Genome analysis

Episo: quantitative estimation of RNA 5-methylcytosine at isoform level by high-throughput sequencing of RNA treated with bisulfite

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Abstract

Motivation: RNA 5-methylcytosine (m⁵C) is a type of post-transcriptional modification that may be involved in numerous biological processes and tumorigenesis. RNA m⁵C can be profiled at single-nucleotide resolution by high-throughput sequencing of RNA treated with bisulfite (RNA-BisSeq). However, the exploration of transcriptome-wide profile and potential function of m⁵C in splicing remains to be elucidated due to lack of isoform level m⁵C quantification tool.

Results: We developed a computational package to quantify Epitranscriptomal RNA m⁵C at the transcript isoform level (named Episo). Episo consists of three tools: mapper, quant and Bisulfitefq, for mapping, quantifying and simulating RNA-BisSeq data, respectively. The high accuracy of Episo was validated using an improved m⁵C-specific methylated RNA immunoprecipitation (meRIP) protocol, as well as a set of in silico experiments. By applying Episo to public human and mouse RNA-BisSeq data, we found that the RNA m⁵C is not evenly distributed among the transcript isoforms, implying the m⁵C may subject to be regulated at isoform level.

Availability and implementation: Episo is released under the GNU GPLv3 license. The resource code Episo is freely accessible from https://github.com/liujunfengtop/Episo (with Tophat/cufflink) and https://github.com/liujunfengtop/Episo/tree/master/Episo_Kallisto (with Kallisto).

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Supplementary information: Supplementary data are available at Bioinformatics online.

1 Introduction

Post-transcriptional modifications in RNAs have drawn much attention in recent literature (Chi, 2017), as rapidly growing evidence has suggested that reversible RNA modifications may be a new layer of epigenetic regulation in gene expression (Zhao et al., 2017), and its disruption may lead to life-threatening disease like cancer (Frye et al., 2016; Popis et al., 2016). 5-Methylcytosine is a type of chemical modification on the nucleotides that can be found in both DNA and RNA. To identify transcriptome-wide m⁵C, several advanced techniques have been employed, including high-throughput sequencing of RNA treated with bisulfite (RNA-BisSeq) (Schaefer et al., 2010; Squires et al., 2012), RNA m⁵C-RNA immunoprecipitation (RIP) (Jayaseelan et al., 2011), 5-azacytidine-mediated RNA immunoprecipitation (Aza-IP) (Khoddami and Cairns, 2013) and methylation individual-nucleotide-resolution crosslinking and immunoprecipitation (miCLIP) (Hussain et al., 2013a, b). Among these, RNA-BisSeq is considered the gold standard for RNA m⁵C (Amort et al., 2017; Hussain et al., 2013a, b; Squires et al., 2012; Yang et al., 2017). Hereinafter in this article, m⁵C only refers to RNA m⁵C, unless otherwise indicated.

The transcriptome-wide function of m⁵C is just starting to be investigated (Edelheit et al., 2013; Hussain et al., 2013a, b; Shelton et al., 2016). It is clear that m⁵C is distributed widely over protein coding and non-coding RNAs in human (Squires et al., 2012) and mouse (Amort et al., 2017), as well as enriched in CG-rich regions and the initiation site of coding regions (Yang et al., 2017). RNA m⁵C is conserved from bacteria to mammals and plants, and its functions have been suggested to include structural and metabolic stabilization and translational regulation (Blanco et al., 2014; Burgess et al., 2015; Chen et al., 2019; Gabriel Torres et al., 2014; Popis et al., 2016; Schaefer et al., 2010; Schwartz et al., 2013;
Squires et al., 2012; Yang et al., 2019). At the transcription level, alternate splicing is a predominant contributor to the diversity of protein types in higher eukaryotic cells (Black, 2003). Any changes in this regulated process could result in severe phenotypes (Pan et al., 2008). However, both the distribution of m⁵C among the transcript isoforms and its functional relevance to the splicing process have been barely investigated.

In order to study the relationship between m⁵C and splicing, a tool that is able to quantify m⁵C at an isoform level is necessary. However, to the best of our knowledge, such method has yet been found in literature. There are several tools for RNA-BisSeq or Aza-IP data analysis (Bormann et al., 2018; Legrand et al., 2017; Liang et al., 2016; Rieder et al., 2016). meRanTK is a toolkit composed of tools for RNA-BisSeq read mapping, methylation calling and differentially methylation identification (Rieder et al., 2016). BS-RNA maps and annotates RNA-BisSeq data with more attention on the 'dovetailing' reads (Liang et al., 2016). BisRNA considers the possible artifact that may be stochastically introduced by the experiment (Legrand et al., 2017). BisAMP is more specific to targeted RNA m⁵C analysis (Bormann et al., 2018). However, none of above tools supports the analysis of RNA-BisSeq data at isoform level.

