Human ALKBH3-induced m^{1}A demethylation increases the CSF-1 mRNA stability in breast and ovarian cancer cells

Ho-Hyung Woo[a]* and **Setsuko K. Chambers**[a,b]

[a]*The University of Arizona Cancer Center, Tucson, AZ 85724, USA*

[b]*Department of Obstetrics and Gynecology, College of Medicine, The University of Arizona, Tucson, AZ 85724, USA*

**Corresponding author. E-mail address*: hhwoo@u.arizona.edu (H.H. Woo).

ABSTRACT

In ovarian and breast cancers, the actions of the cytokine CSF-1 lead to poor prognosis. CSF-1 expression can be regulated post-transcriptionally. RNA methylation is another layer of posttranscriptional regulation. The methylation of N^1 atom of adenine (m^1A) results in a conformational change of RNA which regulates translational efficiency. Our study indicates that the m^1A is also involved in the CSF-1 mRNA decay. The alteration of ALKBH3 expression, an m^1A demethylase, regulates the CSF-1 mRNA stability. Demethylation of m^1A by ALKBH3 increases the half-life of CSF-1 mRNA without affecting the translation efficiency. The m^1A in CSF-1 mRNA is mapped in the 5'UTR near the translation initiation site. YTHDF2, a known m^6A reader which interacts with the CCR4-NOT deadenylation complex, is not the reader of m^1A-containing CSF-1 mRNA. Overexpression of ALKBH3 increases CSF-1 expression and the degree of cancer cell invasiveness without affecting cell proliferation or migration. Collectively, we showed that CSF-1 mRNA decay can be regulated at an epigenetic level, and that alteration of the N^1-methylation status leads to phenotypic changes in cancer cell behavior.

Keywords:

ALKBH3;

m^1A demethylation;

CSF-1 mRNA;

RNA stability;

Ovarian cancer;

Breast cancer

1. Introduction

For posttranscriptional regulation of mRNA expression, several factors are involved including the RNA conformation determined by RNA-protein and RNA-RNA interactions. Recent study indicates that the RNA chemical modification is also involved in determining the translation efficiency and RNA decay [1]. More than 170 chemical modifications of RNA are identified [2,3,4]. Among them, N^6 methylation of adenine (m^6A) is the most studied. The m^6A is found mostly in the coding region and the 3'UTR of mRNA [5,6]. On a global level, 0.2-0.5% of adenine is m^6A [7]. In contrast, N^1 methylation of adenine (m^1A) is less abundant, *i.e.,* on a global level, 0.02% of adenine is m^1A. The m^1A is found mostly in the GC-rich sequence with highly structured 5'UTR near the translation initiation site (TIS) of mRNA [8,9]. On average, only one m^1A is found per mRNA, compared with 3-5 m^6As per mRNA [6].

Adenine methyltransferases (*i.e.,* writer) methylate mRNA, while adenine demethylase (*i.e.,* eraser) demethylates it [10,11]. For N^6 methylation of adenine, METTL3, METTL14, and WTAP are writers and form a methylation complex [12]. In contrast, a writer for N^1 methylation of adenine is not yet identified [13]. FTO and ALKBH5 are erasers and demethylate m^6A.

For m^1A demethylation, ALKBH3 is an eraser [9,14,15]. ALKBH3 is an alkylation damage repair enzyme (*i.e.,* dealkylase), which demethylates 1-methyladenine (m^1A) and 3-methylcytosine (m^3C) in RNA and single-stranded DNA [14,15]. Presence of m^1A in a highly structured 5'UTR near the TIS of mRNA correlates with increased translation [8,9,16]. The positive charge introduced by m^1A may alter secondary structure in the region, resulting in the increased accessibility of the translation machinery. In contrast, there is no report yet about the involvement of m^1A in determining the RNA stability. However, the fact that ALKBH3 can

demethylate m^1A in RNA may protect RNA from alkylation damage [17,18], otherwise m^1A RNA may go to the decay pathway.

The m^6A in RNA is involved in translation efficiency [19] as well as RNA stability [20,21]. YTHDF1, an m^6A reader, interacts with translation machinery and enhances translation efficiency [22]. Meanwhile, another m^6A reader, YTHDF2 induces the degradation of m^6A-containing RNAs by recruiting the CCR4-NOT deadenylation complex [23]. YTHDC1, a nuclear m^6A reader, regulates mRNA splicing through recruiting pre-mRNA splicing factor SRSF3 [24]. HNRNPA2B1 is another nuclear m^6A reader and mediates alternative splicing [25].

The cytokine macrophage colony-stimulating factor, CSF-1, activates the CSF-1R (receptor tyrosine kinase encoded by proto-oncogene *c-fms*), thereby promoting metastatic spread of several cancers. In epithelial ovarian cancer, overexpression of CSF-1/CSF-1R is associated with poor prognosis and enhances invasive and metastatic behavior [26,27,46]. In breast cancer, both CSF-1R [28] and CSF-1 [29] are associated with poor overall survival. CSF-1 can be regulated at a post-transcriptional level [30], however epigenetic mechanisms in its regulation have not been previously explored. There is need for an improved understanding of how CSF-1 could be regulated in cancer.

