• Review Article •

Recent progress in N6-methyladenosine modification in ocular surface diseases

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Received: 2022-07-03 Accepted: 2023-03-01

Abstract

● N6-methyladenosine (m6A) modification is a reversible process promoted by "writers", inhibited by "erasers", and processed by "readers". During the last decade, increasing emphasis has been placed on the underlying roles of m6A modification owing to their great importance in biological significance. The abnormal regulation of m6A modification will lead to aberrant cellular behavior and various diseases. Recently, studies have demonstrated that m6A modification is closely associated with the genesis and progression of ocular surface diseases (OSDs). This review focus on the role of m6A modification and research progress in OSDs including fungal keratitis, herpes simplex keratitis, immune-related keratoconjunctival diseases, pterygium, ocular chemical burns, and Graves' ophthalmopathy, which may provide new insights into and prospective applications for OSDs.

● **KEYWORDS:** N6-methyladenosine; m6A modification; epigenetics; ocular surface diseases

DOI:10.18240/ijo.2023.04.20

Citation: Wang W, Zou J. Recent progress in N6-methyladenosine modification in ocular surface diseases. *Int J Ophthalmol* 2023;16(4):645-651

INTRODUCTION

Ocular surface diseases (OSDs) comprise a spectrum of disorders that are characterized by abnormalities in the structure and function of the conjunctiva, cornea, and glandular network[1]. Traumatic, chemical, surgical and inflammatory damage can cause a variety of pathological changes, such as corneal conjunctivalization, neovascularization, limbal stem cell deficiency, dry eye disease, and subsequent visual dysfunction[2].

Epigenetics involves a phenomenon that modulates heritable gene expression without altering the sequence of DNA. RNA methylation is a widely prevalent epigenetic modification, as well as DNA methylation, histone modification, non-coding RNA modification, and chromatin remodeling[3]. Several crucial modifications of messenger RNA (mRNA) that maintain its stability include N6-methyladenosine (m6A), N1-methyladenosine, and 5-methylcytosine[4]. m6A, the most prevalent epigenetic modification of mRNAs, is widely distributed in various forms of RNAs, such as rRNAs, circRNAs, snRNAs, miRNAs, and lncRNAs[5]. m6A modification is a dynamic and reversible process whose regulation is accomplished by three categories of proteins: "writers", "erasers", and "readers".

m6A has been directly linked to a variety of diseases, and early research has mostly focused on its biological role in embryonic development and tumorigenesis[6-8]. Over the last several years, growing numbers of scientists have begun to emphasize the significance of m6A modification in OSDs. Here, we present a review of the biological process of m6A modification and summarize the latest research progress of m6A modification in OSDs, including fungal keratitis (FK), herpes simplex keratitis (HSK), immune-related keratoconjunctival diseases [*e.g.*, systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA)], pterygium, ocular chemical burns, and Graves' ophthalmopathy (GO).

M6A MODIFICATION

m6A is a methylation modification that occurs on the sixth nitrogen (N) atom of adenine (A) in RNA[9]. At present, the biological significance of m6A modification has been identified, which is linked to virtually all aspects of mRNA activity and metabolism, such as biogenesis, alternative splicing, 3'-end processing, nuclear export, translation, localization, and decay[10]. The aberrant m6A modification disrupts these RNA metabolic processes, which influence the hematological, neurological, respiratory, gastrointestinal, and gynecological systems[11].

Evidence indicates that m6A modification may play a critical role in the regulation of several immune-related processes, including proliferation, differentiation, and activation of immune cells[12]. Considering these findings, m6A is possibly

involved in the occurrence, progression, and outcome of immune-related disorders, including viral infections, cancers, and inflammatory and autoimmune disorders. Meanwhile, m6A modification acts as a double-edged sword in cancer and participates in the promotion and inhibition of tumorigenesis: it promotes tumorigenesis in certain cancers and inhibits tumor progression in others through its dual-directional modulatory functions[13].

Writers The m6A writers are multi-subunit methyltransferase complexes that direct the installation of m6A at specific locations in target mRNAs. Methyltransferase-like 3 protein (METTL3), also known as MT-A70, is considered the primary critical methyltransferase for m6A methylation. It is located in splicing facto r-rich nuclear speckles, in which it contributes directly to mRNA splicing[14-15]. Methyltransferase-like 14 protein (METTL14) is a homologue of METTL3 and shares almost 43% sequence homology with METTL3[16]. METTL3 and METTL14 are two core subunits of the writer complex that display independent catalytic activity and exert synergistic effects through the formation of stable heterodimers[17]. Wilm's tumor-1-associated protein (WTAP) interacts with these heterodimers and subsequently localizes them into nuclear speckles to affect cellular m6A deposition[18]. Numerous auxiliary subunits are necessary for the efficient installation of the m6A modification and determine the specific types of the writers, including METTL16, METTL5, Cbl proto-oncogene-like 1, Vir-like m6A methyltransferase-associated, zinc finger CCCH-type containing 13, and RNA-binding motif protein 15[11].

