PRISM: methylation pattern-based, reference-free inference of subclonal makeup

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Abstract

Motivation: Characterizing cancer subclones is crucial for the ultimate conquest of cancer. Thus, a number of bioinformatic tools have been developed to infer heterogeneous tumor populations based on genomic signatures such as mutations and copy number variations. Despite accumulating evidence for the significance of global DNA methylation reprogramming in certain cancer types including myeloid malignancies, none of the bioinformatic tools are designed to exploit subclonally reprogrammed methylation patterns to reveal constituent populations of a tumor. In accordance with the notion of global methylation reprogramming, our preliminary observations on acute myeloid leukemia (AML) samples implied the existence of subclonally occurring focal methylation aberrance throughout the genome.

Results: We present PRISM, a tool for inferring the composition of epigenetically distinct subclones of a tumor solely from methylation patterns obtained by reduced representation bisulfite sequencing. PRISM adopts DNA methyltransferase 1-like hidden Markov model-based in silico proofreading for the correction of erroneous methylation patterns. With error-corrected methylation patterns, PRISM focuses on a short individual genomic region harboring dichotomous patterns that can be split into fully methylated and unmethylated patterns. Frequencies of such two patterns form a sufficient statistic for subclonal abundance. A set of statistics collected from each genomic region is modeled with a beta-binomial mixture. Fitting the mixture with expectation-maximization algorithm finally provides inferred composition of subclones. Applying PRISM for two AML samples, we demonstrate that PRISM could infer the evolutionary history of malignant samples from an epigenetic point of view.

Availability and implementation: PRISM is freely available on GitHub (https://github.com/dohlee/prism).

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Supplementary information: Supplementary data are available at Bioinformatics online.

1 Introduction

The concept of clonal evolution in cancer (Nowell, 1976) has revolutionized our understanding of cancer biology throughout various subjects including progression of cancer (Merlo et al., 2006), recurrence (Yates et al., 2017), metastasis (Gundem et al., 2015; Turajlic and Swanton, 2016; Yates et al., 2017) and treatment response (Almendro et al., 2014; Kreso et al., 2013). Evolution of the cancer subclones often results in a tumor composed of several genetically or epigenetically distinct subclones. Therefore, the subclonal diversity arising from clonal evolution has long been acknowledged as one of the prominent causes of intratumor heterogeneity (ITH). ITH intuitively reflects the adaptive capacity of a tumor to survive changing conditions. Thus, its utility as a biomarker predicting the aggressiveness of a tumor has been widely studied.

Next-generation sequencing (NGS) has offered us an excellent opportunity to interrogate ITH at an unprecedented resolution. Accordingly, there have been several important approaches to define ITH measures in various omics level, including genomic (Mroz and Rocco, 2013), transcriptomic (Park et al., 2016) and methylomic (Landau et al., 2014) level. These ITH measures were proven to have remarkable clinical potentials. However, the best precision and utilization of ITH can be achieved through the direct characterization of cancer subclone itself. Formally, this problem of characterizing constituent subclones of a bulk tumor only from its molecular
signature is often referred to as ‘subclonal inference’. A critical application of subclonal inference is the reconstruction of the evolutionary history of a tumor (Gerlinger et al., 2012), which harbors great potential for the precision medicine when exploited (Hiley et al., 2014). Most existing methods for subclonal inference such as ABSOLUTE (Carter et al., 2012), THetA2 (Oesper et al., 2014), SciClone (Miller et al., 2014) or PyClone (Roth et al., 2014) utilize prevalence of a subclonal genomic variation including somatic copy number alteration (CNA) or single nucleotide variant (SNV) as a proxy of subclonal abundance.

Meanwhile, clonal evolution accompanied by the genome-wide, dynamic reprogramming of DNA methylation has received increasing attention in recent years (Brocks et al., 2014; Ferrando and Lopez-Ortin, 2017; Li et al., 2016; Mazor et al., 2016), inspiring researchers to develop methods to inspect the methylicom evolution in cancer (Barrett et al., 2017; Li et al., 2014). However, applications of those methods are limited to detecting methylation patterns that are likely to have undergone subclonal expansion, and none of them directly aims to uncover the subclonal population structure of a tumor sample, thereby enabling the inference of methylome-based evolutionary tree of the subclones. Therefore, we present a bioinformatic tool named PRISM as a solution for the epigenetic subclonal inference problem. Motivated by the strategy taken by mutation-based subclonal inference algorithms, which takes advantage of the dichotomousness of variant and reference alleles, PRISM tackles the problem by focusing on the particular genomic region harboring dichotomous groups of methylation patterns, namely fully methylated and unmethylated patterns.

