Original Article

Human cancer cells compensate the genes unfavorable for translation by N^6-methyladenosine modification and enhance their translation efficiency

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Background: N^6-methyladenosine (m^6A) is the methylation of RNA adenosines that participates in multiple biological processes, such as facilitating translation of host genes via the reader protein YTHDF1. The core writer protein of m^6A in humans is METTL3.

Methods: We utilized YTHDF1 target genes and normal or si-*METTL3* NGS (next-generation sequencing) data from HeLa cells generated by a previous work and collected known human oncogenes from a website. We evaluated the translation capability of these m^6A genes or oncogenes by comparing their mRNA lengths and codon usage bias. Additionally, we calculated the translation efficiency of all genes expressed in the normal or si-*METTL3* HeLa cells using NGS data.

Results: The m^6A genes are enriched in oncogenes compared to the non-m^6A genes. We observed significantly longer mRNA lengths for the m^6A genes, especially for the oncogenes. We also observed stronger codon usage bias for the m^6A genes than for the non-m^6A genes. We provided evidence that the longer mRNA lengths and stronger codon bias were unfavorable for translation. However, this disadvantage was compensated by m^6A modification because the m^6A genes but not the non-m^6A genes showed higher translation efficiencies in normal cells than in si-*METTL3* cells.

Conclusions: HeLa cells compensate for genes unfavorable for translation by m^6A modification and enhance their translation efficiency. This compensation could originally have been designed for oncogenes, since we observed enrichment of m^6A genes in the oncogenes. If oncogenes modified by m^6A obtain higher translation efficiencies and eventually facilitate cancer cell proliferation, then this strategy may be used by cancers for rapid cell growth.

Keywords: N^6-methyladenosine (m^6A); translation efficiency; oncogenes; mRNA length; codon usage bias

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Introduction

RNA modification is omnipresent in all living organisms and cells. More than one hundred types of RNA modifications are known (1), of which the two most prevalent in the animal kingdom are A-to-I RNA editing and N^6-methyladenosine (m^6A). A-to-I RNA editing is most commonly observed in coding mRNAs from more primitive organisms (2-5) or repetitive sequences in mammals (6-8), whereas m^6A is widespread in the transcriptomes of many animal species (9-17). In humans, tens of thousands of m^6A sites have been identified in cell lines, such as HeLa cells (13,14); typically, these sites are enriched around stop codons

and have the consensus sequence context GRAC (R = A or G; A = methylated A) (14). Proteins interacting with m^{6}A include the m^{6}A writers (METTL3 and METTL14), readers (YTH domain proteins) and erasers (18). Functional studies have shown that m^{6}A can affect mRNA stability (10,13) or promote translation of host genes (10,14) according to the different readers that bind to m^{6}A sites. Interestingly, in human HeLa cells, m^{6}A sites bound by the reader protein YTHDF1 increase the translation efficiency of host genes (14), whereas m^{6}A sites bound by another reader (IGF2BP) can either facilitate translation or stabilize host mRNAs (10). A handful of studies have discussed the potential relationship between the m^{6}A modification and cancer (10,19-21), but no study has systematically investigated how m^{6}A can affect the translation of global oncogenes (rather than particular oncogenes). Furthermore, the overall relationship (or overlap) between m^{6}A genes and oncogenes is unreported. We believe that this basic information is important for determining whether m^{6}A genes and oncogenes are mutually favored or avoided. Importantly, even researchers who mention the enhanced translation efficiency of oncogenes caused by m^{6}A have not given a "biological" reason why translation of the target genes should be elevated. Specifically, why should m^{6}A facilitate the translation of oncogenes if the oncogenes are already optimized for a high translation efficiency? With the development of next-generation sequencing (NGS) techniques, NGS big data and many bioinformatics tools have been commonly applied to cancer studies (22,23). In this study, by utilizing the YTHDF1 target genes and the normal or si-*METTL3* NGS data from HeLa cells generated by a previous work (14), we demonstrate that the m^{6}A genes in HeLa cells originally are unfavorable for translation due to their significantly longer mRNA lengths and stronger codon usage bias. However, the translation of these target genes is compensated by the m^{6}A modification, because the translation efficiency of the m^{6}A genes is significantly higher in normal cells (with m^{6}A) than in si-*METTL3* cells (no m^{6}A). Furthermore, we found enrichment of m^{6}A genes in human oncogenes, and these oncogenes were even less suitable for translation. Our results suggest that the compensation of translation by m^{6}A may originally have been designed for those oncogenes to help cancer cell growth. Alternatively, the m^{6}A modification at least facilitates the translation of target genes that originally are unfavorable for translation. This strategy may be used by cancer cells to increase the protein quantity of a particular set of genes and eventually achieve rapid cell growth. This study deepened our understanding of the role played by m^{6}A modification in cancer cells and revealed why m^{6}A genes and some oncogenes need methylation to enhance their translation. We also provided novel insights into a potential method to suppress oncogenes in cancer cells.

Methods

Data collection

We collected the m^{6}A peaks bound by the reader protein YTHDF1 reported in a previous study (14). The list of oncogenes was downloaded from the latest version of the Cancer Gene Census website (CGC, https://cancer.sanger.ac.uk/census/). NGS mRNA-Seq and Ribo-Seq data from normal or si-*METTL3* HeLa cells were obtained from the same study (14). Adenosines within the GRAC (R = A or G; A = methylated A) motif in m^{6}A peaks were defined as m^{6}A sites in HeLa cells.