Erasers m6A erasers are a group of proteins that can exert demethylation activity, which removes m6A modifications. Fat mass and obesity-associated protein (FTO), the originally discovered eraser, belongs to the non-heme Fe(II)- and α-KG-dependent dioxygenase AlkB family, which catalyzes a wide range of biological oxidative demethylation of m6A[19]. FTO is predominantly localized in the nucleus, and it is responsible for demethylating mRNA at m6A sites by approximately 5% to 10%, as well as being detected in cytoplasm[19-20].

ALKB homolog 5 (ALKBH5) is the second eraser identified to date. Its demethylation activity profoundly influences nuclear mRNA export, RNA metabolism, and the assembly of mRNA processing factors in nuclear speckles[21]. Recently, flavin mononucleotide has been reported to be a novel artificial small-molecule demethylase that mediates the photochemical demethylation of m6A residues in RNA[22].

Readers The identification and characterization of m6A readers have contributed to a deeper understanding of the precise regulation of m6A biological functions. The readers consist of YTH domain-containing family proteins (YTHDF1/2/3 and YTHDC1/2), the heterogeneous nuclear ribonucleoprotein (HNRNPA2B1, HNRNPC, and HNRNPG), insulin-like growth factor 2 mRNA binding protein 1/2/3, and eukaryotic initiation factor 3, which collaboratively regulate the process of RNA export, translation, and degradation[14,23]. Because m6A modification requires readers to carry out their biological functions, the same modification will produce reverse biological effects when binding to different readers. Consequently, each group of readers can alter mRNA transcription differently, and these groups jointly influence the cellular functions to produce alterations in physiological conditions[13]. Once regulation is mismanaged, various diseases can occur.

M6A MODIFICATION IN OCULAR SURFACE DISEASES

Fungal Keratitis FK is a form of serious microbial keratitis caused by opportunistic pathogenic fungi and can result in visual impairment and even blindness[24]. Among the infectious keratitis cases in China, over 60% of cases are due to fungal sources, with Fusarium being the most common pathogen[25]. According to recently obtained high-throughput sequencing results, FK markedly alters not only the levels of both mRNA and miRNA but m6A levels as well[26-27].

A study conducted by Hu and Lin[28] investigated the role of m6A modification in experimental FK. By inoculating mice with *Fusarium solani* (*F. solani*), they created a murine model of FK and found that, in the *F. solani*-treated group, the overall m6A levels in corneal tissue were increased in comparison with those in the controls. An analysis of Western blots and immunofluorescence staining demonstrated a notable increase in the expression of METTL3 in mice with *F. solani*. However, the expression levels of FTO, ALKBH5, WTAP, and KIAA1429 were not significantly different between the two groups. Additionally, 1137 mRNAs were examined for differences in m6A modifications, 780 of which were hypermethylated and 357 were hypomethylated. Based on Kyoto Encyclopedia of Genes and Genomes (KEGG) and Western blot, FK is associated with enriched m6A-methylated mRNAs in the PI3K-Akt pathway, along with elevated phosphorylation levels of Akt and PI3K. As expected, the knockdown of METTL3 inactivated PI3K/AKT pathway and inhibited the expression of multiple inflammatory cytokines[29]. Thus, METTL3 is shown to be a pro-inflammatory protein in Fusarium solani-infected corneal tissue, and it has the potential to be a diagnostic and therapeutic target for FK in the future.

Herpes Simplex Keratitis HSK is a blinding disease characteristic of recurrent infections in the cornea that is mainly caused by herpes simplex virus type 1 (HSV-1). After the primary infection, HSV tends to establish a latent infection in the trigeminal ganglion. The reactivation of HSV could be triggered by stress, fever, ultraviolet light exposure, and

long-term local use of topical corticosteroids, resulting in a recurrence of HSK[30]. Recurrent HSK manifests a variety of clinical symptoms, among which are epithelial keratitis, stromal keratitis, endothelial keratitis, and neurotrophic keratopathy[31]. It is generally accepted that topical antivirals are effective at reducing the disease severity and temporal courses of recurrent HSK, while some cases of poor treatment outcomes continue to be reported.