2 Problem formulation and approach

2.1 Terminologies used in this research

In this section, we define terminologies used throughout the research. To help understand the terminologies, a schematic illustration is in Figure 1A. We define an epigenetic subclone as a clonal population of cells harboring distinct regional methylation patterns that exclusively belong to that subclone. Also, the exclusive subclonal methylation pattern will be referred to as fingerprint methylation pattern or fingerprint pattern for convenience. We defined an epilocus as a short genomic region (typically ~100 bp) at which a group of reduced representation bisulfite sequencing (RRBS) reads was mapped, and subsequently a fingerprint epilocus is defined as an epilocus harboring fingerprint pattern. From each fingerprint epilocus, a fraction of sequencing reads supporting fingerprint pattern can be calculated and this fraction will be denoted as fraction of fingerprint pattern (FF) (Fig. 1C). This value will serve as an estimator of subclonal abundance harboring the particular fingerprint patterns, and will be used for the intuitive illustration of the core algorithm of PRISM.

2.2 Overview of algorithm

The main approaches taken by PRISM are based on the following notions. Each of them is separately discussed in the upcoming sections.

1. The applicability of PRISM heavily depends on the existence of fingerprint epiloci, which indeed is not a well-established conception. A growing body of evidence for the role of genome-wide DNA methylation reprogramming in cancer and our empirical observations on fingerprint epiloci justify the existence of fingerprint epiloci. Consequently, epigenetic subclones, if they exist, can be traced with a sufficient number of fingerprint methylation patterns.

2. Before the PRISM analysis, methylation patterns undergo in silico proofreading which corrects for the erroneous methylation states. It is based on DNA methyltransferase 1 (DNMT1)-like hidden Markov model (HMM) that is designed to mimic the DNA methylation maintenance process of DNMT1. Notably, in silico proofreading serves as an effective calibrator of estimated subclonal abundance, and it also increases the number of fingerprint epiloci enough for inferring epigenetic subclones as shown in Section 4.1.

3. The estimate of subclonal abundance is drawn from individual ‘one-versus-all the other’ binary pattern decomposition problem for each fingerprint epilocus. If there are k epigenetic subclones, these subclonal abundance estimates will be clustered around k genuine subclonal abundances. Thus, the problem can be viewed as a k-mixture decomposition problem, which is modeled as a beta-binomial mixture and solved by the expectation-maximization (EM) algorithm.

2.2.1 Existence of fingerprint epiloci

Using the prevalence of subclonal variant as an estimate of subclonal abundance has been a successful strategy for mutation-based subclonal inference (Miller et al., 2014; Roth et al., 2014). PRISM adopts a similar approach, by considering methylation fingerprint patterns as subclonal variants. Therefore, justifying the existence of fingerprint epiloci is crucial for the feasibility of PRISM.

Our point of view on the epigenetic subclonal inference problem is shown in Figure 1A. Aberration of methylation in cancer is characterized by genome-wide hypomethylation, as well as focal hypermethylation at regions including CpG islands (Baylin and Jones, 2011). Also, the global alterations of DNA methylation have been extensively studied in cancer, especially in leukemias (Heller et al., 2016; Oakes et al., 2016). Although defective epigenetic regulators are thought to affect DNA methylation reprogramming, the precise mechanism of the phenomenon still needs to be clarified. Nevertheless, accumulating evidence suggests that the methylation landscape of cancer evolves under selective pressure (Mazor et al., 2016), implying that epigenetic subclones expand due to the increased fitness conferred by reprogrammed methylomic profile. Given the existence of epigenetic subclones, we postulated that distinct methylation patterns (e.g. regional hypermethylation) that uniquely define the subclone could be found by scrutinizing their methylation profiles. Therefore, the region with those distinct methylation patterns (i.e. fingerprint epilocus) became the principal unit of analysis in PRISM workflow because it could be treated as evidence of epigenetic subclone.