Annotation of m^{6}A sites

We annotated the m^{6}A sites using the hg19 human genome downloaded from the UCSC Genome Browser (genome.ucsc.edu). If an m^{6}A site hit multiple isoforms of the same gene, then the transcript with the longest CDS (canonical transcript) was retained. The canonical transcript of each gene was defined by the SnpEff software (24). An m^{6}A modification that did not hit any genes was annotated as intergenic.

Processing of the next-generation sequencing data

We aligned the NGS reads (mRNA-Seq and Ribo-Seq from the normal and si-*METTL3* cells) to the hg19 reference genome using STAR (25). The uniquely mapped reads were kept for downstream analysis. The read counts of each gene in each sample were calculated by htseq-count (26). In the gene expression analysis, the canonical transcript of each gene was chosen, and all reads that overlapped with exon regions were counted.

Calculating differences in translation efficiency

We counted the reads within CDS regions of the canonical transcripts of each gene. The translation efficiency (TE) was defined as the ratio of normalized Ribo-Seq and mRNA-

Seq read counts. Here, using the mRNA-Seq and Ribo-Seq counts from the normal (Control) and si-*METTL3* (Treated) conditions, we employed xtail (27) to detect differences in the translation efficiency between the Control and Treated samples. The TE of the Control and Treated conditions and the $\log_2$TE fold change (FC) were given by the software. Genes with a $\log_2$TE FC <0 represent genes with a downregulated TE following si-*METTL3* treatment.

Gene ontology (GO) enrichment

The GO analysis was performed using DAVID (28). Highly expressed genes (raw read count >100) in normal HeLa cells were used as background genes.

Conservation analysis

The conservation level of genomic positions is measured by the phyloP score (downloaded from the UCSC Genome Browser, genome.ucsc.edu). Briefly, sites with higher conservation levels have higher phyloP scores. For the comparison of the conservation levels of m^{6}A+ and m^{6}A– sites in coding regions, sites in different codon positions were compared separately.

Codon usage bias

The protocol used to calculate codon usage bias was described in an earlier study (29). The codon bias of a gene is calculated by the deviation (chi-square) of the A/T content of synonymous codons from that of the intronic regions. Higher deviation indicates a stronger codon bias for a gene. The codon bias of a particular codon is the correlation coefficient between the codon frequency within a synonymous codon family and the deviation (Chi-square value) of each gene (29). A higher correlation coefficient suggests stronger bias for a codon.

Statistical analysis

All statistical analyses were conducted in the R environment (http://www.R-project.org/).

Results

m^{6}A methylome in human HeLa cells

We retrieved the m^{6}A peaks (YTHDF1 target) identified in HeLa cells from a previous study (14) and extracted all adenosine sites within the GRAC (R = A or G; A = methylated A) motif according to the instructions in the literature. Adenosines located in the GRAC context in m^{6}A peaks were regarded as m^{6}A sites (*Figure 1A*). In total, we obtained 18,276 unique m^{6}A sites. We annotated these m^{6}A sites according to the reference genome hg19, and the canonical transcript of each gene was chosen if a site was located in multiple isoforms (Methods). The majority of m^{6}A sites were located in CDS regions, and the 3'UTRs (untranslated regions) also contained a large fraction of m^{6}A sites (*Figure 1B*). Apart from a few m^{6}A sites in intergenic regions, most of the sites were assigned to 6,025 unique human genes. The m^{6}A genes were significantly enriched in transcription factors based on the GO enrichment analysis (*Figure 1C*, only GO terms with FDR values <0.05 were listed), which agreed well with known concepts.

The m^{6}A modification was previously reported to increase the translation efficiency of host genes (14,17). Since m^{6}A events (most of which are located around stop codons) have been reported to help recruit translation initiation factors and facilitate translation, the exact position of an m^{6}A site on a mRNA may not be important as long as the methylation event takes place on this mRNA (in the CDS, UTRs or around stop codons). To investigate whether the particular m^{6}A position was important, we sought potential differences between m^{6}A sites (m^{6}A+) and comparable non-m^{6}A sites (m^{6}A–). The m^{6}A– sites were defined as unmethylated adenosines within GRAC motifs in m^{6}A genes (in HeLa cells). We found that the m^{6}A+ sites in coding regions largely subjected to natural selection were not more conserved than the m^{6}A– sites at the genome level (*Figure 1D* and Methods). This result agreed with an earlier study, which reported that generally human m^{6}A sites were non-conserved (30). The particular m^{6}A positions may not be important; otherwise, they would be preserved by natural selection and exhibit high conservation levels. Furthermore, we compared codons containing m^{6}A+ and m^{6}A– sites in CDSs. Due to the constraint of the GRAC sequence context, the m^{6}A sites were only found in a small set of codons, and the m^{6}A+ sites did not show any striking enrichment compared to the m^{6}A– sites (*Figure 1E*). Again, we could not deduce any putative function of the m^{6}A positions from this result, suggesting that the m^{6}A modifications might not exert their function at the "intra-gene" level. Instead, as many previous studies have revealed (14,17), the major function of m^{6}A is to increase the translation efficiency of host genes.

