Direct quantification of 3' terminal 2'-O-methylation of small RNAs by RT-qPCR

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ABSTRACT

Modification of nucleotides significantly increases the diversity of functional nucleic acids. As one of the most common modifications of RNAs, methylation of the 2'-hydroxyl-group of ribonucleotides (2'-O-methylation) has been found in various RNAs in eukaryotes. However, due to the lack of an efficient method for quantifying small RNA 3' terminal 2'-O-methylation, it is difficult to monitor the dynamic change of 3' terminal 2'-O-methylation during various biological processes. Capitalizing on the finding that 3' terminal RNA 2'-O-methylation can inhibit the activity of poly(A) polymerase, an enzyme that can add the poly(A)-tail to RNA, we develop a method by which the 2'-O-methylation level of small RNAs, such as microRNAs (miRNAs) and Piwi-interacting RNAs (piRNAs), can be directly quantified based on the poly(A)-tailed RT-qPCR technique. With this method, we successfully determine the 2'-O-methylation level of miRNAs in *Arabidopsis thaliana* and mouse lung tissue, piRNA in human seminal plasma, and monitor the alteration of miRNA 2'-O-methylation in *Drosophila* Schneider 2 cells after knockdown of *Drosophila* methyltransferase protein Hua enhancer 1 (DmHen-1).

Keywords: methylation; RT-qPCR; miRNA

INTRODUCTION

Nucleic acid modification is ubiquitous across many species and yields diversification of DNA and RNA (Limbach et al. 1994). Since the first pseudouridine was discovered 50 yr ago (Cohn 1960), more than 100 different RNA modifications have been identified in different organisms (Motorin and Helm 2011; Machnicka et al. 2013). Modified RNA molecules include tRNA, rRNA, mRNA, and a variety of small RNAs such as plant microRNAs (miRNAs). Among these various modifications, 2'-O-methylation (2'Ome), found in various RNAs in eukaryotes as well as bacteria and archaea, is one of the most common modifications of RNA. Recent studies indicate that different small RNAs in insects and mammals, including small interfering RNAs (siRNAs) and piRNAs, have 3' terminal 2'Ome (Kirino and Mourelatos 2007a; Shen et al. 2012).

It has been suggested that methyltransferase protein Hua enhancer 1 (HEN1) mediates the 3' terminal 2'Ome

of miRNAs, siRNAs, and piRNAs (Horwich et al. 2007; Kirino and Mourelatos 2007b; Ohara et al. 2007; Saito et al. 2007). In plants, nearly all small RNAs are 2'-O-methylated, regardless of the AGO proteins to which they bind (Yang et al. 2006). *Arabidopsis* HEN1 mediates the methylation of double-stranded small RNAs before their loading into AGO proteins (Yu et al. 2005). In *Drosophila*, the termini of siRNAs and piRNAs are methylated by DmHen1, the *Drosophila* homolog of HEN1 (Ghildiyal and Zamore 2009). Unlike plant HEN1, DmHen1 acts on a single strand in RISC but not on an RNA duplex (Horwich et al. 2007). Also in *Drosophila*, the small RNAs bearing the 2'-O-methylation at their 3' terminal end were generally found to be bound to AGO2 instead of AGO1 (Ameres et al. 2010). Although the exact role of 3' terminal 2'Ome for small RNAs remains unclear, it has been shown that 3' terminal 2'Ome can protect small RNAs from the 3' end uridylation and 3' to 5' exonucleolytic degradation (Li et al. 2005; Ji

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and Chen 2012). A previous study shows that poxviruses-encoded poly(A) polymerase mediates 3' polyadenylation of host miRNAs, resulting in the degradation of miRNAs (Backes et al. 2012), and 3' end 2'Ome may protect small RNAs from degradation during viral infection.

