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REVIEW

The Epitranscriptome: A New Era in Epigenetics

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ABSTRACT

Epigenetics, which is defined as changes independent of DNA sequence, cause permanent or transient changes in the expression of the genes and play critical roles in embryogenesis, genetic imprinting, development and cancer. The role of epigenetics in biology is so important that some researchers refer to these modified bases as the fifth and sixth bases of the genetic code. However, there are few studies on RNA modifications summarized in this review under the title of epitranscriptome. N6-methyladenosine is one of the most common mRNA modifications in eukaryotes. Enzymes in-volved in this modification, its biological functions and mechanisms are still being discovered. Understanding the role of N6-methyladenosine on the regulation of gene expression and developmental process will enable the elucidation of epigen- etic regulation at the RNA level and its effects on protein synthesis. In this re- view; recent developments, functions of N6-methyladenosine mRNA methylation and its rela- tion with cancer are discussed.

Keywords: RNA methylation, N6-methyladenosine, epitranscriptome, gene expression.

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INTRODUCTION

Epitranscriptome is a reversible, chemical mark on RNA molecule that affects the regulation of gene expression. It has crucial roles in biolog-
ical processes such as ribosome formation, mRNA stabilization, RNA folding, protein synthesis and cell differentiation. It may also contribute to cancer, obesity and other related diseases [1-2].

Discovery of m^{6}A mRNA Methylation

The acceptance of N6-methyladenosine (m6A) as a widespread mRNA modification, has been achieved with the discovery of new tools and methods that allow detection of m6A regions. For example, m6A immunoblotting has shown that m6A levels differ among RNA molecules obtained from different tis-sues and cancer cell lines, and the highest m6A ex-pression is observed in the brain, heart, and kidney. While, m6A-containing mRNAs are low in the fetal
brain, they increase during development, and reach the highest levels in adults [3]. In the light of these information, it is thought that m6A is a tissue specific mRNA regulator [4].

Methylated transcriptome was first defined by methylated RNA immunoprecipitation followed by sequencing (MeRIP-Seq) [3]. This method, also known as

m6A-Sequencing (m6A-Seq), is a new gen- eration sequencing technique that is based on the determination of the nucleotide sequence follow- ing precipitation of m6A-containing RNA fragments [3]. A large number of m6A regions were identified in mRNAs isolated from different tissues and cell lines by MeRIP-Seq analysis [5]. These are the first results proving that m6A is a widespread mRNA modification [3]. It has been determined that m6A is present in approximately 25% of all transcripts. Therefore, this modification is not essential for the biogenesis or processing of mRNA [4]. However, it has been found that the transcript length is import-ant for m6A distribution and m6A is mostly found in evolutionary conserved regions [3-5]. Thus, it is thought that this modification has functional and regulatory roles in mRNA [4]. Despite the fact that 94.5% of the m6A found in brain tissue are found in mRNAs, the rest of is located in non-coding RNAs such as X inactive specific transcript (XIST) and HOX transcript antisense RNA (HOTAIR) [3]. This informa-tion suggests that non-coding RNAs are also an im- portant target for m6A modification [4]. MeRIP- Seq analysis revealed that m6A residues were accumu-lated in specific regions of mRNA. Most m6A regions are located around the stop codon. In addi-tion, some mRNAs contain m6A residues in the 5' untranslated region (UTR) and since mRNAs con-taining m6A residues in 5'UTRs show tissue-specif-ic differences, it is thought that m6A mediated con-trol occurs more frequently in some tissues [3-5-6]. However, a tissue that is free from m6A regions was not found until this time [3]. Residues of m6A are also present in the 3 'UTR and in coding sequenc-es however, the frequency of these residues is low-er than those found around the stop codon. PolyA tail does not contain any m6A residue [3]. Although, many regulatory elements such as miRNAs and reg- ulatory proteins known to bind 3 'UTR [7-8], residues of m6A are located around the 5' UTR and stop co-don, suggesting that this modification has differ-ent regulatory functions [4]. MeRIP-Seq analysis re-vealed that GAC and AAC conserved motifs, partic-ularly GAC, were rich in the m6A residues [3]. In vitro methylation studies have shown that methylation is often involved in GAC-containing RNAs [9] and that a mutation causing GAC-GAU transformation is suf-ficient to inhibit adenosine methylation [10]. In addi-tion, the fact that this modification is reversible distinguishes it from other modifications [1-4].