The interaction between virus and host is regulated by m6A modification[32]. In the 1970s, Moss *et al*[33] first reported that HSV-1 mRNAs were modified by m6A. Recently, a number of studies on DNA and RNA viruses have revealed the significance of m6A modifications in regulating alternative RNA splicing[34] and host antiviral responses[35]. Srinivas *et al*[36] found that, in human fibroblasts, HSV-1 facilitated a remarkable redistribution of the nuclear m6A machinery as it proceeded through the infection cycle. METTL3 and METTL14 were distributed in the cytoplasm, while WTAP was retained within the nucleus. Other methyltransferase complex subunits, as well as YTHDC1 and ALKBH5, were redistributed in the same manner. In order to accomplish these changes, the HSV-1 IE protein ICP27 is required, which is an essential viral regulator of host mRNA that facilitates the transport of intron-less viral mRNAs. These results suggested that HSV-1 infection strongly antagonizes the activity of m6A modification, thereby favoring viral replication efficiency.

Wang *et al*[37] found that, upon HSV-1 virus infection, HNRNPA2B1 combines with viral DNA in the nucleus to form a homodimer and translocates to the cytoplasm, where it triggers the TBK1-IRF3 pathway and initiates an immune response. In addition, through the inhibitory effect of hnRNPA2B1 on FTO demethylation, CGAS, IFI16 and STING mRNAs exhibit enhanced m6A modification and nucleoplasmic transport. Therefore, hnRNPA2B1 promotes further enhancement of the host's antiviral innate immunity.

Although there is no direct evidence to support that m6A modification is associated with the pathogenesis, progression, or prognosis of HSK, it can be concluded from the above studies that m6A likely plays a significant role in HSK.

Immune-Related Keratoconjunctival Diseases Immune-related keratitis is categorized into primary immune keratitis and keratitis mediated by systemic autoimmune diseases. Primary immune keratitis comprises Mooren's ulcer[38], interstitial keratitis[39], and vernal keratoconjunctivitis[40] as well as keratoconjunctivitis sicca[41]. A Meta-analysis reported that ocular involvement was prevalent in 18% of cases of RA and 31% of SLE cases[42]. The common systemic autoimmune diseases that manifest in the ocular surface include RA, SLE, connective tissue disorders (Sjögren's syndrome, scleroderma, and relapsing polychondritis), and vasculitis (giant cell arteritis, granulomatosis with polyangiitis, and Behcet's disease)[43-44]. Patients with ocular involvement of autoimmune disorders most commonly have keratoconjunctivitis that can result in corneal infections and ulcerations[45]. Rarely, the autoimmune inflammatory mediators can also directly attack the peripheral corneal stroma, leading to peripheral ulcerative keratitis, which is a non-infectious ulcer associated with epithelial defects[46]. Until now, little has been known about the exact cellular and molecular mechanisms in immune-related keratoconjunctival disorders. It is widely accepted that epigenetic pathways play fundamental roles in both the innate and adaptive immune systems. Wang *et al*[47] reported that m6A tightly controls the maturation, activation and function of immune cells by enhancing the translation of specific leucocyte differentiation antigens, suggesting that m6A modification may be involved in regulating the pathogenesis of autoimmune diseases.

RA, a chronic autoimmune disorder with high levels of disability, involves reduplicated small joint destruction, particularly in the hands and feet. The underlying etiological factors of RA remain unclear, but they are probably associated with genetic susceptibility, environmental factors, and epigenetics[48]. Various cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-17, are involved in the processes of inflammation, joint destruction, and certain comorbidities in RA progression[49]. Recent investigations have revealed the critical importance that m6A plays in RA pathological process. Wang *et al*[50] conducted a study to elucidate the function and potential mechanism of METTL3 in RA pathogenesis. They found that METTL3 expression in peripheral blood mononuclear cells isolated from RA patients was positively correlated with biochemical indicators, such as C-reactive protein and erythrocyte sedimentation rate, two commonly used metrics to assess the activity of RA. In addition, lipopolysaccharide-induced inflammation of pTHP-1 macrophages could contribute to METTL3 activation and biological functions, whereas METTL3 overexpression effectively inhibits inflammatory mediators by lipopolysaccharide stimulation through the transcriptional factor nuclear factor (NF)-κB. These findings revealed that METTL3 may be a novel biomarker for RA diagnosis and treatment.