CSF-1 is encoded by a single gene located in chromosome 5. CSF-1 transcript precursor is alternatively spliced to generate four major mRNA variants and proteins [27,31]. Among them, the variant-1 and -4 are the most abundant mRNAs in breast and ovarian cancer cells. The only difference between the variant-1 and -4 transcripts is in the 3'UTR, *i.e.,* the 3'UTR of variant-1 is derived from exon-10, while the 3'UTR of variant-4 is derived from exon-9. However, the variant-1 and -4 of CSF-1 mRNAs encode a same ~61 kDa protein. This monomer is glycosylated and forms heterodimer which is the major form of secreted CSF-1 in ovarian cancer.

Since the 5'UTR of CSF-1 mRNA is GC-rich and the m^1A is found mostly in the GC-rich sequence, we studied whether the adenine in this region is N^1-methylated. We hypothesize that CSF-1 mRNA decay can be regulated at an epigenetic level, and that alteration of the N^1-methylation status would lead to phenotypic changes in cancer cell behavior.

In this study, we mapped an m^1A in the 5'UTR near the TIS of CSF-1 mRNA. Demethylation of the m^1A by ALKBH3 stabilizes the CSF-1 mRNA and increases the CSF-1 protein level in breast and ovarian cancer cells. This is the first report of involvement of m^1A in determining the mRNA stability. We also found an inverse correlation between the presence of m^1A and CSF-1 expression in breast and ovarian cancer cells. We show that overexpression of ALKBH3 increases the CSF-1 expression and the degree of invasion *in vitro* without affecting cell proliferation or migration.

2. Materials and Methods

2.1.Cell Culture

Hey ovarian cancer cells were cultured in DMEM/F12 (Mediatech) supplemented with 10% fetal bovine serum. BT20 breast cancer cells were cultured in MEM supplemented with 10% fetal bovine serum.

2.2. qRT-PCR

First cDNA was synthesized by pdN_6. For quantification of CSF-1 mRNA variants, following primers were used for qPCR.

Primer-1F, CTG GGC TCC CTG CTG TTG TTG GTC TG

Primer-1R; TTT GGC ACG AGG TCT CCA TCT GAC TGT C

Primer-2F; GGG CCA ACA GCC GGC AGA TGT AAC TGG

Priemr-2R; TAG AAC AAG AGG CCT CCG ACG GCC AGC

Primer-3F; ACA GCT TTG CTG AAT GCT CCA GCC AAG G

Primer-3R; ACC TGT AGA ACA AGA GGC CTC CGA CGG

Primer-4F; CAT CAC CTG AAC CTA CTG AAG TTG TGT G

Primer-4R; CAG GGG TGA GGA ACA ATA GGG TCA CAG C

2.3.Immunoblot

For CSF-1 detection, CSF-1 monoclonal antibody (Santa Cruz, sc-365779) was used. For ALKBH3 detection, ALKBH3 monoclonal antibody (Santa Cruz, sc-376520) was used.

2.4.ALKBH3 overexpression and silencing

To alter the ALKBH3 expression, either overexpression or silencing by shRNA was applied in breast and ovarian cancer cells. ALKBH3 (Origene, RC212873) was overexpressed. For silencing of ALKBH3, shRNA (Origene, TR306757) was used.

2.5. RNA half-life study

To determine CSF-1 mRNA half-life in BT20 breast cancer cells, actinomycin-D (Act-D) chase experiments were performed with 5 μg/ml of Act D (Sigma) added to inhibit new transcription. Cells were harvested at 0 h, 0.5 h, 1 h, 2 h, 4 h, and 6 h after Act D treatment, total cellular RNA extracted using Trizol (Invitrogen), and was fractionated in 1% agarose gel for northern blot analysis. ^{32}P-labeled CSF-1 antisense riboprobe was used for northern hybridization.

CSF-1 mRNA half-lives were also calculated after qRT-PCR, normalized to GAPDH mRNA, values were plotted, and the time period required for a given transcript to decrease to one-half of the initial abundance was calculated. GAPDH mRNA is not affected by ALKBH3, and has a long half-life (>18 hrs). Three independent experiments were performed.

2.6. Polysome profile

Polyribosome profiling was described previously [32]. BT20 or Hey cells were grown ~80% confluence in five 10 cm plates. After cycloheximide treatment for 15 min, cells were harvested, lysed, and fractionated by sucrose density gradient ultracentrifugation. RNA in each fraction was isolated by Trizol. The RNA was primed by pdN_6 and quantified by qRT-PCR to get the amounts of mRNAs associated with polyribosomes. For analysis of the relative distributions of CSF-1 and GAPDH mRNAs in polyribosome gradients, C_t values from individual fractions 1-11 were each subtracted from the C_t value from fraction 1 for CSF-1 and GAPDH mRNAs, as fraction 1 had the largest C_t values (that is, the lowest CSF-1 and GAPDH mRNA abundances). The resulting ΔC_t numbers were converted into fold differences. The abundance of each mRNA as a percentage of the total from all 11 fractions was then calculated. GAPDH mRNA was used as a control for estimation of relative distribution of CSF-1 mRNA in polyribosome gradients. N=3.