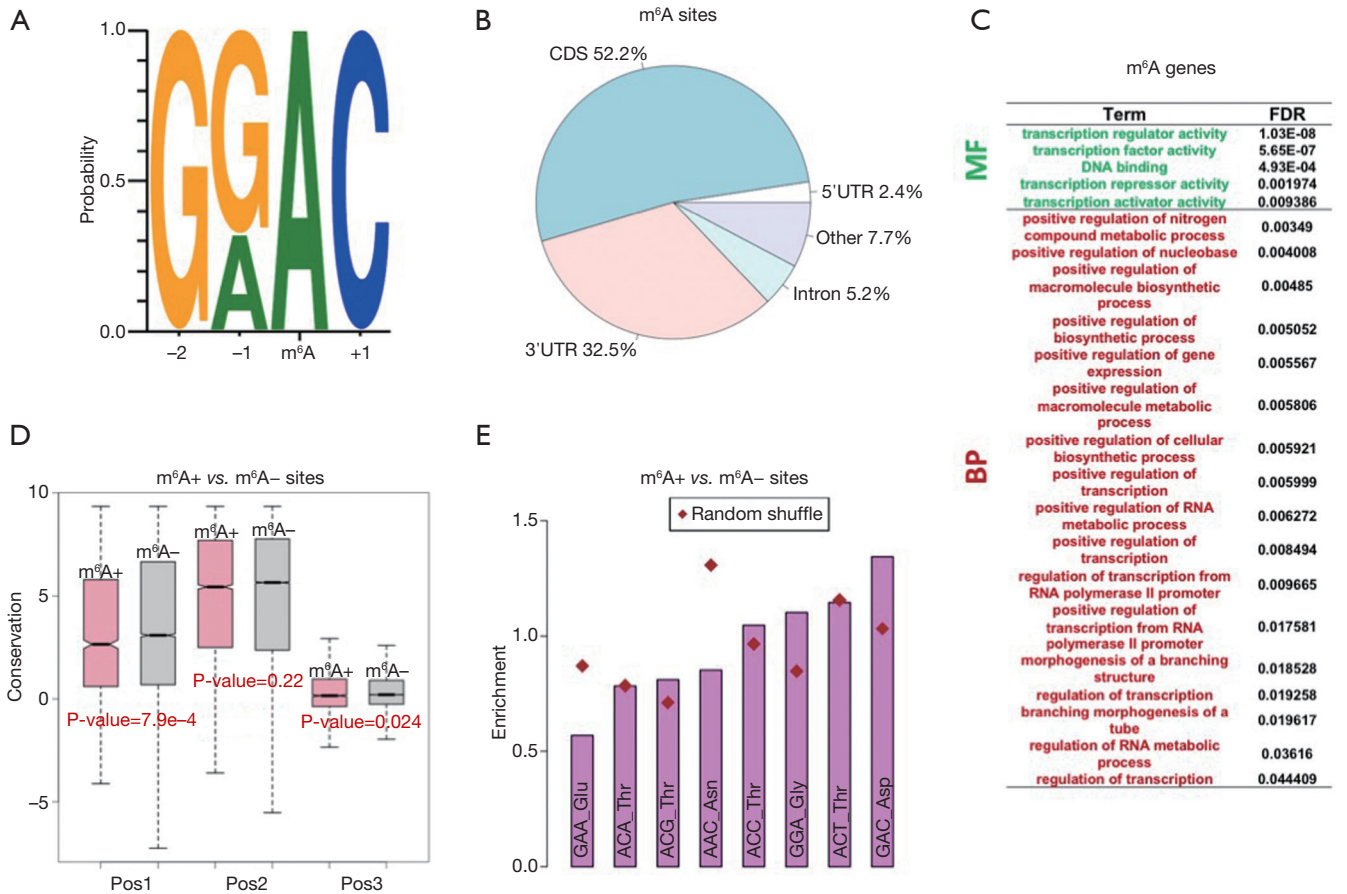

Figure 1 Landscape of m^{6}A sites (YTHDF1 targets) reported in human HeLa cells. (A) The 4-mer motif around m^{6}A sites in HeLa cells; (B) the genomic annotations of m^{6}A sites in HeLa cells; (C) gene ontology (GO) of highly expressed m^{6}A genes in HeLa cells; (D) conservation level (phyloP score) of m^{6}A+ and m^{6}A− sites in the coding regions of m^{6}A genes. "pos" represents the position of the m^{6}A sites on a codon. The Wilcoxon rank sum test was used to calculate the P values; (E) enrichment of codons and amino acids at m^{6}A+ sites. "Random shuffle" represents the enrichment observed by shuffling the m^{6}A+ sites among all GRAC motifs.

Crosstalk between m^{6}A genes and oncogenes

m^{6}A methylation events are known to promote host gene translation (14,17), and this mechanism does not rely much on the exact position of the methylation sites. Next, we investigated whether human cancer cells could enhance the translation of oncogenes via m^{6}A modification.

