ARTICLE

RNA methylation in lymphoid malignancies

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N^6-methyl-adenosine (m^6A) modification is the most abundant internal modification in mammalian RNA and plays an important role in gene expression mechanisms such as mRNA splicing, nuclear export, transcript stability and translational efficiency. The extent and the significance of m^6A modifications in pathological conditions, especially hematological malignancies such as lymphomas are not known. In this study, the global m^6A methylation by an immunocapture method in normal B cells (GMO 6990), and in the B cell neoplastic cells such as diffuse large B cell lymphoma (DB), Burkitt's lymphoma (Raji) and a relapsed pre-B cell acute lymphoblastic leukemia (ALL) (NALM6) cells are discussed. Variable mRNA expression of RNA methylases such as methyltransferase like 3 (METTL3) and Wilms tumor 1 associated protein (WTAP) that methylates internal adenosine residues in mRNA was observed in normal and lymphoma cells as quantified by qRT-PCR. Further, an mRNA demethylase, Fat mass and obesity associated enzyme (FTO) mRNA expression was found to be high in lymphoma cells compared to normal B cells. A similar trend was also observed between normal and ALL patients at diagnosis. Overall, our studies suggest that altered RNA methylation and enzymes controlling this process may be of significance in the pathology of lymphoid malignancies.

Keywords: B cell; lymphoma; RNA methylation; demethylase

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Introduction

Lymphoid neoplasms are the fourth most common cancer and sixth leading cause of cancer death in the United States [1]. Non-Hodgkin lymphoma (NHL) is a subtype of lymphoid neoplasm that includes B cell acute lymphoblastic leukemia (pre B ALL), diffuse large B cell lymphoma (DLBCL) and Burkitt's lymphoma (BL). The incidence of pre-B ALL has increased since 2003 and primarily affects the pediatric population [1]. DLBCL is much more common in adults and the incidence increases with age. BL is relatively rare, but it is one of the most common types of childhood lymphoma [1] and can also occur in older adults. At the molecular level, Non-Hodgkin lymphoma presents with heterogeneous features including chromatin modifications and epigenetic alterations such as methylation of DNA that contribute to lymphomagenesis [2]. Akin to DNA modification, RNA methylation (e.g. N^6-methyl adenosine, m^6A) is also present in various RNA including mRNA and long non-coding RNA (lncRNA) [3], and may constitute another layer of epigenetic regulation of gene expression in lymphoma.

Table 1. Different primers used in this study

Genes	Primers
METTL3	F: 5'-CTACAGGATGATGGCTTTCTC- 3'
	R: 5'-ATTTCATCTACCCGTTCATACC-3'
WTAP	F: 5'-ATGGCGAAGTGTCGAATG-3
	R- 5'-GTACTCTGCATACCCTCTACT-3'
FTO	F: 5'-GGCTAGTGACTGCTGTATTG-3'
	R- 5'-GATAGAAGCTACAGACGAATGG-3'
ALKBH5	F: 5'-TGGTCCTGACCTCTCTTATC-3'
	R: 5'- CTACCACACACCACAACAA-3'

The most abundant internal mRNA modification in eukaryotes is N^6-methyl adenosine (m^6A). An estimated three to five m^6A sites were reported to be present in one single mRNA [4]. RNA methylation is non-randomly distributed and enriched around transcription start sites, long internal exons and stop codons [5]. Also, m^6A methylation is highly enriched in the last coding sequences of the majority of genes and this modification leads to widespread effects on gene expression, development and protein termination [6]. Although certain mRNA regions are enriched with m^6A modification the function of such enrichment in RNA is currently unknown. The biological functions attributed to m^6A modification on mRNA include modification of mRNA splicing, nuclear export, stability and translational efficiency [5], indicating that methylation is correlated with unique regulatory functions. Exactly, how the m^6A distribution varies in mRNA of normal vs diseased cells needs to be determined.