Although 2'Ome methylation is the most common modifications of RNAs, it is difficult to quantify the level of 2'Ome methylation in small RNAs including miRNAs and piRNAs (Aschenbrenner and Marx 2016). Presently, the identification of small RNA 2'Ome mainly relies on sodium periodate oxidation and northern blot or mass spectrometry (Yu et al. 2005; Ohara et al. 2007). These methods do identify the presence of 2'Ome in small RNAs but yield little quantitative information. Moreover, these technologies are labor intensive and time-consuming. Although a direct and site-specific quantification of RNA 2'Ome has been recently developed for long RNA fragments such as rRNA (Aschenbrenner and Marx 2016), there is no method available for directly quantifying the 3' terminal 2'Ome of small RNAs. With the observation of the ubiquity of 3' terminal 2'Ome modifications across a wide variety of RNAs, there is an urgent need for a direct method to quantify the 3' terminal 2'Ome level of small RNAs.

In the present study, we capitalize on the observation that 2'Ome of the terminal nucleotide can inhibit the activity of poly(A) polymerase and develop a poly(A)-tail RT-qPCR method for directly quantifying the 3' terminal 2'Ome level of small RNAs. Using this novel method, we have accurately quantified the level of 3' terminal 2'Ome of miRNAs extracted from *Arabidopsis thaliana* and mouse lung tissue, as well as piRNA from human seminal plasma.

RESULTS

Poly(A)-tailed RT-qPCR discriminates 2'-O-methylated miRNA from unmethylated miRNA

Through comparing the effect of various modifications on the 3' terminal nucleotide of a small RNA on yeast poly(A) polymerase-catalyzed reaction, Yang et al. (2006) found that among all modifications including 2'deoxy, 2'Ome, 3'deoxy, and 3'Ome, only 2'Ome significantly inhibited poly(A) polymerase activity. Capitalizing on this finding, we used poly(A)-tailed RT-qPCR to amplify equal amounts of 2'Ome miR-21 and unmethylated miR-21. We compared the result obtained from poly(A)-tailed RT-qPCR with that derived from the standard stem-loop primer RT-qPCR. The schematic diagram of stem-loop primer RT-qPCR and poly(A)-tailed RT-qPCR is shown in Figure 1A. The poly(A)-tailed RT-qPCR results clearly indicated a significant amplification delay on 2'Ome miR-21 compared with unmethylated miR-21, which is opposite to the stem-loop primer RT-qPCR result showing no significant difference between 2'Ome miR-21 and unmethylated miR-21 (Fig. 1B–D).

To exploit the concentration range of these two RT-qPCR methods to detect the percentage of 2'Ome in individual miRNA, we also serially diluted the synthetic miR-21 to concentrations ranging from 10^{-3} μ M to 10^{-9} μ M, and evaluated the level of amplification with poly(A)-tailed RT-qPCR and stem-loop primer RT-qPCR, respectively. Both poly(A)-tailed RT-qPCR and stem-loop primer RT-qPCR showed a linear correlation between Ct and miRNA concentration (Fig. 1E,F). In this system, the water background, representing a blank control for RT-qPCR, was around 10^{-9} μ M; the minimal concentration of miR-21 detected by poly(A)-tailed RT-qPCR was thus about 10 fM. The same strategy was applied to the mixture of methylated or unmethylated miR-16 and the result further indicated that poly(A)-tailed RT-qPCR had a higher Ct value when the percentage of miR-16 2'Ome increased (Supplemental Fig. S1).

Δ Ct method for direct quantification of short RNA 2'-O-methylation

Since poly(A)-tailed RT-qPCR but not stem-loop primer RT-qPCR shows significant amplification delay in methylated miRNAs, we hypothesized that the methylation ratio of a certain miRNA in a certain sample could be detected using these two systems. Therefore, according to the equation:

$$x = \frac{E_S^{Ct_{stdS}-Ct_S} - E_T^{Ct_{stdT}-Ct_T}}{E_T^{Ct_{stdT}-Ct_T-a}}.$$

