Differential Stability of Thymidylate Synthase 3'-Untranslated Region Polymorphic Variants Regulated by AUF1*

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Rudolf Pullmann, Jr.‡, Kutb Abdelmohsen‡, Ashish Lal‡, Jennifer L. Martindale‡, Robert D. Ladner†, and Myriam Gorospe§

From the ‡Laboratory of Cellular and Molecular Biology, NIA-IRP, National Institutes of Health, Baltimore, Maryland 21224 and §Norris Comprehensive Cancer Center, University of Southern California, Los Angeles, California 90033

A 6-nucleotide insertion (I)/deletion (D) polymorphism in the 3'-untranslated region of the thymidylate synthase gene was shown to influence mRNA stability, but the molecular basis of this effect has not been elucidated. Here, studies of both endogenous and ectopically expressed thymidylate synthase alleles revealed that the mRNA-binding, decay-promoting protein AUF1 has higher affinity for allele D mRNA. AUF1 overexpression preferentially suppressed D allele mRNA levels, whereas AUF1 silencing selectively elevated D allele mRNA levels. Our results illustrate the functional consequences of ribonucleoprotein associations involving a polymorphic RNA sequence and uncover a novel mechanism of action for non-coding RNA polymorphisms.

Post-transcriptional gene regulatory processes, including altered mRNA turnover, have emerged as key mechanisms controlling gene expression in physiologic and pathologic situations (1). These control mechanisms involve the association of trans factors, including RNA-binding proteins (RBPs) and noncoding RNA, with cis sequences present on the mRNA (2, 3). An important subset of regulatory ribonucleoprotein (RNP) associations relies on the presence of cis RNA sequences that are uracil (U)- or adenine (A)-rich (also known as AREs), present in the 5’- or 3’-untranslated regions (UTRs) of the mRNA. Indeed, many ARE-bearing mRNAs encode proteins involved in immune function, proliferation, differentiation, development, the stress response, and malignant transformation (4, 5). A number of ARE-binding RBPs that influence mRNA stability have been described, including RBPs that stabilize target mRNAs, such as the Hu proteins (6), destabilize target mRNAs, including BRF1, KSRP, and TTP (7–9), or have a dual function, such as NF90 and AUF1 (10–12).

The thymidylate synthase (TS) mRNA has AREs in its 3'-UTR. A key enzyme in the de novo synthesis of thymidylate, TS has been a major target protein for chemotherapeutic intervention for decades (13). Given its involvement in folate and homocysteine synthesis, TS is also a critical gene in embryonic development (14). The regulation of TS expression occurs at multiple levels, including regulated transcription and translation (15, 16). A polymorphism consisting of the deletion (D)/insertion (I) of a 6-bp stretch (TTAAAG) in the TS 3’-UTR was recently shown to appear with a frequency for the D allele of ~30–40% in Caucasians (17). This polymorphism was found to be associated with inflammatory conditions (rheumatoid arthritis) and several malignancies, such as colorectal and lung carcinomas, and non-Hodgkin lymphoma (18–21). It was also found to increase the risk for developmental malformations such as spina bifida (22).

In a recent study, the D allele showed decreased mRNA stability in vitro compared with the I allele. Associated with this finding, colorectal tumors from D allele carriers had lower intratumoral TS mRNA levels (23). Here, we investigated the mechanisms governing the expression of D and I alleles. An analysis of RBPs that might confer differential expression to the polymorphic TS variants revealed that AUF1 preferentially bound the D allele and thereby lowered its steady-state levels, whereas it displayed significantly lower affinity for the I allele, whose levels were comparatively elevated. This report demonstrates the differential stability of mRNAs resulting from RNP associations of naturally occurring polymorphic sequences.

