m^6)a RNA Methylation: The Implications for Health and Disease

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Abstract

The recent resurgence of interest in m^6)a RNA modification is a highly reactive and fluid modification which can respond rapidly to a broad variety of stimuli, and translate these signals into cellular activity. The little information that has been established on its functional capacity has opened up many new avenues of research and has tremendous implications for several fields of study. Here we outline the breakthroughs which have led to the resurgence of interest in this modification and discuss the effects and potential they represent in terms of control in the immune system, viral replication and infection, as well as the occurrence and progression of cancer.

Introduction

Analysis of nucleic acid modifications and their explicit effects on epigenetic status is a rapidly expanding research arena, one that is growing in stature and importance. Until recently this field had focussed on changes in the chemistry of DNA and the actions of histone proteins and their subsequent modifications. A few key discoveries are set to add a whole new RNA dimension to this exciting field of research. RNA methylation has entered the fray, specifically N-methyl-6-Adenosine – also known as m^6)a or N6-methyladenosine. Although this is not the only RNA modification, over 100 have been identified; it is by far the most abundant [1]. Not only is this modification found extensively in mRNAs it is also found in non-coding RNAs (ncRNAs), including long ncRNAs (IncRNAs) [2]. The m^6)a modification has been demonstrated to be an internal modification predominantly found in mature mRNA transcripts [2]. Analysis of these transcripts revealed m^6)a modifications on mRNA transcripts which encoded genes involved in transcriptional regulation, RNA metabolism and signalling cascades [2]. The number of 3’ UTRs of mRNA transcripts demonstrating m^6)a methylation appear to be above average at two thirds, it should be noted however that accuracy of site detection and methylation state is likely to be more accurate for highly expressed genes than genes with low copy numbers. Around 30% of genes contain microRNA (miRNA) binding sites in the 3’UTRs [3]. Interestingly, where these occur simultaneously the m^6)a modification is found closer to the stop codon, whereas the miRNA binding site appears to be at the opposite end [2]. Without further information this proximity may well be circumstantial and irrelevant, however the occurrence of both together is higher than would be expected by chance alone. Even more intriguingly, some miRNAs themselves contain a m^6)a recognition site which may represent a mechanism for their regulation [4]. The m^6)a modification has been demonstrated in a broad range of species including humans [5], mice [5], virii [6,7], yeast [8-10], bacteria [11] and plants [12]. The methylation reaction is catalysed by a conserved mechanism, based around a multi-component enzymatic complex [13] and counterbalanced by a series of demethylases [14]. Although this multi-component complex has not been elucidated as yet, we postulate that it will behave in a similar manner to other complexes in that it will contain a RNA binding protein (specific for the recognition sequence), although multiple proteins are possible due to the variability of the recognition sequence (12 possible permutations) it seems likely that only 1 or 2 binding proteins are responsible for the core of the complex. Larger numbers of co-factors will determine addition/removal of methylation, leading to several possible complex assemblies. It is also likely that other binding proteins may enhance or repress binding to the recognition sequence or overlapping sites, for example FTO binding sites [15], thus determining which sites are methylated or non-methylated. The recognition sequence for the m^6)a methylation site is significantly different from other RNA modifications, this sequence has been identified as RRACH (where R = G or A and H = A, C or U) [7,16-20]. Its occurrence has been estimated at 1 in 2000 bases in humans [2,5]. It has been found to be located proximal to stop codons and is highly enriched in the 3’ untranslated region [2]. Like most methylation modifications of nucleotides S-adenosylmethionine (SAM) acts as a methyl group donor for the reaction [21], linking the methylation of RNA to the dynamic equilibrium of this molecule and its associated enzymes. Due to the critical nature of SAM for methylation of both RNA and DNA it is maintained in a highly regulated equilibrium which responds rapidly to cellular factors to maintain idyllic levels. This also suggests that SAM levels are likely to be a driving force for methylation levels via enzyme activity, where availability of SAM drives methylation and low levels of SAM would stimulate demethylation. This is supported by a recently published model suggesting relative methylation levels is determined by
enzymes [22], the same group also suggest that absolute methylation levels are driven by both enzyme levels/activity and transcription regulation. This model is a little simplistic in its overview as SAM is central to a range of methylation processes not just m⁶A; a more likely model will involve more transcriptional and translational control of methylation co-factors for both relative and absolute m⁶A methylation levels.

Renewed interest in RNA epigenetics

Unlike its more mature sibling, DNA methylation, RNA methylation has not been the subject of intense research or discussion [23]; however, that is about to change. Despite its discovery in the 1970s [24], the m⁶A RNA modification has been mostly neglected until recent times, with research focussing on its occurrence [17] and sequence specificity [16,20] rather than its function.