We calculated the methylation ratio by performing RT-qPCR (formula derivation refers to Materials and Methods section) and then plugging Ct values into the equation. In this experiment, we mixed synthetic methylated MIR168 (2'Ome MIR168) with unmethylated MIR168 in a different ratio and then detected the mixed samples with both stem-loop primer RT-qPCR and poly(A)-tailed RT-qPCR. For MIR168 mixed samples with different methylation ratios, the stem-loop primer Ct values were almost the same (Fig. 2A), but the tailing Ct values varied with methylation ratios (Fig. 2B). By plugging the Ct values into the equation, we can calculate the determined methylation ratio (Fig. 2C). We named this method the Δ Ct method for methylation ratio detection. Next, we used the same Δ Ct method to analyze the percentage of MIR168 methylation in *Arabidopsis thaliana*. In this experiment, total RNA was extracted from *Arabidopsis thaliana*. As shown in Figure 2D, we measured the Ct value of MIR168 extracted from *Arabidopsis thaliana*, synthetic unmethylated MIR168, and 2'Ome MIR168 using stem-loop primer RT-qPCR and poly(A)-tailed RT-qPCR, respectively. According to the Δ Ct method, we calculated the percentage of MIR168 methylation in *Arabidopsis thaliana* to be 83% (Fig. 2E). Serving as either a positive or negative control,

FIGURE 1. Amplification delay on 2'OMe miR-21 by poly(A)-tailed RT-qPCR. (A) Schematic diagram of stem-loop primer reverse transcription and poly(A)-tailed reverse transcription. Black line represents template miRNA. Green line represents the stem-loop primers or universal primers. Red cross represents methylation site. (B) The amplification curve of poly(A)-tailed RT-qPCR for unmethylated miR-21 and 2'OMe miR-21. Note that there is a significant amplification delay by poly(A)-tailed RT-qPCR between unmethylated miR-21 and 2'OMe miR-21. (C) The amplification curve of stem-loop primer RT-qPCR for unmethylated miR-21 and 2'OMe miR-21. Note that there is no significant amplification delay by stem-loop primer RT-qPCR between unmethylated miR-21 and 2'OMe miR-21. (D) Ct value of stem-loop primer RT-qPCR and poly(A)-tailed RT-qPCR for unmethylated miR-21 and 2'OMe miR-21. Data were presented as scatter plots ($n = 9$). (E) The standard curve of stem-loop primer RT-qPCR for miR-21. RNA concentration represents the synthetic miR-21 concentration before reverse transcription. (F) The standard curve of poly(A)-tailed RT-qPCR for miR-21. RNA concentration represented the synthetic miR-21 concentration before reverse transcription. In panels E and F, the concentration ranges from 10^{-3} to 10^{-9} μM. The upper left shows the regression equation and the goodness-of-fit.

synthetic 2'OMe MIR168 or unmethylated MIR168 showed perfect or negative methylation, respectively.

Nonlinear regression method for direct quantification of miRNA 2'-O-methylation

Given that plant miRNAs generally are all 3' terminal methylated, the methylation ratio of 83% for *Arabidopsis thaliana* MIR168 detected by our method seemed quite low.

After analyzing the Ct value of various miRNAs for methylation ratio measurement, we observed that the relationship between the Ct value of poly(A)-tailed RT-qPCR and methylation of miRNA fitted more into a second order polynomial curve. As shown in Figure 3A, the relationship between the 2'OMe level of MIR68 and the Ct value of tailed RT-qPCR displayed a perfect second order polynomial curve. The results showed the goodness-of-fit of MIR168 2'OMe versus the Ct value of tailed RT-qPCR by

FIGURE 2. Quantification of miRNA methylation ratio by ΔC_t method. (A,B) Stem-loop primer RT-qPCR (A) and poly(A)-tailed RT-qPCR (B) C_t values of different methylation ratio synthetic MIR168. By substituting C_t values in the formula, we calculated the methylation ratio for each synthetic sample ($n=6$). (C) Accuracy of ΔC_t method in detecting various 2'-Ome levels of synthetic MIR168. (D) Stem-loop primer RT-qPCR and poly(A)-tailed RT-qPCR C_t values of MIR168 in *Arabidopsis thaliana*, synthetic 2'-Ome (methylated), and unmethylated MIR168 ($n=9$). (E) Quantification of the methylation ratio of MIR168 in *Arabidopsis thaliana* by ΔC_t method. The detected percentage of 2'-Ome in MIR168 is indicated on the top of the column. Data are presented as Mean $\pm$ SE ($n=3$).

