. The Illumina HumanMethylation450 BeadChip has been used extensively as a cost-effective way to profile nearly half a million CpG sites across the human genome. Here we present DiffVar, a novel method to test for differential variability between sample groups. DiffVar employs an empirical Bayes model framework that can take into account any experimental design and is robust to outliers. We applied DiffVar to several datasets from The Cancer Genome Atlas, as well as an aging dataset. DiffVar is available in the *missMethyl* Bioconductor R package.

Background

DNA methylation is crucial for normal embryonic development with roughly 3% to 6% of all cytosines methylated in normal human DNA [1]. However, methylation changes are known to accumulate with age [2], with up to 30% of CpG sites changing methylation status within the first 1.5 years of life [3]. In addition, aberrant methylation patterning is associated with many diseases. In particular, in cancer cells, disruption of normal methylation events are very common with the number of genes undergoing CpG island promoter hyper-methylation increasing during tumour development, combined with an extensive loss of DNA methylation in other genomic regions [1,4]. This phenomenon is not consistent across all cancers, however; distinct DNA methylation patterns have been observed between subtypes of the same cancer [5-7]. Akalin *et al.* [5] showed that one subtype of acute myeloid leukaemia shows widespread hyper-methylation in promoter regions and CpG islands neighbouring the transcription start site of genes, while a second subtype displayed extensive loss of methylation at an almost mutually exclusive set of CpGs in introns and intergenic CpG islands and shores. Abnormal methylation events can potentially silence tumour suppressor or growth regulatory genes, activating novel pathways that

contribute to tumour progression [8]. However, epigenetic changes are potentially reversible, as it is possible to reactivate genes that have been silenced by methylation [1], making them an attractive therapeutic target. Hence the study of DNA methylation in cancer remains an important topic of interest with much still to be discovered.

Cancer is a heterogeneous disease. Within each type of cancer there is the potential for tumour growth to be driven by perturbations in many different molecular pathways, and these perturbations will vary from individual to individual. Epigenetic instability or the loss of epigenetic control of important genomic domains can lead to increased methylation variability in cancer, which may contribute to tumour heterogeneity [9]. In a large study profiling 1505 CpG sites of 1628 human samples, of which 1054 were tumours, Fernandez *et al.* [10] observed little variation in the DNA methylation patterns of normal tissue, while the established tumours showed greater CpG methylation heterogeneity. Hansen *et al.* [9] proposed that increased epigenetic heterogeneity in cancer could underlie the ability of cancer cells to adapt rapidly to changing environments. Hence studying the heterogeneity of cancers could lead to better understanding of tumourigenesis.

As mentioned, it has been shown that DNA methylation patterns are associated with an individual's age [2]. Interestingly, both tumour development and aging are processes that result in the global loss of genome-wide

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DNA methylation combined with gains in CpG island promoter methylation [11,12]. Hence it has been speculated that the accumulation of epigenetic alterations during aging might contribute to tumorigenesis [12].

Several technologies are available for profiling DNA methylation, based on both arrays and sequencing. While the cost of next-generation sequencing has dramatically decreased, it is still too expensive for many large studies to profile widespread methylation. The introduction of the Illumina Infinium HumanMethylation450 BeadChip is a more affordable alternative for measuring genome-wide DNA methylation at 482,421 CpG sites. Consequently, thousands of tumour and normal tissues have been profiled using these arrays, with numerous cancer data sets now publicly available through The Cancer Genome Atlas (TCGA) [13,14]. Similarly, these arrays are being used to profile methylation differences between disease cases and normal controls in so-called epigenome-wide association studies [15].

To date, the main focus when analysing DNA methylation data has been detecting CpG sites that are differentially methylated between groups. Methods to detect differences in means for high-dimensional biological data are well established and include approaches such as those taken in the Bioconductor [16] software packages *limma* [17], *minfi* [18], *edgeR* [19] and *DESeq* [20]. A CpG site that is statistically significantly differentially methylated between groups (for example, cancer versus normal) will have different group means; however, the measurements within each group will tend to be quite consistent (Figure 1A). Recently, several papers have observed consistent methylation between normal samples and highly variable methylation between cancer samples, arguing that identifying features that differ in terms of variability may be just as relevant or important as differential methylation for understanding disease phenotypes [21-25]. In other words, there is interest in identifying differentially variable CpG sites, where the samples in one group have consistent methylation values and the samples in the other group have highly variable methylation values (Figure 1B).

Methods for detecting differential variability in high-dimensional 'omics data have not been well addressed in the literature to date. Jaffe *et al.* [24] have a sophisticated method to detect differential variability over regions, specifically developed for the CHARM array [26]. Bar *et al.* [27,28] propose a three-component mixture model on the ratio of the sample variances from treatment and control groups. The method aims to separate features that are not differentially variable, have inflated variance in the treatment group, or inflated variance in the control group. In addition, they performed empirical Bayes shrinkage on the inflation factors to stabilise the estimates. Other attempts for determining CpG site-wise

differential variability include the F test [9,29] and the Bartlett test [25]. Unfortunately, the F test and Bartlett test are known to be highly sensitive to outliers [30]. While Hansen *et al.* [9] did not address the issue of outliers in the data when using an F test for equality of variances, Ho *et al.* [29] performed an outlier removal step prior to testing for differential variability. By contrast, Teschendorff and Widschwendter [25] implemented the Bartlett test and claimed that features that are differentially variable due to outliers are of interest. Their application is specific to the early stages of carcinogenesis, where they hypothesised that outliers may play an important role. It has been observed, however, that outliers are often a result of technical and biological artefacts rather than being biologically relevant to disease. For example, a technical artefact could arise due to processes surrounding the technology and biological artefacts could include stromal contamination of a tumour sample. Microarrays can suffer from spatial artefacts [31] and outliers arising from sample specific GC content biases have been reported in RNA sequencing data [32]. Mislabelled samples can also lead to outlying observations [33]. Hence, a method that successfully identifies differentially variable sites with a broader distribution of methylation values, such as that in Figure 1B, is desirable. Furthermore, the F test and Bartlett test assume that the data are normally distributed, which is not the case for methylation data [34].

Here we present a new method for detecting differential variability for individual CpG sites in methylation data. Our approach is inspired by Levene's z-test [35]. It is a simple and computationally efficient test that is robust against non-normality and outliers. A major advantage of our method is that it is suitable for any experimental design; it is not limited to a two-group scenario. The method, called *DiffVar*, is available as a function in the *missMethyl* Bioconductor R package, and depends on the *limma* framework. We applied *DiffVar* to several publicly available cancer data sets from TCGA, as well as a publicly available aging data set [2]. When we applied *DiffVar* to the cancer data sets from TCGA, we observed that a large proportion of the top differentially variable CpG sites are found in CpG islands. Interestingly, the top differentially variable CpG islands tend to differ from cancer to cancer. We further found that the 10,000 top-ranked differentially variable CpG sites have very little overlap with the 10,000 top-ranked differentially methylated CpG sites, consistent with the findings by Teschendorff and Widschwendter [25]. Applying *DiffVar* to an aging data set revealed that centenarians have highly variable methylation compared to newborns and approximately 17% of the differentially variable CpGs were also differentially methylated.