2.7. Purification of CSF-1 mRNA

CSF-1 mRNA was purified from total RNA by 5'-Biotin-Linked CSF-1 specific antisense oligos and Dynabeads MyOne Streptavidin C1.

Following 5'-Biotin-Linked CSF-1 antisense RNAs were used.

Primer 1; 5'Biotin-CTC TCA TTC AGT CAA GGG TCT GCG GGT TGA

Primer 2; 5'Biotin-ACC TTT ACG GGA AAT CAG GCC GAC GCT GTC

Primer 3; 5'Biotin-ATG CAG AAA CCT GGG CGA ACA ACA CAG CCT

2.8. Methylated RNA immunoprecipitation sequencing (MeRIP-seq)

m^1A sequencing was followed as previously described [33,34] with modifications. Purified CSF-1 mRNA was immunoprecipitated by anti-1-methyladenosine mAb (MBL, D345-3) and then fractionated to 100-150 nt in size using RNA fragmentation module (NEB). After dephosphorylation, 3'-adapter was ligated by T4 RNA ligase 2 (NEB). CSF-1 mRNA was reverse transcribed by M-MuLV reverse transcriptase (RNase H^-) (Promega). RNA was removed by alkaline hydrolysis and the cDNA was purified using MinElute PCR purification kit (Qiagen) before sequencing.

2.9. RNA Primer Extension

The primer extension was performed as previously reported [34] with modifications. To detect m^1A^{-29}, purified CSF-1 mRNA was mixed with 10 ug 5′-Biotin labeled primer (m^1A^{-29}-primer: 5'Biotin-ACG GGC AGC TGG GTC CCG GCC GGG C) in a 10 μL solution. The RNA mixture was then heated at 80°C for 5 min and slowly cooled to 25°C for 1 h. The RT mixture (containing M-MuLV reverse transcriptase [RNase H^-] and 0.8 μL 1 mM dATP, dTTP, dCTP, and dGTP mix in 15 μl final volume) was then added into the RNA mixture. The reverse transcription was carried out at 52°C for 1 h and then the RNA template was digested with 2 M NaOH. The reverse transcribed products were separated by 20% Urea-PAGE and then transferred to Nylon membrane, detected by Streptavidin-HRP antibody. For primer extension of A^{-15} and A^{-11}, 5′-Biotin labeled primers were used (A^{-15}-primer: 5'Biotin-CCC GGC GCG GTC ATA CGG GCA GCT, A^{-11}-primer: 5'Biotin-GGC GCC CGG CGC GGT CAT ACG GGC).

2.10. IP-qRT-PCR

Immunoprecipitation (IP) of the endogenous mRNP complex was done by the protocol described previously with modifications [32]. After formaldehyde crosslink, cytoplasmic proteins were extracted using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce). For YTHDF2 IP, 5 μg rabbit polyclonal anti-human YTHDF2 antibody (Proteintech 24744-1-AP) was used. A reaction containing normal rabbit IgG (Sigma) served as a negative control. For m^6A IP, 5 μg mouse monoclonal m^6A antibody (Synaptic Systems 202 111) was used. A reaction containing normal mouse IgG (Sigma) served as a negative control.

2.11. Cell Proliferation, Invasion, and Migration Assay

The cell proliferation assay was done by WST-1 assay reagent (Cell Biolabs). Cells, $1x10^3$ per well in a 96-well plate, were seeded. Cells were stained by WST-1 for every 24 h up to 148 h.

The Membrane Migration and Invasion Culture System (Cell Biolabs) was used to quantitate the degree of migration and invasion of BT20 and Hey cells transfected ALKBH3 overexpressing or silencing by shRNA. Assay details were followed by company protocols. For invasion assay, cells, $3x10^5$ per well in a 24-well plate, were seeded onto 8-μm pore filters coated with a human defined basement membrane matrix and incubated for 48 h. Similarly, directed motility assay of BT20 and Hey cells was performed. Fibronectin (25 μg/ml, Fisher) was added as a chemoattractant in the lower wells of the chamber, above which uncoated 8-μm pore filters were placed. Cells, $3x10^4$ per well in a 24-well plate, were seeded onto uncoated 8-

μm pore filters and incubated for 20 h. The results were reported as mean percent invasion ± SD or migration ± SD. Each experiment was done with triplicates.

2.12. Statistical Analyses

Data are depicted as mean±SD from at least three independent experiments. Exact *n* values are provided in the figure legends. The unpaired two-way *t*-test, nonlinear regression analysis, and one way ANOVA were performed using SigmaStat (Jandel Scientific Corp.). $P<0.05$ was considered statistically significant.

3. Results

3.1. *ALKBH3 increases the expression of CSF-1 mRNAs and protein*

CSF-1 has the four major alternatively spliced transcripts (Fig. 1A). Among them, the variants-1 and -4 (var-1 and -4) encode a same ~61 kDa protein. Together the var-1 and var-4 transcripts compose >96% of CSF-1 transcripts in BT20 breast cancer cells (Fig. 1B) and Hey ovarian cancer cells (Fig. 1C). Since the var-1 and -4 are the major transcripts, this report is focused on these two transcripts.