A new surge of interest was sparked by the discovery that the obesity related (fat mass and obesity related (Fto)) gene was capable of demethylating RNA [14]. Since then many new studies identifying novel functions of the m⁶A modification and outlining more members of the methylation/demethylation machinery have been conducted. In this regard, a major component of the m⁶A methylation machinery, METTL3 has been identified as have other candidates for the enzyme complex which drives m⁶A RNA methylation, METTL14 [25], KIAA1429 [26] and WTAP [27]. Furthermore, the activity of a second demethylase, ALKBH5 has also been established. This resurgence in interest has also been accelerated by overcoming a major obstacle in the detection of m⁶A [28], the development of novel techniques and adaptations of old ones, reviewed by Chandola et al. [2014] [29]. These have allowed a greater parity of the occurrence and fluidity of this epigenetic modification [30]. There is growing evidence that these RNA modifications can respond rapidly to a variety of stimuli, particularly stress [31,32]. Recently, FTO has been further implicated in the complicated control of RNA epigenetics by evidence which suggests it may catalyse further reactions oxidising the N(6)-methyladenosine to form the intermediate product N(6)-hydroxymethyladenosine, which is followed by the formation of the further oxidised product N(6)-formyladenosine [33]. These modifications represent novel short term (half-life ~ 3 hours [33]) epigenetic changes which may have far reaching implications; they may modulate the effects of the m⁶A modification or may aid in its demethylation, similarly to DNA methylation.

Similarly to DNA methylation, it has been shown that m⁶A RNA methylation can influence gene expression [5,34,35], its regulatory role has been emphasised by its occurrence in only a portion of transcripts [17], with some demonstrating no m⁶A modifications at all [36]. This modification has also been suggested to have other key functions, which include RNA splicing [5,14,37], targeting mRNA for degradation [38], regulating RNA stability by modulating binding of RNA binding proteins [5,39,40], translational control [41], meiosis [8] and cellular differentiation [12]. The consequences of experimental retardation of the methyltransferase system are disparate and range from apoptosis in humans [42], developmental arrest in plants [12] and defective gametogenesis in yeast [9] and fruit flies [43]. Looking for condition specific alterations in m⁶A sites and levels using an activated immune response in bone-marrow derived dendritic cells (BMDCs), embryonic and adult mouse brains, fibroblasts undergoing reprogramming (to induced pluripotent stem cells, iPSCs) and differentiating human embryonic stem cells (ESCs) revealed that methylation profiles were fairly consistent, after controlling for increased specific protein expression under the above conditions [26]. This suggests a similar basal role shared throughout the body. Intriguingly, sites for m⁶A modification are significantly enriched on lower expression genes, with constitutively expressed housekeeping genes (translation, mitochondrial, splicing and chromatin regulation) demonstrating a complete absence of m⁶A methylation [26]. However, the system used for these studies may not be suitable for quantification of levels, while the actual sites may be consistent the occupancy may not be. Mouse embryonic stem cell (mESC) Mettl3 and Mettl14 knockdown studies led to a similar phenotype: lack of m⁶A RNA methylation and loss of their self-renewal capability, furthermore, these studies implicated human antigen R (HuR) and microRNA in this process [44].

m⁶A RNA methylation and the brain

The epigenetic status of the brain has been shown to be critical for both development and neurological disorders, including neurodegenerative diseases. DNA methylation has also been shown to be critical in brain development and function [45]. Aberrant cytosine methylation on tRNAs leads to neuro-developmental disorders in mice [46]. m⁶A methylation is also found in tRNAs [47], as well as rRNA [48], the relationship between tRNAs and neural development hints at a potential role for m⁶A methylation in neural development as well. A mouse based study demonstrated a high level of enrichment of m⁶A in liver, kidney and brain [2]. This would suggest that the presence of m⁶A methylation is essential for the development and/or functions of these organs. Levels of this modification in the brain are low during embryogenesis and dramatically increase by adulthood [2]; this suggests that m⁶A plays a role in neuronal maturation and normal functioning of the adult brain. Furthermore, the enrichment in brain and concomitant increases in levels associated with ageing suggests a role in neurodegenerative diseases, for example Alzheimer's disease and Parkinson's disease, however no data is available to support this hypothesis to date. A highly intriguing finding is that a greater percentage of target transcripts for the most highly expressed miRNAs found in the brain contain m⁶A modifications [2]. This raises an interesting question: does m⁶A methylation affect miRNA expression, or do miRNAs influence methylation of their own transcripts? A large number of unique highly likely m⁶A sites were identified in both in the embryonic and adult brain, however closer inspection revealed that this was most likely due to increased expression of particular genes that carry the m⁶A modification [26]. Although it appears the number of sites is not increased in the brain, the levels of m⁶A modified mRNA are increased; this suggests a role for m⁶A in neural function and maturity as the genes that were upregulated and carried m⁶A modifications were involved in neural processes.
m6A RNA methylation and the immune system