EXPERIMENTAL PROCEDURES

Cell Culture, Transfections, and RNA Interference—Human colorectal carcinoma RKO cells were cultured in minimal essential medium. All transfections were carried out using Lipofectamine 2000 (Invitrogen) using plasmid vectors to silence AUF1 (pSILENCER-AUF15) (24) and to express AUF1 isoforms (10) and pGL3-TS 3’-UTR D or I alleles (pLuc(D); pLuc(I)) (23); pcDNA3.1 was transfected as a control. Small interfering RNAs (siRNA) targeting HuR and a control siRNA were previously reported (24). A plasmid vector to overexpress HuR (pHuR-TAP) and the corresponding control vector (pTAP) have also been reported (25). Cells were harvested 48 h after transfection and used for further analysis.

PCR Analyses—Conventional PCR was performed after reverse transcription (RT) of total RNA isolated from RKO,
HeLa, and HepG2 cells employing primers that were previously described (17); PCR products were resolved by electrophoresis in ethidium bromide-stained 3% agarose gels. For quantitative PCR (qPCR) analysis, reverse-transcribed RNA (using SSII-RT and either random hexamers or oligo(dT); Invitrogen) was used in PCR amplification reactions using SYBR Green PCR master mix (Applied Biosystems). For the quantification of endogenous TS (BC_002567) alleles, allele-specific antisense primers (GCAACATATAAAAAGCCTATAAA for D, GCAACATAAATTTCTACACTATAAC for I) and a common sense primer (GTGCTTTATGCGGACACTTTACTTATTTAAGGT) were used; the annealing temperature was set at 57 °C. The resulting PCR fragments were verified by sequencing. The levels of expressed luciferase chimeric constructs pLuc(D) and pLuc(I) were measured using primers TCAAGAGGACGCACTGTGGTG and GGTTGAGCAGAGGATGGAT. The data were normalized to GAPDH mRNA as described (24).

To measure the half-life of chimeric TS alleles, cells were treated with 4 μg/ml actinomycin D to block de novo transcription, RNA was collected at the times indicated, and RT-qPCR was performed to measure the levels of Luc(D) and Luc(I) mRNAs. These values were normalized to the amount of GAPDH mRNA in each sample and represented as the percentage of mRNA levels present at time 0, before adding actinomycin D. Using a semi-logarithmic scale, mRNA half-life was calculated as the time required for a given transcript to be reduced to one-half (50%, horizontal dashed line in Figs. 4B and 5C) of its initial abundance.

Western Blotting—Whole-cell lysates were size fractionated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes. Monoclonal antibodies recognizing HuR, hnRNPC1/C2, and β-tubulin were from Santa Cruz Biotechnology; a monoclonal antibody against NF90 was from BD Biosciences; a β-actin antibody was from Abcam; and a polyclonal antibody against AUF1 was from Upstate Biotechnology. Following secondary antibody incubations, signals were detected by enhanced chemiluminescence (Amersham Biosciences).

Biotin Pulldown—For in vitro synthesis of biotinylated transcripts, cDNA was used as template for PCR reactions whereby the T7 RNA polymerase promoter sequence was added to the 5'-end of all fragments. For biotin pulldown analysis, forward oligomers contained the T7 RNA polymerase promoter sequence (CCTCAGACCTTTGCTCA (forward); GGTTGAGCAGAGGTCATTTATT (reverse)). Primers used for the synthesis of sequences 981–1283 of GAPDH (NM_002046) were (T7) CCTCAGACCTTTGCTCA (forward); GGTTGAGCAGAGGTCATTTATT (reverse). Primers used for the amplification of 3'-UTR transcripts were (T7) GGGTGCTTTCAAAGGAGCTTG (forward); CGAATGCAACACTTTCTTTAATTTAAGGT and TGGTGTTACTCAGCTCTCCCTCA for transcript 1, (T7) TCAGTTATTTTATTTTATTATTGTCTTTTTTC for transcript 2, (T7) TGAGGAGAATGAAATGTATGTGCA and TGGTGTTACTCAGCTCTCCCTCA for transcript 3, and (T7) CTCAAATCTGAGGAGCCTGAG and CagaTgca-
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A