Furthermore, we have encountered some empirical observations on fingerprint epiloci in several, if not all, clinical samples from acute myeloid leukemia (AML) patients. Our preliminary analysis revealed that the fingerprint epiloci are uniformly distributed throughout the genome (Supplementary Fig. S1), thereby supporting our supposition that fingerprint epiloci arise from global DNA methylation reprogramming. More concrete examples of these findings are going to be discussed in Section 4.3.

2.2.2 In silico proofreading of methylation pattern based on DNMT1-like HMM

DNA methylation data sequenced by RRBS protocol suffers from diverse sources of errors such as the relatively high error rate of DNMT1 that is responsible for DNA methylation maintenance, or
incomplete bisulfite conversion. Meanwhile, PRISM utilizes the exact count of specific methylation patterns, which is vulnerable to even small amounts of biased errors. Thus, techniques used for subclonal inference at mutation level (Miller et al., 2014; Roth et al., 2014) are not directly applicable to PRISM. Motivated by the successful application of in silico error correction algorithm of DNA sequencing data for fragment assembly (Pevzner et al., 2001), we developed a novel in silico proofreading algorithm for methylation patterns to overcome this difficulty.

Computational modeling of DNMT1 enzymology is at the core of our methylation error correction algorithm (Figs 1B and 2A). We called this model as DNMT1-like HMM. It is indeed a generative model, which produces approximate copies of template methylation pattern that may contain some errors in them (Fig. 2B). Having established the generative model for DNA methylation maintenance, conversely, we can infer the template methylation pattern from the observed erroneous methylation patterns (Fig. 2B). This inference constitutes the essence of our error correction algorithm which is referred to as in silico proofreading of methylation patterns.

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Fig. 1. Workflow of PRISM. (A) The concept of fingerprint epilocus. White and black circles denote unmethylated and methylated CpGs, respectively. Assume a bulk tumor comprising four epigenetic subclones (denoted by green, red, blue, sky blue cells) with relative abundance as shown in the figure. We expect fingerprint pattern (illustrated as four consecutive black circles) of the four subclones to be found throughout the genome. Inside the chromosome ideogram, fingerprint epiloci are shown as vertical bars with corresponding colors. Shown below are some detailed examples of fingerprint epiloci with the status of methylation patterns. For simplicity, only fully methylated fingerprint patterns are considered as fingerprint pattern in the figure. However, fully unmethylated fingerprint pattern will also be taken into account in the post hoc processing step of actual analyses. (B) Preprocessing step. Initially, RRBS mapping result is used for epiloci extraction, and optional pre-filtering is done to facilitate downstream steps. Methylation patterns obtained by RRBS are susceptible to error (red circles) for various reasons. Therefore, DNMT1-like HMM-based in silico proofreading attempts to correct for these errors. Obvious non-fingerprint epiloci will be subsequently discarded. Finally, we obtain analysis-ready fingerprint epiloci for the main analysis of PRISM. (C) Binary pattern decomposition problem. Analysis-ready fingerprint epiloci consist of nearly dichotomous groups of methylation patterns, namely, fully methylated and unmethylated patterns. Separate counting of fingerprint and non-fingerprint patterns gives estimates for the relative abundance of subclones. (D) Beta-binomial mixture model fitting and model selection. All the solutions of binary pattern decomposition problems are merged into a single beta-binomial mixture problem. Each k-cluster model is fit by EM algorithm, where k = 1, 2, 15 by default. Among the model fits, the model that best explains the data are chosen by selecting the model with the minimum BIC. Finally, the subclonal inference result is obtained, and used for further analyses such as functional annotation.
2.2.3 k-mixture decomposition for epigenetic subclonal inference

The core analysis of PRISM can be viewed as a k-mixture decomposition problem after the HMM-based proofreading of methylation patterns. In this section, we give brief illustration of k-mixture decomposition problem. Algorithmic details of k-mixture decomposition are described in Section 3.2.

Subclonal abundance estimates drawn from binary pattern decomposition problems.

Decomposing methylation patterns for each of fingerprint epiloci generates an estimate of subclonal abundance, even though we do not know from which subclone the fingerprint pattern is originated.