The m^6A modification of mRNA functions as a dynamic mark that is post-transcriptionally installed and erased by m^6A methylases and demethylases. Also, more than one methylase or demethylase has been reported to be involved in m^6A modification [7]. Methyl transferase like-3 (METTL3/MT-A70), is an RNA methylase that installs m^6A modification in mammalian mRNA as a part of methyltransferase complex [8]. METTL3 is observed both in the nucleus and cytoplasm, suggesting that RNA methylation could occur in both cell compartments [9, 10]. As mentioned above, m^6A modification is important for several cellular functions, stem cell differentiation, and RNA editing [11, 12]. On the other hand, m^6A demethylases are also important for the dynamic regulation of RNA m^6A modification. Fat mass and obesity associated (FTO) enzyme was reported to possess RNA demethylase function [13] and is one of the enzymes largely explored with respect to m^6A modification. FTO preferentially removes methyl groups from single stranded RNA (ssRNA) by means of oxidation, but it can also target double stranded RNA, albeit with low affinity [13, 14]. Thus, both METTL3 and FTO play an important role in RNA regulation and processing. Alteration in the quantity or efficiency of these molecules will affect RNA processing and could help drive cancer phenotypes.

It is hypothesized that the m^6A content in mRNA from lymphoma cells will be lower compared to normal cells and the enzyme(s) involved in m^6A demethylation may be higher in lymphoid malignancies. To test our hypothesis, we explored the total m^6A methylation profiles in various lymphoid malignant cells and normal B cells by direct extraction of mRNA and utilizing an immunocapture method with antibodies specific for N^6-methyladenosine (m^6A). We also investigated the mRNA expression of factors controlling m^6A modifications in these cells. Our preliminary results indicate a lower mRNA methylation trend in different lymphoma cells as well as an increased expression of m^6A demethylase suggesting that alteration in m^6A modification may play an important role in lymphoid malignancies.

Materials and methods

Cell lines. Diffuse large B-cell lymphoma (DB), Burkitt's lymphoma (Raji), relapsed Pre-B acute lymphoblastic cells (NALM6) were maintained in complete RPMI media with 10% FBS. Normal B cells (GM06990) were maintained in complete RPMI media containing 15% FBS. Only cells in the early passages (<5) were used in our experiments. All lymphoma cells were tested for authenticity using STR (IDEXX BioResearch, West Sacramento, CA), interspecies contamination, and mycoplasma contamination.

mRNA isolation. The cellular mRNA was prepared using the GeneElute direct mRNA miniprep kit (Sigma, St Louis, MO) as described in the kit protocol. In brief, the cells (10^6 cells each) were lysed and homogenized on ice followed by digestion with proteinase K to eliminate RNase. Subsequently, after addition of sodium chloride the polyadenylated RNA was captured on oligo (dt) polystyrene beads and eluted using 10 mM Tris-HCl (pH 7.4). To avoid any trace contamination of DNA, the samples were treated with RNase-free DNAse1 (Qiagen, Germantown, MD). Further, in order to inhibit residual RNase in the sample, RNasin ribonuclease inhibitor (50-100 U) (Promega, Madison, WI) was added to the sample preparations according to the manufacturer's protocol.

Enzyme-Linked Immunosorbent Assay (ELISA). The *m^6A* methylation in mRNA was quantified using a specific

