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ALK-mediated post-transcriptional regulation: focus on RNA-binding proteins

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TABLE OF CONTENTS

1. ABSTRACT

Extensive research has been carried out in the past two decades to provide insights into the molecular mechanisms by which the Nucleophosmin-Anaplastic Lymphoma Kinase (NPM-ALK) exerts its oncogenic effects. These studies led to the concept that NPM-ALK acts at the transcriptional level through the activation of several transcription factors downstream of many different signaling pathways including JAK3/STAT3, PI3K/AKT and RAS/ERK. Nevertheless, the discovery of several RNA-binding proteins (RBPs) within ALK interactome suggested an additional and complementary role of this oncogenic kinase at the post-transcriptional level. This review gives emerging views in ALK-mediated post-transcriptional regulation with a focus on RBPs that are associated with ALK. We will summarize the capacity of NPM-ALK in modulating the biological properties of RBPs and then discuss the role of cytoplasmic aggregates, called AGs for "ALK granules", which are observed in anaplastic large cell lymphoma (ALCL) expressing the ALK kinase. AGs contain polyadenylated mRNAs and numerous RBPs but are distinct from processing bodies (PBs) and stress granules (SGs), two well-known discrete cytoplasmic sites involved in mRNA fate.

2. INTRODUCTION

The control of mRNA abundance starts with transcription and is followed by multiple post-transcriptional processes that affect virtually all steps of mRNA life cycle ranging from pre-mRNA splicing to mRNA degradation (1). In cancer research, the role of post-transcriptional gene regulation has been gaining a lot of momentum over the last few years. Today, it has become clear that cancer development is dictated not only by aberrant transcriptional events and signaling pathways but also by altered post-transcriptional regulation of genes involved in the control of cell proliferation, differentiation, invasion, metastasis, apoptosis, and angiogenesis (2–4). Due to their key regulatory roles in RNA fate, RBPs have thus emerged as fundamental players in tumor development. The identification of several RBPs within ALK-interacting partners or ALK downstream targets opens new avenues to understand the role of ALK in controlling RNA metabolism