The influence of Fto and the m6A modification on the DA signalling system gives it a broad reach, not least in the function and activity of the immune system. For instance the D2-like receptors, which are particularly influenced by m6A modifications [53], are found in greater levels on memory and effector CD4 T lymphocytes than their naive counterparts [64]. Furthermore, this influence extends to the development of T cells, particularly in the thymus [65]. The D2-like DA receptors are also capable of attenuating NK cell activity [66]. These receptors are considered to be more important for the modulation of T lymphocyte function than D1-like DA receptors [67], this indicates a greater degree of control over T lymphocytes may be exerted by the m6A modification and its machinery than had previously been thought possible. Dopamine has also been shown to upregulate interleukin (IL)-6 and IL-8 in keratinocytes [68], indicating a broader control over inflammation. D2-like receptors have been demonstrated to exhibit an influence over chronic neuroinflammation [69] which implicates the m6A modification in neurodegeneration and its associated disorders. WTAP influences cellular immune responses in patients with leukemia [70], administration of a vaccine based on WTAP also results in localised skin inflammation around the injection sites [71], providing further evidence for a role of m6A modification in the modulation of inflammation.

Interestingly, BMDCs stimulated with lipopolysaccharide (LPS) demonstrated the presence of a large number of highly likely m6A sites these related to increased expression of proteins involved in the immune response [26]. This indicates a key role for this modification in the direct modulation of immune response elements and thus the immune system, particularly an active response. It has also been demonstrated that LPS challenge down-regulates FTO mRNA levels, at least in the liver this is in conjunction with a reduction of Toll-like receptor (TLR) 2 and 4 levels [72].

The TLRs recognise a variety of antigens from infectious agents to damaged cells from the host organism and initiate a response [73]. Three of these recognise RNA: TLR3 recognises double stranded RNA (dsRNA) [74,75], whereas TLR7 and TLR8 recognise single stranded RNA (ssRNA) [76,77] or double stranded short interfering RNAs (siRNAs) [78]. It has now been shown that methylated RNAs, including the m6A modification, are significantly less immunogenic [79]. This may be part of the self-recognition machinery to prevent TLRs from activating upon binding of native mRNAs.

<table>
<thead>
<tr>
<th>Gene/Protein</th>
<th>Proposed Function in m6A</th>
<th>Related diseases/disorders</th>
</tr>
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<tbody>
<tr>
<td>FTO</td>
<td>Demethylation</td>
<td>Parkinson's diseases, Attention Deficit Disorder, addiction, epilepsy, viral infection, colorectal cancer, endometrial cancer, stomach cancer, prostate cancer, breast cancer and pancreatic cancer</td>
</tr>
<tr>
<td>ALKBH5</td>
<td>Demethylation</td>
<td>Cancer (other family members implicated in bladder cancer)</td>
</tr>
<tr>
<td>WTAP</td>
<td>Methylation</td>
<td>Leukemia, localised inflammation</td>
</tr>
<tr>
<td>METTL3</td>
<td>Methylation</td>
<td>Cancer (via DNA damage repair pathways)</td>
</tr>
<tr>
<td>METTL14</td>
<td>Methylation</td>
<td>Cancer (via regulation of self-renewal capacity)</td>
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Table 1: Summary of known m6A methylation enzymes and their relationship with disease/disorders
Viral use of m<sup>6</sup>A RNA methylation

The lack of immune response to methylated RNA via the TLR pathways provides an ideal sanctuary for RNA based virii. The use of the m<sup>6</sup>A modification on their genome would prevent detection by the TLRs which are part of the frontline defence against pathogens [80]. It is therefore unsurprising to find some RNA based viruses which utilise this modification, for example Rous Sarcoma Virus (RSV) contains 7 m<sup>6</sup>A methylation sites in its genome [6] and simian virus 40 (SV40) has more than 10 sites [7]. This modification has also been identified in B77 avian sarcoma virus [81] and Adenovirus type-2 [82]. It would also appear that m<sup>6</sup>A RNA methylation is more densely utilised in these viral genomes than the mammalian genome with frequencies ranging from 1 in 400 found in adenovirus type-2 [82] to 1 in 1000 found in RSV [83,84]. The m<sup>6</sup>A modifications in SV40 were not only found in the SV40 nuclear RNA, but also specifically on the 16S and 19S viral messengers [7], this may be a modification to protect or localise these mRNAs and the nuclear RNA in host cells. Data from Adenovirus type-2 demonstrates conservation of the m<sup>6</sup>A modification between the nuclear resident viral genome and the mRNA transcripts it generates [85]. Furthermore, as mentioned previously these modifications may be an evolutionary attempt to evade the host immune system by avoiding detection by TLRs. Given the critical nature of the m<sup>6</sup>A methylation to some virii, it is likely that these directly or indirectly influence host m<sup>6</sup>A methylation/demethylation to further their own ends; the restricted size of the viral genomes would suggest that this is accomplished via viral co-factors or viral driven expression of host co-factors.