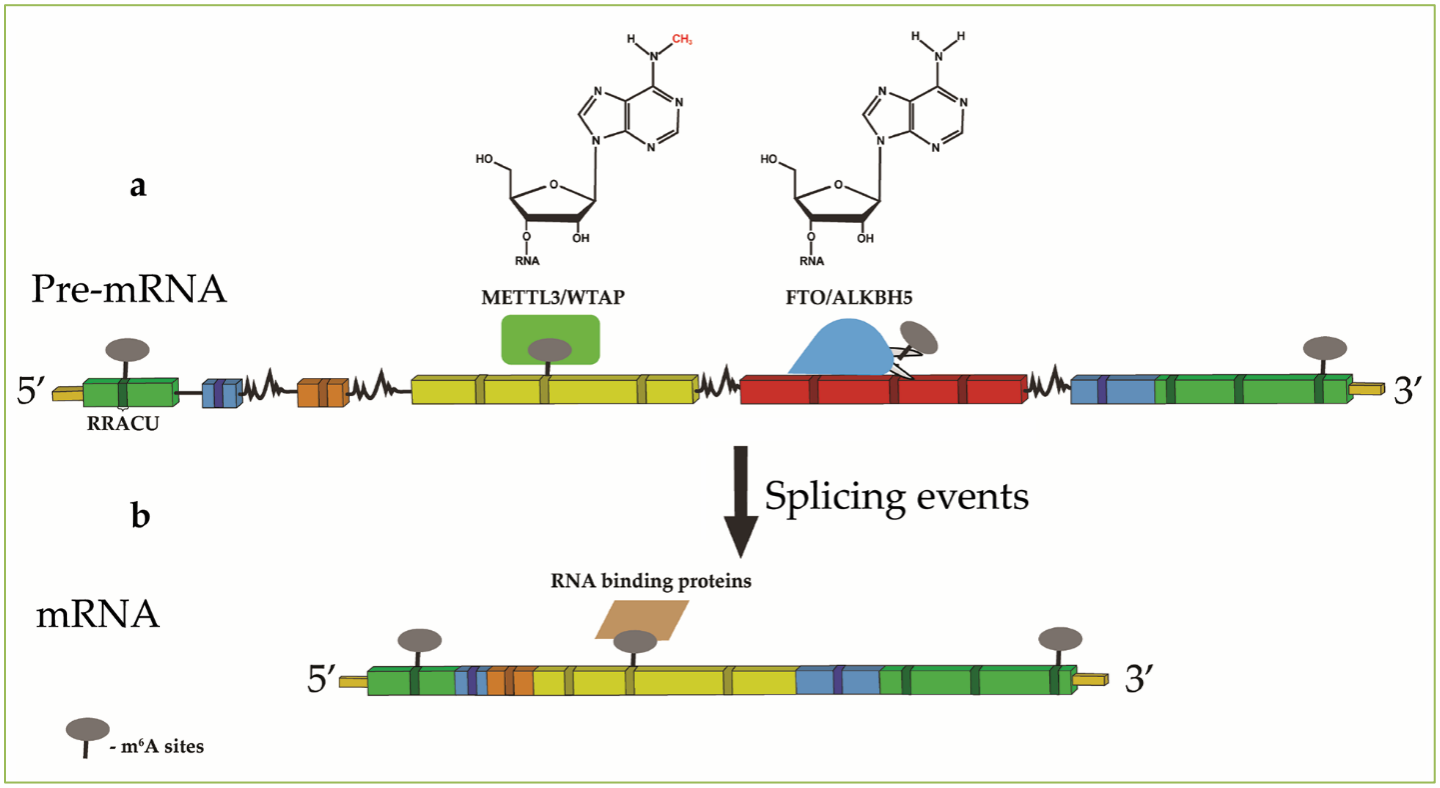

Figure 1. RNA m^{6}A methylation. a) m^{6}A methylation in RNA facilitated by methylase enzymes such as *METTL3* and *WTAP*. Demethylases such as *FTO* and *ALKBH5* removes the methyl groups from the adenosine base. b) m^{6}A sites in mRNA can influence the interaction of the RNA binding proteins. RRACU, methylation consensus region (vertical lines); m^{6}A, grey pins; exons, thick colored bars; introns, thin lines.