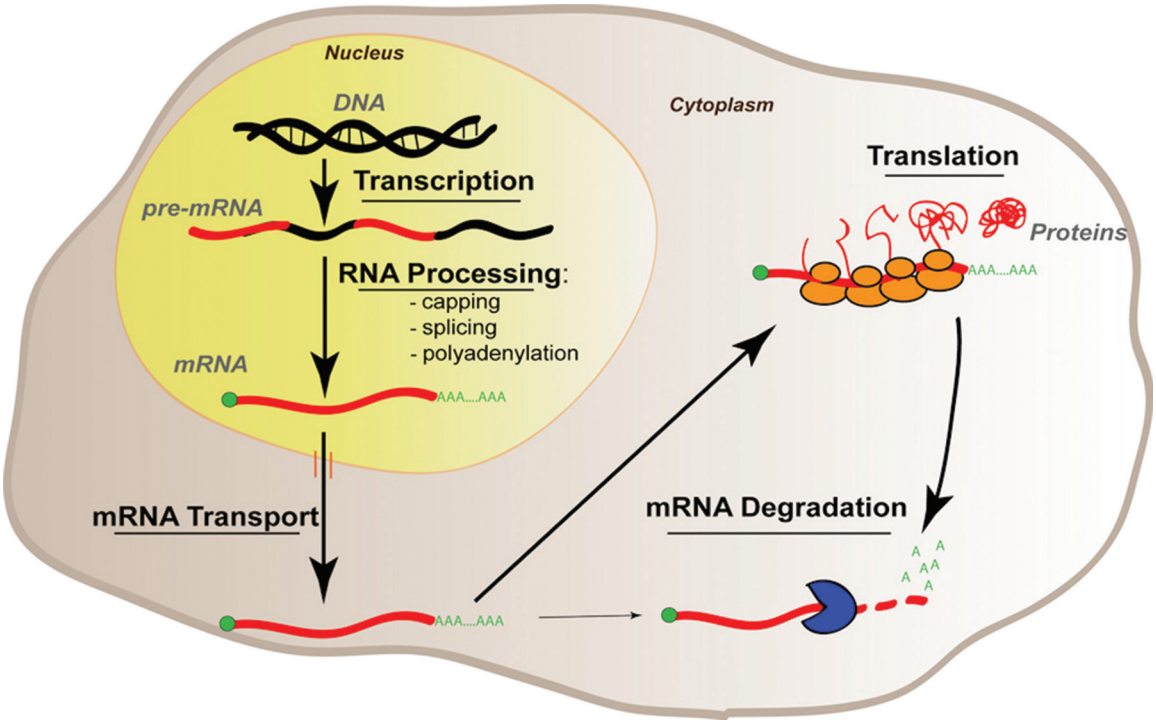

Figure 1. Schematic of mRNA fate from synthesis to degradation in eukaryotic cells. In eukaryotes, mRNAs are first synthesized in the nucleus as pre-mRNAs that are subjected to 5'-end capping, splicing and 3'-end polyadenylation. Once pre- mRNA processing is complete, mature mRNAs are primed for export, travel to the nuclear envelope and translocate into the cytoplasm where they serve as the blueprints for protein synthesis by ribosomes and then are degraded. Different sets of RBPs (not shown) are associated to the mRNA at each different time points and in different compartments, thereby regulating the fate of their target in a time- and space-dependent way.

and to consider RBPs as promising therapeutic targets in ALK-induced tumorigenesis.

3. RBPS AND THEIR ROLE IN POST-TRANSCRIPTIONAL REGULATION: A BRIEF OVERVIEW

mRNA is not naked in the cell: it is associated with a large variety of RBPs to form messenger ribonucleoprotein complexes (mRNPs). These dynamic particles influence every aspect in the processing of transcripts, including pre-mRNA splicing, polyadenylation, nuclear export, mRNA decay and translation (5, 6) (Figure 1).

3.1. Cis-regulatory elements

RBPs often interact with defined cis-regulatory elements, namely specific sequences or secondary structures typically found in the untranslated regions (UTRs) of target mRNAs (7, 8), organizing functionally related mRNAs into "regulons" (9). Their regulatory actions may be positive or negative, depending on the protein, the mRNA and the biological context. In the 5'-UTR, ribose methylation of the cap structure as well as 5' terminal polypyrimidine sequences or secondary structures such as internal ribosome entry sites (IRESs) control protein expression (10). In the 3'-UTR, sequence elements such as AU- rich elements (AREs) represent the most common cis-acting destabilizing elements identified so far in mammalian cells that can modulate the stability of mRNAs, their translational efficiency and localization (11-13). ARE-containing mRNAs (ARE- mRNAs) have been shown to represent 5-8% of cellular mRNAs and are mostly transcribed from early response genes encoding proto-oncogenes (such as c-myc), cyclins (such as cyclin D1, A2 and B1), growth factors (such as VEGF) or cytokines (such as TNF-alpha) (14, 15). AREs are recognized by specific ARE-binding proteins (ARE-BPs), including BRF1, TTP, KSRP and AUF1/hnRNPD (see below), that bind directly or indirectly to the cis-acting elements and promote the deadenylation and degradation of the mRNA (16). Only few ARE-BPs protect their target ARE-mRNAs from degradation, possibly by antagonizing the recruitment of competing mRNA-destabilizing factors: Hu antigen R (HuR) (see below) is a representative example of such proteins (17).

3.2. RBPs and microRNAs interplays

Evidence accumulated over the past ten years has revealed that RBPs share functional interactions with microRNAs (miRNAs), another fundamental class of regulatory molecules that act post-transcriptionally,

uncovering a new level of complexity of gene expression regulation, particularly in cancer cells (for recent review, see(18)). RBPs can indeed regulate the expression of specific miRNAs (19) or exert a widespread effect on miRNAs via the modulation of a key protein involved in miRNA production or function (20, 21). Functionally, RBPs can either act in synergy with miRNAs to achieve mRNA destabilization and/or translational inhibition (22, 23) or exert the opposing effects, thus inhibiting miRNAs action and resulting in the protection of specific target mRNAs (24, 25).