Specifically, ‘dichotomous’ epiloci will only be considered as fingerprint epiloci where most of its methylation patterns are fully methylated or unmethylated, and this binarization significantly facilitates the whole workflow. Therefore, for each fingerprint epilocus, we can reduce the problem into a ‘binary’ pattern decomposition problem (one subclone versus all the other subclones) (Fig. 1C). It is because there are only two types of methylation patterns, i.e., fingerprint pattern from one subclone and non-fingerprint pattern from all the other subclones. To simplify the approach, we regard fully methylated patterns as fingerprint patterns. With that simplification, finally, a binary pattern decomposition results in two values for each fingerprint epilocus i: the number of fingerprint patterns (mi) and non-fingerprint patterns (ui). These two values (mi, ui) together constitute a sufficient statistic for underlying subclonal abundance, where the maximum likelihood estimator of subclonal abundance is m̂i = (mi + ui) which is equivalent to FF.

3 Methods

3.1 Preprocessing of RRBS mapping result

3.1.1 Extraction of epiloci and methylation patterns from RRBS data

Epiloci and methylation patterns assigned to them were extracted from RRBS mapping data. Mapped reads harboring the same set of CpGs are grouped, and the read groups carrying at least d reads with at least c CpGs were retained for further analysis. By default, d was set to 20, and c was set to 4. Since subsequent in silico proofreading is a resource-intensive step, we allowed an optional pre-filtering before in silico proofreading step if a sufficient amount of data is given. A read group was pre-filtered out if it did not meet any of the following criteria, which is rather lenient: (i) The two most frequent patterns together should be fully methylated or unmethylated. (ii) The two most frequent patterns should account for >50% (by default) of reads mapped at the epilocus.

3.1.2 DNMT1-like generative HMM-based in silico proofreading of methylation patterns

We suggest that the enzymology of DNMT1 can be elegantly modeled by HMM, as demonstrated in Figure 2A. Suppose a situation in which DNMT1 tries to maintain methylation patterns on hemimethylated DNA duplex. There are two possible states of DNMT1 enzyme with respect to the target DNA: DNMT1 attached to DNA and detached from DNA. Accordingly, our DNMT1-like HMM consists of two hidden states, attached (a) and detached (d) states. To account for the processive methylation of DNMT1, we introduced two parameters, processivity of DNMT1 (p) and recruitment efficiency of DNMT1 to DNA (q) (Supplementary Fig. S2A and B). For example, the probability of transiting from state a to a is p, and from state d to d is 1 − q. Then we modeled DNMT1 copying the methylation status from existing methylation pattern on the
template strand (template pattern) to the daughter strand by unrolling the HMM configuration by the length of template pattern (Supplementary Fig. S2C). For each CpG site, emission probability of observed methylation status depends on both corresponding hidden state and methylation state of template pattern. In attached state, probabilities of emitting observed methylation status \(m\) from template methylation status \(t\) are defined with the error rate of DNMT1 \(e_a\) as follows:

\[
e_{a,t} (m) = \begin{cases} 1 - e_a & \text{if } t = o \\ e_a & \text{if } t \neq o \end{cases}
\]

For example, the probability that methylated cytosine \(m\) is emitted from attached state \(a\) and unmethylated template cytosine \(b\) is represented as \(e_{a,b}(m)\) and its value is \(e_b\). Emission probabilities from the detached state are defined as follows:

\[
e_{d,m} (o) = \begin{cases} 1 - e_d & \text{if } o = u \\ e_d & \text{if } o = m \end{cases}
\]

\[
e_{d,b} (m) = \begin{cases} 1 - e_b & \text{if } o = u \\ e_b & \text{if } o = m \end{cases}
\]

here \(e_d\) and \(e_b\) denote the overall sequencing error rate and bisulfite conversion error rate, respectively.

Given the template methylation pattern, we could compute the probability of any methylation pattern using forward algorithm. These DNMT1-like HMM-based pattern probabilities were used in the hard-EM algorithm for subsequent template inference, which finally results in the set of the most likely template methylation patterns. The details of the forward algorithm and the hard-EM algorithm used for DNMT1-like HMM-based in silico proofreading is provided in Supplementary Information.