The 5'UTRs near the TIS (-130 ~ +1) of CSF-1 var-1 and -4 transcripts are GC-rich (81%). Since ALKBH3 demethylates m^1A in mRNA [37,38] and the m^1A is found mostly in the GC-rich sequence near the TIS of mRNA [7,8], we determined to study the influence of ALKBH3 on CSF-1 mRNA expression. In order to determine the relative influence of ALKBH3 on the expression of CSF-1, ALKBH3 was overexpressed (OE) or silenced by shRNA in BT20 breast and Hey ovarian cancer cells. We determined the effects of altering the level of ALKBH3 on the level of CSF-1 mRNA and protein in BT20 (Fig. 1D-I) and Hey cells (Fig. 1J-O). The overexpression (OE) of ALKBH3 increases the level of CSF-1 var-1 mRNA by 3-fold (Fig. 1D) and var-4 by 4.5-fold in BT20 cells (Fig. 1E). At the same time, the CSF-1 protein level increased by 7-fold compared to empty vector-transfected BT20 cells (empty) (Fig. 1H). We confirmed the increased ALKBH3 protein level by ALKBH3 overexpression (Fig. 1H). Similar effect was observed in Hey cells (Fig. 1J-O). The overexpression (OE) of ALKBH3 increases the CSF-1 var-1 mRNA level by 11.9-fold (Fig. 1J) and var-4 by 10-fold (Fig. 1K) compared to the empty vector-transfected Hey cells (empty). By immunoblot, we showed that the overexpression

of ALKBH3 increased the level of CSF-1 protein by 3.65-fold compared to the empty vector transfected Hey cells (Fig. 1N).

We also silenced the ALKBH3 expression with shRNA in BT20 cells (Fig. 1F,G,I). Silencing of ALKBH3 decreases the CSF-1 var-1 mRNA level by 1.8-fold (Fig. 1F) and var-4 by 1.8-fold (Fig. 1G) compared to the scrambled shRNA transfected BT20 cells. By immunoblot, we showed that silencing of ALKBH3 with shRNA decreased the level of CSF-1 protein by 1.9-fold compared to the scrambled shRNA transfected BT20 cells (Fig. 1I). As expected, we confirmed that ALKBH3 protein level was decreased in response to ALKBH3 silencing (Fig. 1I). Similar effect was observed in Hey cells (Fig. 1L,M,O). Silencing of ALKBH3 decreases the CSF-1 var-1 mRNA level by 14.3-fold (Fig. 1L) and var-4 by 21.5-fold (Fig. 1M) compared to the scrambled shRNA transfected Hey cells. By immunoblot, we showed that silencing of ALKBH3 decreased the level of CSF-1 protein by 1.9-fold compared to the scrambled shRNA transfected Hey cells (Fig. 1O).

Similar effects were also observed in MDA-MB-231 breast cancer cells and SKOV3 ovarian cancer cells (Supplementary data Fig. S1).

Taken together, these results indicate that ALKBH3 regulates CSF-1 at both the mRNA and protein levels, in both breast and ovarian cancer cells.

3.2. ALKBH3 increases the half-life of CSF-1 mRNAs

We next studied whether ALKBH3 stabilizes the CSF-1 mRNAs. Compared to the cells transfected with the empty vector, BT20 and Hey cells overexpressing (OE) ALKBH3 showed

an increase in the steady-state levels of CSF-1 mRNAs, and cells in which ALKBH3 were silenced with shRNA showed a decrease in the steady-state levels of CSF-1 mRNAs (Fig. 1).

CSF-1 mRNA expression levels were determined by northern analysis (Fig. 2A and B) and qRT-PCR (Fig. 2E and F). GAPDH mRNA, which has long half-life (>20 h) and is not influenced by ALKBH3 (Fig. 2C), was used as an internal loading control. We studied the effects of altering ALKBH3 levels on CSF-1 mRNA half-life in BT20 cells. As shown in Fig. 2, at least part of the 3-fold increase in the steady-state level of CSF-1 mRNA var-1 in BT20 cells seen on overexpression (OE) of ALKBH3 (Fig. 1D) is due to an increase in the stability of CSF-1 mRNA var-1 (Fig. 2A,E,G). When the empty vector treatment groups in BT20 cells were compared with ALKBH3 overexpressed groups (OE), the half-life of CSF-1 mRNA var-1 increased from 4 h to >6 h (two-tailed t-test, n=3, $P<0.001$). These results underscore the stabilizing influence of ALKBH3 on CSF-1 mRNA var-1. Similar effect was also observed in the CSF-1 mRNA var-4 (Fig. 2B,F,H). When the empty vector treatment groups in BT20 cells were compared with ALKBH3 overexpressed groups (OE), the half-life of CSF-1 mRNA var-4 increased from 4 h to >6 h (two-tailed t-test, n=3, $P<0.001$).