3.3. Regulation of RBPs expression and activity

RBPs are universal in living cells and their functions are largely dependent on their abundance and subcellular distribution (6, 26). Many RBPs are aberrantly expressed in cancer cells and have thus a cancer-specific regulatory activity (2, 27, 28). In this way, HuR has been reported to have an exacerbated activity in many epithelial cancers (such as colon, ovarian, breast cancers) due to either its overexpression, its cytoplasmic delocalization or an increase in its binding affinity toward its target RNAs (4, 29–31). Indeed, this nucleocytoplasmic protein is predominantly nuclear in normal cells, but abundantly expressed in the cytoplasm of malignant cells. Elevated cytoplasmic levels of HuR correlates with advanced clinico-pathological parameters, a criterion that serves as a prognostic factor of poor clinical outcome in some cancer types (32, 33).

RBPs are the targets of several signaling pathways that alter their activity under physiological conditions. For example, the DNA/RNA-binding protein PSF (detailed below) has been reported to be targeted by different serine, threonine or tyrosine kinases (such as PKC (34), GSK3 (35), MNKs (36) or BRK (37)), resulting especially in the modification of its transcriptional repressor activity or its ability to control nuclear mRNA processing (35). Also, HuR transport across the nuclear membrane and, hence, its ability to stabilize and/or modulate the translation of many of its target mRNAs is influenced by various kinases, including CDK1, CHK2, AMPK, p38, PKC alpha and PKC delta (38). Under pathological conditions, alterations of signaling pathways can modify the phosphorylation status of some RBPs, resulting in an alteration of their activity toward their target mRNAs. Such a hypothesis has been confirmed in ALK-expressing cells where the phosphorylation status of three RBPs, namely PSF, AUF1 and HuR has been shown to be altered, specifically on tyrosine residues.

4. ALK: A MODULATOR OF RBPS ACTIVITY

Given the emerging crucial role of RBPs in cancer, the potent and constitutive tyrosine kinase activity of ALK and the numerous signaling pathways it activates, a focus has recently been done on some RBPs in ALK-positive cancer cells, more particularly in ALCL. Indeed, thanks to the establishment of the “interactome” of ALK, defined by ALK immunoprecipitation assays followed by mass spectrometry analyses, two different groups identified six DNA/RNA-binding proteins: PSF, Nucleolin, FUS/TLS, AWS, p54nrb and AUF1, as new partners of ALK (39, 40). To date, among these RBPs, only two, PSF (39) and AUF1 (40), have been subjected to more detailed studies in ALK-positive ALCL. Recently, the impact of ALK on the behavior of another abundantly expressed RBP, HuR, has been more precisely defined (41).

4.1. PSF

PSF (polypyrimidine tract-binding protein (PTB)-associated splicing factor) is a nuclear protein associated with speckles and the nuclear membrane and matrix (42), which possesses a diverse set of functions thanks to its DNA- and RNA- binding properties (43). While PSF acts as a transcriptional repressor and is involved in the unwinding and annealing of chromatin during DNA replication or repair steps, it also plays a role in pre-mRNA splicing, nuclear retention of promiscuously edited RNAs and in cytoplasmic mRNA stability (42, 43). Using ALK immunoprecipitation assays performed in different human ALK-positive human ALCL cell lines and primary lymph node tissues, Galietta *et al.* have shown that PSF interacts with ALK (39). Their association requires an active ALK kinase domain and correlates with the phosphorylation of PSF on Y293. This tyrosine phosphorylation is proposed to favor the partial re-localization of PSF from the nucleus to the cytoplasm of ALK-positive cells, thus impairing its nuclear-associated functions. Moreover, overexpression of PSF specifically hampers cell growth, increases apoptosis and reduces the clonogenic potential of NPM- ALK-expressing cells, suggesting that PSF might play a role in NPM-ALK-mediated transformation through mechanisms that remain to be further investigated (39).

4.2. AUF1

While AUF1 is one of the best characterized ARE-BPs implicated in the decay of ARE-mRNAs, some of its four isoforms can also adopt a stabilizing activity depending on the cellular context (45). In ALK-expressing cells, we established that the functional switch of AUF1 from a destabilizing to a stabilizing protein was correlated to its phosphorylation on tyrosine residues (40). Indeed, ALK immunoprecipitation from human ALCL cell lines or murine ALK-transformed NIH3T3 cells led to the identification of AUF1 as a new partner of ALK and extensive 2D gel analysis revealed increased AUF1 phosphorylation on tyrosine residues (predominantly the p45 isoform) in NPM-ALK expressing cells (40). Interestingly, AUF1 and ALK partnership was confirmed independently by immunofluorescence analysis of ALK-transformed NIH3T3 or ALK-positive ALCL cells, showing the simultaneous presence of AUF1 and NPM-ALK in specific cytoplasmic foci (called AGs for ALK-granules,) that are not observed in cells expressing a kinase defective ALK form (40) (see