Interestingly, there are several RNA based viruses which produce mRNAs that have been shown not to possess any m<sup>6</sup>A modifications; these include tobacco mosaic virus [83], reovirus [84], vaccinia virus [86], cytoplasmic polyhedrosis virus [87] and Newcastle disease virus [88]. Investigations into the occurrence and influence of this modification in viral genomes peaked in the 1970s, given the latest reports of the occurrence and functionality of m<sup>6</sup>A RNA methylation perhaps a new wave of research will investigate the incorporation of this epigenetic mark in virii – is it a protective measure against internal degradation, a mechanism to avoid the host immune system or an enhancer to ensure translation of viral mRNAs to enhance the lytic phase.

Cancer and m<sup>6</sup>A RNA methylation

Given that m<sup>6</sup>A modifications are found on many housekeeping genes which influence translation, energy production/usage and differentiation and that it can stabilise mRNAs it is highly likely that this modification will be involved in cancer at some juncture; whether this is a key step in the formation, advancement or malignancy of tumours has yet to be seen. It is however, evident that this modification will become the subject of significant levels of research and spirit some heated debates in the near future. Perhaps it will lead to new understanding, diagnosis and treatments for a variety of cancer subtypes.

Evidence for the involvement of m<sup>6</sup>A mRNA modifications in cancer is already beginning to accumulate, although most is circumstantial at this point. For instance, widely disparate levels are evident in a variety of cancer cell lines [2], this indicates that this normally fairly consistent modification is highly disrupted, at least in some cancers, whether this is cause or effect remains to be seen. Additionally, increasing levels of SAM can inhibit the growth of some human gastric cancer cells; this effect is mediated through a reduction in mRNA levels, and thus protein levels, of c-myc and urokinase plasminogen activator (uPA) [89]. Although levels of SAM will affect many forms of methylation, its effects on mRNA expression may indicate a role for m<sup>6</sup>A in targeting specific mRNAs for degradation thus lowering their levels; it would require further experimentation to fully elucidate the effects of SAM on these cells. Intriguingly, it has been shown that inhibition of the m<sup>6</sup>A modification leads to a prolonged circadian cycle [90] and that disruption of the circadian clock may be associated with the development of cancer in some cases [91].

Evidence for the involvement of the m<sup>6</sup>A RNA methylation associated machinery is more prolific, WTAP is a key antigen in leukemia [70], FTO is mutated in ~82% of colorectal cancer cases, 100% of colorectal carcinoma cell cultures, 41.7% of endometrial carcinomas and 6.7% of stomach cancer cases [92]. Allelic variants of FTO have also been associated with prostate cancer risk [93], breast cancer [94,95] and pancreatic cancer [96]. The demonstration that METTL3 is phosphorylated when DNA damage is detected [97] indicates it may play a role in the cellular reaction to DNA damage. Although no direct evidence has been presented, the role of these pathways in many tumour types suggests that METTL3 may play a role in tumorigenesis. Similarly, no direct evidence exists for a role of ALKBH5 in tumourigenesis, however other members of its family, namely ALKBH2 and ALKBH8, have been implicated in bladder cancer progression [98,99].

Viral driven tumourigenesis is not uncommon, for example human papillomavirus [100], with the potential for RNA viruses to evade the immune system using the m<sup>6</sup>A modification there is the opportunity for this process to occur unabated. The activation of TLR signalling also induces several anti-cancer proteins and is considered a target for cancer therapy [101]. Furthermore, modification of RNA bases has been shown to enhance/perturb TLR signalling pathways [100,102], leading to the possibility that RNA modification plays a role in cancer progression [29].

The influence of other epigenetic modifications in tumourigenesis is unquestionable; in fact DNA methylation disruption is considered one of the hallmarks of cancer [103]. These changes are so prolific in tumourigenesis that DNA epigenetic modifications, DNA methylation and histone acetylation/deacetylation, are already being targeted for use in cancer therapy [104], perhaps RNA epigenetics is the next avenue for investigation in the pursuit of novel therapeutics.
References