nonlinear regression analysis as 0.9953 (Fig. 3B), which was significantly higher than that of linear regression analysis (0.9605, Fig. 2C). We next tested the mixture of synthetic methylated or unmethylated miR-21 and miR-16, and found that the relationship between their 2'-Ome levels and C_t values of tailed RT-qPCR all fit better in a second order polynomial curve (Fig. 3B), suggesting that the second order polynomial curve may be suitable for all short RNAs. To determine the working concentration range of this nonlinear regression analysis, we examined miR-21 at different concentrations. The result showed that the goodness-of-fit of miR-21 2'-Ome versus the C_t value of tailed RT-qPCR was 0.9608 and 0.9834 when the concentration of miR-21 was 100 and 1 pM, respectively (Fig. 3B), suggesting that nonlinear regression analysis has a wide working range of miRNA concentrations. To distinguish from the linear ΔC_t method for methylation ratio detection, we named this method as a nonlinear regression method. Next, we further compared the accuracy of these two methods in detecting miRNA 2'-Ome levels in a mixture of synthetic methylated and unmethylated miRNAs. As shown in Supplemental Figure S2, the nonlinear regression method (left panels) displayed higher accuracy in measuring the 2'-Ome level of MIR168 (100 pM), miR-21

(100 pM), and miR-21 (1 pM) than that of the linear ΔC_t method (right panels). A significantly higher goodness-of-fit ($R^2=0.9935$ for MIR168 and $R^2=0.975$ for miR-21) was obtained by the nonlinear regression method compared to that by the ΔC_t method ($R^2=0.8231$ for MIR168 and $R^2=0.7756$ for miR-21) when miRNA concentration was 100 pM (Supplemental Fig. S2A,B). Similar results were obtained when miRNA concentration was 1 pM (Supplemental Fig. S2C).

Next, we applied this nonlinear regression method to assess the methylation percentage of individual small RNAs in a real biological sample. In this experiment, synthetic methylated and unmethylated oligonucleotides were mixed according to different ratios as standard samples for establishing the standard curve, respectively. The methylation ratio of MIR168 in *Arabidopsis thaliana*, miR-16 in mouse lung tissue, and piR-31068 in human seminal plasma was analyzed according to the respective standard curve. In this experiment, the total RNAs from *Arabidopsis thaliana*, mouse lung tissue, and human seminal plasma were extracted as previously described (Chen et al. 2008; Hong et al. 2016). Based on individual standard curves, we detected the 2'-Ome level of MIR168 in *Arabidopsis thaliana* as 98% (Fig. 4A). By the same

FIGURE 3. Ct value of poly(A)-tailed RT-qPCR and methylation ratio fit of a quadratic curve. (A) Scatter plot of tailing Ct and methylation fraction for synthetic MIR168. The distribution of plots presents a curve. (B) Regression curve of Ct value and methylation ratio for different miRNAs at different concentrations. Upper left is 100 pM MIR168, upper right is 100 pM miR-16, lower left is 100 pM miR-21, and lower right is 1 pM miR-21. Data are presented as Mean $\pm$ SE ($n = 9$).

method, we obtained the 2'Ome level of miR-21 in mouse lung tissue as -0.2% (Fig. 4B) and piR-31068 in human seminal plasma as 99% (Fig. 4C), respectively. This result suggests that *Arabidopsis thaliana* MIR168 and human piR-31068 are almost completely 2'-O-methylated at 3' terminus, whereas mouse miR-21 is not 2'-O-methylated at all.