3.1.3 Identification of fingerprint epiloci

After correcting for the errors, we discarded an epilocus from further analysis if it did not meet any of the following criteria, which is stricter than pre-filtering criteria: (i) The two most frequent patterns should be fully methylated or unmethylated. (ii) The two most frequent patterns should account for >80% of reads mapped at the epilocus.

3.2 PRISM model

3.2.1 Establishment of PRISM model

We let \(m_i\) and \(u_i\) denote the number of reads at fingerprint epiloci \(i\) which supports methylated patterns and unmethylated patterns, respectively (Supplementary Fig. S3). We modeled two values, \(m_i + u_i\) and \(m_i\) with beta-binomial distribution parameterized by \(x\) and \(\beta\). The model for fingerprint epiloci \(i\) became as follows:

\[
m_i \sim \text{BetaBinomial}(m_i + u_i, x, \beta)
\]

We attempted to solve beta-binomial mixture model and derive \(K\) clusters from \(E\) fingerprint epiloci in total, where \(K\) is defined a priori. More specifically, for a cluster \(k\), independent parameters \(z_k\) and \(\beta_k\) were introduced, and the optimal values of \(z_k\) and \(\beta_k\) were determined by EM algorithm.

For the following descriptions, we let superscripts in parenthesis denote the iteration number of EM algorithm. All of the cluster weight \(\pi_k^{(n)}\) were initialized with 1/K. For reasonable initialization of \(z_k^{(1)}\) and \(\beta_k^{(1)}\), we fit Gaussian mixture model with values of \(m_i/(m_i + u_i)\), or FFs. Fitting Gaussian mixture model gives mean and variance for each cluster \(k\), denoted by \(\mu_k\) and \(\sigma_k^2\), respectively. We could initialize \(z_k^{(1)}\) and \(\beta_k^{(1)}\) using the result of Gaussian mixture fit as follows:

\[
z_k^{(1)} = \frac{1 - \mu_k - 1}{\sigma_k^2} \mu_k^2
\]

\[
\beta_k^{(1)} = \frac{1}{\mu_k - 1}
\]

In the E-step, \(L_k^{(n)}\), the likelihood of \(z_k^{(n)}\) and \(\beta_k^{(n)}\) with regard to epilocus \(i\) was computed with beta-binomial probability mass function. Assume we are at iteration \(n\) of EM algorithm.

\[
L_k^{(n)} = \Gamma(m_i + u_i + 1) / \left( \Gamma(m_i + 1) \Gamma(u_i + 1) \right) \left( z_k^{(n)} \right)^{m_i} \left( 1 - z_k^{(n)} \right)^{u_i}
\]

Accordingly, the posterior probability of epilocus \(i\) being assigned to cluster \(k\) \(p_{ik}^{(n)}\) can be computed as follows:

\[
p_{ik}^{(n)} = \frac{\pi_k^{(n)} L_k^{(n)}}{E_{ij}^{(n)}}
\]

In the M-step, \(\pi_k^{(n+1)}\), \(z_k^{(n+1)}\) and \(\beta_k^{(n+1)}\) are computed. The maximum likelihood estimation of \(\pi_k^{(n+1)}\) is straightforward:

\[
\pi_k^{(n+1)} = \frac{E_{ij}^{(n)}}{E}
\]

However, the maximum likelihood estimation of \(z_k^{(n+1)}\) and \(\beta_k^{(n+1)}\) given \(m_i\) and \(u_i\), which is not trivial, is done through Newton’s iteration (Minka, 2000). A more detailed explanation of the maximum likelihood estimation of \(z_k^{(n+1)}\) and \(\beta_k^{(n+1)}\) is demonstrated in Supplementary Information.

The termination condition of the EM iteration is described as follows. For each iteration, we computed log likelihood \(l^{(n)}\):

\[
l^{(n)} = \sum_i \log \sum_k p_{ik}^{(n)}
\]

If \(\left| l^{(n)} - l^{(n-1)} \right| < 0.001\), the EM iteration was terminated.

3.2.2 Model selection

We used Bayesian information criterion (BIC) (Schwarz et al., 1978) to select the model with optimal number of clusters. BIC of a model with \(K\) clusters \((M_K)\) is defined as

\[
\text{BIC}(M_K) = (3K - 1) \log E - 2 \sum_i \log \sum_k p_{ik}
\]

Since there are 2\(K\) free parameters for \(x\) and \(\beta\), and \(K - 1\) free parameters for \(\pi\), \(M_K \) has 3\(K\) - 1 free parameters in total. In practice, PRISM selects the model with minimum BIC among \(M_1\) through \(M_{15}\) by default.