3′UTR

(D) D allele 3′UTR
(I) I allele 3′UTR

GAPDH 3′UTR

p45
p42
p40
p37

AUF1
NF90
HuR
hnRNPC1/C2

B

3′UTR

1
1010
1122
2
1134
3
1255
4D
1233
4I
1397
1427
1499
1465

TTAAAG

1 2 3 4D 4I

GAPDH 3′UTR

AUF1
NF90
HuR

C

Relative TS mRNA levels in IP

IP: AUF1  HuR  NF90  C1/C2  IgG

D  I
RESULTS AND DISCUSSION

AUF1 Preferentially Associates with TS Polymorphic Allele D—A 6-bp D/I polymorphism at the distal end of the TS mRNA 3′-UTR (Fig. 1A) was shown previously to influence the rate of mRNA decay, with the D allele mRNA displaying a significantly shorter half-life than the I allele mRNA (23). A recent screen revealed representative cell types of each polymorphic group, D/D, D/I, I/I; the human colorectal carcinoma cell line RKO was heterozygous for the polymorphism and was thus chosen for further analysis (Fig. 1B). In RKO cells, the relative basal levels of allele D mRNA, measured by RT followed by qPCR, were ~63% of the levels of allele I mRNA (Fig. 1C).

To identify RBPs preferentially binding either of the allelic forms, biotinylated RNAs spanning the entire TS 3′-UTR with or without the insertion (I and D, respectively) were used in pulldown assays followed by detection of associated RBPs by Western blot analysis (Fig. 2A and “Experimental Procedures”). As shown, several RBPs exhibited binding to both the D and I alleles, including AUF1, NF90, HuR, and hnRNP C1/C2. However, only the mRNA decay-promoting AUF1 displayed preferential affinity for one allele (D) relative to the other (Fig. 2A). Binding was low or undetectable in reactions using a negative control biotinylated RNA comprising the GAPDH 3′-UTR (Fig. 2A). To define the regions implicated in these RNP associations, a series of biotinylated transcripts spanning the 3′-UTR were prepared and tested in biotin pulldown assays. As shown (Fig. 2B), AUF1 predominantly associated with transcript 4 (containing the polymorphism), but it bound the D allelic transcript (4D) more robustly than it bound the I allelic transcript (4I). NF90 preferentially associated with transcript 2, whereas the HuR RNP complexed with transcripts 2 and 3 at levels that were only slightly above background. Interestingly, fragment 3, which had elevated AU content, was not a strongly preferred target of these RBPs. Despite the general affinity of AUF1 for RNAs with elevated AU residues, TS 3′-UTR fragment 4 is not

GAACACTTCTTATTATAG for transcripts 4D and 4I. Biotinylated RNAs were synthesized using the MaxiScript T7 kit (Ambion). For biotin pulldown analysis, whole-cell lysates (40 μg each, prepared as described) (24) were incubated with purified biotinylated transcripts for 1 h at 25 °C. Complexes were isolated with paramagnetic streptavidin-conjugated Dynabeads ( Dynal), and bound proteins in the pulldown material were analyzed by Western blotting.

Immunoprecipitation—For immunoprecipitation of endogenous RNA-protein complexes from cytoplasmic extracts (400 μg), lysates were incubated (1 h, 4 °C) with Protein A-Sepharose beads (Sigma) that had been precoated with 30 μg of IgG1 (BD Biosciences), antibodies recognizing AUF1 (Upstate Biotechnology), NF90 (BD Biosciences), HuR or hnRNP C1/C2 (Santa Cruz Biotechnology). Beads were washed and pulldown material processed as described (24). RNA was extracted in the presence of Glycoblue (Ambion) and subsequently used for RT-qPCR analysis.