To address this question, first we searched for known human oncogenes from the Cancer Gene Census (CGC, https://cancer.sanger.ac.uk/census/). We downloaded 719 human oncogenes from the latest version of the CGC website. A total of 335 of these 719 oncogenes were methylated in HeLa cells (*Figure 2A*). Furthermore, considering the mRNA expression levels, we extracted genes with a raw read count >100 in HeLa cells (see Methods for detail). A total of 4,968 of the 6,025 m^{6}A genes and 478 of the 719 oncogenes were highly expressed in HeLa cells. The 4,968 m^{6}A genes and 478 oncogenes included 283 overlapping genes (*Figure 2B*). Thus, more than half of the highly expressed oncogenes were methylated. Then, we examined the gene ontology of the highly expressed oncogenes. We found that these oncogenes were enriched in the transcriptional regulation and metabolism categories (*Figure 2C*), which agreed with the known features of m^{6}A genes. Next, we calculated the number and density of m^{6}A sites in oncogenes and other genes. To exclude the potential bias caused by mRNA length (as longer genes tend to bear more m^{6}A sites by chance), we ranked all genes into five groups with decreasing mRNA length. Within each bin, we compared the number of m^{6}A sites (*Figure 2D*, top) and

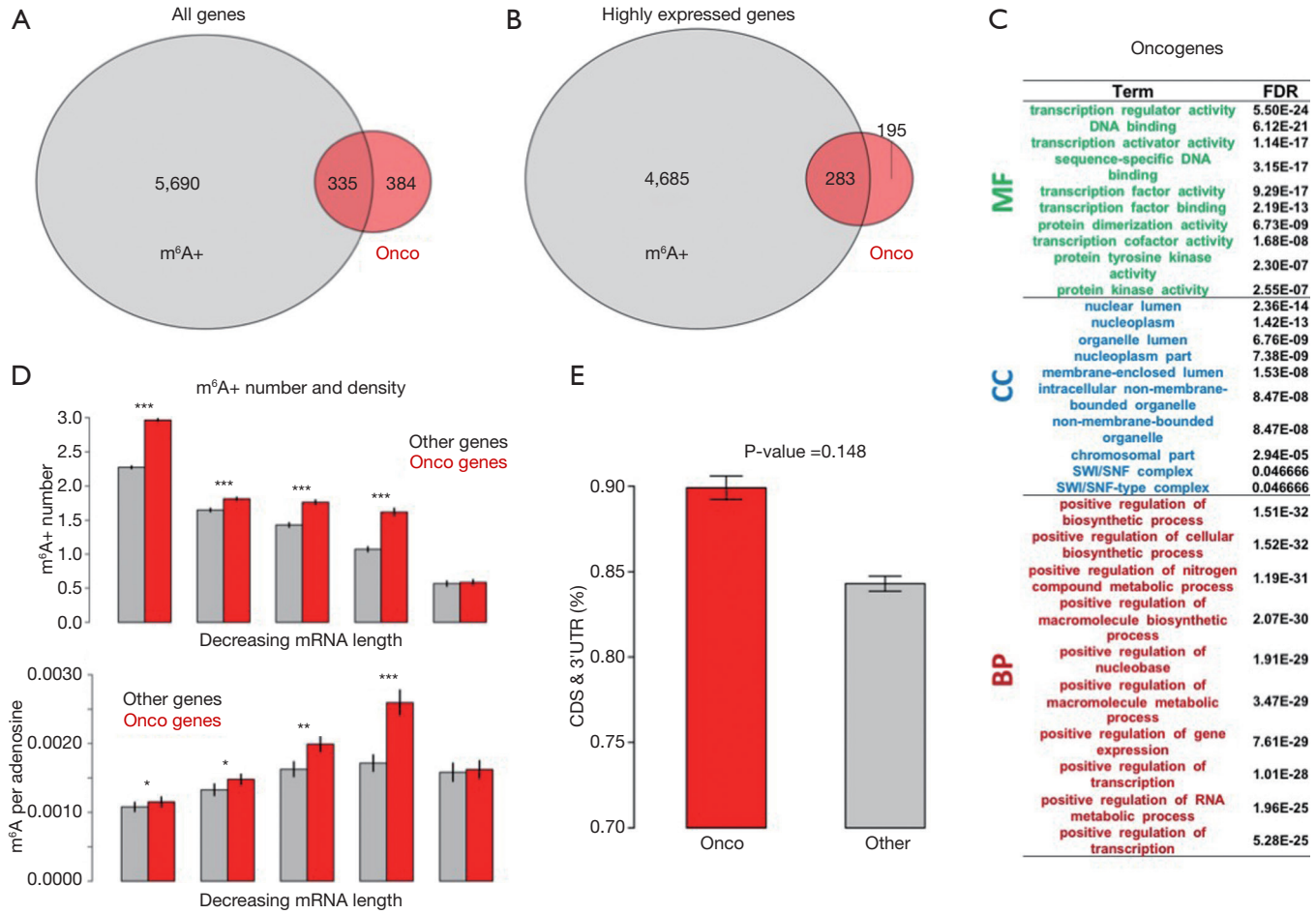

Figure 2 Crosstalk between m^{6}A genes and human oncogenes. (A) Overlap between all m^{6}A genes and human oncogenes; (B) overlap between highly expressed m^{6}A genes and oncogenes in HeLa cells; (C) gene ontology (GO) of highly expressed oncogenes in HeLa cells; (D) the numbers (top) and densities (bottom) of m^{6}A sites in oncogenes and other genes. To exclude the potential bias caused by mRNA length, we ranked all genes into five groups with decreasing mRNA length. Within each bin, we compared the number of m^{6}A sites (top) and the density of m^{6}A sites (bottom) in oncogenes versus other genes. Wilcoxon rank sum test was used to calculate the P values; (E) fractions of m^{6}A sites located in the CDSs and 3'UTRs of the oncogenes or non-oncogenes. Fisher's exact test was used to calculate the P values. In this Figure, m^{6}A+ represents m^{6}A genes, and m^{6}A– represents non-m^{6}A genes.

the density of m^{6}A sites (*Figure 2D*, bottom) in oncogenes versus other genes. The m^{6}A density is defined as m^{6}A sites per adenosine, which canceled the bias introduced by gene length. Strikingly, our results show that both the m^{6}A number and density is generally higher in oncogenes than other genes (*Figure 2D*). This pattern indicates a potential functional role of the m^{6}A modification in oncogenes.