3.2.3 Joint analysis of multiple samples from single tumor

Subclonal inference generally benefits from joint analysis of multiple sequencing data from the same tumor because different samples often have different mixing proportion of subclones which increases the chance of separation for two subclones that could not be separated by investigating a single sample due to their similar relative abundance. Thus many of existing subclone detection tools are able to analyze several samples jointly to increase their resolution of subclone detection (Miller et al., 2014; Roth et al., 2014). PRISM can also be applied for two or more samples from a single tumor.
3.2.4 Post hoc processing of clusters and copy number-aware analysis

After selecting the optimal model, PRISM excludes uninformative clusters that seem to have arisen from outliers. It also checks whether it should treat several clusters as single subclones by taking account of the unmethylated fingerprint, by merging ‘reflected’ clusters. Also, PRISM can utilize CNA information to obtain corrected methylation pattern counts which reflect methylation patterns originating from copy number-gained segments. For further details of post hoc processing step and copy number-aware analysis, please see Supplementary Information.

3.3 Data retrieval and processing

Methylated and unmethylated cell line RRBS data (Barrett et al., 2017) were downloaded from European Nucleotide Archive under accession number PRJEB21102. MCF10A-Er-Src cell line, GM06990 B-lymphocyte cell line and T-47D cell line RRBS data were downloaded from Sequence Read Archive under run accession SRR222434, SRR531452 and SRR222532, respectively. We also downloaded public AML RRBS data along with corresponding whole exome sequencing (WES) data from dbGaP under accession phs001027.v2.p1. For the full description of the data processing step, please refer to Supplementary Information.

4 Results and discussion

4.1 Impact of in silico proofreading on PRISM analysis

We assessed the impact of in silico proofreading on the accuracy of the estimated size of subclones (Fig. 3). For that, we obtained two raw RRBS data representing methylation states of the fully methylated cell line and fully unmethylated cell line. We mixed two raw RRBS data to simulate a mixture of epigenetically homogeneous cells. Each of the two raw data were subsampled with 10%, 20%, …, 90% of reads to generate benchmark mixtures of the two cell lines. We then concatenated corresponding pairs of subsampled raw data such that their mixing ratio (MR) would sum up to 100%. For example, we joined 30%-subsampled fully methylated cell line RRBS data and 70%-subsampled fully unmethylated cell line RRBS data together. This entire step was repeated for 10 times. We then examined the accuracy of MR estimates given by PRISM, with or without in silico proofreading.

Running PRISM without in silico proofreading resulted in markedly biased estimations, which consistently underestimated MR (Fig. 3A). The worst estimation was for the MR of 20%, where the ratio between estimated MR and true MR was 0.86. The estimations after in silico proofreading were more ‘calibrated’ to correct estimations (Fig. 3B), and the ratio between the worst estimation of MR and true MR was 0.96 for MR of 20%. In silico proofreading seems to correct for the biased error rate of methylation patterns, which is significantly higher for fully methylated patterns than for fully unmethylated patterns (Supplementary Fig. S4). Meanwhile, we were also possible to show that in silico proofreading facilitated PRISM analysis by considerably increasing the number of fingerprint epiloci for the inference of epigenetic subclones with marginal introduction of artificial fingerprint epiloci (Supplementary Fig. S5).

We generated more realistic benchmark mixtures of cells by mixing cell line RRBS data established from various tissues to evaluate the performance of PRISM in practical situations. In particular, three cell line RRBS data were carefully chosen from ENCODE project (Varley et al., 2013): (i) MCF10A-Er-Src cell line which is derived from non-tumorigenic epithelial cells of the mammary gland, (ii) GM06990 B-lymphocyte cell line from lymphoblastoid and (iii) T-47D cell line established from mammary ductal carcinoma. Epigenomic reprogramming plays a crucial role in development, shaping distinct methylation landscape for each cell type from different cell lineage. Therefore, PRISM should be able to detect global DNA methylation reprogramming event in order to distinguish a lymphocyte cell line (GM06990) from two epithelial cell lines (MCF10A-Er-Src and T-47D). Moreover, we asked whether PRISM could accurately separate non-cancerous (MCF10A-Er-Src) and cancerous (T-47D) cell lines, where both of them were derived from the mammary gland.