Another study by Shi *et al*[51] focused on the functions that METTL3 performed in the process of inflammation and cell viability, proliferation, invasion, and migration of fibroblast-like synoviocytes (FLSs). In synovial tissues from RA patients, METTL3 expression was significantly up-regulated, and its overexpression led to the release of massive inflammatory mediators in FLSs. Additionally, METTL3 was shown to facilitate activation and inflammatory reactions in FLSs by the NF-κB signaling pathway, which accelerated the progression and outcome of RA.

SLE is a relapsing chronic multisystemic autoimmune disease mediated by autoantibodies that adversely affects vital organs, including the skin, brain, kidneys, eyes, and joints. In recent years, m6A modification has been proven to function critically in the progression of SLE. Studies conducted by Luo *et al*[52-53] demonstrated that, in peripheral blood isolated from SLE patients, the levels of mRNA for METTL14, ALKBH5 and YTHDF2 were significantly decreased by quantitative real-time polymerase chain reaction (qRT-PCR). This low expression was associated with elevated C-reactive protein and declined complement 3, which suggested a correlation with disease activity in SLE. Moreover, ALKBH5 mRNA levels were inversely associated with anti-dsDNA levels and positively correlated with white blood cell count. Importantly, the results of the logistic regression analysis demonstrated that low expression of ALKBH5 and YTHDF2 in peripheral blood was associated with the development of SLE, which can be used as a biomarker to assess SLE activity and confirm diagnosis.

Although there are rare reports of research on m6A modification in immune-related keratoconjunctival diseases, based on the findings of the studies described above, there are plentiful applications for further research.

Pterygium Pterygium is a wing-shaped, superficial external fibrovascular conjunctival tissue that generally grows toward the cornea surface and is usually situated on the nasal side, which can lead to astigmatism and even vision loss[54]. The pathogenesis of pterygium has not been completely elucidated. In histopathology, pterygium specimens are classified by squamous metaplasia of the conjunctival epithelium, goblet cell hyperplasia, disruption of Bowman's layer, and degeneration of subconjunctival collagen and elastin fibers[55]. Jiang *et al*[56] explored the potential relationship between m6A modifications and pterygium-related genes. Based on a comprehensive data and bioinformatical analysis of the m6A-modified RNA sequence, pterygium and normal conjunctiva were compared for differences in expression of m6A methylation. It was observed that 458 m6A peaks were down-regulated and 1301 peaks were up-regulated in the mRNA of pterygium compared to normal conjunctival tissues. This appears to be predominantly caused by METTL3, which acts as a crucial regulator in pterygium. KEGG pathway analysis indicated that the genes up-regulated in m6A modification were linked to the Hippo signal transduction pathway, while the down-regulated genes were associated with the Notch signal transduction pathway. By regulating the Hippo and Notch signaling pathways, m6A modification can influence important biological phenomena, including cell proliferation, migration, apoptosis, and cell cycle, thus leading to pathological changes in the conjunctival tissue. Additionally, five genes (DSP, MXRA5, ARHGAP35, TMEM43, and OLFML2A) have been identified from comprehensive analysis of RNA sequence results as being closely correlated with the progression of pterygium. However, it remains to be discovered and further clarified what the specific functions of m6A-related genes are in pterygium.

Ocular Chemical Burns Approximately 22% of ocular injuries result from chemical burns. Two-thirds of chemical burns are caused by alkalis, while the rest are caused by acids and alcohols[57]. Alkaline agents, in general, penetrate more deeply than acids. Once the epithelium is compromised, alkaline solutions induce cellular membrane lysis with penetration into the deep tissue layers, causing tissue ischemia and necrosis. Conversely, proteins are denatured in acidic solutions, which causes coagulative necrosis and builds barriers that prevent deeper penetration of tissues[58]. In the late stages of severe cases, ocular alkali injuries can trigger a cascade of events leading to blindness, including corneal opacity, ulcer perforation, neovascularization, pseudopterygium growth, and symblepharon[59].