To address this issue, we developed a probabilistic model to quantify Epitranscriptomal RNA m⁵C at the transcript isoform level (named Episo), which utilizes single-nucleotide resolution m⁵C data from RNA-BisSeq. Episo consists of three tools: mapper, quant and Bisulfitetf, for mapping, quantifying and simulating RNA-BisSeq data, respectively. Both in silico and wet experiments showed that the prediction of Episo is highly accurate. By applying Episo to recently published m⁵C data (Yang et al., 2017), we generated the first transcript isoform level m⁵C profiles for HeLa cells and four mouse tissues. We found that the distribution of m⁵C in the transcript isoforms was remarkably dissimilar to random shuffled data, suggesting that there may exist a latent regulatory layer for m⁵C at isoform level.

2 Materials and methods

Reference genome and transcriptome for human and mouse, version GRCh37 and GRCm38, respectively, were downloaded from the BIG Data Center under accession number PRJCA000315 (Members, 2017).

Episo consists of three tools: mapper, quant and Bisulfitetf, for mapping, quantifying and simulating RNA-BisSeq data, respectively (Fig. 1). Before reads mapping, Episo has all low-quality bases and adaptor sequences removed by Cutadapt (Martin, 2011).

The mapper maps RNA-BisSeq reads to the reference genome and reference transcriptome. We adopted the mapping strategy used in Bismark to map RNA-BisSeq reads with modifications (Krueger and Andrews, 2011). First, all RNA-BisSeq reads were C-to-T and G-to-A transformed, and the resultant data were denoted as BSC-T and BSG-A, respectively. Second, the reference transcriptome was also C-to-T and G-to-A transformed, and the transformed references were denoted as RefC-T and RefG-A, respectively. Last, the four types of mapping (BSC-T versus RefC-T, BSC-T versus RefG-A, BSG-A versus RefC-T and BSG-A versus RefG-A) were performed by Bowtie (version 1.1.2, see Fig. 1). The uniquely mapped reads, i.e. those that were uniquely mapped to a genome locus in at least one of four above mappings, but not necessarily mapped to a unique transcript, were used in subsequent processes.

The quant tool quantifies m⁵C level at transcription isoform level from RNA-BisSeq data. The quant tool consists of two steps. The first step estimates transcription level from RNA-BisSeq data. To accomplish this, quant constructs a virtual RNA-seq dataset, i.e. for all RNA-BisSeq reads that contain unmethylated cytosines, quant transforms them back to their native cytosine states. With such virtual RNA-seq data, quant estimates gene transcription level using third party tools, which has two choice in current implement, i.e. Tophat (version 2.1.0)/Cufflink (version 2.2.1) (Trapnell et al., 2009, 2010) and Kallisto (version 0.44.0) (Bray et al., 2016). The users can also replace them with any favorite tools easily by making input data format acceptable for Episo. For example, Episo takes fragments per kilobase of transcript per million mapped reads (FPKM) as the default input format, while the output of Kallisto is estimated transcripts per million (TPM). So, we need to convert TPM to FPKM using the following formula.

$$\text{TPM} = \left( \frac{\text{FPKM}}{\sum \text{FPKM}} \right) \times 10^6$$  (1)

We present the performance and data analysis using the results from Tophat/Cufflink in this article; however, the results are essentially similar when using Kallisto.

The second step of quant estimates the RNA m⁵C level at each putative methylation site in the isoforms. We define the methylation rate at global, isoform and single-nucleotide levels as follows. The global methylation rate is the proportion of cytosine sites that have been methylated in all examined RNAs. This rate is estimated by directly counting the unconverted cytosines in RNA-BisSeq data. The methylation rate at isoform level is defined as $R_{iso}/R_{c}$, where $R_{iso}$ denotes the RNAs that carry at least one methylated cytosine site, and $R_{c}$ denotes all RNAs of the given isoform iso. The methylation rate of a single-nucleotide at the level of a given isoform is denoted as $R_{m,iso}$, where $R_{m,iso}$ denotes the RNAs from the methylated cytosine sites, and $R_{iso}$ denotes all RNAs of the given isoform that carry this cytosine site.

To estimate the RNA m⁵C rate at isoform level, one needs to estimate the probability that a read $r$ in $R$ was generated from a given isoform $t$ in $T$, where $R$ denote a set of RNA-BisSeq reads and $T$ denote a set of isoform(s). Let’s denote this probability as $P(r,t)$. One way to calculate this probability was showed by Trapnell et al. (2009),
Effectively length of an isoform, the maximum likelihood estimation and 95% confidence interval of Episo: estimation of RNA m5C at isoform level

We assume it originated from isoform t and denote the reads that cover the given cytosine site, and let $R$ denote the reads that carry at least one m5C. Moreover, $quant$ can be easily derived from formula (2). It is the $r_t$ and the 95% confidence interval needed for Episo; however, users can take these two values from any third party tools. In the current release of Episo, the users can either choose Kallisto (version 0.44.0) (Bray et al., 2016) or TopHat (version 2.1.0)/Cufflink (version 2.2.1) (Trapnell et al., 2010) for these two inputs.