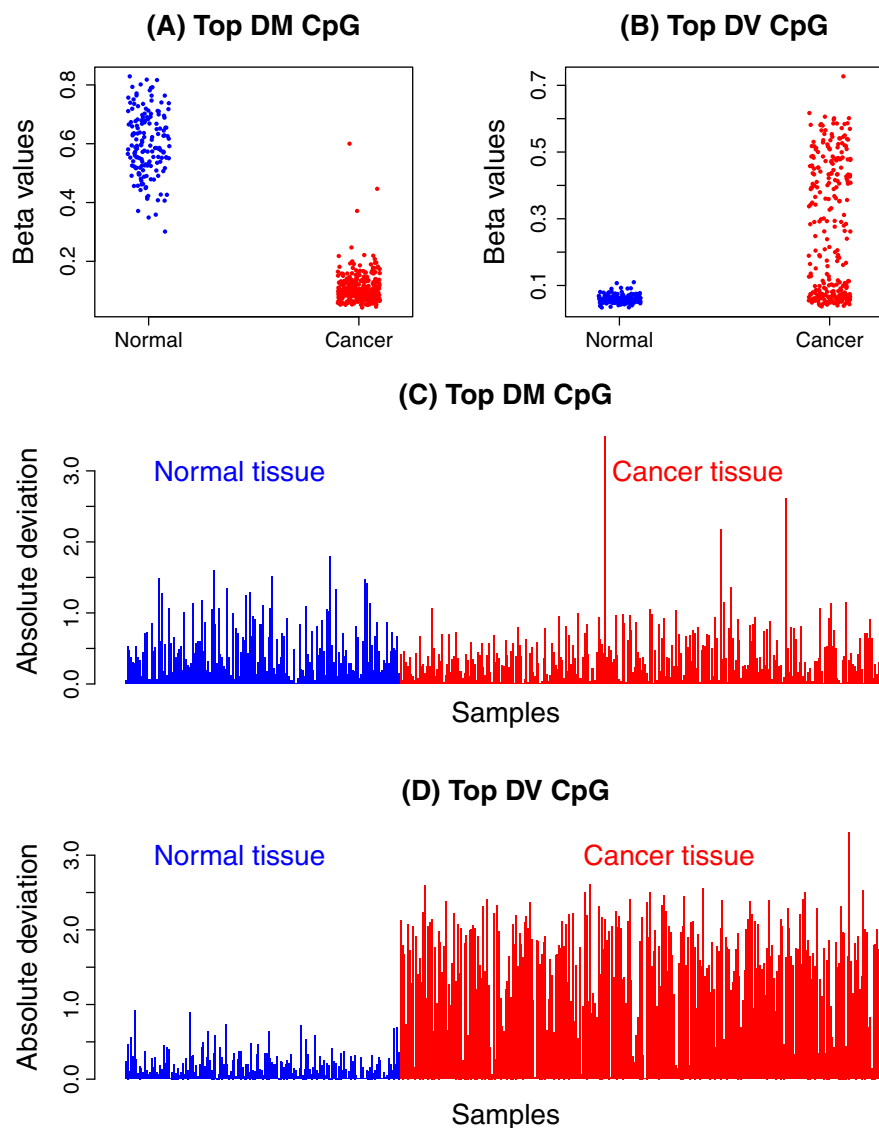


Figure 1 Differential methylation (DM) and differential variability (DV) in the kidney cancer methylation data set. (A) and (C) show the β values and absolute deviations of the most significantly differentially methylated CpG site between the normal and cancer samples. There is an obvious shift in mean between cancer and normal (A), but in (C) the variability in both groups looks very similar. (B) and (D) show the β values and absolute deviations of the most significantly differentially variable CpG site between the normal and cancer samples. It is clear that the variability of the cancer and normal groups are very different, with very large deviations in the cancer group and consistently small deviations in the normal group apparent in (D). DM, differential methylation; DV differential variability.

Results

DiffVar: a new method to identify differentially variable features

The focus of this paper is on methylation data from Illumina's Infinium HumanMethylation450 BeadChip, although our method can be applied to any high-dimensional data such as gene expression data. The output from the array consists of two measurements for each CpG site, the methylated and unmethylated intensity signal. These two measurements can be used to calculate either β values, which

capture the proportion of methylation at each CpG site, or M values, defined as the \log_2 ratio of the methylated to unmethylated intensity. Since M values do not display the severe heteroscedasticity that occurs with β values [34], DiffVar is performed on M values. If only β values are available they can be transformed into M values using a logit transformation, $M = \text{logit}(\beta)$. Similarly M values can be converted back to β values for interpretation and visualisation. For more details on the array and raw data see Materials and methods.

As mentioned, our method is inspired by Levene's *z*-test [35]. Intuitively, a measure of variability can be thought of as the distance of each point within a group from the group mean. Figure 1C and D show examples of the CpG sites from Figure 1A and B across a large kidney cancer and normal data set from TCGA (see Materials and methods). Each sample is plotted as a bar with the height of the bar equal to the absolute deviation from the group mean. Highly variable groups will be characterised by consistently large deviations (Figure 1D, red bars representing the cancer group) and low variability groups will have consistently small deviations (closer to zero) about the group mean (Figure 1D, blue bars representing the normal group). To determine if one group is more variable than another, we can simply perform a *t*-test on the absolute or squared deviations of the *M* values from the group mean. This tests the null hypothesis that the group variances are equal. CpG sites that have deviations consistently larger in one group compared to another group will be identified as significantly differentially variable. Figure 1C shows the absolute deviations from the group means for the top differentially methylated CpG site. Although there is a shift in mean *M* values between the two groups, the variations about the mean are similar in both groups; hence this CpG site will not be significantly differentially variable.

In high-dimensional data, it is well known that a simple *t*-test can result in many false discoveries, hence we employ an empirical Bayes modelling framework to stabilise the *t*-statistics [36]. Additional variables, which may influence the variability within groups, can be taken into account within the linear modelling framework. This ensures that any experiment that can be summarised by a design matrix can be accommodated. Unequal sample sizes are taken into account by multiplying the absolute or squared deviations from the group mean by a leverage factor, $n_k/(n_k + 1)$, where n_k is the sample size for group *k*. Moderated *t*-statistics [36] are computed and Benjamini and Hochberg false discovery rates (FDR) [37] are reported. In this manner, a list of differentially variable CpG sites is obtained. For a more formal definition of the statistical model, see Additional file 1.

Simulation study

DiffVar correctly controls type I error rate

Two strategies were used to determine type I error rate control. The first strategy generated *M* values under a hierarchical model while the second strategy involved re-sampling data from the kidney cancer data set, using only the non-diseased samples. Details of the simulations are provided in Materials and methods. Briefly, a two-group scenario with a sample size of 50 in each group was simulated. *M* values were generated for 10,000 CpG

sites and with the same variance for each group. CpG sites were tested for differential variability using the F test, Bartlett's test, *DiffVar* with absolute deviations, and *DiffVar* with squared deviations. The numbers of significant raw *P* values at cut-offs of 0.001, 0.01, 0.05 and 0.1 were counted for each of 1000 simulations. Table 1 summarises the results. Under the hierarchical model, the type I error rates are very close to the nominal *P* value for all tests, showing that all tests have good type I error rate control in this scenario, although *DiffVar* with squared deviations appears conservative at 0.001. For the resampled data, the Bartlett test and the F test lose type I error rate control for all nominal *P* values (Table 1). This is likely due to real data containing technical and biological artefacts that produce outliers. By contrast, *DiffVar* with absolute and squared deviations maintains type I error rate control over all nominal *P* values. *DiffVar* with squared deviations is conservative at nominal *P* values of 0.001 and 0.01.

DiffVar is robust to outliers

To explore the effect of outliers further, the simulations under the hierarchical model were modified to incorporate 200 CpGs with a single outlier by randomly selecting one sample and substituting the maximum *M* value over all 10,000 simulated *M* values. From Table 2 it is again apparent that the Bartlett test and F test are extremely

Table 1 Comparing the type I error rate for the four different methods

Data generated under hierarchical model				
Test method	Nominal <i>P</i> value			
	0.001	0.01	0.05	0.1
Bartlett	0.001032	0.01014	0.0504	0.1006
F test	0.001011	0.01003	0.05001	0.1001
<i>DiffVar</i> (abs)	0.0010029	0.01023	0.05094	0.1036
<i>DiffVar</i> (sq)	0.0007118	0.009800	0.0499	0.1018
Resampled data from kidney non-diseased samples				
Test method	Nominal <i>P</i> value			
	0.001	0.01	0.05	0.1
Bartlett	0.009	0.0257	0.07715	0.1331
F test	0.0089	0.0253	0.0763	0.1319
<i>DiffVar</i> (abs)	0.001	0.0104	0.0523	0.105
<i>DiffVar</i> (sq)	0.0004	0.00665	0.0448	0.0981

Data were generated in two ways: under a hierarchical model and by randomly selecting the non-diseased kidney samples. Median type I error rates are reported for 1000 simulations with no differentially variable or differentially methylated features. For simulations generated under the hierarchical model, the standard deviation with which the error rate is estimated ranges from approximately 0.00024 for rates near 0.001 to 0.0029 for rates near 0.1 with no notable difference between the methods. For the resampled data, the standard deviation ranges from approximately 0.0026 for rates near 0.001 to 0.026 for rates near 0.1 with *DiffVar* (sq) being noticeably less variable than the other methods. abs, absolute deviations; sq, squared deviations.