To study the effect of ALKBH3 down-regulation on the expression of CSF-1 mRNAs, we used shRNA that targeted ALKBH3. Reducing the level of ALKBH3 with shRNA decreased the steady-state levels of CSF-1 mRNAs seen in BT20 cells (Fig. 1F and G). Here we find that the stability of the CSF-1 transcript was also influenced by ALKBH3 abundance: silencing of ALKBH3 by shRNA decreased the half-life of CSF-1 mRNA var-1 from 4 h to 0.7 h (two-tailed t-test, n=3, $P=0.001$) (Fig. 2A,E,G; shRNA). This finding of change of CSF-1 mRNA stability by ALKBH3 contributes but does not entirely explain the magnitude of ALKBH3's post-transcriptional regulation of CSF-1 in BT20 cells. Similar effect was also observed in CSF-1

mRNA var-4 (Fig. 2B,F,H). When the empty vector treatment groups in BT20 cells were compared with ALKBH3 down-regulated groups (shRNA), the half-life of CSF-1 mRNA var-4 decreased from 4 h to 0.9 h (two-tailed t-test, n=3, $P<0.001$).

Taken together our data indicates that ALKBH3 stabilizes CSF-1 mRNAs var-1 and var-4.

3.3. ALKBH3 has little effects on CSF-1 mRNA translation

Since alteration of ALKBH3 expression also has effects on the CSF-1 protein level, we tested whether ALKBH3 affects translation of CSF-1 mRNAs. Polysome profiles of cytoplasmic lysates from BT20 and Hey cells were generated by sucrose density gradient centrifugation (Fig. 3). The polysome profiles were similar between ALKBH3-OE, ALKBH3-shRNA, empty vector, and scrambled shRNA cells (Fig. 3G).

In BT20 cells transfected with the empty vector, 44.4% of CSF-1 mRNA var-1 and 48.3% of var-4 cosedimented with polysomes (Fig. 3A and B, fractions 6-11). Upon overexpression (OE) of ALKBH3, 42.4% of CSF-1 mRNA var-1 and 48% of var-4 cosedimented with polysomes (Fig. 3A and B). In BT20 cells transfected with the scrambled shRNA, 41.7% of CSF-1 mRNA var-1 and 47.4% of var-4 cosedimented with polysomes (Fig. 3C and D). Upon silencing (shRNA) of ALKBH3, 44% of CSF-1 mRNA var-1 and 53.6% of var-4 cosedimented with polysomes (Fig. 3C and D). ALKBH3 overexpression or silencing had little effect upon the distribution of CSF-1 mRNAs. The distribution of GAPDH mRNA, which served as controls, was not changed (Fig. 3E and F). Similar effects were also observed in Hey cells (Supplementary data Fig. S2).

Taken together, these results indicate that ALKBH3 has little effect on the translation efficiency of CSF-1 mRNA, but primarily regulates CSF-1 mRNA stability.

3.4.Assessment of ALKBH3 mutant phenotype

Using BT20 breast cancer cells and Hey ovarian cancer cells, we performed *in vitro* loss-of-function analyses by silencing ALKBH3 (shRNA) and gain-of-function analyses by over-expressing (OE) ALKBH3. Alteration of ALKBH3 expression did not have significant effect on cell proliferation *in vitro* in BT20 (Fig. 4A and B) and Hey cells (Fig. 4C and D) (for overexpression; nonlinear regression, P=0.5885 for BT20, P=0.9752 for Hey) (for silencing; nonlinear regression, P=0.5462 for BT20, P=0.6501 for Hey).

Effects of alteration of ALKBH3 expression on directed motility of BT20 and Hey cells was studied (Fig. 4E and G) using fibronectin as a chemoattractant. Both overexpression (OE) and silencing (shRNA) of ALKBH3 did not have significant effect on the degree of motility, compared to control empty vector and scrambled shRNA cells (One way ANOVA, BT20, n=3, P=0.143 for overexpression, P=0.059 for silencing; Fig. 4E) (One way ANOVA, Hey, n=3, P=0.103 for overexpression, P=0.234 for silencing; Fig. 4G).

In vitro invasion of these cells through a human extracellular matrix was also studied using a transwell assay. Overexpression (OE) of ALKBH3 increased the degree of invasion of BT20 and Hey cells by 1.4-fold and 1.6-fold, respectively, compared to control empty vector cells (One way ANOVA, BT20, n=3, P=0.002; Fig. 4F) (One way ANOVA, Hey, n=3, P=0.007; Fig. 4H). In contrast, silencing (shRNA) of ALKBH3 did not have significant effect on the degree of

invasion in both cell types compared to scrambled shRNA cells (One way ANOVA, BT20, n=3, P=0.627; Fig. 4F) (One way ANOVA, Hey, n=3, P=0.654; Fig. 4H).

Taken together, we conclude that overexpression of ALKBH3 increases the degree of invasion of BT20 and Hey cells without altering the cell proliferation or migration. A potential explanation for the lack of effect of ALKBH3 silencing on invasion may be the low level of endogenous ALKBH3 in these cell types (Fig. 1).