below, paragraph 5.2.). Finally, NPM-ALK-expressing cells (either murine or human) were less sensitive to transcription inhibition (Actinomycin D treatment) than control cells or cells that express the kinase defective NPM-ALK mutant. The extent of their survival was correlated with increased half-life of some AUF1 mRNA targets, such as different *cyclins* (A2, B1, D1, D3) and *c-myc* mRNA. In conclusion, we postulated that ALK-mediated modification of AUF1 activity or availability in specific cell compartments leads to a decrease in ARE-mRNAs turnover, which favors enhanced cell survival and proliferation and thus contributes to malignancy (40).

4.3. HuR

HuR is the only member of the Elav/Hu proteins family to be ubiquitously expressed (46). Besides its mRNA stabilizing role, HuR modulates the translation efficiency of some of its target mRNAs, although the precise mechanisms underlying both effects remain to be elucidated (47). HuR has a large range of ARE-mRNA targets such as *cyclin B1, cyclin D1, MCL-1, Bcl-2, VEGF, HIF-1 alpha, TNF alpha, Cox2, Snail or MMP9,* ranking it as a dainty regulator of proliferation, apoptosis, migration, inflammation and angiogenesis, which are key processes altered in oncogenesis (48). We have recently shown that ALK could modulate the activity of HuR in ALCL cell lines (41). Indeed, we performed co-immunoprecipitation assays and found that ALK and HuR are present within the same complex. We further confirmed this association by confocal analysis that showed colocalization of both proteins in AGs present in the cytoplasm of ALK-expressing cells (ALCL or NIH3T3) (see below, paragraph 5.2.) (41). We then observed that HuR was phosphorylated on tyrosine residues in ALK-positive ALCL. *In vitro* kinase assays finally showed that ALK could directly phosphorylate HuR on tyrosine residues. In parallel, our study revealed that NPM-ALK activity enhances the binding of HuR to the 3'UTR of *C/EBPbeta* ARE-containing mRNA. This mRNA encodes a transcription factor required to induce ALK-positive ALCL transformation by enhancing growth and survival (49). HuR-increased affinity toward *C/EBPbeta* mRNA leads to its enhanced stabilization and recruitment into actively translating polysomes, promoting accrued expression of this crucial transcription factor in ALK-positive cells (41).

Altogether, these data suggest that part of NPM-ALK oncogenic properties relies on its ability to modify PSF, HuR and AUF1 activities and hence to alter expression of key actors of transformation.

5. ALK- GRANULES: CHARACTERIZATION AND POSSIBLE FUNCTION

5.1. Stress granules (SGs) and Processing bodies (PBs)

Over the past decades, an increasing number of reports have revealed that a key aspect of gene regulation relies on cytoplasmic control of mRNA degradation and translation. In particular, intensive study of labile and highly mobile non - membranous cytoplasmic granules has pointed to cell's ability to form dynamic cytoplasmic domains by means of transient mRNPs aggregation (50, 51). According to their name, SGs appear under stress conditions and form microscopically visible cytoplasmic structures as a result of stress-induced translation arrest and polysomes disassembly (52). SGs contain mRNAs and many components of stalled 48S pre-initiation complexes (including the small, but not large, 40S ribosomal subunit and translation pre-initiation factors, such as eIF2 alpha and eIF4E). The dynamic nature of SGs suggests that they are sites of mRNA triage, wherein individual mRNAs are dynamically sorted for storage, degradation, or translation during stress and recovery (52). On the contrary, PBs are present in the cytoplasm of unstressed cells but they are further induced in response to stresses or other conditions that lead to the inhibition of translation (53). They concentrate many players of the translational repression and mRNA decay machinery (such as the 5' to 3' exonuclease XRN1 and the decapping enzyme complex Dcp1/Dcp2) as well as most actors of miRNA repression pathway (54). For these reasons, it is believed that PBs play a fundamental role in mRNA turnover. However, not all mRNAs which enter PBs are degraded, as it has been demonstrated that some mRNAs can exit PBs and re-initiate translation (55). Although SGs and PBs are structurally distinct, they may fuse under certain stimuli, leading to a potential exchange of their protein content and a re-assortment of their jailed mRNAs (50, 51).