Table 2 Comparing the type I error rate for the four different methods with 200 outliers

Test method	Nominal <i>P</i> value			
	0.001	0.01	0.05	0.1
Bartlett	0.01379	0.02601	0.06781	0.1179
F test	0.01467	0.02663	0.06767	0.1172
DiffVar (abs)	0.0009701	0.01006	0.05085	0.1019
DiffVar (sq)	0.0005373	0.008198	0.04880	0.1011

Median type I error rates are reported for 1000 simulations with no differentially variable or differentially methylated features, but with 200 outliers incorporated in the data. The standard deviation with which the error rate is estimated ranges from approximately 0.0003 for rates near 0.001 to 0.003 for rates near 0.1 with the Bartlett test and F test more variable than DiffVar at nominal *P* values of 0.001 (standard deviation approximately 0.002 compared to 0.0003) and 0.01 (standard deviation approximately 0.0018 compared to 0.0010). abs, absolute deviations; sq, squared deviations.

sensitive to outliers and have lost all type I error rate control, particularly for smaller nominal *P* values. DiffVar with absolute or squared deviations is robust to outliers and maintains type I error control, although DiffVar with squared deviations is once again conservative at nominal rates of 0.001 and 0.01. Figure 2A shows the number of CpGs with outliers in the top 500 features ranked by each method. Although the F and Bartlett tests produce different *P* values, they have similar ranking by *P* value, resulting in overlapping curves in Figure 2A,B. It is immediately apparent that the top-ranked features using Bartlett and F *P* values are produced by CpGs with outliers. In contrast, DiffVar using either squared or absolute deviations does not preferentially rank the CpG sites containing outliers near the top of the list (Figure 2A).

DiffVar produces fewer false positives in the presence of both outliers and truly differentially variable features

To add complexity to the simulations, we incorporated 1000 differentially variable features in the simulated data in addition to the 200 outliers. The variability of the 1000 CpGs in the second group was roughly twice that of the variability in the first group (see Materials and methods). Figure 2B shows receiver operating characteristic (ROC) curves for the four methods. It is clear that the outliers severely affect the F and Bartlett tests' ability to detect the true differentially variable features. The DiffVar tests always detect more true positives and fewer false positives than the F and Bartlett tests, with very little difference between absolute or squared deviations. The dip in the curves corresponding to the F and Bartlett tests shows that the outliers are ranked above truly differentially variable CpGs. DiffVar correctly ranks truly differentially variable features at the top of the list and is not affected by outliers.

Sample size considerations for testing differential variability

To estimate the group variances accurately, larger sample sizes are needed than for accurately estimating group means. We investigated a range of sample sizes that would enable reliable detection of differentially variable features. Detecting differential variability depends on sample size as well as how large the variability in one group is compared to the other. We assessed the effect of sample size for DiffVar in two ways. Firstly, through the use of simulations, and secondly, by subsetting the kidney cancer data set, which contains 283 cancer tumours and 160 normal samples.

Initially, we simulated 50,000 CpGs with 200 outliers and 5000 CpGs sites differentially variable between two groups with sample sizes of 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100. We simulated three scenarios with the 5000 differentially variable CpGs having twice, five times and ten times the variability in Group 2 compared to Group 1, and generated 1000 such data sets for each scenario. Total numbers of differentially variable features were assessed at 5% FDR cut-off for the four different methods, with the numbers of false discoveries and true discoveries recorded for each data set.

In addition, we used real data from the kidney cancer versus normal tissue data set to investigate the effect of sample size. We took a conservative approach to determining the true differentially variable CpGs by taking the intersection of the significant CpGs at 1% FDR using DiffVar (abs), the F test and the Bartlett test when analysing the full data set. We randomly sampled 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 samples for each group and re-analysed the subsetted data to see how many true differentially variable features were recovered at 5% FDR. To estimate the FDR, we classed any significantly differentially variable CpG not in the list of true differentially variable CpGs as a false discovery. We repeated the sampling procedure 100 times for each distinct sample size.

The results for the simulated and real data are displayed in Figure 2C,D,E,F and Additional file 2: Figure S1. Across all simulation scenarios a similar pattern emerges. While the F and Bartlett tests tend to have greater power to detect true differentially variable CpGs, this comes at a price of more false discoveries, particularly at small sample sizes (Figure 2C,D,E,F and Additional file 2: Figure S1). DiffVar (abs) and DiffVar (sq) control the FDR correctly at all samples sizes; however, DiffVar (sq) is conservative for sample sizes below 40 (Figure 2C,E and Additional file 2: Figure S1A,C). For data where the variability in Group 2 is roughly twice that of Group 1, larger sample sizes are needed to detect differentially variable CpGs (Additional file 2: Figure S1B). For data where the variability in Group 2 is roughly ten times that of Group 1, sample sizes as low as $n = 20$ show

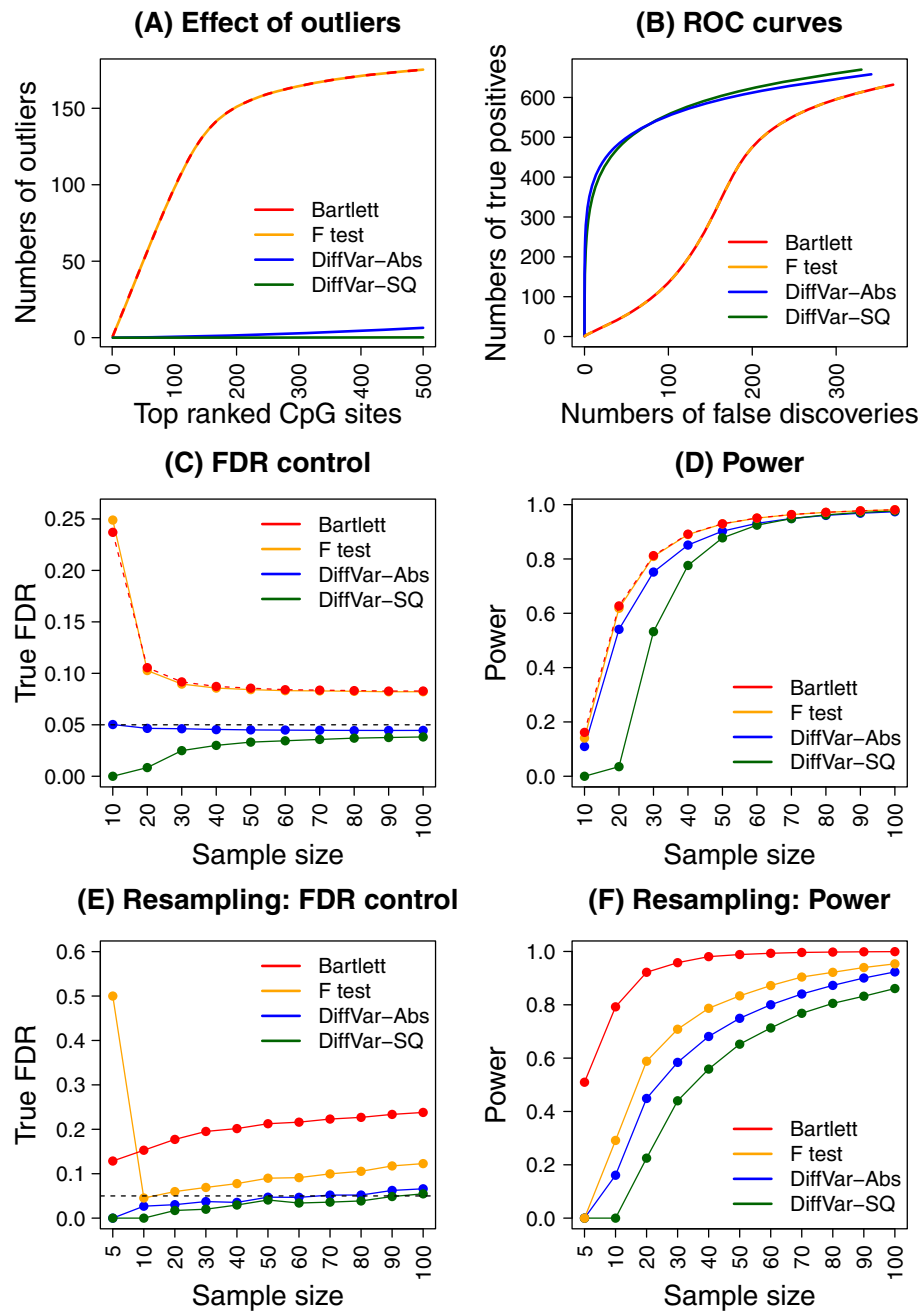


Figure 2 Performance of the four different methods using simulations. Results shown in plots (A-D) are averaged over 1000 simulated data sets and results shown in plots (E) and (F) are averaged over 100 resampled data sets at each sample size. (A) Cumulative numbers of differentially variable CpG probes containing outlier observations when ranking by the four different methods. There are no differentially variable CpGs simulated but 200 outliers are included in the data. (B) ROC curves when 200 outliers and 1000 differentially variable CpGs are present in the simulated data. (C) Control of the FDR of the four methods at a 5% nominal FDR cut-off (horizontal dashed black line) over ten different sample sizes. This simulation contains 1000 CpGs that are roughly five times more variable in Group 2 compared with Group 1. (D) The power to detect differentially variable features at ten different sample sizes when Group 2 is roughly five times more variable than Group 1. (E) Control of the FDR using resampled kidney cancer data sets at 11 different sample sizes. (F) The power to detect differentially variable features using resampled kidney cancer data sets at 11 different sample sizes. Abs, absolute deviations; FDR, false discovery rate; ROC, receiver operating characteristic; SQ, squared deviations.