Now, we estimate the RNA m5C level for a given isoform t (denoted as $M_t$). Let $R_t$ denote the reads that carry at least one methylated cytosine, i.e. unconverted cytosines after bisulfite treatment. We define the relative transcription level of methylated isoform, i.e. the proportion of reads from isoform $l$ in $R_t$ is denoted as $\rho_t$. Following logic identical to that in formula (2), we can calculate the maximum likelihood estimation and 95% confidence interval of $\rho_t$. To simplify the derivation, we assumed the independence between transcription and post-transcriptional modification, i.e. $\rho_1 \perp \rho_2$, $M_t$ can be estimated according to the delta method, as

$$M_t \approx m \left( \mu + \frac{\sigma^2}{\mu^3} \right)$$

where $m$ and $n$ denote the number of reads in $R_t$ and $R$, respectively, $\mu$ and $\sigma^2$ denote the estimated $m$ and $\sigma^2$, respectively, and $\rho_t$ is the variance of $\rho_t$. Moreover, $quant$ can estimate the m5C level for a subset of isoforms of a given gene. Let $B_t$ be a subset containing $k$ isoforms from gene $A_t$; then, the RNA m5C level in $B_t$ can be estimated as

$$M_{B_t} \approx \frac{m}{n} \sum_{i=1}^{k} \rho_i \left( \frac{\rho_i}{\rho_i} + \frac{\sigma_i^2}{\rho_i^3} \right)$$

Finally, we estimate the RNA m5C level for a given cytosine site. Let $R_t$ denote the reads that carry the given cytosine site, and let $R$ denote the reads that carry methylated cytosine at the given site. Following logic identical to that in formulas (2)–(5), the RNA m5C of a given cytosine site on an isoform or a subset of isoforms can be estimated.

The Bisulfitefq simulates bisulfite treatments, and the sequencing process were simulated by the FluxSimulator with default parameters (Montgomery et al., 2010). Bisulfitefq consists of Bisulfitefq-reads, Bisulfitefq-fragment and Bisulfitefq-fragment-multivariate. The Bisulfitefq-reads component generates RNA-BisSeq data with a given global m5C level, i.e. it randomly transforms a given proportion of cytosines to thymines in the input reads. The Bisulfitefq-fragment component transforms all cytosines in a given proportion of randomly selected reads into thymines. The Bisulfitefq-fragment-multivariate can then simulate an epitranscriptome with multiple m5C levels between isoforms. It can take up to three m5C levels as input parameters. For each m5C level, it transforms all cytosines in the given proportion of randomly selected reads from one assigned isoform into thymines. For example, let a gene A have three isoforms, namely A1, A2 and A3, while P1, P2 and P3 are three m5C levels we want to simulate for the isoforms, respectively. The Bisulfitefq-fragment–multivariate transforms all cytosines with 1-P1, 1-P2 and 1-P3 of randomly selected reads from isoform A1, A2 and A3, respectively, into thymines.

Gene ontology (GO) analysis of RNA m5C-containing genes was performed using DAVID (Dennis et al., 2003), and the sequence motifs of the RNA m5C sites were discovered using MEME (Bailey et al., 2009).

The human cervical carcinoma cell line HeLa are cultured in DMEM high glucose medium (HyClone, SH30243.01) with 10% FBS (BioWest, S1380-500), and 1% Penicillin–Streptomycin (Corning, 30002283). Cell identity was verified by STR analysis (DNA fingerprinting), and mycoplasma contamination was regularly tested for cell cultures.

The regions spanning nt 914–1465 of E.coli 16S rRNA sequence was amplified by PCR with a forward primer harboring a T promoter sequence. 0.5 μg production was used as template for in vitro transcription with MEGAScript Kit (Promega, P1440) according to the protocol. The transcribed RNA was treated 15 min at 37°C with 1 μL RQ1 RNase-free DNase and purified using RNA clean kit (ZYMO RESEARCH R1015) following the protocol. The spike-in RNA was subpackaged and stored in RNase-free water at -80°C. Following the manufacturer’s recommendations, about 3 × 10^7 HeLa cells were treated with 6 ml TRIzol Reagent (Invitrogen, 15596026) for total RNA isolation. Isolated RNA was then subjected to two rounds of poly(A) RNA enrichment using fresh Dynabeads (Ambion, 61006) and treated with 1U of DNase I (Thermo Scientific, 00383793) for 15 min at 37°C. The mRNA was cleaned using RNA clean kit (ZYMO RESEARCH R1015) and concentration was determined in NanoDrop (Thermo Scientific, NanoDrop 1000) by measuring absorbance at 260 and 280 nm.