In this experiment, it should be noted that merely mixing the raw data will result in an undesired result because the sequencing libraries were prepared separately, so the cleavage site of restriction enzyme may differ between samples. Furthermore, the sequencing depth of the same epilocus will be strikingly different, which may affect the proportion of reads severely when a mixture is generated. Therefore, we mixed them with a deliberate approach as follows. The three raw RRBS data were independently processed and mapped to the reference genome. Then the epiloci which appear in all of three alignment results and have 20 or more mapped reads were retained for the mixing procedure. For each epilocus, simulated sequencing depth $d$ was sampled from $NegBin(5, 0.03)$ with constraint $d \geq 20$. We randomly sampled MRs ($P_1$, $P_2$, $P_3$) from $Dirichlet(3, 3, 3)$, and for each epilocus, $[P_1, d]$ reads were sampled from each of the three data. The entire mixing step was repeated two times to generate two independent mixtures (Supplementary Table S1).

We supposed each cell line as a putative subclone in the mixture, and let PRISM estimate the number and abundance of the subclones only from their mixed methylation patterns in the two mixtures. PRISM identified four subclones (Fig. 4A). Regarding the average FF of each cluster as MR estimate, we observed that the resulting MR estimates of subclones 1, 2 and 3 reasonably represented the true MRs (Fig. 4B and Supplementary Table S2). For subclone 4, which was unexpected, we could not draw a concrete conclusion on whether it was an artifact of sequencing procedures, or it was a
4.3 Detection of epigenetic subclones in AML dataset

We next applied PRISM for AML data to test if PRISM can draw clinically meaningful observations. For each subject, a couple of samples were taken at two time points (diagnosis and relapse) and sequenced by RRBS. Two-sample joint analysis were done, which resulted in 3.13 inferred subclones on average (Supplementary Fig. S6). In this section, we describe our explanatory analyses for subject AML-105 and AML-109, since both of them had five inferred subclones, which seemed to be sufficient to reveal intriguing clonal dynamics. Microscopic inspection of data providers revealed that both of the samples had relatively normal cytogenetic properties, except for AML-105 relapse sample, which had a small fraction (~10%) of cells harboring genomic deletion in q-arm of chromosome 7. Moreover, no significant CNA was detected from WES data of those samples (Supplementary Fig. S7). Therefore, we concluded that the CNA of the samples would not affect our analysis.

For AML-105, initially, diagnosis and relapse samples were analyzed separately (Fig. 5A). Four and three putative epigenetic subclones were found, respectively. However, the two-sample joint analysis identified five epigenetic subclones (Fig. 5B), emphasizing the necessity of multi-sample joint analysis to achieve a reasonable resolution of subclonal inference. Interestingly, independent analysis of variant allele frequency (VAF) from WES data (Supplementary Fig. S8A) revealed that the subclonal abundance inferred by VAFs of heterozygous somatic mutations within isocitrate dehydrogenase 2 (IDH2) and DNA methyltransferase 3 alpha (DNMT3A) was concordant with the subclonal abundance estimates of subclone 2 (0.61–0.84) identified by PRISM. This indirectly suggests that somatic mutations in IDH2 and DNMT3A may have served as driver events that accelerate subsequent epigenomic evolution, given the role of IDH2 (Kernytsky et al., 2015) and DNMT3A (Yang et al., 2015) as epigenetic regulators (Fig. 5E). Additionally, possible epigenetic drivers of subclonal expansion were identified by functionally annotating identified subclones (Fig. 5C). We used a set of 68 recurrently mutated genes in AML (Metzeler et al., 2016) (Supplementary Table S3) since we assumed that the epigenetic aberration of these genes would also be critical for the progression of AML. Subclone 3 harbored intensively altered methylation (hereafter referred to as epi-mutation) at Wilms’ tumor 1 (WT1), whose overexpression and mutation have significant implications in AML (Menssen et al., 1995; Miwa et al., 1992). What is most striking is that dysregulated WT1 has recently been known to alter the methylation landscape of cells by impeding the activity of TET2 (Rampal and Figueroa, 2016). Epi-mutations in DNMT3A characterized subclones 1 and 3, while subclone 1 was annotated with additional gene, GATA binding protein 2 (GATA2). One potential pitfall of PRISM is that it cannot avoid reporting a cluster of epiloci arisen from genomic imprinting. However, it can simply overcome by annotating subclones with known imprinted genes and excluding the subclone showing a considerably high proportion of imprinted epiloci (Fig. 5D). A curated list of imprinted genes was obtained from Geneimprint (Falls et al., 1999). Therefore, subclone 4 was
excluded from further analyses since 20.9% of its epiloci were known to be imprinted. Finally, we could infer one of the possible evolutionary histories of subclones regarding both genomic and epigenomic events that the subclones underwent (Fig. 5E).