It has been shown that the termini of siRNAs and piRNAs in *Drosophila* are methylated by DmHen1, the *Drosophila* homolog of HEN1 (Ghildiyal and Zamore 2009). To test whether this direct RT-qPCR method can monitor the alteration of miRNA methylation, we knocked down DmHen1 expression in *Drosophila* Schneider 2 cells via siRNA. Three DmHen1 siRNA (DmHen1-siRNA1, 2, and 3) and scramble oligonucleotides were obtained and transfected into *Drosophila* Schneider 2 cells separately or in a combined fashion (DmHen1-siRNA1-3). As shown in Figure 5A, the expression level of *Drosophila* DmHen1 was significantly reduced by three DmHen1 siRNAs but not scramble oligonucleotides. When combining three DmHen1 siRNAs, the *Drosophila* DmHen1 level in *Drosophila* Schneider 2 cells was decreased to $\sim 30\%$ of the original level. After extracting RNA from the cells and performing the stem-loop RT-qPCR and tailed RT-qPCR, we calculated the 2'Ome level of Dme-miR-263-5p in various *Drosophila* Schneider 2 cells by the nonlinear regression method. The result showed that the 2'Ome level of Dme-miR-263-5p in *Drosophila* Schneider 2 cells treated with DmHen1-siRNA1 or DmHen1-siRNA1-3 was reduced from $103.3 \pm 6.1\%$ to $73.2 \pm 1.7\%$ and $49.3 \pm 5.0\%$, respectively (Fig. 5B). The decrease of the Dme-miR-263-5p 2'Ome level is positively associated with the reduction of DmHen1 in *Drosophila* Schneider 2 cells.

DISCUSSION

Accumulating evidence has suggested that plant miRNAs play important roles in various biological processes in not only plants, but also in mammals as exogenous miRNAs derived from food (Carrington and Ambros 2003; Zhang et al. 2012). Given that plant miRNAs, as well as piRNA in animals, are 2'-O-methylated at the 3' end nucleotide, accurate detection of the 2'-O-methylation of small RNAs is of great importance for further understanding their biological functions. In this study, by capitalizing on the finding that 2'-O-methylation of miRNAs at the 3' terminal nucleotide can inhibit the activity of poly(A) polymerase, an enzyme essential for RT-qPCR assay, we develop a poly(A)-tailed RT-qPCR method by which the 2'Ome level of small RNAs such as miRNAs and piRNAs can be directly quantified. Using this method, we directly quantified the level of 3' terminal 2'Ome of miRNAs extracted from plant and mouse lung tissue, as well as piRNA from human seminal plasma. Furthermore, we successfully monitored the alteration of miRNA 2'-O-methylation in *Drosophila* Schneider 2 cells after decreasing DmHen-1 expression via DmHen-1-specific siRNA.

The current method for identifying the 2'-O-methylation of small RNA is sodium periodate oxidation combined with northern blot or mass spectrometry (Yu et al. 2005; Ohara et al. 2007). Although these methods are definitive tools to study small RNA methylation, they often require a large amount of RNA and are time-consuming. Therefore, to develop new methods to quantitatively analyze small RNA methylation, several approaches have been pursued. In many cases, these methods are limited to qualitative analysis rather than quantitative analysis of small RNA

FIGURE 4. The 2'Ome level of MIR168 in *Arabidopsis thaliana*, miR-16 in mouse lung tissue, and piR-31068 in human seminal plasma detected by nonlinear regression method. (A) The 2'Ome level of *Arabidopsis thaliana* MIR168 and synthetic methylated or unmethylated MIR168 detected by nonlinear regression method. (B) The 2'Ome level of mouse lung miR-16 and synthetic 2'Ome (methylated) or unmethylated miR-16 detected by nonlinear regression method. (C) The 2'Ome level of piR-31068 in human seminal plasma and synthetic methylated or unmethylated piR-31068 detected by nonlinear regression method. In panels A–C, the detected methylation ratios are indicated on the top of the column, while the standard curves of Ct versus methylation for MIR168, miR-16, and piR-31068 are shown in the upper right, respectively. Data are presented as Mean $\pm$ SE ($n=3$ or 9 as indicated).