more than 80% power to detect differential variability (Additional file 2: Figure S1D).

A similar story emerges when looking at the kidney cancer data. When $n = 5$, the Bartlett test is the only test to recover significant CpGs in the resampled data (Figure 2F); however, it shows inflated FDRs across all sample sizes (Figure 2E). DiffVar (abs) has almost perfect FDR control across all sample sizes; however, it lacks power when $n = 5$. A minimum sample size of 10 appears necessary to recover differentially variable CpGs (Figure 2F). For both the simulated and real data sets we find a trade-off between control of the FDR and power but DiffVar (abs) gives the best compromise with many fewer false positives.

Application to TCGA methylation data sets

Top differentially methylated and differentially variable CpG sites have little overlap

We analysed the kidney cancer, lung adenocarcinoma and prostate cancer versus normal tissue methylation data from TCGA (see Materials and methods for sample details). All samples were hybridised to Illumina's Infinium HumanMethylation450 arrays. The data were read into R using the *minfi* [18] and *illuminaio* [38] Bioconductor packages, the raw intensities normalised using SWAN [39] and M values and β values calculated. Differential methylation was assessed using moderated t -statistics on the M values using the *limma* [17] Bioconductor R package. Differential variability was determined using DiffVar with absolute deviations. Significantly differentially methylated CpGs were identified as those with FDR $< 5\%$ and difference in mean β at least 0.1. Significantly differentially variable CpGs were identified as those having FDR $< 5\%$, as well as having a variability ratio of at least 5. In other words, the magnitude of the variance in one group had to be at least five times the magnitude of the variance in the second group.

Under these criteria, there were 59,271 significantly differentially methylated CpGs in the kidney cancer data set, 77,588 in the lung cancer data set and 71,361 in the prostate cancer data set. The proportions of these that were hyper-methylated in cancer were 47% in the kidney cancer data set, 47% in the lung cancer data set and 70% in the prostate cancer data set (Figure 3A). DiffVar detected 109,529 differentially variable CpGs in the kidney cancer data set, 146,453 in the lung cancer data set and 22,001 in the prostate cancer data set (Figure 3B), with the vast majority of these more variable in cancers compared with normal samples (kidney: 99.9%, lung: 99.8% and prostate: 95%).

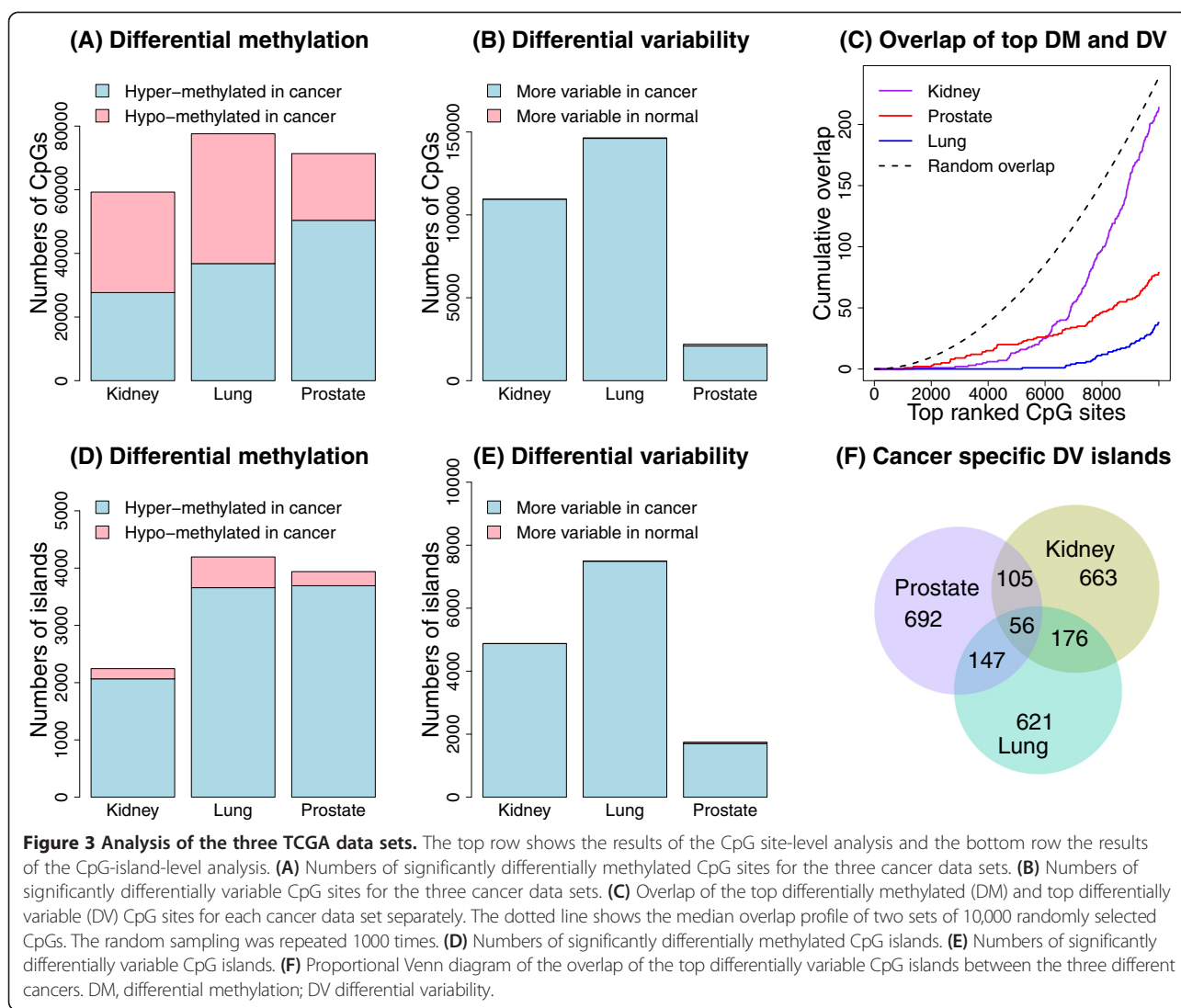
Restricting to only the most highly ranked significantly differentially methylated and differentially variable CpGs, we found very little overlap between the top-ranked 10,000 CpG sites (Figure 3C). This phenomenon was

observed across all cancer data sets, with the degree of overlap between differentially methylated and differentially variable CpGs lower than expected by chance (Figure 3C). Kidney cancer had the most overlap (2%) and lung cancer had the least overlap (0.5%). This implies that the most significantly differentially methylated and differentially variable CpG sites are different in the three cancer data sets analysed.