**PRISM** revealed much intriguing clonal dynamics for AML-109 (Fig. 6). Three subclones were found in each of the separate analysis of diagnosis and relapse sample (Fig. 6A), while joint analysis of the two longitudinal samples revealed five subclones (Fig. 6B). Similarly, independent whole exome analysis revealed a novel subclone at relapse that was not detectable at diagnosis (Supplementary Fig. S8B). The novel subclone carried somatic mutations in four AML-related genes, including **WT1** and **IDH2**. Interestingly, functional annotation again discovered altered methylation of **WT1** (Fig. 6C). Regarding subclone 4 as a representative of the whole clonal population, **WT1** methylation alteration was clonal rather than subclonal in this case; in other words, it was deemed to have occurred at the onset of the malignancy. Furthermore, subclone carrying epiloci at **DNMT3A** (subclone 3) showed dramatic increase in its relative abundance (from 0.062 to 0.921). It implies that subclone 3 had a remarkable evolutionary advantage over other subclones in the tumor niche established by anti-cancer drugs, presumably due to the combined effect of genomic and epigenomic variations. Subclone 1 was excluded from the inference of evolutionary history because of its significantly high proportion of imprinted epiloci (Fig. 6D). The reconstructed subclonal evolutionary trajectory (Fig. 6E) verified **WT1** epi-mutation as an early event in clonal evolution, followed by **MPL**, **JAK2** and also **DNMT3A** epi-mutation accompanied by several mutations.

**5 Conclusion**

While it has long been considered that the evolution of the cancer genome is the primary factor constituting inherent heterogeneity of a tumor, the evolution of epigenome brings up another dimension to defining the heterogeneity. Whether the genomic and epigenomic evolution occur co-ordinately or independently is still obscure, and even seem to be case-dependent (Li et al., 2016), which is reproduced by comparing PRISM and two SNV-based subclonal inference methods (Supplementary Fig. S9). Nevertheless, investigating the epigenomic evolutionary history of a tumor at the resolution of cancer subclone provides valuable insight into the epigenetic mechanism of the progression of the malignancy. It also offers novel implications for the history of clonal evolution and helps design the therapeutic strategy.

Despite the limitation of our research that we could not provide experimental evidence of the existence of the subclones, we showed that the inference of epigenetic subclonal population structure was possible by focusing on the fingerprint epiloci that seem to have arisen from global DNA methylation reprogramming. Analyzing clonal dynamics of the two AML samples implied the significance of interplay between epigenetic regulators such as **WT1**, **IDH2** and **DNMT3A** in clonal evolution. Moreover, by combining information of genomic variation, we could gain valuable insight into the simultaneous evolution of the genomic and epigenomic landscapes. Consequently, we carefully suggest that the development of epigenomic subclonal inference algorithm brings us one step closer to the multi-omics level characterization.
of cancer subclones, which is one of the ultimate goals of molecular oncology.

Funding

This research is supported by Next-Generation Information Computing Development Program through the National Research Foundation of Korea(NRF) funded by the Ministry of Science, ICT(No.NRF-2017M3CAA7065887), the Collaborative Genome Program for Fostering New Post-Genome Industry of the National Research Foundation (NRF) funded by the Ministry of Science and ICT (MSIT) (No.NRF2014M3CA9A3063541), and a grant of the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea (grant number : HI15C3224).

Conflict of Interest: none declared.

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