Thirty microgram purified mRNA was mixed with 50 ng spike-in RNA. The mixture was divided equally into three portions. Four microgram anti-m5C antibody (1.36 μg/ml, Diagenode, C15200081), 2 μg random 25nt oligonucleotides and 50 μl Dynabeads Protein G (Novex, 10007D) was incubated in 300 μl IP buffer [10 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.05% Triton-X(v/v)] at 4°C for 2 h on a rotating wheel. The same procedure was performed using Mouse IgG (1 μg/ml, Diagenode, C15400001) as control. We used 250 μl IP buffer to wash bead–antibody complexes three times, then added RNA mixture and finally brought to 250 μl with IP buffer. The RNA mixtures were incubated at 4°C overnight (>12 h) with 1 μl RNasin (Invitrogen, N8080119) on a rotating wheel. The RNA–antibody–beads complexes were gentle washed three times using IP buffer and incubated in 300 μl elution buffer (5 mM Tris–HCl pH 7.5, 1 mM EDTA, 0.05%SDS, 80 μg Proteinase K) for 1 h at 50°C. After removing beads complexes, the eluted production was cleaned with RNA clean kit (ZYMO RESEARCH R1015). We repeated the beads–antibody incubation, RNA complexes incubation and RNA–antibody–beads complexes elution processes for five times. The supernatant was collected in a new tube and cleaned with RNA clean kit (ZYMO RESEARCH R1015). The supernatant concentration was determined in nanodrop by measuring absorbance at 260 and 280 nm.

Five tubes of cleaned eluted MeRIP production and 300 ng supernatant RNA were reverse transcribed using SuperScript III reverse transcriptase (Invitrogen 18080-093). Each tube contains 11 μl RNA solution, 1 μl dNTPs and 1 μl random 6 bp Hexamers. The tubes were incubated at 65°C for 5 min and 8°C at least 1 min. Four microlitre 5× First strand buffer, 1 μl 0.1 M DTT, 1 μl RNasin and 1 μl SuperScript III reverse transcriptase were mixed and added the mixture into each tube. The tubes were incubated at 55°C for 60 min followed by 70°C for 15 min. The reverse transcribed products were stored at -20°C.

The spike-in RNA was used as internal unspecific binding control to normalize binding of RNA between IgG control and m5C sample. Five tubes of reverse transcribed MeRIP products were mixed and measured for the enrichment using 2 M M_Ambers method by comparing the anti-m5C sample with the anti-IgG sample. Data were expressed as the expression of target genes relative to the spike-in control in the anti-m5C sample compared with the anti-IgG sample. PCR primers can be found in the Supplementary Table S1.
If our model is correct, we should expect

\[
\text{Predicted MR rate} = \frac{\text{m5C level from isoform A}}{\text{m5C level from isoform B}}
\]

where

\[
\begin{align*}
\text{Observed MR rate} & = \frac{\text{observed m5C level in isoform A}}{\text{observed m5C level in isoform B}} \\
\text{m5C counts from isoform A} & = \text{IgG counts from isoform A} \\
\text{m5C counts from isoform B} & = \text{FCmRIP(isoform A)} \\
\text{IgG counts from isoform B} & = \text{FCmRIP(isoform B)}
\end{align*}
\]

3 Results

We present an RNA-BisSeq data analysis package, named Episo, to quantify m^5C at the transcript isoform level. Episo consists of three tools: named mapper, quant and BisulfiteEq, for mapping, quantifying and simulating RNA-BisSeq data, respectively (Fig. 1). The detailed algorithm can be found in the Section 2.

3.1 In silico assessment of Episo

To in silico assess the performance of Episo, we simulated paired-end RNA-BisSeq data with three global methylation rates, 0.1, 1 and 10% (about 23 million 101-bp length paired-end reads for each methylation rate) using BisulfiteEq. Comparing to meRanTK, the mapping rates of mapper were consistently higher at all three methylation rates tested (86.6% versus 80.72%). Next, we assessed the accuracy of quant at global and single-nucleotide level. The quant accurately estimated the relative global RNA m^5C rates at all nucleotide contexts, i.e. CpG, CHG and CHH, and at all the three methylation rates (Supplementary Table S2). Because Episo, to the best of our knowledge, is the first computational tool that enables isoform level quantification of m^5C, there are no existing methods to compare with. Thus, to further assess the accuracy of quant at isoform level, we simulated RNA-BisSeq data with 10, 5 and 1% m^5C isoform level methylation (Fig. 2A). These three methylation levels were examined because most methylated transcript isoforms were estimated carrying 1–10 percent m^5C in real samples (Fig. 2B). Because the m^5C level is so low, when gene expression level is also low, the data would be too noisy to make meaningful predictions, e.g. the average differences are 0.2076, 0.4211 and 0.4763 compared with simulated levels of 10, 5, 1%, respectively (Supplementary Fig. S1). We only considered the transcripts that have sufficient expression level, i.e. fragments per kilobase per million reads (FPKM) >2 in our tests. We found that the average differences between estimated and simulated m^5C levels were minor (the average differences are 0.0070, 0.0013 and 0.0006 compared with simulated levels of 10, 5, 1%, respectively, Fig. 2A). To assess the accuracy of quant at single-nucleotide level, we simulated RNA-BisSeq data with 60, 40 and 10% level single-nucleotide m^5C methylation. These three methylation levels were tested because most real methylated cytosines have an m^5C level between 10 and 60% in real samples (Fig. 2C). In this dataset, we also found that the average differences between the estimated and simulated RNA m^5C levels were nearly zero (the average differences are 0.0027, 0.0011 and 0.0013, when the simulated levels are 60, 40 and 10%, respectively, Fig. 2A). Last, Episo was found to predict isoform m^5C levels specifically and sensitively in tested methylation level (Supplementary Fig. S2).