5.2. ALK-granules (AGs): novel mRNP cytoplasmic structures

Analysis of HuR and AUF1 subcellular localization in NPM-ALK-expressing cells led to the discovery of new cytoplasmic structures called AGs, for ALK-granules (40, 41, 56, 57). AGs not only contain the ALK tyrosine kinase and the two ARE-BPs, HuR and AUF1, but also polyadenylated mRNAs and many other components of SGs and PBs with which they constantly and dynamically interact (56, 57). AGs formed independently of the cellular context, being observed in different human ALK-expressing ALCL cell lines as well as in NIH3T3 cells expressing either NPM-ALK or other ALK fusion proteins (56). Using kinase defective NPM-ALK-mutant and an ALK-specific inhibitor, we showed that an active phosphorylated form of NPM-ALK is required for AGs formation (40, 56). As shown by electron microscopy, AGs are round shaped and their size varies between 200 and 500 nm in diameter (57). Video-microscopy revealed that AGs move along the microtubules network with kinetics similar to PBs with which they frequently contact (56). However, in normal cell culture conditions, AGs do not contain PB components although some of PB markers (DCP1, EDC3, LSM1), but not the XRN1 exonuclease, re-localize to AGs upon inhibition of translation.

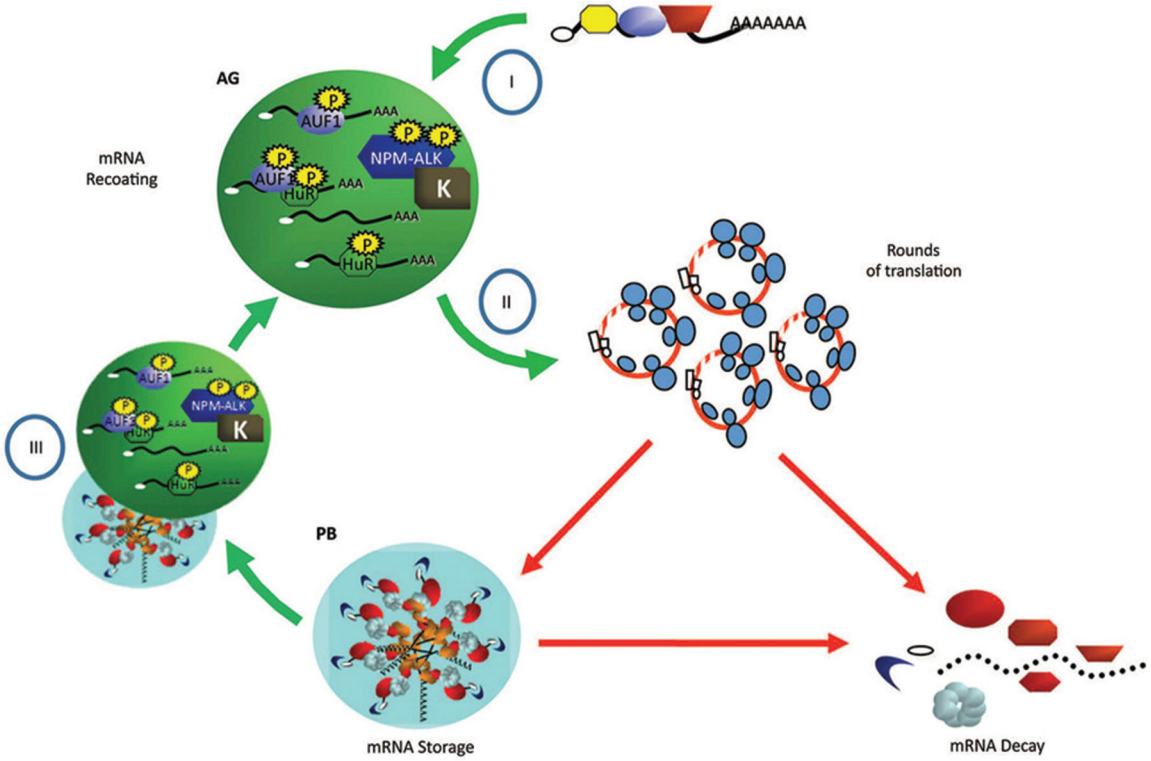

Figure 2. mRNA cycle within dynamic cytoplasmic structures in ALK-positive ALCL (from (57)). Instead of being degraded in the cytosol, some mRNAs either nascent or exiting polysomes can be, according to their protein coating, selectively captured by AGs (I). Thanks to NPM-ALK or its associated kinases (K), they are recoated and stored or directed to polysomes where they are actively translated (II). Upon completion of translation, they are either degraded in the cytosol or trapped in PBs where they can again be selectively captured by AGs during AGs-PBs contacts (III) and re-enter the cycle of translation.