3.5.Mapping m^1A in the 5'UTR near the TIS of CSF-1 mRNA

Since ALKBH3 demethylates m^1A in RNAs, and m^1A is found mostly in the GC-rich sequence near the TIS [8,9], we mapped m^1A in the CSF-1 mRNA which is GC-rich in the 5'UTR. To map the m^1A in the CSF-1 mRNA, methylated RNA immunoprecipitation sequencing (MeRIP-seq) was adapted. First, CSF-1 mRNA is isolated from total RNA using the biotin-labeled antisense oligos and streptavidin resin (Fig. 5A). MeRIP-seq locates the m^1A in the 5'UTR (-38 ~ -1) close to the TIS (Fig. 5B). The GC content is unusually high in this region of CSF-1 mRNA (90% GC), which is a landmark for m^1A peak area [8]. There are 3 adenines (A^{-29}, A^{-15}, and A^{-11}) found in this region.

To further locate m^1A in the 5'UTR of CSF-1 mRNA, m^1A-induced reverse transcription (RT) arrest in the CSF-1 mRNA using the primer extension assay was performed. RT was arrested at m^1A^{-29}, but not at A^{-15} nor A^{-11} (Fig. 5C), indicating m^1A^{-29} is a true methylation residue.

Quantification of m^1A^{-29} in CSF-1 mRNA was done in ALKBH3 overexpressed (OE) and silenced (shRNA) BT20 cells. m^1A^{-29} CSF-1 mRNA was immunoprecipitated by m^1A

monoclonal antibody and qRT-PCR was performed. First, CSF-1 mRNA was immunoprecipitated by 20-fold more than the IgG control in BT20 cells (Fig. 5D). Next, we compared the degree of m^1A methylation in CSF-1 mRNA between the ALKBH3 overexpressed (OE) and silenced (shRNA) BT20 cells (Fig. 5E). There was 3-fold difference in m^1A level, *i.e.,* the ALKBH3 overexpressed BT20 cells has 3-fold less m^1A in CSF-1 mRNA than that in ALKBH3 silenced BT20 cells.

Taken together, our data indicates that the A^{-29} in CSF-1 mRNA is N^1-methylated. ALKBH3 demethylates the m^1A^{-29} in CSF-1 mRNA.

3.6.Inverse correlation between the m^1A level and CSF-1 expression in breast and ovarian cancer cells

To find out the associations between expression of ALKBH3 and CSF-1, and between the degree of methylation of the m^1A^{-29} of CSF-1 mRNA and the steady-state levels of CSF-1 mRNA and protein in breast and ovarian cancer cells, we compared the expression level of ALKBH3 and CSF-1 in MCF10A normal breast, BT20, MDA-MB-231 breast cancer cells, and Bix3, Hey, SKOV3 ovarian cancer cells (Fig. 6).

Expression of ALKBH3 and CSF-1 proteins are correlated in both breast and ovarian cancer cells, *i.e.,* expression of both proteins are low in MCF10A and Bix3 cells comparing to the BT20, MDA-MB-231, SKOV3, and Hey cells (Fig. 6A-D). The CSF-1 mRNA levels are also well correlated to the CSF-1 protein levels (Fig. 6E).

To check the degree of m^1A level in CSF-1 mRNA, purified CSF-1 mRNA is immunoprecipitated by m^1A monoclonal antibody and then qRT-PCR is performed. The % of m^1A CSF-1 mRNA is calculated by dividing the total input CSF-1 mRNA. The degree of m^1A

level is inversely correlated to the CSF-1 mRNA and protein levels (Fig. 6F), *i.e.,* the level of m^1A in CSF-1 mRNA is higher in MCF10A and Bix3 cells comparing to the other breast and ovarian cancer cells.

We conclude that the presence of m^1A decreases the steady-state level of CSF-1 mRNAs and the degree of m^1A demethylation is correlated with ALKBH3 expression in breast and ovarian cancer cells.

3.7. YTHDF2 is not the reader of m^1A in CSF-1 mRNA

YTHDF2 is a reader of m^6A and destabilizes m^6A-containing RNA by recruiting the CCR4-NOT deadenylation complex [23]. Since the m^1A destabilizes CSF-1 mRNA (Fig. 1 and 2), we determined whether YTHDF2 is a reader of m^1A in CSF-1 mRNA.

ALKBH3-silenced BT20 cells, which have a higher degree of m^1A in CSF-1 mRNA (Fig. 5E), was immununoprecipitated with YTHDF2 (Fig. 7A) and qRT-PCR was performed. CSF-1 mRNA was not co-immunoprecipitated with YTHDF2 (Fig. 7B,C); *i.e.,* no difference in CSF-1 mRNA var-1 (Fig. 7B) and var-4 (Fig. 7C) levels were observed between YTHDF2-IP and IgG-IP.

Next, we checked the m^6A status in CSF-1 mRNA in BT20 cells. The purified CSF-1 mRNA was not immunoprecipitated with the m^6A antibody (Fig. 7D for var-1, and 7E for var-4).

Taken together, we conclude that YTHDF2 is not the reader of m^1A nor does CSF-1 mRNA have measureable m^6A.