3.2 Experimental assessment of Episo

To experimentally assess the accuracy of Episo’s prediction by MeRIP followed by qPCR (Fig. 2D and Supplementary Fig. S3), we applied Episo to RNA-BisSeq data of HeLa cells and predicted m^5C level of transcripts at isoform level. To distinguish the methylation levels between isoforms easily, we looked for genes satisfied all the following conditions for experimental validation. First, this gene should have and only have one Guanine site that its corresponding cytosine site in the RNA production be methylated. Second, the gene generates at least two isoforms that carrying the methylated site. Third, the predicted methylation level at the site is no less than 0.1 in tested isoforms, because most methylated transcript isoforms were estimated carrying 1–10 percent m^5C in real samples (Fig. 2B). Because the m^5C level is so low, when gene expression level is also low, the data would be too noisy to make meaningful predictions, e.g. the average differences are 0.2076, 0.4211 and 0.4763 compared with simulated levels of 10, 5, 1%, respectively (Supplementary Fig. S1). We only considered the transcripts that have sufficient expression level, i.e. fragments per kilobase per million reads (FPKM) >2 in our tests. We found that the average differences between estimated and simulated m^5C levels were minor (the average differences are 0.0070, 0.0013 and 0.0006 compared with simulated levels of 10, 5, 1%, respectively, Fig. 2A). To assess the accuracy of quant at single-nucleotide level, we simulated RNA-BisSeq data with 60, 40 and 10% level single-nucleotide m^5C methylation. These three methylation levels were tested because most real methylated cytosines have an m^5C level between 10 and 60% in real samples (Fig. 2C). In this dataset, we also found that the average differences between the estimated and simulated RNA m^5C levels were nearly zero (the average differences are 0.0027, 0.0011 and 0.0013, when the simulated levels are 60, 40 and 10%, respectively, Fig. 2A). Last, Episo was found to predict isoform m^5C levels specifically and sensitively in tested methylation level (Supplementary Fig. S2).

Fig. 2. The accuracy of Episo and the distribution of estimated m^5C levels in human cells and mouse tissues. (A) The distribution of estimation errors of Episo. The difference of m^5C levels between the simulated and Episo estimated data is shown at the resolution of RNA isoforms and single cytosine. At both resolutions, the comparisons were made at three m^5C levels that covered the main range of estimations in real data from human cells and mouse tissues as shown in (B) and (C). (B) The distribution of estimated m^5C levels at the resolution of RNA isoforms. In the mouse tissues tested, the m^5C level is significantly higher in brain than that in the other three tissues. The '*' indicates significant difference of P-value < 0.001. (C) The distribution of estimated m^5C levels at the resolution of single cytosine. For both metrics, we found no significant different among mouse tissues tested. (D) Experiment design for PK3R2. The unique exons for isoform PK3R2-002 and PK3R2-004 are marked in pink shadow and the primers design are indicated as red arrows. The unique methylated cytosine is marked with red flag. See Supplementary Figure S3 for PRKCA and TUBGCP2. (E) The comparison of the predicted MR ratio with the observed MR ratio. The hollow points and horizontal whiskers represent the expectation and 95% confident interval of predicted MR rate, respectively. The Y coordinates for the hollow points are the mean of experimental data. The solid points and vertical boxplots represents the experimental measured MR rates and their distributions of six replicates (three independent experiments with two replicates each), respectively. The diagonal line is Y = X. (Color version of this figure is available at Bioinformatics online.)
Therefore, the distinct m$_5$C pattern in mouse brain and HeLa cells (Supplementary Fig. S4). However, this weak correlation may only hold for transcripts with distribution diversity of m$_5$C over isoforms between real data and transcriptomic data (1.34, KS test, P-value <2.2e-16 for all the samples tested). The CV is a statistic measures the diversity of a distribution, and is calculated as the absolute FPKMs of isoforms divided by the average FPKM of the isoforms.

### Uneven m$_5$C distribution among tissues at isoform level

To explore the distribution of m$_5$C level in real samples, we applied quant to recently published RNA-Seq data in human (HeLa) and mouse (liver, kidney, heart and brain). At the isoform level, although the overall m$_5$C is low in all the samples tested, it is indeed higher in HeLa cells and mouse brain than other tissues and the difference is moderate (The median m$_5$C level in isoforms of HeLa cells, mouse liver, kidney, heart and brain were estimated as 0.035, 0.027, 0.025, 0.023 and 0.035, respectively) (Fig. 2B). Intriguingly, the distribution of m$_5$C at single-nucleotide level is undistinguishable in all mouse tissues and HeLa cells (Fig. 2C), suggesting the presence of more methylated cytosine sites in HeLa cells and mouse brain compared to other mouse tissues.