Enzymes That Have a Role in mRNA Methylation

The enzymes involved in methylation and demeth-ylation are separated into three groups (Table). Writers that methylate the adenine, erasers that cat-alyze the removal of this methylation, and readers that mediate the functions of m6A [1]. N6-adenosine-methyltransferase 70 kDa subunit is a protein that acts as a methyltransferase in a multienzyme com-plex and is encoded from methyltransferase like 3 (ME T TL3) gene [11]. Enzyme purification exper-iments have shown that this enzyme is specific for GAC and AAC sequences in single-stranded RNA [12-13]. In addition, other studies have identified homo-logs of this enzyme in plants (mRNA adenosine methylase, MTA) (inducer[14], Saccharomyces cerevisiae of meiosis 4, Ime4) [15] and Drosophila melanogaster (Ime4) [16]. Recently, it has been determined that METTL3 interacts directly with methyltransferase like 14 (METTL14) to form a heterodimeric meth- yltransferase enzyme complex in the cell and meth- ylate GAC sequence [17]. In a study carried out in mouse embryonic stem cells by suppressing Mettl3 or Mettl14 genes, it was shown that the amount of m6A were decreased [18] thereby, it has been veri- fied that METTL3 and METTL14 target mRNA for methylation [4]. Studies in yeast and Arabidopsis thaliana have shown for the first time that Mum2 and FIP37, mammalian Wilms Tumor 1-Associated Protein (WTAP) homologs, interact with the METTL3-METTL14 complex [14-19]. Although WTAP does not have the methyltransferase activity, it has been found that it provides the localization of the enzyme complex in the nuclear speckles [17-20]. It is thought that methylation occurs in the nucleus due to the fact that METTL3, METTL14, and WTAP locate in nuclear speckles and methyltransferase activity is detected in nuclear extracts [21]. However, in ad- dition to metyltransferase activity in cytosolic ex- tracts [9], METTL3 was observed in cytoplasm and perinuclear regions [22]. Therefore, it is thought that methylation occurs both in the nucleus and in the cytoplasm. Methylation in the nucleus plays a role in the processing of the mRNA in the nucleus, while the methylation in the cytoplasm controls other pro- cesses in the cell such as translation, differentiationetc. [4].

After the discovery of enzymes that methylate mRNA, endogenous demethylases that remove methyl group have been discovered. The presence of enzymes capable of removing m6A suggests that m6A is a dynamic modification and its effect is re- versible [4]. The first identified m6A demethylase, fat mass and obesity-associated protein (FTO) [23], leads to the formation of a hydroxymethyl by the hydrox- ylation of the methyl groups on the bases. This hy- droxymethyl becomes quite unstable when bound to nitrogen atoms and spontaneously decomposes formaldehyde thereby, causes demethylation [24]. a-ketoglutarate-dependent dioxygenase alkB ho- mologue 5 (ALKBH5), which is another m6A demeth- ylase, is required for spermatogenesis in mice, and expressed in the testis [25]. While ALKBH5 shows nuclear localization [25], FTO is also found in cytosol [26]. Therefore, it is thought that FTO may target ma- ture mRNAs while ALKBH5 targets pre-mRNAs in the nucleus [4]. m6A can both induce and block the RNA- protein interactions. Besides, the role of reader pro- teins named YTH N6-Methyladenosine RNA Binding Proteins (YTHDF1, YTHDF2, and YTHDF3) that have identified in mammalian cell extracts, is only to me- diate the functions of m6A [27-28].