5.3. A hypothesis for AGs function

Under stress conditions (such as heat shock or arsenite treatment), SGs are formed in ALK-positive cells, and in some cases, they are in close proximity with AGs (56). However, a significant decrease in the number and size of SGs were observed in NPM-ALK-expressing cells compared to control cells (56). Theoretically, if SGs were storage centers, their limited number in ALK-positive cells would have led to accelerated mRNA degradation and decreased translation, but that was not the case. Indeed, NPM-ALK expressing cells showed less inhibition of translation of a subset of mRNAs than control cells (57). Sucrose density fractionation and microarray analysis revealed that a majority of the AG-enriched transcripts were also enriched in the polysomal fractions, suggesting that they were actively translated. For instance, *C/EBPbeta* mRNA that is a well-known target of HuR (41), as well as many mRNAs encoding proteins implicated in cell proliferation and apoptosis, were found stabilized and actively translated in ALK-positive ALCL (57). Thus, mRNAs are not simply stored in AGs, but possibly recoated and sorted to be efficiently addressed to polysomes (Figure 2). In conclusion, we propose that AGs might correspond to SGs in non-stressed but transformed cells, being therefore able to de-sensitize the cells to external stimuli and stresses, promoting their survival and proliferation under conditions where normal cells undergo cell arrest and apoptosis (57). This hypothesis raises the possibility that AG-like structures might be present in cells transformed by other oncogenic kinases, contributing similarly to their malignancy.

6. CONCLUSIONS

While post-transcriptional regulation of gene expression is now considered as a mechanism of central importance in cancer research, it has been largely overlooked in the scope of ALK-expressing tumors. Data accumulated in the past recent years and summarized in this review have largely enriched and complexified our vision of the molecular mechanisms by which NPM-ALK modulate gene expression. New evidence has been indeed added through the characterization of three functionally distinct RBPs with NPM-ALK mediated altered activities (39–41) and the identification of AGs, which concentrate and protect specific mRNAs from decay (57). Our knowledge on this particular field of post-transcriptional processes will certainly increase with the careful analysis of additional data of NPM-ALK interactome and phosphoproteome that revealed several other RBPs

involved in RNA regulation and processing (58, 59), and additional in-depth studies of their potential interplay with miRNAs (see paragraph 3.2.).

Given the large implication of RBPs in networks of RNA-protein and protein-protein interactions that control RNA fate, it comes with no surprise that alterations of RBPs functions or expression impact many different genes and pathways leading to a broad spectrum of complex human disorders, including cancer. In particular, HuR has been shown to be overexpressed in most human solid tumors as well as in ALCL (41). Its function, and more particularly its ability to bind AREs, is also altered by post-translational modifications, directly executed by ALK or several other kinases (summarized in this review). Thus, the modulation of mRNA/RBPs interactions or RBPs post-translational modifications could open new avenues for potential universal therapies covering ALK-positive tumors and many other diseases.

7. ACKNOWLEDGEMENTS

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Abbreviations: NPM: Nucleophosmin; ALK: Anaplastic Lymphoma Kinase; RBPs: RNA-binding proteins; ALCL: Anaplastic Large Cell Lymphoma; AGs: ALK granules; PBs: processing bodies; SGs: stress granules; mRNPs: messenger ribonucleoprotein complexes; UTRs: untranslated regions; IRESs: internal ribosome entry sites; AREs: AU-rich elements; ARE-mRNAs: ARE-containing mRNAs; AUF1: AU-rich element binding factor1; HuR: Hu antigen R; ARE-BPs: ARE-binding proteins; miRNAs: microRNAs; PSF: polypyrimidine tract-binding protein (PTB)-associated splicing factor; TTP:tristetrapolin.

Key Words: ALCL, NPM-ALK, Post-transcriptional regulation, RBPs, Review

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