N^6-methyladenosine capture antibody according to the manufacturer's protocol (Abcam, Cambridge, MA). Briefly, from the mRNA isolated from the above step 150-200 ng was added to the wells in a 96 well plate containing the binding solution (80 µl). Positive and negative controls (supplied by the manufacturer) were also processed in parallel. Subsequently, the capture antibody was added to the wells and incubated for 90 min. After washing the wells using the wash buffer the detection antibody was added to the wells and incubated for 30 min. After further washing, the enhancer solution was added to the wells and incubated for additional 30 min. For signal detection, the developer solution was added and incubated for 10 min. After addition of a stop solution the absorbance (450 nm) was measured in a microplate reader. The results are depicted as m^{6}A content in sample cellular mRNA's. Two technical replicates were performed for each sample.

Real-time quantitative PCR (qRT-PCR) analysis. Total RNA from different lymphoma and normal cells were extracted using RNeasy mini kit (Qiagen, Valencia, CA). cDNA was generated from total RNA using cDNA synthesis kit (Bio-rad, Hercules, CA). Quantitative real-time PCR (qRT-PCR), was performed with CFX Connect and a Sybr Green reaction (Biorad). The data was analyzed using $2^{-\Delta\Delta CT}$ method to assess fold change in mRNA expression. Gene expression levels were normalized against 18sRNA for individual samples. The primer sequences for the genes used in this study are depicted in Table 1.

Collection of patient samples. Bone marrow samples from normal healthy donors and pre-B lymphoblastic leukemia patients (pre-B ALL) (de-identified) were obtained upon ethical approval by the Institutional Review Board, University of Missouri. ALL patient samples contained at least 88% blasts. The mononuclear cells from the bone marrow samples were isolated as described previously [15].

RNA-seq analysis. Total RNA was isolated from the normal and ALL patient samples. RNA-seq libraries were constructed using NEBNext® UltraTM Directional RNA Library Prep Kit for Illumina® (New England Biolabs). The sequencing was done using Illumina HiSeq 2000 (1x100 bp reads).

Processing and RNA-seq read mapping. 100 bp single-end RNA-seq reads were obtained from Illumina HiSeq 2000. Sequence files were generated in FASTQ format. RNA-seq data were analyzed using Perl programming on Unix/Linux based system (64-bit computer running Linux; 4 GB of RAM). The quality score of RNA-seq reads was obtained by using the FastX-Toolkit v. 0.0.13. Reads were