Table :m6A regulators in humans

Protein	Functional classification	m^{6}A associated biological function(s)	References
Methyltransferase-like 3 (METTL3)	Catalytic subunit of m^{6}A methyltransferase	Installs m^{6}A; promotes translation independently of its catalytic activity	Bokar et al.1997, Liu et al.2014
Methyltransferase-like 14 (METTL14)	A core subunit of m^{6}A methyltransferase	A key component for m^{6}A installation	Liu et al.2014
Wilms tumour 1 associated protein (WTAP)	Regulatory subunit of m^{6}A methyltransferase	Facilitates m^{6}A installation	Liu et al.2014, Ping et al.2014
KIAA1429	Regulatory subunit of m^{6}A methyltransferase	Facilitates m^{6}A installation	Schwartz et al.2014
Fat mass and obesity-associated (FTO)	m^{6}A demethylase	mRNA splicing, translation and adipogenesis	Jia et al.2011, Zhou et al.2015, Merkstein et al.2015
AlkB homologue 5 (ALKBH5)	m^{6}A demethylase	mRNA nuclear processing, mRNA export, promotes stemness phenotype of breast cancer stem cells	Zheng et al.2013, Zhou et al.2015, Zhang et al.2016
Heterogeneous nuclear ribonucleoprotein A2/B1 (HNRNPA2B1)	m^{6}A reader	mRNA splicing, miRNA biogenesis	Alarcon et al.2015
Heterogeneous nuclear ribonucleoprotein C (HNRNPC)	m6A reader that recognizes m^{6}A-induced structural changes	m^{6}A structural switch, mRNA splicing	Liu et al.2015
YTH domain-containing 1 (YTHDC1)	Direct m^{6}A reader	mRNA splicing, transcriptional silencing	Xu et al.2014, Xiao et al.2016
YTH m^{6}A-binding protein 1 (YTHDF1)	Direct m^{6}A reader	Translation initiation	Wang et al.2014,
			Wang et al.2015
YTH m^{6}A-binding protein 2 (YTHDF2)	Direct m^{6}A reader	mRNA decay	Wang et al.2014
YTH m^{6}A-binding protein 3 (YTHDF3)	Direct m^{6}A reader	Unknown	Wang et al.2014

Effects of mrNA methylation on celluar Activity

Adenosine methylation controls the activity of mRNA biogenesis, stability, splicing, transcription and trans-lation through the reader proteins (Figure 1).

1. mrNA stabilization

In several studies, m6A was reported to accelerate mRNA degradation. It was shown that the stability of the mRNAs increases in Mettl3 and Mettl14 knockout mouse embryonic stem cells, so researchers have suggest-ed that m6A takes the place of HuR, which is an mRNA stabilizer. In vitro studies showed that m6A inhibits the binding of HuR and causes mRNA degradation [18].

2. mrNA splicing

Studies have shown that the majority of the METTL3 binding sites reside in introns and that exon skipping and intron retention which occur in mouse embry- onic stem cells in the absence of METTL3 suggest- ed that m6A is associated with alternative splicing [29]. mRNAs that have alternative splice isoforms were found to have a higher methylation

rate and a greater number of METTL3 binding sites [5-20]. As a result, m6A is thought to contribute to alter- native splicing; however, the relationship between m6A and splicing control is not yet fully understood. It has also been found that FTO prevents the bind- ing of serine-arginine-rich splicing factor (SRSF) by removing m6A around splice sites and consequently modulates alternative splicing [30].

3. Nuclear Export and Translation of mrNA

It is thought that m6A plays a role in the nuclear export of mRNA. It was observed that the lack of METTL3 inhibited the mRNA export [31] while the absence of ALKBH5 increased it [25-30]. In addition to that, conflicting results have been obtained re- garding the effect of m6A on translation. In some studies, it was shown that methylated mRNA has higher translational activity than non-methylated mRNA [32], whereas methylation has been shown to reduce translation efficiency in another study [33]. Therefore, it is thought that the effect of m6A on protein production varies between different mR- NAs [4]. The beginning of the translation is also reg- ulated via YTHDF1. It recognizes m6A located in the 3' end of mRNA and enhances translational activi- ty by recruiting translation initiation factors [34]. Recent studies have shown that eukaryotic initia- tion factor 3 (eIF3) enhances translation by interact- ing with mRNAs which contain m6A at 5 'UTRs [35- 36]. It is known that YTHDF1 and YTHDF2 promote translation.