CpG islands are highly variable in cancer samples compared to normal tissue samples

We next investigated the genomic context of differentially variable probes. We found that the top differentially variable CpG sites were mostly in CpG islands. Furthermore there was a greater proportion of differentially variable sites in CpG islands compared to differentially methylated CpG sites. This was consistent across the three cancer data sets (Figure 4A, Additional file 2: Figures S2A and S3A). An interesting observation for the kidney cancer data set was that the CpG islands were over-represented among differentially variable CpG sites but under-represented among differentially methylated CpG sites. The lung and prostate cancer data sets had more CpG islands represented among the top-ranked differentially methylated CpG sites; however, there was always a greater proportion of CpG islands represented in the top differentially variable CpG sites. Hansen *et al.* [9] found increased variability of almost all CpG sites (islands, shores and distal to islands region) in their custom-made CHARM array. However, when analysing a publicly available colorectal cancer versus normal mucosa data from the Illumina Infinium HumanMethylation27 BeadChip array, they found that differentially variable CpGs were over-represented in sites far from islands and shores, and under-represented in islands. This contrasts with our findings, and may be due to differences in the genomic region composition of the different arrays, differences in sample size (22 matched colorectal cancer versus normal mucosa), differences in analysis strategy and potentially differences in tissue type.

This led us to examine the proportion of CpG sites in shores, shelves and open seas, based on University of California, Santa Cruz (UCSC), annotation (Figure 4B, C, D, Additional file 2: Figures S2B, C, D and S3B, C, D). A CpG that is not in an island, shore or shelf was classed as being in an open sea. In the kidney cancer data set there is a striking difference in the proportion of differentially methylated and differentially variable CpG sites in open seas (Figure 4B), with a large number of differentially methylated CpG sites appearing in these regions. There are fewer CpG sites in shelves and shores represented in the array, hence interpretation of these regions is more difficult. In the lung and prostate cancer data sets, the differences between top differentially methylated



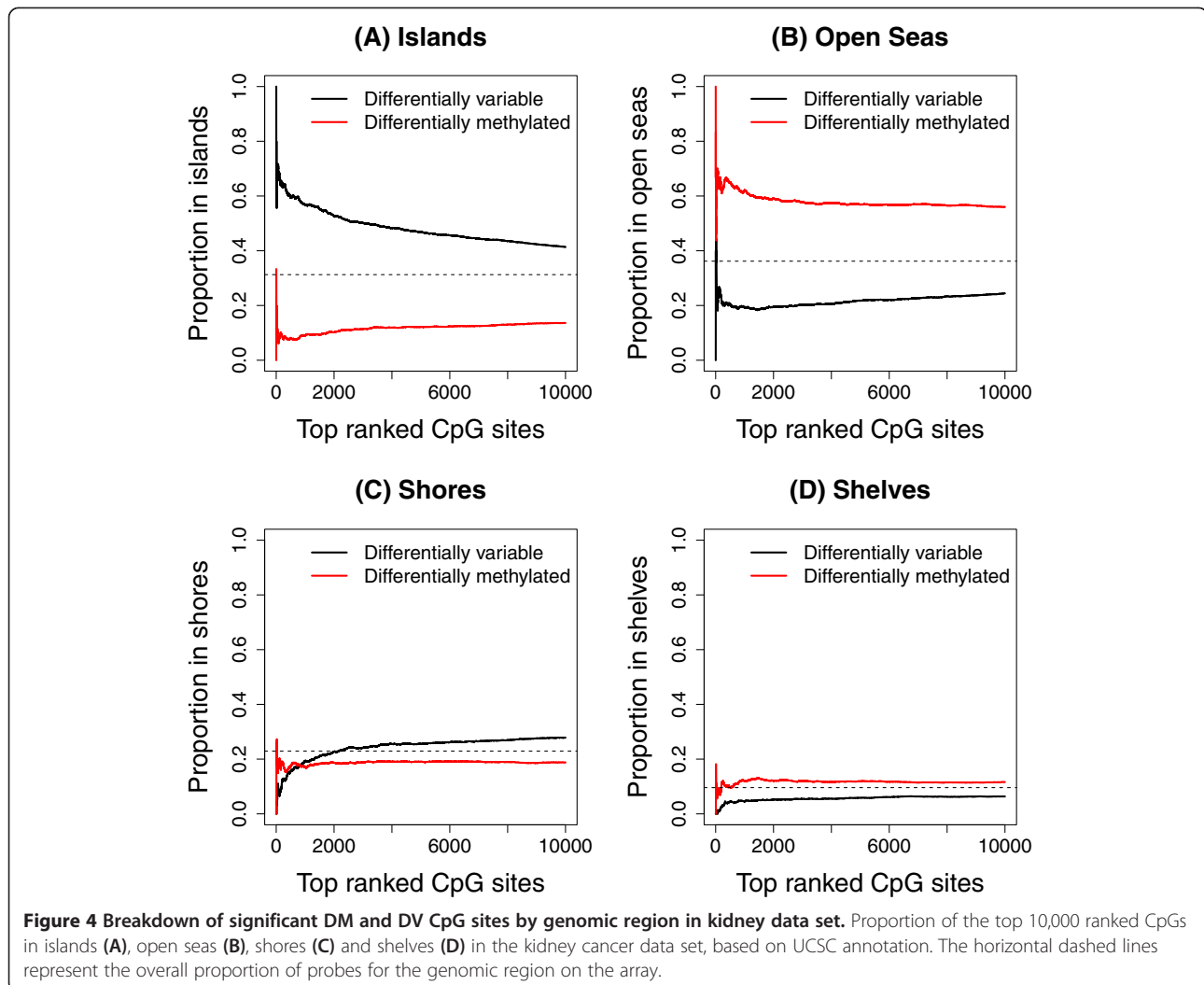
and top differentially variable CpG sites in shores, shelves and open seas are not as striking.

To gain additional insight into the differentially variable and differentially methylated CpG sites that are in open seas, we tested whether ENCODE-defined regulatory regions [40] were over- or under-represented (see Materials and methods for further details). Of the nine human cell types that have these regulatory regions characterised, normal lung fibroblasts are the only tissue relevant to any of our data sets. For the significantly differentially variable CpGs in open seas in the lung cancer data set, we found that strong enhancers, weak enhancers, active promoters and weakly transcribed regions were over-represented, and heterochromatin/low signal regions were under-represented. For the significantly differentially methylated CpGs in open seas, strong enhancers and active promoters were over-represented and heterochromatin/low signal

regions were under-represented. The results are displayed in Additional file 3: Table S1.

Top differentially variable CpG islands tend to differ between cancer types

Due to the interesting finding that the differentially variable CpG sites are over-represented in CpG islands, we proceeded with a CpG-island-level analysis by averaging the intensities of the CpG sites across each CpG island. Just over 30% of the probes on the array interrogate CpG islands, with 25,744 unique islands represented. Even though between individuals the CpG sites are highly variable, within an individual, the M values of the CpG sites in a CpG island are highly correlated (Additional file 2: Figure S4). There were 2245 significantly differentially methylated CpG islands in the kidney data set, 4195 in the lung data set and 3939 in the