4. Discussion

Recent advances reveal the involvement of RNA epitranscriptome in posttranscriptional regulation [1,35,36]. The N^6-methylation of adenine (m^6A) in mRNA regulates the translation efficiency as well as the RNA stability [5]. Similarly, the N^1-methylation of adenine (m^1A) was reported to enhance the translation efficiency [8,9,16]. For the first time, we report that the m^1A is also involved in determining the mRNA stability. Our results demonstrate that ALKBH3, an m^1A demethylase, stabilizes and increases the half-life of CSF-1 mRNA. Furthermore, ALKBH3-induced m^1A demethylation does not influence the translation efficiency of CSF-1 mRNA, indicating the primary effect of m^1A in CSF-1 mRNA stability in breast and ovarian cancer cells. The m^1A is mapped in the GC-rich sequence near the TIS of CSF-1 mRNA, which is in agreement with the other m^1A containing mRNAs [8,9]. Since ALKBH3 is dealkylase [14,15], which demethylates m^1A and m^3C in RNA and single-stranded DNA, other factors may also contribute to the CSF-1 mRNA decay.

YTHDF2 is a reader of m^6A and recruits the CCR4-NOT deadenylation complex to destabilize the m^6A-containing RNA [23]. However, YTHDF2 does not interact with the m^1A-containing CSF-1 mRNA (Fig. 7), indicating that YTHDF2 is not a reader of m^1A. In addition, CSF-1 mRNA does not contain the m^6A in breast cancer cells (Fig. 7). Our results indicate that the destabilization of m^1A-containing CSF-1 mRNA is mediated by unknown reader(s). Since m^1A is located in the 5'UTR of CSF-1 mRNA, the unknown reader may recruit the 5' to 3' exonucleases [37,38,39].

ALKBH3 RNA expression is observed in a variety of cancers, including breast and ovarian cancers [40]. Overexpression of ALKBH3 is also seen in renal cell carcinoma [41], pancreatic cancer [42], non-small-cell lung cancer [43], and prostate cancer [44]. The silencing of ALKBH3

expression results in apoptosis, suppression of cell proliferation, and inhibition of angiogenesis of pancreatic cancer cells [42]. Furthermore, ALKBH3 knockdown induces cell cycle arrest or apoptosis depending on the TP53 gene status in NSCLC cells [45]. In contrast, the overexpression of ALKBH3 increased anchorage-independent growth and invasiveness of pancreatic cancer cells.

Our data also showed similar effects in invasiveness, *i.e.,* overexpression of ALKBH3 increases the degree of invasion of BT20 and Hey cells (Fig. 4F, H). In contrast, there were no significant effects on cell proliferation or migration (Fig. 4). This discrepancy with previous reports may be caused by different cell types, *i.e.,* relatively low expression of endogenous ALKBH3 in BT20 and Hey cells (Fig. 1), and incomplete silencing by shRNA. Indeed, CSF-1 variant-1 and -4 transcripts are modulated by ALKBH3 overexpression and silencing at a much stronger extent in Hey cells when compared with BT20 cells (Fig. 1). The reason for this differential effect may be related to different cellular metabolism and gene expressions.

The ALKBH3 expression is strongly correlated with that of CSF-1 in breast and ovarian cancer cells, *i.e.,* the expression of ALKBH3 and CSF-1 is lower in MCF10A normal breast cells and higher in breast and ovarian cancer cells (Fig. 6). Conversely, m^1A in CSF-1 mRNA is higher in MCF10A normal breast cells and lower in breast and ovarian cancer cells (Fig. 6). This result indicates that the m^1A level in CSF-1 mRNA is inversely correlated with the breast and more invasive ovarian cancer cell phenotypes [26].

In conclusion, the m^1A is another layer of posttranscriptional regulation of CSF-1 mRNA, in which the m^1A is involved in destabilization of CSF-1 mRNA. Demethylation of m^1A by ALKBH3 increases the CSF-1 expression in breast and ovarian cancer cells. We showed that CSF-1 mRNA decay can be regulated at an epigenetic level, and that alteration of the N^1-

methylation status leads to phenotypic changes in cancer cell behavior. For future study, m^1A readers, those which destabilize CSF-1 mRNA and others which alter the translation efficiency, need to be identified.

Competing interests

The authors declare that they have no competing interests.

Authors' Contribution

H.H.W. conceived the study, designed and performed the experiments, interpreted the results and wrote the manuscript. S.K.C. interpreted the results and wrote the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found on line.