FIGURE 5. The 2'Ome level of *Drosophila* Dme-miR-263-5p in *Drosophila* Schneider 2 cells with or without silencing DmHen1. (A) Reduction of DmHen1 in *Drosophila* Schneider 2 cells via DmHen1 siRNAs analyzed by western blotting. (B) The 2'Ome level of Dme-miR-263-5p in *Drosophila* Schneider 2 cells treated with DmHen1 siRNAs was decreased compared to that in *Drosophila* Schneider 2 cells treated with scramble oligonucleotides. The detected methylation ratios of *Drosophila* Dme-miR-263-5p and synthetic methylated or unmethylated Dme-miR-263-5p are indicated on the top of the column, while the standard curves of Ct versus methylation for Dme-miR-263-5p are shown in the upper right. Data are presented as Mean $\pm$ SE ($n = 3$).

methylation. The main problem of quantifying small RNA methylation is the bias against the RNA 3' terminal 2'-O-methylation by polyadenylate polymerase and T4 RNA ligase, two enzymes that are required for RNA labeling or ligation in commercial miRNA profiling assays. Since polyadenylate polymerase and T4 RNA ligase activity is inhibited by the RNA 3' terminal 2'-O-methylation (Yang et al. 2006; Munafo and Robb 2010), the normal RT-qPCR assay with commercial miRNA probes will produce lower detection results of 2'Ome miRNAs. To minimize this bias related to RNA 3' end 2'-O-methylation, Shen et al. (2012) invented a label-free miRNA profiling method, which makes the high-throughput sequencing more accurate. To overcome the impairment of polymerization reactions for plant miRNAs, which 2'-O-methylation at the 3' end nucleotide limits their capacity as primers for polymerization reactions, Wang et al. (2017) designed an elegant three-way junction structure-based isothermal exponential amplification reaction (3WJ-EXPAR), which provides a non-biased technique for detecting plant miRNAs with 3' terminal 2'Ome. Using this 3WJ-EXPAR system, they accurately

detected the level of methylated plant miRNAs in laboratory conditions.

In contrast to these approaches, we capitalized on the bias of poly(A) polymerase against 3' terminal RNA 2'-O-methylation, and developed a poly(A)-tailed RT-qPCR method for directly detecting 2'Ome levels of miRNAs and piRNAs. For individual methylated miRNA or piRNA, poly(A)-tailed RT-qPCR assay results in a higher Ct value than the standard stem-loop miRNA RT-qPCR assay. In fact, more methylation for a small RNA means a larger difference of Ct between two RT-qPCR methods. Therefore, through analyzing the Ct difference we can determine the percentage of 3' terminal 2'Ome for individual miRNA or piRNA. A similar method has been developed for assaying the methylation level of long RNA fragments such as rRNA (Aschenbrenner and Marx 2016).

Although this direct RT-qPCR assay provides a rapid and accurate method for measuring the level of 3' terminal 2'Ome for small RNAs in general, it also has limitations in application. When the 2'Ome level of individual miRNAs is low, for example, <20%, the Ct value and methylation

ratio are not positively correlated. In other words, this method cannot accurately detect samples with a low 2'-Ome level. This may be due to the relatively low efficiency of poly(A)-tailed RT-qPCR assays for methylated small RNA. To accurately quantify the low 2'-Ome level of small RNAs, a non-biased RT-qPCR assay might be required. It is also worth mentioning that this method may not work accurately in quantification of long RNA 3'-terminal 2'-O-methylation since our method just discriminates 3' terminal 2'-Ome and non-2'-Ome small RNAs. As internal ribonucleoside 2'-Ome modification does significantly influence the activity of poly(A) polymerase by adding poly(A) tails, our method would consider the internal ribonucleoside 2'-Ome modifications as nonmethylated 3' terminal small RNAs.