We began to search for differences between the oncogenes and non-oncogenes (termed other genes) or between the m^{6}A genes and non-m^{6}A genes. First, we compared the m^{6}A site distribution of the oncogenes and the remaining genes among the methylated gene set. We calculated the proportion of m^{6}A sites that were located in CDSs and 3'UTRs. Intriguingly, the oncogenes showed a remarkably higher fraction of m^{6}A sites in the CDSs and 3'UTRs than the other genes (*Figure 2E*). Since YTHDF1 binding of m^{6}A sites facilitate translation via recruitment of initiation factors and circularization of host mRNAs (14), the m^{6}A modifications on CDSs and 3'UTRs are likely to assist with the recruitment and circularization processes (than those modifications in regions such as the 5'UTR).

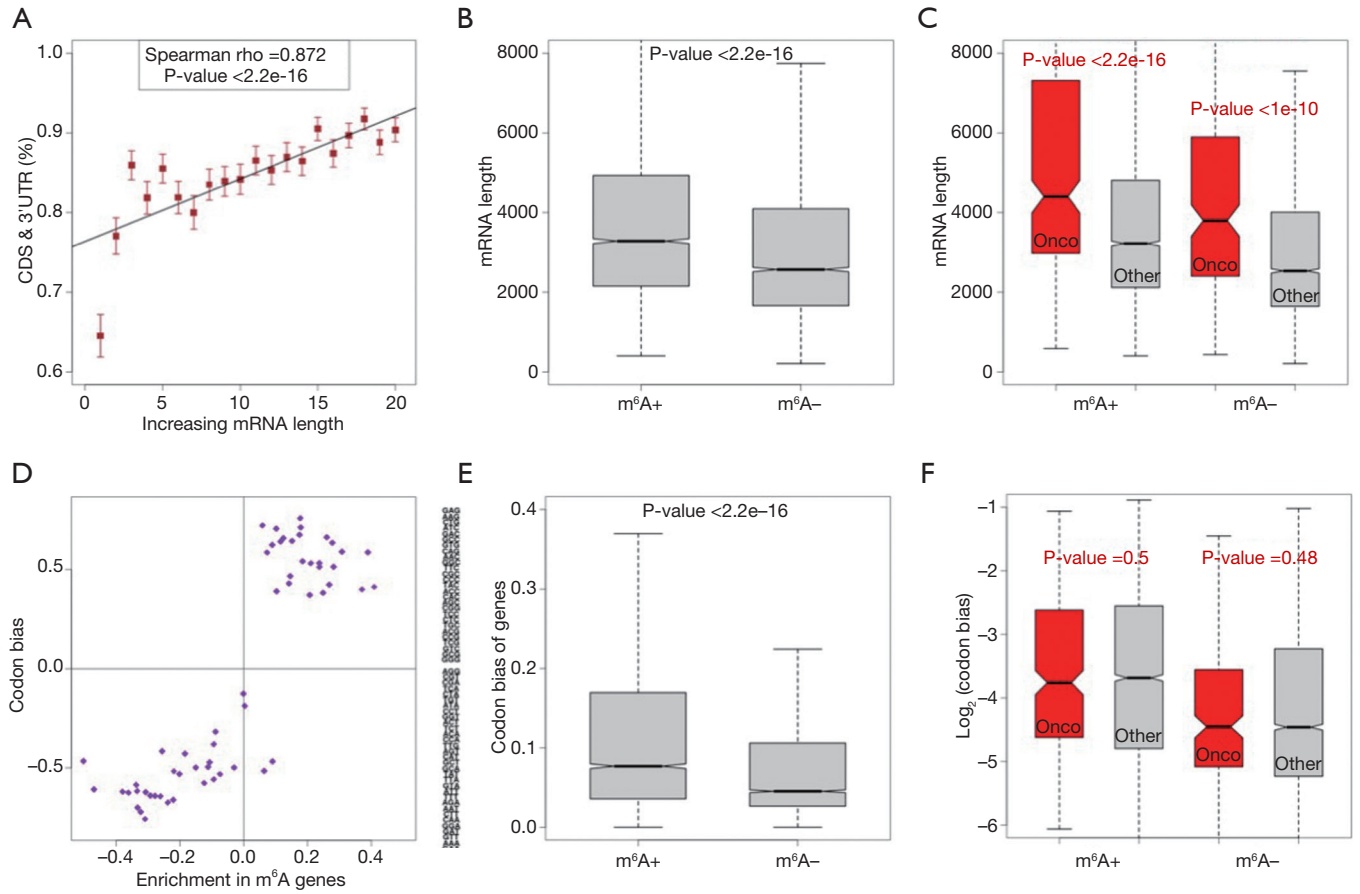

Figure 3 m^{6}A genes, including oncogenes, are unfavorable for translation. (A) Correlation between the mRNA length and the fraction of m^{6}A sites in the CDS and 3'UTR. The highly expressed genes in HeLa cells were divided into twenty bins with increasing mRNA lengths (X-axis); (B) the mRNA lengths of the m^{6}A genes and non-m^{6}A genes. The Wilcoxon rank sum test was used to calculate the P values; (C) the mRNA lengths of oncogenes and non-oncogenes and of m^{6}A and non-m^{6}A genes. The Wilcoxon rank sum test was used to calculate the P values; (D) codon bias of codons enriched in m^{6}A genes compared to non-m^{6}A genes. The codons are listed on the right in the order of codon bias; (E) codon bias of m^{6}A genes and non-m^{6}A genes. The Wilcoxon rank sum test was used to calculate the P values; (F) codon bias of oncogenes and non-oncogenes in the m^{6}A or non-m^{6}A genes. The Wilcoxon rank sum test was used to calculate the P values. m^{6}A+ represents m^{6}A genes, and m^{6}A– represents non-m^{6}A genes.

m^{6}A genes, including oncogenes, are unfavorable for translation

Circularization of mRNA is important for translation. The possibility that the mRNA length may be an important factor that influences circularization is intuitive. We speculated that the longer genes had more difficulty with circularization and therefore were less favorable for translation (this assumption is tested in the next section). Among the highly expressed m^{6}A genes in HeLa cells, we globally profiled the relationship between the mRNA length (the genes were divided into bins) and the fraction of m^{6}A sites in the CDSs and 3'UTRs. Interestingly, the fraction of m^{6}A sites in the CDSs and 3'UTRs increased with the mRNA length (*Figure 3A*, P<2.2e-16). This result suggests that longer m^{6}A genes have a greater need to promote their translation by methylation. Next, we compared the mRNA lengths of the m^{6}A genes versus non-m^{6}A genes (*Figure 3B*) or oncogenes versus other genes (*Figure 3C*). The results are as follows: (I) m^{6}A genes are significantly longer than non-m^{6}A genes (*Figure 3B*), and (II) among these two gene sets, the oncogenes are significantly longer than the other genes (*Figure 3C*). If longer genes are indeed unfavorable for translation, then the m^{6}A genes (and especially the oncogenes among them) will

suffer from a disadvantage in translation.