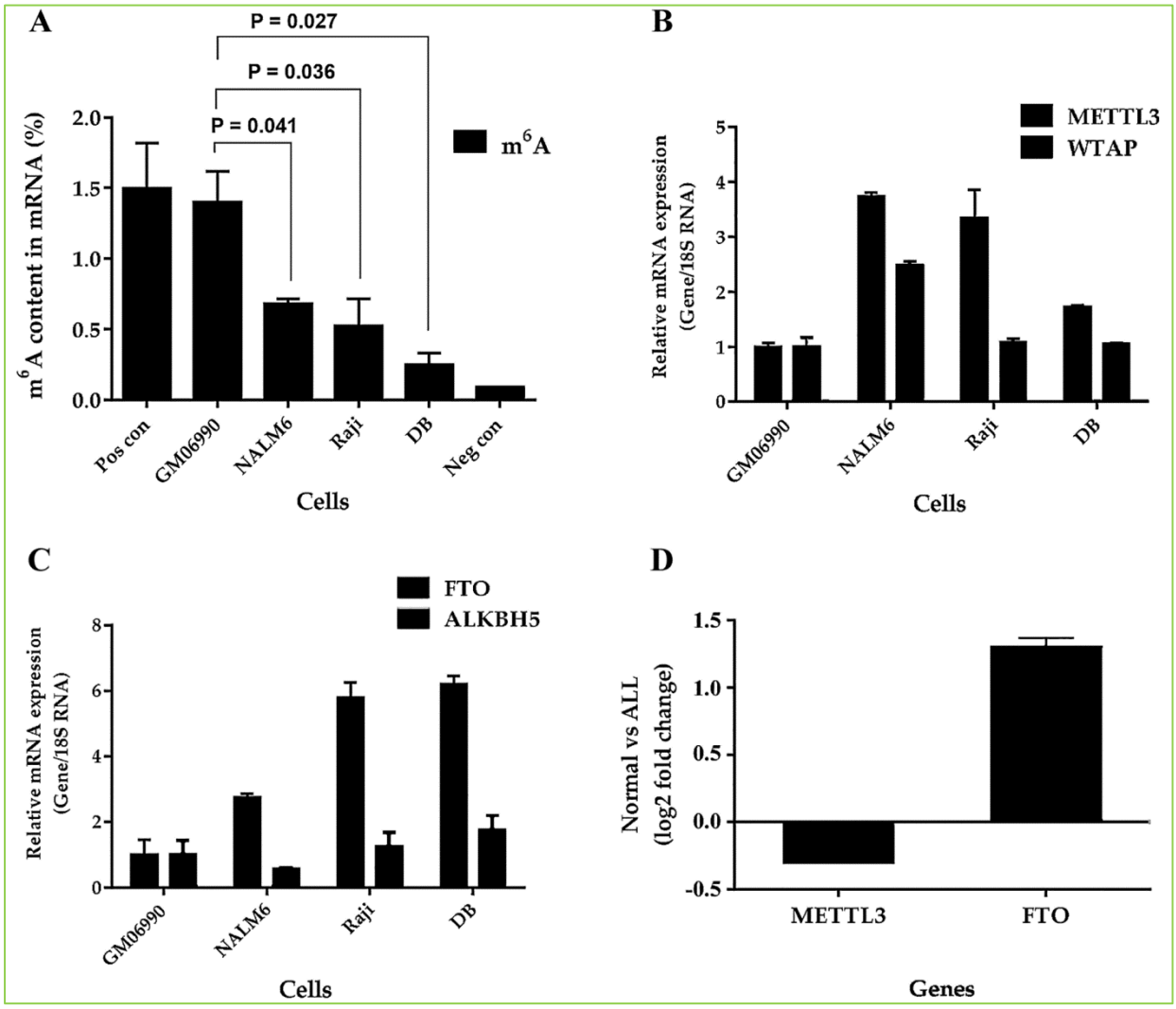

Figure 2. A. m6A content in normal B cells and different lymphoid malignant cells. Immunocapture assay depicting the amount of m6A content in mRNA of the cells analyzed. Pos con – positive control; Neg con-negative control (n=3 for each cells). **B**. qRT-PCR for mRNA expression of m6A modifiers. mRNA expression of a) METTL3 and WTAP. Results are from three experimental replicates. **C.** qRT-PCR for mRNA expression of FTO and ALKBH5. Results are from three experimental replicates. **D.** METTL3 and FTO expression in patient samples. Whole transcriptomic and subsequent bioinformatic analysis (see methods) for RNA expression of normal and ALL patient bone marrow samples (n=8 each) indicated METTL3 is lower (P=0.02) and FTO is higher (P < 0.01) compared to normal.

then processed and aligned to the UCSC *H. sapiens* reference genome (build hg19) using TopHat v1.3.3 [16].

Statistical analysis. All the experiments were performed in triplicates (unless mentioned otherwise) and the data are expressed as mean ±SD. Statistical significance was evaluated using Student's t test. P value <0.05 was considered significant.

Results and discussion

The m^6A modification in mRNA is particularly intriguing (Figure 1), and on average three to five m^6A modifications occur in each mRNA [5]. While the m^6A modification has been shown to be associated with different diseases [4, 17, 18], the m^6A methylation status in lymphoid malignancies is unknown. To determine whether mRNA from normal human B cells and from malignant B cells have variable total m^6A content, mRNA was directly isolated from GM06990 (normal B cells) and B cell lymphoma subtypes (DLBCL (DL), Raji (BL) and NALM6 (pre-B ALL cells from a relapsed patient). Isolated mRNA was processed further to detect total m^6A content by an immunocapture method using an antibody specific for m^6A. The results indicated that m^6A methylation, though variable, is low in all the lymphoma cells tested compared to normal B-cells (Figure 2A), suggesting that alteration in mRNA specific methylation modifications occur in lymphoid malignant cells. Even within the lymphoma cell subtypes, DB cells had the lowest detectable m^6A content compared to other malignant cells