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4. mirNA Binding

Analysis of the MeRIP-Seq data revealed a strong correlation between m6A and miRNA binding sites. 67% of 3'UTRs containing m6A also contain at least one miRNA binding site [3]. It suggests that the presence of the miRNA binding site may trigger the pathways associated with mRNA methylation [4].

5. Circadian Rhythm

mRNA methylation is also thought to be related to circadian rhythm. The circadian rhythm contains a negative feedback loop. The CLOCK proteins reduce the transcription of Clock genes and the post-tran- scriptional regulations play an important role in the control of circadian rhythm. Suppression of Mettl3 expression leads to a decrease in m6A levels in pe- riod circadian clock 2 (PER2) and aryl hydrocarbon nuclear translocator like (ARNTL). Thus, these tran- scripts are retained in the nucleus for a longer peri- od, causing the circadian period to prolong [31].

6. Self-Renewal Capacity and Differentiation of Stem Cells

m6A affects cell differentiation and expression of many transcription factors. During adipogenesis; FTO modulates the levels of m6A and SRSF2 binding. This modification alters the alternative splicing of the genes necessary for adipogenesis [37-38]. In addi- tion, ALKBH5 affects the differentiation of breast can- cer stem cells in humans [39]. When these cells are exposed to hypoxia, demethylation of NANOG is in- duced, and the increase on the stability of transcript leads to the proliferation of cancer stem cells [30]. The effect of m6A changes depending on the transcript that have m6A modification. For instance, if the level of m6A decreases in transcripts that protect plurip- otency, the half-life of these transcripts is prolonged so pluripotency is maintained.

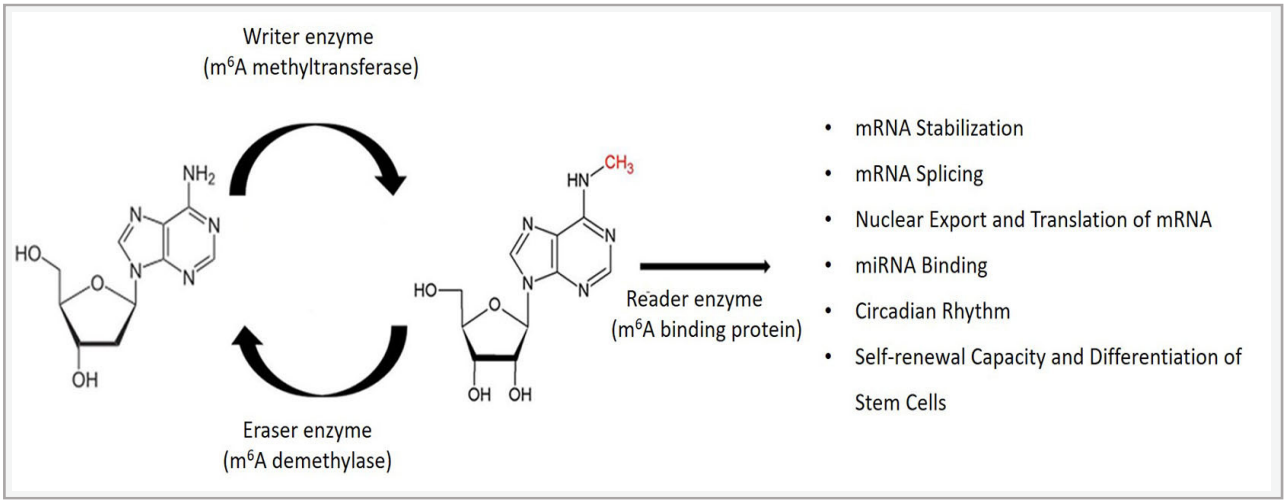

Figure 1: Functions of mRNA methylation

On the other hand, if the level of methylation in the transcripts that provide differentiation is reduced, lineage commitment is triggered. Experimental results indicate that m6A is an important modification in determination of cell fate during differentiation and development [30].