Another factor that can influence the translation rate is (synonymous) codon usage bias. During translation elongation, the rate-limiting step of the decoding process is waiting for the corresponding tRNA of each codon. A relationship should exist between codon usage and the translation efficiency. We followed the method used an early study (29) to calculate the codon bias of each human gene and each codon (see Methods for details). For m^{6}A genes versus non-m^{6}A genes in HeLa cells, we found that the codons enriched in the m^{6}A genes had a stronger bias (*Figure 3D*). At the gene level, the m^{6}A genes had a stronger bias than the non-m^{6}A genes (*Figure 3E*), whereas the oncogenes showed almost no difference in codon bias compared to that of the other genes (*Figure 3F*). If a stronger codon bias is unfavorable for translation (tested in the following section), then the m^{6}A genes should find a solution to neutralize this disadvantage.

m^{6}A methylation facilitates the translation of host genes, including oncogenes

We have proposed that a longer mRNA length and stronger codon bias may be unfavorable for translation and that m^{6}A genes may suffer from these disadvantages. Here, using mRNA-Seq and Ribo-Seq NGS data from HeLa cells generated by a previous study (14), we examined the correlation between the translation efficiency and CDS length (*Figure 4A*) or codon bias (*Figure 4B*). Both variables show a negative correlation with the translation efficiency (P<2.2e-16). These correlations verified our assumption that both long mRNA (CDS) lengths and strong codon bias were unfavorable for translation. Moreover, the length and codon bias did not show any correlations (*Figure 4C*), proving that these two factors might contribute independently to the lower translation efficiency.

A question arises that since m^{6}A genes (especially the oncogenes) are less suitable for translation, can they compensate for these disadvantages by m^{6}A modification? We fully utilized data from normal (Control) and si-*METTL3* (Treated) HeLa cells. Knock down (si-) of the m^{6}A writer gene *METTL3* largely reduced the transcriptome-wide m^{6}A level (14). We compared the translation efficiency of m^{6}A and non-m^{6}A genes in normal or si-*METTL3* HeLa cells. As expected, the m^{6}A genes but not the non-m^{6}A genes showed a reduced translation efficiency in the si-*METTL3* condition (*Figure 4D,E*). Notably, the translation efficiency of the m^{6}A genes was remarkably lower than that of the non-m^{6}A genes when the m^{6}A writer was removed, but the translation efficiencies of the m^{6}A genes were still lower, even with help from m^{6}A (in the normal condition) (*Figure 4D*). Take together with our previous results, we propose that the m^{6}A genes are unfavorable for translation due to their longer lengths and stronger codon bias and that they indeed have low translation efficiencies. With the help of the m^{6}A modification, translation of the target genes is elevated.

Since we showed that m^{6}A genes were enriched in oncogenes (*Figure 2D*), we searched for oncogenes that benefited from m^{6}A modification. We listed the oncogenes with the most decreased translation efficiencies in the si-*METTL3* versus normal condition (*Figure 4F*). This set of oncogenes increased their translation efficiencies through m^{6}A methylation and might play important roles in cancer cell oncogenesis.