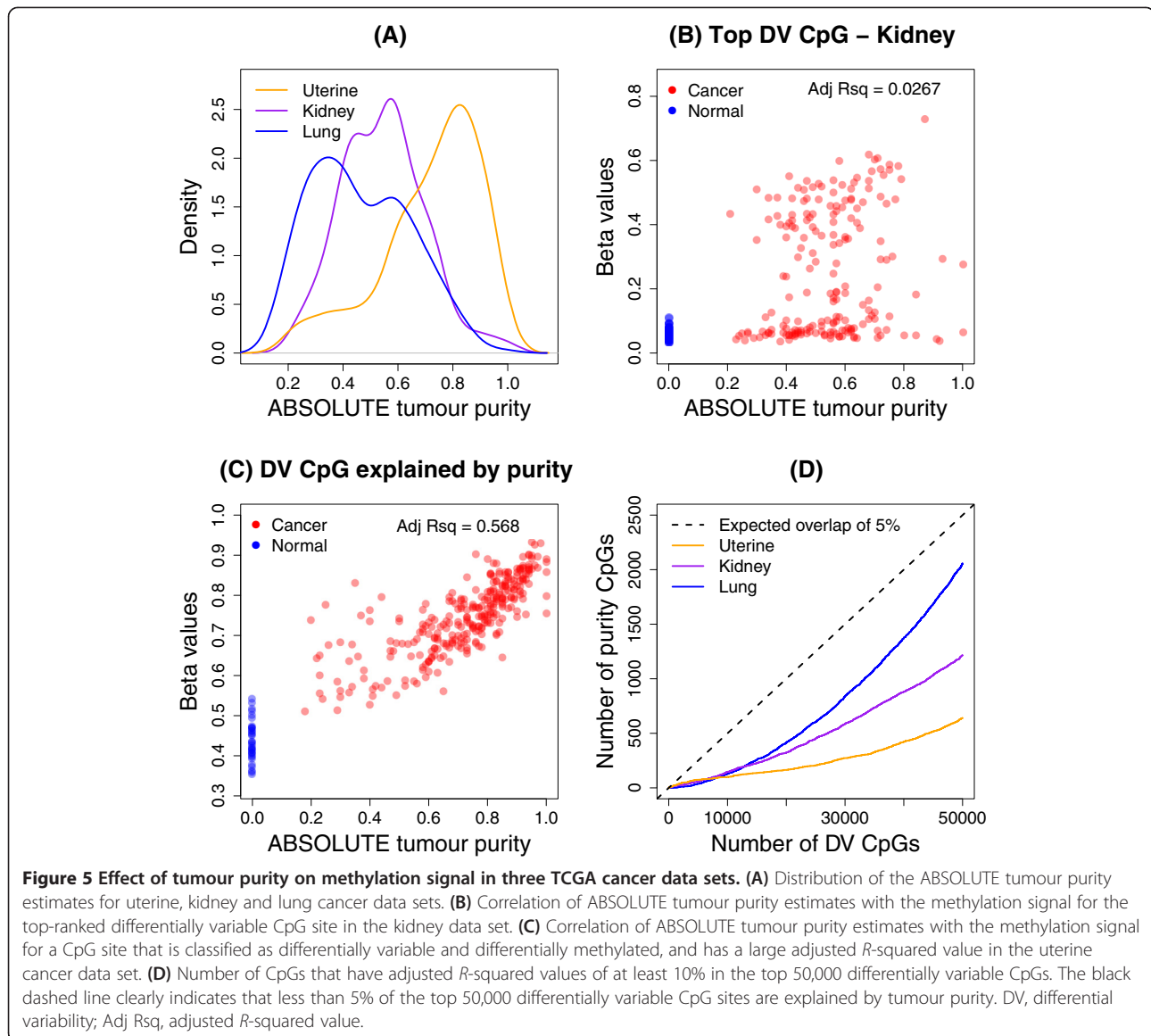


prostate data set. The majority of the significant CpG islands were hyper-methylated in cancer compared to normal tissue (kidney: 92%, lung: 87% and prostate: 94%, Figure 3D). There were 4877 significantly differentially variable CpG islands in the kidney data set, 7495 in the lung data set and 1745 in the prostate data set. We found an even greater proportion of CpG islands were more variable in the cancers compared to normal tissue (kidney: 99.9%, lung: 99.9% and prostate: 98%, Figure 3E). The top 1000 differentially variable CpG islands showed a trend of being unmethylated in the normal samples and becoming methylated in the cancer samples in the kidney data set (Additional file 2: Figure S5). These CpG islands tended to differ between cancer types (Figure 3F). This agrees with the findings by Fernandez *et al.* [10], who showed that the DNA methylation profile is specific to tumour type over 1054 cancer samples, with the tumour profiles characterised by a progressive gain of methylation in CpG-island-associated promoters. In our data sets,

kidney and lung cancer had the most differentially variable CpG islands in common (20%), but across all three cancer data sets the overlap was minimal (approximately 5%).

Infiltrating cells as a cause of CpG variability between cancer samples

One explanation of the increased variability seen in cancer samples is that cancer cells are frequently infiltrated with normal cells. To address this as the underlying cause of differential variability, we performed a thorough analysis relating the methylation levels and tumour purity of the cancer samples in three different cancer data sets (kidney, lung and uterine). We included an extra data set from TCGA (uterine corpus endometrial carcinoma) to get a better understanding of differentially variable CpGs that have the tumour methylation signal explained by tumour purity in low (lung), medium (kidney) and high (uterine) tumour purity contexts (Figure 5A). A differential variability analysis of the uterine data set



had 101,262 CpGs significantly more variable in the cancer samples compared to the normal samples, and 773 CpGs significantly more variable in the normal samples compared to the cancer samples. The proportions of the top 10,000 differentially methylated and differentially variable CpGs in islands, open seas, shores and shelves was similar to the kidney, lung and prostate cancer data sets, with a larger proportion of differentially variable CpGs in CpG islands (Additional file 2: Figure S6).

Carter *et al.* [41] have developed and applied an algorithm (ABSOLUTE) to estimate tumour purity for a number of publicly available data sets, including 11 TCGA cancer data sets, although purity estimates for the prostate cancer samples are not available. There are ABSOLUTE estimates available for 196 samples in the

kidney cancer data set, 236 in the lung data set and 297 samples in the uterine cancer data set. Figure 5A shows a density plot of the ABSOLUTE purity estimates for three cancer data sets: lung (median purity = 0.44), kidney (median purity = 0.56) and uterine (median purity = 0.76).

To assess the effect of tumour purity on methylation levels, we fitted a linear model with methylation level as the response variable and tumour purity as the explanatory variable for the cancer samples only. The adjusted *R*-squared values, which can be interpreted as the proportion of the variation in the methylation signal that is explained by tumour purity, were obtained for each CpG in each cancer data set. We classed CpGs that had an adjusted *R*-squared value greater than 10% to be the CpGs that had the tumour methylation signal best

explained by the purity of each cancer sample. For example, for the top differentially variable CpG in the kidney cancer data set, Figure 5B shows that there is no strong evidence of a relationship between methylation signal and tumour purity (adjusted R -squared = 0.0267). By contrast, Figure 5C shows the relationship for a CpG (cg08395122) that is identified as differentially variable (FDR = 0.0000294, variability ratio = 6.14), differentially methylated (FDR = 2×10^{-41} , $\Delta\beta$ = 0.3), and has a high adjusted R -squared value (0.568) in the uterine cancer data set. For the CpG in Figure 5C, it is clear that the variability between cancer samples is mostly explained by the tumour purity.

The numbers of CpGs on the array that had more than 10% of variability explained by purity were 29,424 for kidney cancer, 47,353 for lung cancer and 25,832 for uterine cancer. However, most of these were not detected as differentially variable between cancer and normal tissue. Of the significantly differentially variable CpGs, 3635 CpGs (3.3%) had at least 10% of the variation explained by tumour purity in kidney, 14,986 (10.2%) in lung and 2745 (2.7%) in uterine cancer. Figure 5D shows the cumulative number of CpGs with at least 10% of the variation explained by tumour purity in the top 50,000 most significantly differentially variable CpGs. The lung cancer samples, which have the lowest median tumour purity, have the largest overlap between top differentially variable CpGs and CpGs with at least 10% of the variation explained by tumour purity, while the uterine cancer samples, which have the highest median tumour purity, show the least overlap.

Application to aging methylation data set

To study differential variability in aging, we used data from Heyn *et al.* [2] to compare methylation in 19 centenarians to 19 newborns. We followed the same analysis strategies used for the cancer data sets to determine significantly differentially methylated and differentially variable CpG sites between centenarian and newborn samples (see Materials and methods). Of the 31,805 significantly differentially methylated CpGs, 9130 (29%) were hyper-methylated in centenarians and 22,675 (71%) were hypo-methylated (Figure 6A). We also detected 34,680 significantly differentially variable CpGs. Intriguingly, although perhaps unsurprisingly, 97% of these were more variable in the centenarians than in the newborns (Figure 6A).

Comparing the 10,000 top-ranked significantly differentially methylated and top-ranked 10,000 differentially variable CpGs, we observed that there was less overlap than expected by chance (Figure 6B). Of the 31,805 total significantly differentially methylated CpGs and 34,680 total significantly differentially variable CpGs, there were 5978 CpGs in common. Of the common CpGs, 5842

(98%) were more variable in centenarians than newborns, with 47% hypo-methylated and 53% hyper-methylated.