It is well known that the transcriptome pattern of brain is distinct from other tissues in mouse (Zheng-Bradley et al., 2010). Therefore, we asked if the distinct m$_5$C pattern in mouse brain stemmed from gene expression level per se. We found a weak negative correlation between RNA m$_5$C at isoform level and its expression (Spearman’s R = -0.31, P <2.2e-16) in mouse brain data. However, this weak correlation may only hold for transcripts with relatively low m$_5$C level because when we limit the analysis to the region of m$_5$C larger than 0.3, the correlation disappeared (Spearman’s R = 0.15, P = 0.26). A similar pattern can be found in other mouse tissues and HeLa cells (Supplementary Fig. S4). Therefore, the distinct m$_5$C pattern in mouse brain and HeLa cells may be largely independent of gene transcription activity.

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Next, we asked whether m$_5$C was evenly distributed in genome. If m$_5$C, in general, is subjected to a certain regulation, we reasoned that the distribution of m$_5$C over isoforms, or between genomic contexts, should deviate from random expectation. Thus, we compared the diversities of m$_5$C as estimated by Episo in real data to the random controls, which were simulated with comparable total expression and methylation levels (about 0.1%) using Bisulfitefq.

First, we found that the RNA m$_5$C in real data is less evenly distributed than random expectation (Fig. 3A). We employed the coefficient of variation (CV) to index the diversity. The CV is a statistic measures the diversity of a distribution, and is defined as the ratio of the standard deviation σ to the mean μ of the distribution and a distribution with a larger CV indicates that it is more diverse than distributions with smaller CVs. We compared the distribution diversity of m$_5$C over isoforms between real data and randomly simulations. The percentages of mRNAs that carrying m$_5$C are comparable between real and simulated control (29, 27, 34, 31 and 40% of mRNAs in the HeLa, liver, kidney, heart and brain, respectively, compared to about 40% in the control, Fig. 3B). The average CV of m$_5$C in HeLa, liver, kidney, heart and brain was 2.28, 2.06, 2.04, 2.08 and 1.94, respectively, higher than the expected CV in simulation (2.08 and 1.94, respectively, higher than the expected CV in simulation (2.08 and 1.94, respectively)). A similar pattern can be found in other mouse tissues and HeLa cells (Supplementary Fig. S4). Therefore, the distinct m$_5$C pattern in mouse brain and HeLa cells may be largely independent of gene transcription activity.

Next, we asked whether m$_5$C was evenly distributed in genome. If m$_5$C, in general, is subjected to a certain regulation, we reasoned that the distribution of m$_5$C over isoforms, or between genomic contexts, should deviate from random expectation. Thus, we compared the diversities of m$_5$C as estimated by Episo in real data to the random controls, which were simulated with comparable total expression and methylation levels (about 0.1%) using Bisulfitefq.

First, we found that the RNA m$_5$C in real data is less evenly distributed than random expectation (Fig. 3A). We employed the coefficient of variation (CV) to index the diversity. The CV is a statistic measures the diversity of a distribution, and is defined as the ratio of the standard deviation σ to the mean μ of the distribution and a distribution with a larger CV indicates that it is more diverse than distributions with smaller CVs. We compared the distribution diversity of m$_5$C over isoforms between real data and randomly simulations. The percentages of mRNAs that carrying m$_5$C are comparable between real and simulated control (29, 27, 34, 31 and 40% of mRNAs in the HeLa, liver, kidney, heart and brain, respectively, compared to about 40% in the control, Fig. 3B). However, for those methylated mRNA in real data, their diversity is much higher than that in control (Fig. 3B and C). The average CV of m$_5$C in HeLa, liver, kidney, heart and brain was 2.28, 2.06, 2.04, 2.08 and 1.94, respectively, higher than the expected CV in simulation (1.34, KS test, P-value <2.2e-16 for all the samples tested). The largest CV in simulation was less than three, while the distribution in real tissues was heavily tailed towards larger CVs. For example, 19, 9, 8, 9 and 7% genes for HeLa, liver, kidney, heart and brain, respectively, had CVs larger than 3 (Fig. 3D). This results suggest, at least these fraction of cytosines are methylated specifically towards certain isoforms and CG dinucleotide.

Next, we wondered whether this larger than expected CVs were also true at single-nucleotide level. Because the CV will be zero if a m$_5$C site was methylated in one isoform, we divided m$_5$C sites into two classes. The two classes, named singletons and multtons, contain the m$_5$C sites that be methylated in only one isoform and multiple isoforms, respectively. We noticed that the CVs in multtons are much smaller than control (Fig. 3C). The average CV of multtons in HeLa, liver, kidney, heart and brain control was 0.87, 0.97, 0.99, 0.98, 1.02 and 1.42 (KS test, P <2.2e-16 for all tissues), respectively. This lower than expected CV implies that, if a cytosine is methylated in multiple isoforms, the methylation tends to be even.