further suggesting a cellular context dependent mode of m^6A modification. This is in keeping with the reports that m^6A modifications have distinct distributions among different tissues indicating each cell type and cell state may have unique m^6A modification patterns [19]. The m^6A modification in mRNA is a dynamic process and may involve in important functions such as cellular homeostasis [17]. Further, in embryonic stem cells (ESC), depletion of m^6A modifications blocked differentiation and led to hyper-naive pluripotent state [7]. Our observations that a decreased m^6A trend in lymphoma cells may be suggestive of continuous cell proliferation and reduced differentiation resulting in more immature lymphocytes, although further studies are needed to confirm this possibility.

The decreased m^6A in lymphoma raised the question whether the factors, specifically the enzymes, involved in the m^6A modifications may be differentially expressed in normal vs lymphoid malignant cells? To explore this, we investigated the gene expression of two mRNA methylases, METTL3 and WTAP as well as two different mRNA demethylases, FTO and ALKBH5 in normal and malignant lymphoid cells. The mRNA expression of methylase enzyme, METTL3, was relatively higher in all the lymphoma cells compared to normal B cells, whereas, WTAP mRNA expression was observed to be high only in NALM6 cells (Figure 2B). While METTL3 and WTAP are required for m^6A modifications, a previous report using siRNA depletion experiments suggested that another enzyme, KIAA1429, associated with methyltransferase complex was required for the full methylation program in mammals [20]. Overall, it appears that mRNA methylation in general requires multiple components as a part of the methyltransferase complex for efficient RNA methylation which again could vary depending on the cellular phenotypes and the disease process.

Gene expression analysis of mRNA demethylases indicated that FTO mRNA expression was higher in all the lymphoma cells tested with increased cellular expression in the following order (DB>Raji>NALM6) (Figure 2C). However, gene expression of ALKBH5, also a demethylase was less compared to FTO in all the lymphoma cells. The mechanisms of demethylation between FTO and ALKBH5 are different. While FTO functions via oxidative demethylation [21], ALKBH5 catalyzes the direct removal of methyl group from m^6A-methylated adenosine. Further, apart from m^6A, other nuclear RNAs were also found to be substrates for ALKBH5 [22]. As per our observations, despite the expression of METTL3 in lymphoma cells the total m^6A content as measured by immunocapture method was found to be low (Figure 2A). We speculate that the decreasing trend of m^6A methylation in lymphoma cells could be in part due to increased expression of FTO in these cells.

To determine if the trends observed in cell lines are consistent in primary cells, data generated from pre-B ALL patients and normal precursor B-cells was analyzed [23]. In these samples the gene expression of METTL3 was lower while FTO was higher in patients diagnosed with ALL compared to healthy donors (Figure 2D), although the m^6A status in these samples needs to be determined. Interestingly, in our studies with relapsed ALL cells (NALM6) we found an increased METTL3 and moderate expression of FTO (Figure 2B &C). While our initial studies shed some light on the m^6A modification in lymphoma cells and status of the enzymes involved in this process, our ongoing and future studies will be focused on the comprehensive roles of m^6A methylation and the factors controlling such modifications in patients with lymphoid malignancies.

Conflicting interests

The authors have declared that no conflict of interests exist.

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Author Contributions

AE did the immunoassays, PCR analysis and other technical aspects of the study. KHT analyzed the clinical data and participated in drafting the manuscript. JB participated in drafting the manuscript. SRK conceived and designed the project, coordinated the study and wrote the manuscript. All authors read and approved the manuscript.

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