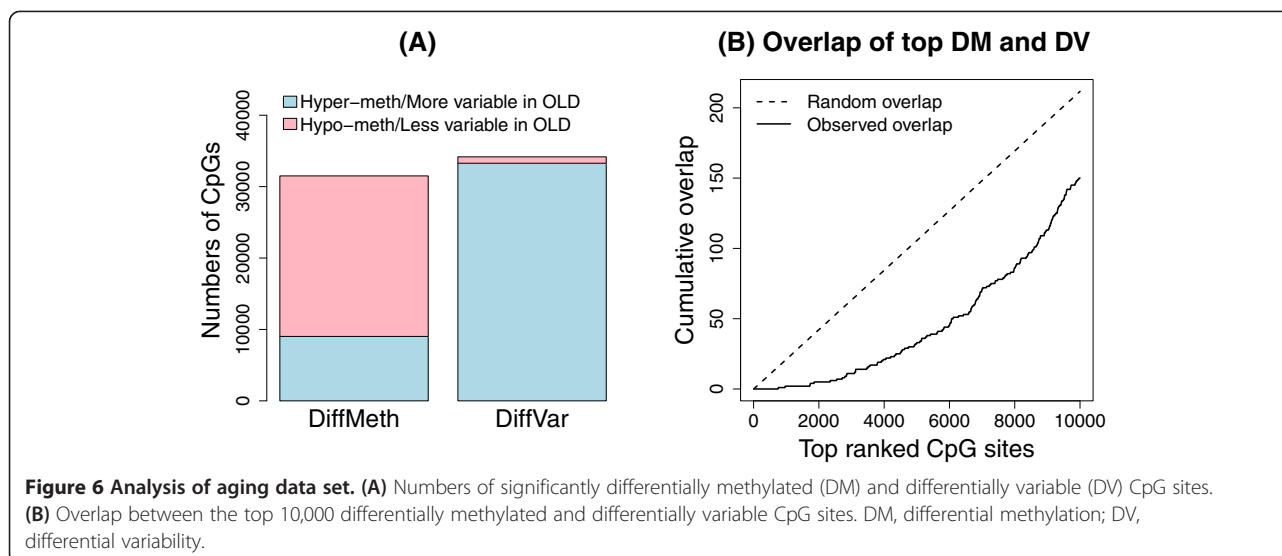
A closer look at the genomic composition of the significantly differentially methylated and differentially variable CpGs revealed that there were more islands and shores represented in the top-ranked 10,000 differentially variable CpGs compared to differentially methylated CpGs (Additional file 2: Figure S7A,C). There were more CpGs in open seas and shelves in the top differentially methylated CpGs than in the top differentially variable CpGs (Additional file 2: Figure S7B,D).

The CpGs that were significantly more variable in the centenarians compared to newborns corresponded to 7068 genes and the CpG sites that were more variable in newborns compared to centenarians corresponded to 484 genes (UCSC annotation). We performed a *GOstats* analysis [42], testing for over-representation of gene ontology (GO) terms amongst the genes associated with differentially variable CpGs. For the CpGs that are more variable in centenarians compared to newborns, many GO categories relating to development were significant (Additional file 4: Table S2, P value cut-off = 0.0001). Far fewer GO terms were significant when testing the genes associated with CpGs that were more variable in newborns compared to centenarians; however, several GO terms related to the major histocompatibility protein complex were significant, as well as a leukocyte-mediated immunity GO term (Additional file 4: Table S2).

Discussion

In this paper we present DiffVar, a new method to detect differential variability between groups of samples. DiffVar is freely available as an R function in the *missMethyl* Bioconductor package. Our test is based on Levene's z -test for variances and incorporates an empirical Bayes modelling framework to deal appropriately with high-dimensional data issues. We find the test holds its size, is robust to outliers and outperforms the F and Bartlett tests in terms of controlling the FDR. For smaller sample sizes, the F and Bartlett tests are more powerful; however, this comes at a price of higher FDRs. DiffVar with absolute deviations represents the best compromise between controlling the FDR and having the power to detect differential variability.

To estimate the variance of a group reliably, more observations are needed than for estimating a mean. We found that for group sizes as low as ten in the kidney cancer data set, there was adequate power to detect differential variability, with the power increasing dramatically up to group sizes of 50 arrays. However, detection of differential variability is also dependent on how large the variability in one group is relative to the other. In



addition to a FDR cut-off, one can specify a threshold on the variability ratio, which is the ratio of the variance in one group compared to the variance in the second group. For example, when analysing the cancer and aging data sets, in addition to the FDR cut-off of 5%, we required a variability ratio of at least five between the two groups for a CpG to be called significantly differentially variable.

We applied our differential variability testing method to several cancer data sets from TCGA as well as a publicly available aging data set. For the cancer data sets, we found that a large proportion of sites showed differential variability and, as expected, were more variable in cancer compared with normal tissue. For the aging data set, we found that a large proportion of CpG sites were more variable in centenarians than newborns. Interestingly, for the top-ranked CpGs in both the cancer and aging data sets, the overlap between differentially variable and differentially methylated CpGs was less than expected by chance.

In all cancer data sets we showed that CpG islands were enriched for differential variability. This is important because methylation of CpG islands in cancer cells is known to contribute to gene silencing [43]. CpG sites that are differentially variable may reflect a loss of epigenetic control that could contribute to tumorigenesis. Another hypothesis is that differentially variable CpG sites capture the heterogeneity between patients whose tumours arose due to the disruption or activation of different biological pathways. For some cancer patients, the methylation status of a differentially variable CpG site is similar to the methylation status in a normal cohort, while for other patients the same CpG site shows aberrant methylation. This type of analysis has the potential to be a starting point to identify clusters of patients

whose tumours arose via similar molecular mechanisms. For the cancer data sets analysed in this paper, we found that only a small proportion of differentially variable CpGs could be explained by tumour purity.

In this paper, we have focused on DNA methylation data that have been generated using Illumina's Infinium HumanMethylation450 BeadChip. DiffVar can also be applied to DNA methylation sequencing data or any set of β or M values from CpG sites or regions. The DiffVar function will transform a matrix of β values into M values by applying a logit transformation. In general, the DiffVar method for testing differential variability can be applied to any 'omics data that use the *limma* pipeline for analysis, such as microarray expression data or RNA-sequencing data. For RNA-sequencing data, DiffVar will perform a *voom* transformation [44] before testing for differential variability. The DiffVar modelling framework has the added benefit of giving the user access to other tools in the *limma* package, for example, testing relative to a threshold (TREAT [45]), as well as the gene set testing functions ROAST [46] and CAMERA [47].

Conclusions

DiffVar is a novel method to test for differential variability in high-throughput genomics data such as methylation arrays or gene expression data. We have shown that DiffVar controls the FDR while being robust to outliers. DiffVar is available as an R function in the *missMethyl* Bioconductor package.

Materials and methods

Illumina Infinium HumanMethylation450 BeadChip

The Illumina Infinium HumanMethylation450 BeadChip allows for the simultaneous measurement of 482,421 CpG sites. There are two types of probes: Infinium I,

which was previously used on the Infinium HumanMethylation27 array and constitutes 135,501 probes; and Infinium II, which is the rest of the probes. The Infinium I design has two probes that measure the methylated and unmethylated states, respectively, whereas the Infinium II design has a single probe that can detect whether a CpG site is methylated or unmethylated. A thorough description of the array design is available in Maksimovic *et al.* [39]. The resulting data are similar to two-colour microarray gene expression data; there is an intensity measurement for both the methylated and unmethylated channels for each CpG site. The main difference is that the dynamic range for expression data is different from the dynamic range for methylation data. SWAN normalisation can be performed on the Infinium I and Infinium II probes within each array to reduce the technical biases inherent in the probe design before statistical analysis [39]. Once normalisation has been performed, β values for each CpG site can be computed as the ratio of the methylated intensity versus the combined methylated plus unmethylated intensity. Du *et al.* [34] recommend the use of M values for statistical analysis, which are calculated as the \log_2 ratio of methylated versus unmethylated intensities. A small offset can be added to the numerator and denominator to stabilise the M values. M values with an offset of 100 for both the numerator and denominator are used in all statistical analyses reported in this paper.

Data sets

Throughout the paper we demonstrate our method with publicly available data sets generated by the TCGA research network [48], as well as an aging data set, all of which had methylation profiled using the Illumina HumanMethylation450 arrays. In all TCGA data sets used, the cancer samples are from solid tumours and the normal samples are from solid normal tissue. The raw data in the form of idat files (called level 1 data on the TCGA website) for the Methylation450 platform were downloaded from the TCGA data portal [49] and sample annotation was downloaded from the Biospecimen Metadata Browser [50] by specifying “Analyte” as the element and “DNA” as the analyte. The clear cell kidney carcinoma versus normal tissue data set (KIRC) has 160 normal samples and 283 cancer samples. The lung adenocarcinoma data set (LUAD) has 427 cancer samples and 31 normal samples. The prostate adenocarcinoma data set (PRAD) has 194 cancer samples and 49 normal samples. The uterine corpus endometrial carcinoma data set (UCEC) has 423 cancer samples and 34 normal samples. The specific sample identifiers and other sample information can be found in Additional file 5: Table S3, Additional file 6: Table S4, Additional file 7: Table S5 and Additional file 8: Table S6. For the

aging data set, methylation profiles were generated from 19 healthy male centenarian peripheral blood samples and 19 male newborn umbilical cord blood samples. The data are available for download from the Gene Expression Omnibus [GEO:GSE30870].