There are 991, 870, 872, 1261 and 2959 singletons in HeLa, mouse liver, kidney, heart and brain, respectively. We asked if they are biased to certain nucleotide type. By comparing the relative frequencies of m$_5$C at three di/tri-nucleotide (CG, CHH and CHG) contexts between these singletons and control, we detected a strong bias towards CG in all tissue types we tested (Fig. 3C). And m$_5$C is also enriched at CHH in brain. GO analysis of genes with CV > 0 showed that they were enriched for several post-translational modifications, while motif analysis on singletons showed that they were enriched for several post-translational modifications.
enriched in the simple repeats region (Supplementary Figs 55 and S6). Together, at the whole gene level, the above analysis implies that the distribution of mC may be isoform-specific, while at single-nucleotide level, it may be more CG specific.

4 Discussion

In this work, we described a computational tool, Episo, to quantify the RNA mC At isoform level and single cytosine sites. The ability to distinguish mC level between isoforms of the same gene distinguishes Episo from existing tools, e.g. meRanTK. To the best of our knowledge, Episo is currently the only method with this feature. The accuracy of Episo mainly depends on the expression level of the host gene and the methylation level per se (Fig. 2). Although the expected estimation error is almost zero, variation cannot be completely ignored. Given the low mC level in most real samples, ultra-high estimation error is almost zero, variation cannot be completely ignored. Given the low mC level in most real samples, ultra-high estimation error is almost zero, variation cannot be completely ignored. Given the low mC level in most real samples, ultra-high estimation error is almost zero, variation cannot be completely ignored. Given the low mC level in most real samples, ultra-high estimation error is almost zero, variation cannot be completely ignored. Given the low mC level in most real samples, ultra-high estimation error is almost zero, variation cannot be completely ignored. Given the low mC level in most real samples, ultra-high estimation error is almost zero, variation cannot be completely ignored. Given the low mC level in most real samples, ultra-high estimation error is almost zero, variation cannot be completely ignored. Given the low mC level in most real samples, ultra-high estimation error is almost zero, variation cannot be completely ignored. Given the low mC level in most real samples, ultra-high estimation error is almost zero, variation cannot be completely ignored. Given the low mC level in most real samples, ultra-high estimation error is almost zero, variation cannot be completely ignored. Given the low mC level in most real samples, ultra-high estimation error is almost zero, variation cannot be completely ignored. Given the low mC level in most real samples, ultra-high estimation error is almost zero, variation cannot be completely ignored.

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David Martin performed English language editorial services.

Author contributions

Z.Z. conceived this project. J.F.L. developed Episo, J.F.L. and Z.Z. analyzed data, Z.A., J.L. and F.L. performed the experiments, and Z.Z., F.L. and J.F.L. prepared the manuscript. All authors read and approved the final manuscript.