MATERIAL AND METHODS

Synthetic miRNA oligonucleotides and primers

Synthetic miRNA oligonucleotides include nonmethylated miRNA oligonucleotides, 2'-Ome miRNA oligonucleotides (GenePharma) for each miRNA. Primers include stem-loop RT primer, miRNA sense primer, and universal RNA primer (URP) (GenScript) for each miRNA. Oligonucleotides and primers powder were dissolved in DEPC water to generate 20 μ M and 100 nM/ μ L stock solution and stored in -70°C , respectively. For miRNA methylation detection, the oligonucleotides were diluted into 100 pM concentration before use. For stem-loop primer RT-qPCR calibration, the reaction concentration is 0.1–1.0 μ M. The sequences of all used oligonucleotides and primers are listed in [Supplemental Table S1](#).

RNA extraction

RNAs were extracted with TRI Reagent (Sigma-Aldrich). A total of 50–100 mg tissue or cell samples were grinded with 1 mL TRI Reagent in a tissue grinding apparatus. Lung tissues were obtained from 6-wk-old male Balb/c mice. Human semen samples were collected from healthy donors in Jinling Hospital and Nanjing Drum Tower Hospital, Nanjing University with signed consent forms, and the seminal plasma was obtained by centrifuging semen samples at 3000 rpm for 5 min and collecting supernatant. For RNA extraction, 100 μ L supernatant was mixed with 1 mL TRI Reagent. After grinding on ice, samples were incubated at room temperature for 5 min, supplemented with 200 μ L chloroform, vortexed, and centrifuged at 12,000g, 4°C for 15 min. The supernatant was transferred to a new 1.5 mL tube, and then supplemented with 500 μ L isopropanol and incubated at -20°C for 1 h followed by centrifugation at 16,000g, 4°C for 20 min. After discarding supernatant, the pellet was washed with 1 mL 75% RNase free EtOH, centrifuged again at 16,000g, 4°C for 20 min. The pellet was then air-dried and dissolved in 20–50 μ L DEPC water.

Cell culture, siRNA transfections, and western blotting

Drosophila Schneider 2 (S2) cells (ATCC, Schneider's *Drosophila* Line 2) were cultured in Schneider's *Drosophila* Medium (Gibco)

supplemented with 10% fetal bovine serum (Gibco) at 25°C . For transfections, cells were seeded at 8×10^5 cells/well in a 6-well dish, and then cells were transfected with DmHen-1 siRNAs (at a final concentration of 100 nM) using a Cellfectin II Reagent (Invitrogen). The siRNA sequences are as follows: DmHen1-siRNA1: sense: 5'-3' GCUGGAAGGAUCAGAUUAATT, antisense: 5'-3' UUAUUCUGAUCCUCCAGCTT, DmHen1-siRNA2: sense: 5'-3' GCAAACCCAAU AUCGAUAATT, antisense: 5'-3' UUAUC GAUUAUGGGUUUGCTT, DmHen1-siRNA3: sense: 5'-3' CCUC CCAGGAUGAUGCUUUTT, antisense: 5'-3' AAAGCAUCAUCC UGGGAGGTT. At 48 h post-transfection, cells were harvested for western blotting. After transfection, cells were harvested and dissolved in RIPA lysis buffer (Beyotime, P0013B) for 30 min on ice. Cell lysates were then centrifuged at 12,000g for 20 min at 4°C , and the supernatants were harvested. After mixed with loading buffer and denaturation at high temperature, proteins (30 μ g/lane) were loaded on 10% SDS-PAGE gels. After SDS-PAGE separation, the proteins were transferred onto polyvinylidene difluoride membranes. Incubation with primary antibodies was overnight at 4°C ; secondary antibodies were incubated with the membrane for 120 min at room temperature. The DmHen-1 antibody (Santa Cruz Biotechnology) was used in a 1:500 dilution as a loading control, and β -actin antibody (Cell Signaling) was used at a 1:1000 dilution. Western blot images were analyzed by Image J and normalized by β -actin.

