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Vinther, Jeppe

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No Evidence for N7-Methylation of Guanosine (m<sup>7</sup>G) in Human let-7e

Jeppe Vinther<sup>1</sup>,<sup>*</sup>

<sup>1</sup>Department of Biology, University of Copenhagen, Ole Maaløes Vej 5, DK-2200 Copenhagen N, Denmark
<sup>*</sup>Correspondence: jvinther@bio.ku.dk

In a recent study published in Molecular Cell, it was concluded that “METTL-1 Promotes let-7 MicroRNA Processing via m<sup>7</sup>G Methylation” (Pandolfini et al., 2019). The authors develop two sequencing-based methods for pull-down of internally m<sup>7</sup>G methylated RNA. One of the methods (m<sup>7</sup>G-RIP-seq) is based on RNA immunoprecipitation with an m<sup>7</sup>G-specific antibody. The other (BoRed-seq) takes advantage of selective reduction of m<sup>7</sup>G with NaBH<sub>4</sub> to create abasic sites, which are subsequently biotinylated with N’-aminooxymethylcarboxyhydrazino D-biotin and purified by binding to streptavidin beads. Based on the overlap between enriched RNAs detected by the two methods, the authors identified 32 “high confidence” m<sup>7</sup>G modified microRNAs, including the let-7e microRNA, for which the methylation was validated and located to position 18 in the precursor hairpin using LC-MS/MS applied to an RNase A fragmented sample (Pandolfini et al., 2019). In this Letter, I demonstrate that the LC-MS/MS results presented by Pandolfini and coworkers are misinterpreted, and in fact show that let-7e is unlikely to be m<sup>7</sup>G modified. I also point out issues with the sequencing-based methods of m<sup>7</sup>G detection used by Pandolfini and coworkers, which questions whether the let-7e microRNA, or indeed any other human microRNA, is m<sup>7</sup>G modified.

Our lab recently published a method for detection of internal m<sup>7</sup>G modifications based on NaBH<sub>4</sub> reduction and subsequent mapping of mutations in sequencing data (Enroth et al., 2019). The methylation of let-7e described by Pandolfini and coworkers is not supported by our data. In contrast, we demonstrate that let-7e is not m<sup>7</sup>G modified in human HeLa cells (Enroth et al., 2019). In our publication, we also noted that RNase A cleavage of human LSU rRNA produces an RNA fragment identical in sequence to the let-7e fragment analyzed by Pandolfini and coworkers. Strikingly, this fragment has an U60 snoRNA-guided 2’ OMe modification exactly at the position reported by Pandolfini and coworkers to be m<sup>7</sup>G modified (Krogh et al., 2016; Taoka et al., 2018). This raises the possibility that Pandolfini and coworkers are detecting the LSU rRNA fragment rather than the let-7e fragment.

In MS analysis, the positive charge on m<sup>7</sup>G makes m<sup>7</sup>G modified RNA positions especially prone to base loss, which previously has been exploited to validate m<sup>7</sup>G modifications (Guymon et al., 2006; Wong et al., 2013). Therefore, I calculated the expected m/z values based on either G or m<sup>7</sup>G loss for the relevant sub-fragments observed by Pandolfini and coworkers in their MS/MS analysis (Figure S1A). Comparison with the MS/MS spectrum obtained by Pandolfini and coworkers (Figure S1B) did not provide any clear evidence for m<sup>7</sup>G loss, whereas four separate sub-fragments unequivocally support the loss of G (Figure S1C). This demonstrates that the methylated RNase A fragment presented by Pandolfini and coworkers is derived from the LSU rRNA rather than the let-7e microRNA. On the other hand, the non-methylated RNase A fragment, which is also detected by Pandolfini and coworkers, is unlikely to stem from the LSU rRNA because the stoichiometry of the 2’ OMe modification at position 4340 of the LSU rRNA is 99% (Taoka et al., 2018). In the sequencing experiments performed by Pandolfini and coworkers, let-7e is the most highly expressed ncRNA with the potential to produce the RNase A fragment AGGAGGU (Figure S1D). The presented MS evidence therefore suggests that let-7e is not m<sup>7</sup>G modified, which questions the specificity of the BoRed-seq and m<sup>7</sup>G-RIP-seq methods and the validity of the list of “high confidence” m<sup>7</sup>G modified microRNA.

To demonstrate m<sup>7</sup>G-dependent enrichment in the BoRed-seq and m<sup>7</sup>G-RIP-seq methods, Pandolfini and coworkers show enrichment of tRNAs, some of which contain known m<sup>7</sup>G modifications. For m<sup>7</sup>G-RIP-seq, enrichment of the m<sup>7</sup>G-containing SSU rRNA over the LSU rRNA was also demonstrated. The BoRed-seq protocol includes an acid hydrolysis step in the presence of 1.7 mM 7-methyl GTP to create abasic sites from the NaBH<sub>4</sub> reduced RNA. In our hands this treatment is not necessary to give abasic sites (Enroth et al., 2019). Acid treatment in the presence of amines is known to result in RNA strand cleavage via beta-elimination at abasic sites (Küpfner and Leumann, 2007), which would compete with the biotinylation reaction expected to occur in BoRed-seq. I have reanalyzed the sequencing data and for m<sup>7</sup>G modified tRNAs, I find that reads mapping to the 3’ end of tRNAs and terminating at position 47 appear in the BoRed-seq NaBH<sub>4</sub>-treated sample (Figure S1E). This shows that widespread beta-elimination occurs in the BoRed-seq protocol and suggests that the observed enrichment for tRNAs is caused by beta-elimination leading to formation of a 5’ phosphophate (Küpfner and Leumann, 2007) and efficiently sequencing library preparation rather than specific pull-down. This is further supported by the lack of enrichment of the m<sup>7</sup>G modified SSU rRNA compared to the non-m<sup>7</sup>G modified LSU rRNA in the experiment (Figure S1F).

For the m<sup>7</sup>G-RIP-seq experiment, Pandolfini and coworkers show that the m<sup>7</sup>G modified SSU rRNA is enriched compared to the non-modified LSU rRNA in a bioanalyzer trace. At the level of reads mapping to the two rRNAs, I also observe solid enrichment of the
SSU over the LSU rRNA (Figure S1G). The same is true for tRNA known to have m7G modifications, which are enriched when compared to tRNAs without m7G modifications. The “high confidence” set of m7G modified microRNAs are significantly less enriched than m7G modified tRNAs, suggesting that it is problematic to identify m7G modification of microRNAs based on the m7G-RIP-seq data alone (Figure S1G). I also note that the comparison between the m7G-RIP RNA sample and the input RNA fails to control for unspecific binding of RNA to tube, beads, and antibody, although the m7G competitive elution used in the protocol may to some degree mitigate this issue.

Taken together, reanalysis of the data presented by Pandolfini and coworkers demonstrates that let-7e is unlikely to be m7G modified and that the BoRed-seq and m7G-RIP-seq methods are not properly controlled or validated. I therefore conclude that N7-methylation of guanosine in let-7e or any other human microRNA remains to be shown.

**SUPPLEMENTAL INFORMATION**

Supplemental Information can be found online at https://doi.org/10.1016/j.molcel.2020.05.022.

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