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References

Agri, P.F. (2015) The importance of being modified: an unrealized code to RNA structure and function. RNA, 21, 532–534. Amort, T., et al. (2017) Distinct 5-methylcytosine profiles in poly(A) RNA from mouse embryonic stem cells and brain. Genome Biol., 18, 1. Bailey, T.L., et al. (2009) MEME SUITE: tools for motif discovery and searching. Nucleic Acids Res., 37, W202–W208. Black, D.L. (2003) Mechanisms of alternative pre-messenger RNA splicing. Annu. Rev. Biochem., 72, 291–336. Blanco, S., et al. (2014) Aberrant methylation of tRNAs links cellular stress to neuro-developmental disorders. EMBO J., 33, 2020–2039. Bornmann, F., et al. (2018) BisAMP: a web-based pipeline for targeted RNA cytosine-5-methylation analysis. Methods., 156, 121–127. Branton, D., et al. (2008) The potential and challenges of nanopore sequencing. Nat. Biotechnol., 26, 1146–1153. Bray, N.L., et al. (2016)Near-optimal probabilistic RNA-Seq quantification. Nat. Biotechnol., 34, 525–527. Burgess, A.L., et al. (2015) Conservation of RNA and RNA 5-methylcytosine in the kingdom Plantae. BMC Plant Biol., 15, 199. Chen, X., et al. (2019) 5-Methylcytosine promotes pathogenesis of bladder cancer through stabilizing mRNAs. Nat. Cell Biol., 21, 978–990. Chi, K.R. (2017) The RNA code comes into focus. Nature, 542, 503–506. Dennis, G., et al. (2003) DAVID: database for annotation, visualization, and integrated discovery. Genome Biol., 4, P3. Edelheit, S., et al. (2013) Transcriptome-wide mapping of 5-methylcytidine RNA modifications in bacteria, archaea, and yeast reveals mC within archael mRNAs. PLoS Genet., 9, e1003602. Eid, J., et al. (2009) Real-time DNA sequencing from single polymerase molecules. Science, 323, 133–138. Fyre, M., et al. (2016) RNA modifications: what have we learned and where are we headed? Nat. Rev. Genet., 17, 365–372. Gabriel Torres, A., et al. (2014) Role of RNA modifications in human diseases. Trends Mol. Med., 20, 306–314. Huang, T., et al. (2019) Genome-wide identification of mRNA 5-methylcytosine in mammals. Nat. Struct. Mol. Biol., 26, 380–388. Hussain, S., et al. (2013a) Characterizing 5-methylcytosine in the mammalian epitranscriptome. Genome Biol., 14, 215. Hussain, S., et al. (2013b) NSun2-mediated cytosine-5 methylation of vault noncoding RNA determines its processing into regulatory small RNAs. Cell Rep., 4, 255–261. Jayaseelan, S., et al. (2011) RIP: an mRNA localization technique. Methods Mol. Biol., 714, 407–422. Khodhamp, V. and Cairns, B.R. (2013) Identification of direct targets and modified bases of RNA cytosine methyltransferases. Nat. Biotechnol., 31, 458–464. Krueger, F. and Andrews, S.R. (2011) Bismark: a flexible aligner and methylation caller for Bisulfite-Seq applications. Bioinformatics, 27, 1571–1572. Legrand, C., et al. (2017) Statistically robust methylation calling for whole-transcriptome bisulite sequencing reveals distinct methylation patterns for mouse RNAs. Genome Res., 27, 1589–1596. Liang, F., et al. (2016) BS-RNA: an efficient mapping and annotation tool for RNA bisulfite sequencing data. Comput. Biol. Chem., 65, 173–177. Mahmoud, M., et al. (2017) Efficiency of PacBio long read correction by 2nd generation Illumina sequencing. Genomics, 111, 43–49. Martin, M. (2011) Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet J., 17, 10–12. Members, B.I.G., et al. (2017) The B.I.G Data Center: from deposition to integration to translation. Nucleic Acids Res., 45, D13–D24. Montgomery, S.B. and et al. (2010) Transcriptome genetics using second-generation sequencing in a Caucasian population. Nature, 464. Pan, Q., et al. (2008) Deep surveying of alternative splicing complexity in the human transcriptome by high-throughput sequencing. Nat. Genet., 40, 1413–1415. Pop, M., et al. (2016) Posttranscriptional methylation of transfer and ribosomal RNA in stress response pathways, cell differentiation, and cancer. Curr. Opin. Oncol., 28, 65–71.
Rieder, D. et al. (2016) meRanTK: methylated RNA analysis ToolKit. Bioinformatics, 32, 782–785.
Schaefer, M. et al. (2010) RNA methylation by Dnmt2 protects transfer RNAs against stress-induced cleavage. Genes Dev., 24, 1590–1595.
Schwartz, S. et al. (2013) High-resolution mapping reveals a conserved, widespread, dynamic mRNA methylation program in yeast meiosis. Cell, 155, 1409–1421.
Shelton, S. B. et al. (2016) Who watches the watchmen: roles of RNA modifications in the RNA interference pathway. PLoS Genet., 12, e1006139.
Shendure, J. et al. (2017) DNA sequencing at 40: past, present and future. Nature, 550, 345–353.
Song, J. and Yi, C. (2017) Chemical modifications to RNA: a new layer of gene expression regulation. ACS Chem. Biol., 12, 316–325.
Squires, J. E. et al. (2012) Widespread occurrence of 5-methylcytosine in human coding and non-coding RNA. Nucleic Acids Res., 40, 5023–5033.
Trapnell, C. et al. (2009) TopHat: discovering splice junctions with RNA-Seq. Bioinformatics, 25, 1105–1111.
Trapnell, C. et al. (2010) Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. Nat. Biotechnol., 28, 511–U174.
Wang, X. et al. (2016) Structural basis of N-6-adenosine methylation by the METTL3-METTL14 complex. Nature, 534, 375.
Yang, X. et al. (2017) 5-Methylcytosine promotes mRNA export-NSUN2 as the methyltransferase and ALYREF as an m5C reader. Cell Res., 27, 606.
Yang, Y. et al. (2019) RNA 5-methylcytosine facilitates the maternal-to-zygotic transition by preventing maternal mRNA decay. Mol. Cell., 75, 1188–1202.e1111.
Yates, A. et al. (2016) Ensembl 2016. Nucleic Acids Res., 44, D710–D716.
Zhao, B. S. et al. (2017) Post-transcriptional gene regulation by mRNA modifications. Nat. Rev. Mol. Cell Biol., 18, 31–42.
Zhong-Bradley, X. et al. (2010) Large scale comparison of global gene expression patterns in human and mouse. Genome Biol., 11, R124.
Zhou, Y. et al. (2016) SRAMP: prediction of mammalian N-6-methyladenosine (m(6)A) sites based on sequence-derived features. Nucleic Acids Res., 44, e91.