Discussion

m^{6}A methylation participates in many biological processes, of which one of the most well-studied functions is facilitating the translation of host genes via the reader protein YTHDF1 (14). Although several studies have mentioned the role of m^{6}A in oncogenesis (10,19,20), they are either case studies of particular genes or do not systematically investigate the translation of target genes. In this work, we utilized the YTHDF1 target genes and normal or si-*METTL3* NGS data (mRNA-Seq and Ribo-Seq) in HeLa cells generated by a previous work (14) and elucidated the potential function of the m^{6}A modification in cancer cells.

We found that the m^{6}A genes were enriched in oncogenes when compared to non-m^{6}A genes. We observed remarkably longer mRNA lengths for m^{6}A genes, especially the oncogenes among them. We also observed stronger codon usage bias for m^{6}A genes than for non-m^{6}A genes. Lines of evidence revealed that a longer mRNA length and stronger codon bias were unfavorable for translation, because these two factors were significantly negatively correlated with the translation efficiencies of the genes. This finding provides a simple explanation for why m^{6}A genes (or the oncogenes among them) need the m^{6}A modification to enhance their translation. Indeed, the translation efficiency of m^{6}A genes is significantly elevated in normal HeLa cells (Control) compared to that in si-*METTL3* HeLa cells (Treated) where methylation is removed, whereas the translation of non-m^{6}A genes is almost unchanged in the control versus treated cells. In other words, the unfavorable features for translation of m^{6}A genes (or the oncogenes among them) are compensated by the m^{6}A modification (*Figure 5*). Recruitment of initiation