In the acute phase of alkaline injuries, over 50% of corneal limbal ischemia is an influential contributor to the dysfunction of the limbal stem cells[60]. Dai *et al*[61] created a limbal stem cell-specific METTL3 knockout (cKO) mouse model and investigated the function of m6A in repairing corneal injury after alkali burns. On days 7 and 14 after alkali burns, the METTL3 cKO mice had more complete corneal epithelium and less stromal layer neovascularization compared to wild-type mice, suggesting that METTL3 knockdown inhibited pathological vascularization and promoted corneal injury repair. Immunofluorescence results for Ki67 indicated that depletion of METTL3 can promote the proliferation, self-renewal, differentiation, and migration of corneal limbal stem cells, thereby accelerating the corneal epithelial repair after alkali injuries. Furthermore, METTL3 was also found to affect the growth and metabolism of corneal limbal stem cells by regulating AHNAK and DDIT4 expression, which strengthened the role of m6A in the repair of corneal alkali damage. In summary, modulating METTL3 and m6A modification pathways may provide a method for treating corneal diseases. Yao *et al*[62] examined the mechanisms of action and effects of METTL3 and m6A modification during pathological neovascularization by using METTL3-ecKO mice. Immunofluorescence staining exposed a smaller area of corneal neovascularization in METTL3 cKO mice than in the controls, indicating that METTL3 gene silencing has an anti-angiogenic effect on corneal alkali injury. Essentially, the promotion of angiogenesis is governed by abnormal Wnt signaling activated by METTL3. These studies suggested that pathological angiogenesis and damage repair are strictly regulated by m6A modification subsequent to corneal chemical burns.

Graves' Ophthalmopathy GO, also called thyroid-associated ophthalmopathy, is an orbital disorder of autoimmune pathology that predominantly attacks extraocular muscles (EOMs) and is the most common extrathyroidal symptom of Graves' disease[63]. The clinical features of GO are complicated, including double vision, exophthalmos, eyelid retraction, diplopia, restrictive strabismus, exposure keratopathy, and optic neuropathy[64-65]. Relevant evidence suggests that epigenetic modifications contribute to the pathogenesis of GO. Zhu *et al*[66] investigated the role of m6A RNA modification in the pathogenesis of GO. They assessed the data on differential expression of m6A methylation collected from RNA sequence datasets and bioinformatics analyses in EOMs from seven GO patients and five non-GO patients. They found that overall m6A levels as well as the expression of WTAP, YTHDF2, and YTHDC2 were significantly up-regulated in the EOMs of GO patients compared to those of patients without GO. KEGG pathway analysis identified 12 out of 19 differentially expressed mRNA-related biological pathways that were related to inflammatory and immune responses. In addition, a significant up-regulation of genes associated with IL-1, IL-6, IL-8, IL-10, IL-17, interferon (IFN)-γ, and TNF-α was observed in EOMs from patients with GO. Therefore, it can be speculated that m6A-related factors detected in EOM specimens may be involved in GO inflammatory regulatory processes by targeting mRNAs for inflammatory genes. These results suggested a possible relationship between aberrant m6A RNA methylation and the expression of inflammation-related genes as well as relevant signaling pathway activity in GO patients.

CONCLUSION

In this article, we reviewed the underlying mechanisms and biological functions of m6A modification and provided updates on the progress of m6A research in OSDs. m6A modification emerges as a vital epigenetic mechanism of gene transcriptional regulation and relies on the post-transcriptional modification of RNA, which plays a pivotal role in many physiological and pathological processes. Currently, with the development of various antibody-independent detection methods, differential m6A levels can be distinguished in individual RNA regions with high resolution, overcoming the limitations of traditional m6A detection approaches and providing us with a range of options[67-68]. To date, accumulating evidence has revealed the effects of m6A modification on tumors and neurological and cardiovascular diseases, while little research has been conducted regarding the study of m6A modification in ocular surface diseases. Our work expounds upon the regulatory role of m6A, primarily in terms of keratitis, immune-related keratoconjunctival diseases, pterygium, ocular chemical burns, and GO, while it remains unclear whether it contributes to other OSDs. Furthermore, most of the existing evidence has concentrated on the crucial m6A modifying enzymes METTL3, WTAP, ALKBH5, and YTHDF2, whereas the potential connections and profound mechanisms of other enzymes with these OSDs are not fully understood.

According to recent research, m6A modification is critical for the progression of multiple cancers, and targeting dysfunctional m6A is an appealing strategy for cancer therapy, suggesting that m6A modification may also serve as a potential therapeutic target for OSDs. Moreover, inhibitors that target writers, erasers, or readers could be helpful in controlling the disease process. With in-depth insight into the specific functions and potential pathways, m6A modification provides a novel perspective for the guidance of diagnosis and therapy in ocular surface diseases.

ACKNOWLEDGEMENTS

Authors' contributions: All authors contributed to the study's conception and design. Wang W helped to perform the literature search and data analysis and draft the manuscript. All authors read and approved the final manuscript.

Foundation: Supported by Project of Shanghai Science and Technology (No.20ZR1443600).

Conflicts of Interest: Wang W, None; **Zou J,** None.

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