Calibration between nonmethylated and 2'-Ome miRNA oligonucleotides

2'-Ome miRNA and unmethylated miRNA oligonucleotides were diluted into the same concentration, and then subjected to stem-loop primer reverse transcription and RT-qPCR detection. There were at least three replicates in each sample. If the difference value for Ct between two samples is smaller than 0.2, we will consider the calibration is completed. If not, we continue diluting the higher concentration sample according to the Ct value until the Ct difference is smaller than 0.2.

Formula derivation

According to the equation:

$$\text{Methylation fraction} = 1 - E^{-(\Delta C_{T1} - \Delta C_{T2})},$$

with E = PCR efficiency (in an ideal situation, E = 2). In this equation, ΔC_{T1} and ΔC_{T2} represent stem-loop RT-qPCR and poly(A)-tailed RT-qPCR systems, respectively. To obtain more accurate results, we refined an equation as follows:

$$\frac{E_S^{C_{TstdS} - C_{TS}}}{E_T^{C_{TstdT} - C_{TT}}} = (1 - x) + x \cdot E_T^{-a},$$

in which

E_S = stem-loop PCR efficiency;

E_T = tailing PCR efficiency;

x = methylation fraction;

a = ΔC_t between synthetic methylated miRNAs and equal amounts of unmethylated miRNAs with poly(A)-tailed RT-qPCR;

C_{stdS} = Ct value of synthetic oligonucleotide with stem-loop primer PCR (the synthetic oligonucleotides concentration is in the same level with the sample to be detected);

C_{stdT} = Ct value of synthetic oligo nucleotide with poly(A)-tail PCR;

C_S = Ct value of stem-loop primer RT-qPCR for the sample to be tested;

C_T = Ct value of poly(A)-tailed RT-qPCR for the sample to be tested.

Therefore, x can be derived through the solution of this equation:

$$x = \frac{E_S^{C_{stdS}-C_S} - E_T^{C_{stdT}-C_T}}{E_T^{C_{stdT}-C_T-a}}.$$

RT-qPCR for stem-loop primer method and tailing method

There were two systems of RT-qPCR in our research: stem-loop primer system and tailing system. For the stem-loop primer system, which was used for calibration, the reverse transcription system included 0.25 U/ μ L AMV Reverse transcriptase XL (Takara), 0.25 mM dNTP mixture (Takara), and 0.25 μ M stem-loop RT primer according to the vendor's protocol. Samples were kept on ice before running programs. The reverse transcription program was as follows: 16°C for 15 min, 42°C for 30 min, and 85°C for 5 min. The real-time quantitative PCR system was 0.07 U/ μ L rTaq (Takara), 0.2 mM dNTP (Takara), 1 $\times$ EvaGreen (Invitrogen), 0.125 μ M sense primer, 0.125 μ M URP, 1.5 mM $MgCl_2$, 1 $\times$ PCR buffer (Mg^{2+} free). The program was as follows: 95°C for 5 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Each cDNA sample was tested in triplicate. For the tailing system, which was used for detecting methylation proportion in synthetic oligonucleotides as well as sample RNAs, All-in-One miRNA First-Strand cDNA Synthesis Kit (GeneCopoeia), containing poly(A) polymerase and RTase Mix, was used according to the manufacturer's instructions. After obtaining cDNAs, rTaq (Takara), dNTP (Takara), EvaGreen (Invitrogen), sense primer, and Universal Adaptor PCR Primer (GeneCopoeia) were used for RT-qPCR detection. The program was as follows: 95°C for 5 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Each cDNA sample was tested in triplicate.

Nonlinear regression analysis and calculation of methylated proportion

After obtaining tailing Ct values for different methylation ratio synthetic oligonucleotides, the Ct values of miRNAs and their corresponding methylation proportion were fitted into a second order polynomial curve (Excel, Microsoft), such as $y = ax^2 + bx + c$, in which y and x represent Ct value and methylation proportion, respectively. X was then calculated according to the formula.

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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