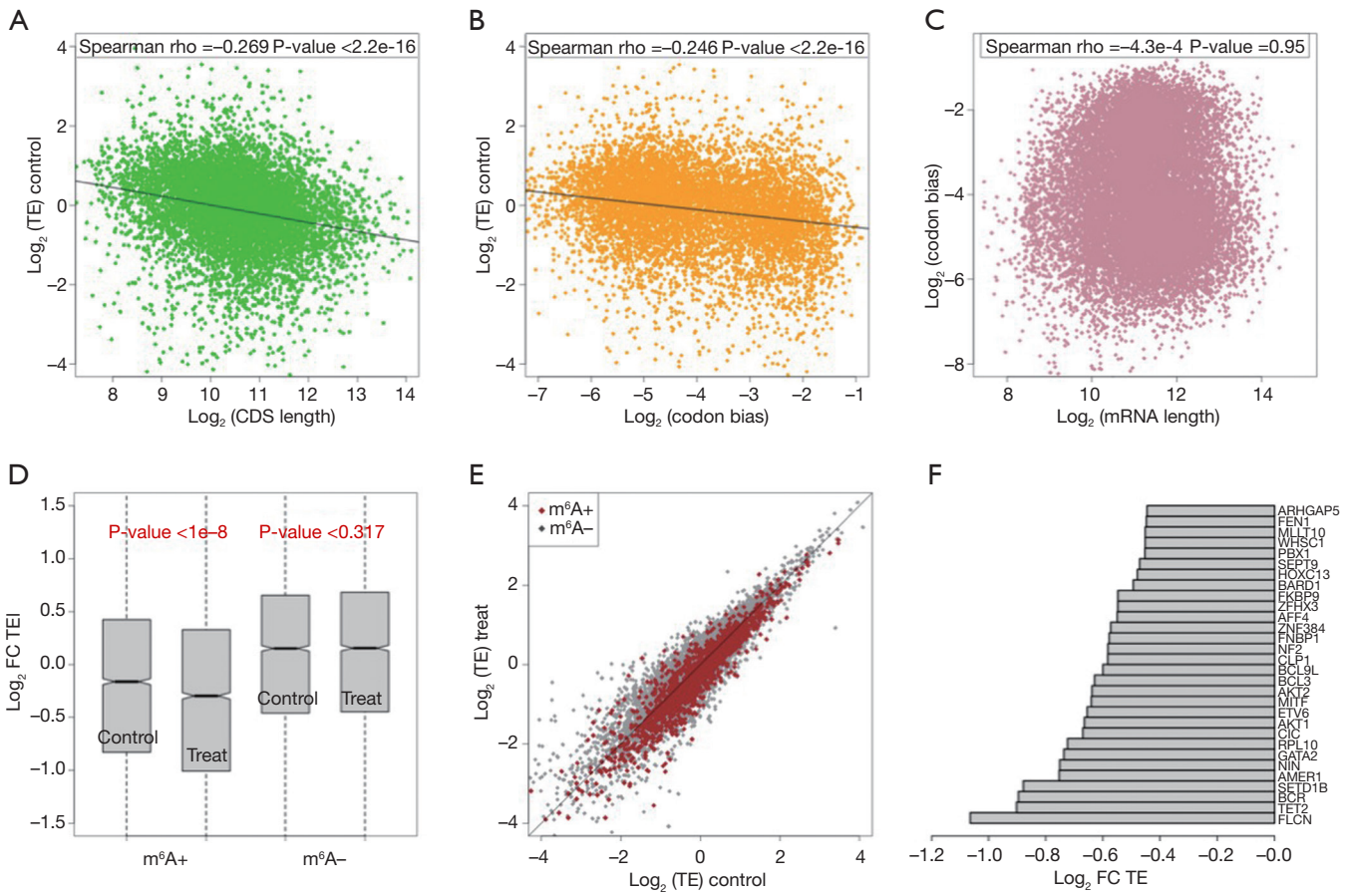

Figure 4 m^{6}A methylation facilitates the translation of host genes, including oncogenes. (A) Correlation between the translation efficiency and CDS length of highly expressed genes in normal HeLa cells; (B) correlation between the translation efficiency and codon bias of highly expressed genes in normal HeLa cells; (C) correlation between codon bias and the CDS length of highly expressed genes in normal HeLa cells; (D) the log2 fold-change of the translation efficiency (TE) in si-*METTL3* versus normal HeLa cells. m^{6}A genes and non-m^{6}A genes were compared separately. The Wilcoxon rank sum test was used to calculate the P values; (E) dot plot displaying the translation efficiency (TE) of m^{6}A genes and non-m^{6}A genes in si-*METTL3* versus normal HeLa cells; (F) genes belonging to both m^{6}A genes and oncogenes; genes with the most decreased translation efficiencies in the si-*METTL3* condition are displayed. In this Figure, m^{6}A+ represents m^{6}A genes, and m^{6}A– represents non-m^{6}A genes.

factors does not directly resolve the codon bias problem, but the increased translation initiation rate will definitely enhance the global translation efficiency of host genes. We should note that the impact of codon usage bias on the mRNA translation efficiency is still debatable (31). However, we do not wish to contradict any previous reports. Conservatively, we declare that the translation efficiency of a gene is negatively correlated with its codon bias, at least for the HeLa cell data used in this study.

Notably, compensation by m^{6}A modification may originally have been designed for oncogenes, since we have observed enrichment of m^{6}A genes in oncogenes. If methylated oncogenes obtain higher translation efficiencies and eventually facilitate cancer cell proliferation, this strategy or mechanism may explain how cancer cells/tissues achieve rapid cell growth. However, direct evidence for this assumption is still lacking. Hopefully, detailed experimental validation will be carried out in the future.

The main contribution of this study is to unveil the enrichment of m^{6}A genes in oncogenes and to clarify why the m^{6}A target is needed to enhance their translation efficiencies by m^{6}A methylation (*Figure 5*). Understanding this relationship is important and should be interesting for the fields of RNA modification, translational regulation and cancer studies.

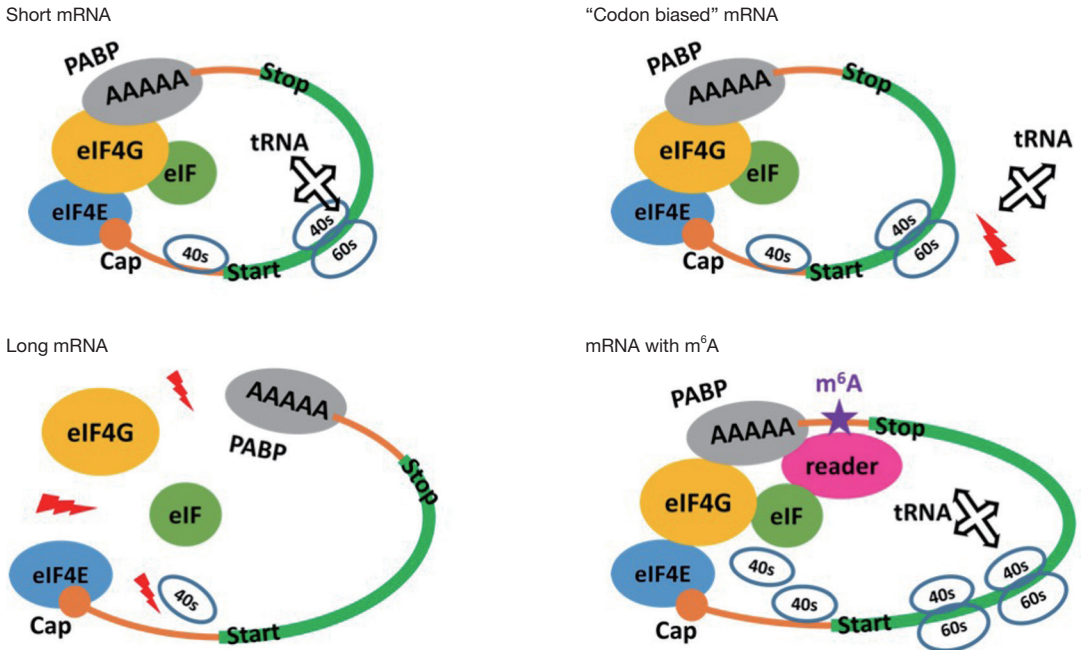

Figure 5 Model illustrating that the features unfavorable for mRNA translation are compensated by m^{6}A methylation. Short mRNAs are favorable for loop formation and translation initiation, whereas long mRNAs are not. Genes with biased codon usage may have a low elongation efficiency due to tRNA availability. With help from the m^{6}A modification and recognition by the m^{6}A reader proteins, translation initiation factors are recruited, and global translation of host genes is enhanced. The eIF (shown in dark green) in the diagram represents a general but not a particular eukaryotic initiation factor.

Conclusions

Our results demonstrate that HeLa cells compensate genes unfavorable for translation by m^{6}A modification and enhance their translation efficiencies (*Figure 5*). These m^{6}A target genes are enriched in oncogenes, and the enhancement of translation of these genes may be related to cancer cell oncogenesis.

Acknowledgments

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Footnote

Conflicts of Interest: Both authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tcr.2019.03.04). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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