Data analysis

The data were read into R from raw idat files using the *minfi* [18] and *illuminaio* [38] packages. For the cancer data sets, CpG sites on the X and Y chromosomes were filtered out. An additional filter was applied to all data sets where CpG sites that had detection $P > 0.01$ in one or more samples were excluded from further analysis. This resulted in 445,378 CpG sites for the kidney cancer analysis, 419,031 CpG sites for the lung cancer analysis, 448,145 CpG sites for the prostate cancer analysis, 421,795 CpG sites for the uterine cancer analysis and 483,615 CpG sites for the aging analysis. The raw intensities were SWAN normalised [39], and finally β and M values were extracted. Statistical analysis was performed on the M values. Differentially methylated CpG sites were determined using moderated t -statistics from the *limma* package [17]. Significantly differentially methylated CpGs were defined as having FDR $< 5\%$ and a difference in mean β level of at least 0.1 between the two groups. Differential variability was assessed using DiffVar with absolute deviations from the *missMethyl* R package. Significantly differentially variable CpGs were defined as having FDR $< 5\%$ and a ratio of group variances of at least 5. Testing for enrichment of GO categories in the aging data set was performed using the R *GOstats* package [42]. The R code for the analysis is available in Additional file 9. The tables of significantly differentially methylated and differentially variable CpGs are available from the authors on request.

Simulations

To check the performance of our method, we generated data sets where the truth is known to gain insight into the type I error rate and FDR control. Data were generated under a hierarchical model whereby the variance for each CpG was first sampled from an inverse chi-square distribution and the M value for each CpG was sampled from a normal distribution with variance equal to the simulated variance. A two-group problem with 50 arrays in each group and 10,000 CpGs was generated in this manner. Then 1000 data sets with no differential variability were simulated by first sampling variances for each CpG site from a scaled inverse chi-square distribution with scaling factor $d_0 s_0^2$ and degrees of freedom d_0 such that the two groups have equal variance. We chose $d_0 = 20$, which represents the scenario where the observed variances are shrunk $20/(50 + 20) = 0.29$ towards the prior variance. In other words, more weight is placed

on the observed variance during the empirical Bayes shrinkage step. We chose $s_0^2 = 0.64$, which is slightly more variable than the hyper-parameter estimates obtained when analysing the larger TCGA cancer data sets (kidney: 0.26, lung: 0.11 and prostate: 0.16). The M values were generated to mimic methylation data by randomly sampling half the CpGs from a normal distribution with mean equal to -2 and variance equal to the sampled variance for the unmethylated CpGs; the remainder, representing methylated CpGs, had M values sampled from a normal distribution with mean equal to 2 and variance equal to the sampled variance. The range of M values this simulation scenario produced is shown in Additional file 2: Figure S8A.

Type I error rates were also assessed using the M values of the non-diseased samples from the kidney cancer data set. First 1000 data sets were generated by randomly sampling 10,000 of the 445,378 CpGs, followed by randomly selecting 100 normal samples, of which 50 were allocated to the normal group and 50 were allocated to the cancer group. Theoretically, for each CpG, the two groups should have equal variance, although additional factors such as inter-individual heterogeneity and outliers will make the data more variable. The sampling strategy should preserve any correlation structure between the CpG sites. The range of M values this produced for one such data set is shown in Additional file 2: Figure S8B.

To assess the impact of outliers, we modified the simulations to include outliers for 200 CpG sites. For each of these 200 CpGs, the M value for one simulated sample was replaced with the maximum simulated M value over all 10,000 CpGs. We introduced differential variability for another 1000 CpGs by simulating larger variances for one of the groups by sampling from a scaled inverse chi-square distribution with scaling factor $d_0 s_0^2$ and degrees of freedom d_0 , where $d_0 = 20$ and $s_0^2 = 1.5$. On average, the variability of the 1000 CpGs in the second group is $1.5/0.64 = 2.34$ times larger than the variability in the first group.

Differential variability was assessed using our method DiffVar, the F test for equality of variances and the Bartlett test. All three tests had two-sided P values computed. Type I error rates and FDRs were calculated and averaged over the 1000 simulations in each simulation scenario.

The simulations to assess the effect of sample size were modified slightly by increasing the numbers of simulated CpGs to 50,000 and allowing 5000 CpGs to be differentially variable. Three different levels of variability were considered: Group 2 was roughly twice, five times and ten times more variable than Group 1. A two-group problem with varying sample size ($n = 10, 20, 30, 40, 50, 60, 70, 80, 90$ and 100) was simulated with 1000 data sets generated for each distinct sample size. The

numbers of truly differentially variable CpGs recovered at a 5% FDR and the numbers of false discoveries were recorded for each sample size. Additional file 10 shows the R code for the simulations. For the subsetting and resampling of the kidney cancer data set, we defined a set of true positives and true negatives in the following way. CpG sites that were differentially variable at 1% FDR using DiffVar (abs), the Bartlett test and F test when analysing the full data set were classed as true positives. All other CpG sites were classed as true negatives. The FDR for each subsetted data set was estimated by counting the number of true negatives that were significantly differentially variable and dividing by the total number of significantly differentially variable CpG sites at a 5% FDR cut-off. Power for each subsetted data set was calculated by counting the number of true positives that were significantly differentially variable at 5% FDR and dividing by the total number of true positives.

Overlap analysis of ENCODE regulatory regions

Ernst *et al.* [40] used a hidden Markov model to segment the genome and computationally predict functional elements for nine human cell types, using chromatin immunoprecipitation sequencing data for nine factors plus input. The chromatin state segmentation annotation is available for download from the ENCODE website [51]. The only relevant tissue where annotation is available is normal lung fibroblasts. We determined whether CpG sites that were significantly differentially variable and in open seas (as opposed to being in islands, shores or shelves), lay in regions that have predicted functional elements using an intersectBed analysis of the lung cancer data set. We calculated the total number of each functional element represented on the array by taking all CpGs in open seas and performing an intersectBed analysis. We then calculated the probability of each functional element being over- or under-represented in the list of significantly differentially variable and differentially methylated CpGs separately using a hypergeometric test. The P values were adjusted for multiple testing using Holm's method [52].

Infiltrating cells analysis

ABSOLUTE tumour purity estimates for the kidney, lung and uterine cancer data sets were downloaded from the Synapse website [53]. Adjusted R -squared values were used to determine the proportion of variation in the tumour methylation signal explained by the tumour sample purity. Adjusted R -squared values were extracted from a linear model fit, regressing the methylation signal on the ABSOLUTE tumour sample purity estimates of the cancer samples for all CpG sites. This was performed in R using the *lm* function. CpG sites that had adjusted R -squared values of more than 10%

were classed as CpGs that had methylation signal explained by tumour purity.

missMethyl Bioconductor package

The DiffVar method is available from Bioconductor in the *missMethyl* package [54].

Additional files

Additional file 1: Statistical model details of DiffVar.

Additional file 2: Additional figures S1–S8.

Additional file 3: Table S1. Results for testing over- and under-representation of ENCODE-defined regulatory regions in significant DM and DV CpGs in open seas in the lung cancer data set.

Additional file 4: Table S2. *GStats* analysis performed on the aging data.

Additional file 5: Table S3. Sample information for the kidney cancer data.

Additional file 6: Table S4. Sample information for the lung adenocarcinoma cancer data.

Additional file 7: Table S5. Sample information for the prostate cancer data.

Additional file 8: Table S6. Sample information for the uterine cancer data.

Additional file 9: R code for analysing the cancer and aging data sets.

Additional file 10: R code for the simulations.

Abbreviations

DM: differential methylation; DV: differential variability; FDR: false discovery rate; GO: gene ontology; ROC: receiver operating characteristic; TCGA: The Cancer Genome Atlas; UCSC: University of California, Santa Cruz.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

AO conceived the idea. BP performed the analysis and wrote the code. AO and BP wrote the paper. Both authors read, edited and approved the final manuscript.

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