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Figure Legends

Fig. 1. (A) Four variants of CSF-1 mRNAs. Var-1 and -4 transcripts encode the same ~61 kDa protein. The only difference between the var-1 and -4 transcripts is in the 3'UTR, in which the 3'UTRs of var-1 transcript derived from exon-10 and of var-4 transcript derived from exon-9. The var-1 and -4 transcripts are major CSF-1 mRNAs in BT20 breast cancer cells (B) and Hey ovarian cancer cells (C). qRT-PCR shows ALKBH3 overexpression (OE) increases the steady state level of CSF-1 var-1 and -4 mRNAs (D, E), and ALKBH3 silencing (shRNA) decreases the steady state level of CSF-1 var-1 and var-4 mRNAs (F, G) in BT20 cells. Immunoblot shows ALKBH3 overexpression (OE) increases CSF-1 protein (H), and ALKBH3 silencing (shRNA) decreases CSF-1 protein (I) in BT20 cells. qRT-PCR shows ALKBH3 overexpression (OE) increases the steady state level of CSF-1 mRNA var-1 and -4 (J, K), and ALKBH3 silencing (shRNA) decreases the steady state level of CSF-1 mRNA var-1 and var-4 (L, M) in Hey cells. Immunoblot shows ALKBH3 overexpression (OE) increases CSF-1 protein (H), and ALKBH3 silencing (shRNA) decreases CSF-1 protein (I) in Hey cells. Data in qRT-PCR is an average of 3 sets of experiments. CSF-1 mRNA level is normalized by GAPDH mRNA. Immunoblot was repeated 7 times with same results.

Fig. 2. Northern blot shows ALKBH3 overexpression (OE) increases, and silencing (shRNA) decreases the half-life of CSF-1 mRNA var-1 (A) and var-4 (B) in BT20 cells. (C) Northern blot shows no influence of ALKBH3 on the half-life of GAPDH mRNA. (D) Ethidium bromide stained northern gel showing equal loading. Band intensity of CSF-1 mRNA var-1 (E) and var-4 (F) in northern blot was normalized by GAPDH mRNA. qRT-PCR confirms the longer half-life

(>6 h) of CSF-1 var-1 (G) and var-4 (H) in ALKBH3 overexpressed (OE), and shorter half-life (<1 h) in ALKBH3 silenced (shRNA) BT20 cells. CSF-1 mRNA level is normalized by GAPDH mRNA in qRT-PCR. Northern blot was repeated 3 times with same results. Data in qRT-PCR is an average of 3 sets of experiments.

Fig. 3. Polysome profile shows no distinct changes of CSF-1 mRNA var-1 (A, C) and var-4 (B, D) distribution in ALKBH3 overexpressed (OE) or silenced (shRNA) BT20 cells. (E, F) ALKBH3 overexpression or silencing does not influence the distribution of GAPDH mRNA in BT20 cells. (G) Polysome profiles of ALKBH3-OE, Empty vector, ALKBH3-shRNA, and Scrambled shRNA in BT20 cells. Data in polysome profile is an average of 3 sets of experiments.

Fig. 4. (A, B) BT20 cells and (C, D) Hey cells were seeded and allowed to grow up to 6 days. After adding the WST-1, cells were then incubated for 2 hours before measurements. (E, G) ALKBH3 has no effects on the degree of motility in BT20 and Hey cells. (F, H) ALKBH3 overexpression regulates *in vitro* invasiveness of BT20 and Hey cells. OE, ALKBH3 overexpression; shRNA, ALKBH3 shRNA. N=3 experiments. *n.s.* = not significant.

Fig. 5. (A) Diagram of methylated RNA immunoprecipitation sequencing (MeRIP-seq). CSF-1 mRNA is purified by biotin-labeled oligos and immunoprecipitated by m^1A monoclonal antibody. (B) MeRIP-seq indicates m^1A is located between -38 ~ -1 of CSF-1 mRNA. (C) RNA primer extension shows that the A^{-29} is m^1A methylated. (D) Purified CSF-1 mRNA from BT20 cells is immunoprecipitated by m^1A antibody. (E) Purified CSF-1 mRNA from ALKBH3 overexpressed (OE) BT20 cells is immunoprecipitated 3-fold less than that from

ALKBH3 silenced (shRNA) BT20 cells. Data in IP-qRT-PCR is an average of 3 sets of experiments. CSF-1 mRNA level is normalized by GAPDH mRNA.

Fig. 6. (A, B) Expression of both ALKBH3 and CSF-1 proteins are correlated in MCF10A normal breast, BT20, MDA-MB-231 breast cancer, and Bix3, SKOV3, Hey ovarian cancer cells. Band intensities of ALKBH3 (C) and CSF-1 proteins (D) are shown. (E) Expression of CSF-1 mRNA var-1 and -4 is correlated with CSF-1 protein levels in breast and ovarian cancer cells. (F) The m^1A in CSF-1 mRNA var-1 and -4 is inversely correlated with ALKBH3 and CSF-1 expression in breast and ovarian cancer cells. Data in qRT-PCR is an average of 3 sets of experiments.

Fig. 7. (A) Immunoprecipitation of YTHDF2 in BT20 (ALKBH3-shRNA) cells. CSF-1 mRNA var-1 (B) and var-4 (C) did not co-immunoprecipitate with YTHDF2. CSF-1 mRNA var-1 (D) and var-4 (E) were not immunoprecipitated by m^6A antibody in BT20 breast cancer cells. Data in IP-qRT-PCR is an average of 3 sets of experiments. CSF-1 mRNA level is normalized by GAPDH mRNA. N = 3 experiments. *n.s.* = not significant.