mRNA Methylation and Cancer

It is now known that adenosine methylation on mRNA is the major epitranscriptomic modification that controls cellular differentiation and pluripoten-c y. At the same time, m6A, which controls gene expression, is thought to play an important role in the development of cancer resulting from uncontrolled cell division [40]. In addition, it has been shown that m6A allows translation to start without the eukaryotic translation initiation factor 4E (eIF4E), which is required for translation under normal conditions [36]. EIF4E-independent translation is known to be active in various disease states, particularly in cancer [40]. The pluripotent stem cells lacking m6A are resistant to differentiation signals. These cells maintain their pluripotency and do not exhibit gene expression profiles of lineage committed cells. It has also been shown that alterations of the m6A levels can alter the differentiation pathways of hematopoietic stem cells. Since these differentiation pathways are associated with solid and hematological malignancies, changes at the m6A level may play a role in the development of cancer [40]. A recent study [39] found an association between changes in m6A levels and abnormal cell differentiation in cancer. Zhang et al. have shown that hypoxia is associated with cancer stem cell formation and that the level of ALKBH5 is increased in breast cancer. ALKB5 is also known to be essential for the acquisition of the phenotype of the breast cancer stem cell. The study suggests that ALKBH5 expression changes m6A level on NANOG mRNA, one of the key transcription factors associated with pluripotency in hypoxic conditions [40]. Hypoxia also induces the expression of zinc finger protein ZNF217, which causes a decrease in m6A levels on both NANOG and KLF4 [39]. Another pluripotent transcription factor, Krueppel-like factor 4 (KLF4), is thought to reduce the level of m6A and lead to the formation of breast cancer cells [40]. Bansal et al. [41] have shown that the W TA P expression increases in 32% of acute myeloid leukemia patients. When W TA P expression in leukemic cell lines is suppressed, apoptosis and differentiation are increased while cell proliferation is decreased [41]. Therefore, the decrease in m6A level is thought to be responsible for the anti-leukemia effects observed in the absence of W TA P. As a result, pluripotency and differentiation related genes are controlled by the m6A levels, and decreases in the lead to defects in the control of these genes [40]. RNA modifications have also important roles in embryonic stem cell maintenance and differentiation, circadian rhythm modification, heat shock response, meiotic progression and neuronal function. However, the function of mRNA modification remains to be determined [42]. In glioblastoma, one of the most deadly primary brain tumor, survival of patients is less than 15 months after diagnosis even with the treatment. As the presence of cancer stem cells make the glioblastoma treatment difficult, new treatment strategies that target these resistant cancer stem cells are urgently needed [43]. Zhang et al. showed that knockdown of METTL3and METTL14 increased growth and self-renewal of glioblastoma stem cells (GSC). In contrast, over-expression of METTL3 or treatment with a chemical inhibitor of the FTO dramatically inhibited GSC growth and self-renewal [44]. In addition, it is also shown that in GSC-grafted animals treatment with a chemical inhibitor of the FTO also suppressed GSC-induced tumorigenesis and prolonged life span. Consequently, in the light of these results m6A mRNA methylation machinery may be promising therapeutic targets for glioblastoma [42].

CONCLUSION

Reversible RNA modifications have the same properties as the epigenetic changes and histone modifications that occur in DNA. Expression levels and posttranslational modifications of enzymes involved in RNA methylation can alter protein production in the cell by constantly reshaping RNA methylome. Studies suggesting miscorrelation between level of mRNA and the amount of protein in the cell also reveal the importance of posttranscriptional control of gene expression. However, although the molecular dynamics of m6A and some of its targets are already known, there are still a number of points that still need to be clarified about their function. For instance, how m6A is shaped according to internal and external signals and their tissue specificity is not yet fully understood. Therefore, by elucidating the effects of m6A on RNA degradation, intracelular transport, translation and splicing; it is thought that the role of epitranscriptome on developmental process and treatment of diseases will also be revealed.

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