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FIGURE 2. Preferential binding of AUF1 to TS D allele mRNA. A, schematic, biotinylated RNA fragments used for pulldown analysis (gray). Whole-cell RKO lysates were incubated with equimolar quantities (~0.9 μg) of biotinylated D, I, or GAPDH 3′-UTR transcripts. The binding of RBPs AUF1, HuR, NF90, and hnRNP C1/C2 was detected by Western blotting. B, partial biotinylated transcripts spanning the TS 3′-UTR were synthesized and tested in biotin pulldown assays (performed as described in panel A). The presence of RBPs AUF1, NF90, and HuR in the resulting RNP complexes was monitored by Western blotting. C, RKO cytoplasmic lysates were used in immunoprecipitation reactions with antibodies recognizing AUF1, HuR, NF90, or hnRNP C1/C2 or immunoprecipitation control reactions employing IgG1. The binding of endogenous RBPs to endogenous D and I mRNA alleles was assessed by RT-PCR using allele-specific primers. Graphs, the means and S.E. from three independent experiments.

FIGURE 3. Overexpression of AUF1 preferentially decreases the expression of D allele mRNA. A, Western blot analysis of the expression of AUF1 and loading control β-tubulin in RKO cells transfected with either empty pcDNA3.1 vector (V) or isomeric-specific AUF1 expression plasmids (alone or in combination (all)); cells were harvested 48 h after transfection. B, cells were transfected as described in panel A and total RNA prepared for analysis of endogenous D and I allele mRNAs using RT-qPCR. C, cells were transfected with either a control plasmid or a plasmid expressing HuR as a fusion protein (pTAP and pHuR-TAP, respectively, and see “Experimental Procedures”). 48 h later, the levels of D and I allele mRNAs were measured by RT-qPCR (left); representative levels of HuR, the fusion protein HuR-TAP, and loading control β-actin as detected by Western blot analysis (right). Graphs represent the means and S.E. from three independent experiments.
particularly adenine-rich, similar to what was seen with another target region within the cyclin D1 mRNA (26).

These RNP complexes were verified in RKO cells by performing immunoprecipitation reactions to isolate endogenous RBPs bound to endogenous TS mRNA alleles. As detected using allele-specific PCR amplification reactions, AUF1 associated preferentially with the D allele mRNA (Fig. 2C), whereas NF90 and HuR did not exhibit a preference for either allelic mRNA. Binding of hnRNP C1/C2 to the TS D and I mRNAs was very low, as evidenced by the fact that the levels of these mRNAs were comparable with the levels of control IgG immunoprecipitation reactions (Fig. 2C). Together, these data indicate that AUF1, NF90, and HuR can bind the TS mRNA but only AUF1 displays strong and preferential binding to the D allele mRNA relative to the I allele mRNA.

AUF1 Overexpression Preferentially Decreases Allele D mRNA Steady-state Levels—AUF1 is expressed in four isoforms (p45, p42, p40, and p37) arising from an alternate splicing of a common pre-mRNA. AUF1 was shown to promote mRNA decay, a process that has been linked to the targeting of AUF1 to the proteasome and the exosome (27, 28). Accordingly, we hypothesized that modulation of AUF1 levels might selectively affect the levels of the D allele mRNA. To test this possibility, we first overexpressed each AUF1 isoform in RKO cells (Fig. 3A) and subsequently evaluated the allelic expression in each transfection group. As shown, the endogenous D allele mRNA was less abundant than the I allele mRNA in control (V) populations (Fig. 3B). Following transfection of AUF1 isoforms, expression of the I allele mRNA decreased modestly, whereas D allele mRNA levels decreased markedly to \( \leq 24\% \) (p45 transfection group) or to less than \( \leq 18\% \) (transfection of all isoforms, p42, p40, or p37) compared with the original D allele mRNA levels. Thus, the relative abundance of D allele mRNA decreased specifically \( \leq 4\text{--}6\text{-fold lower} \) after transfection either with all isoforms or each individual isoform of AUF1 (Fig. 3B). To test whether the effects upon the D allele mRNA were specific to AUF1, HuR was overexpressed and the levels of D and I allele mRNAs measured. As observed (Fig. 3C), ectopic overexpression of HuR led to increases in both D and I allele mRNAs, but their relative abundance was largely unchanged (D was 62% of I in the control group; D was 55% of I in the HuR overexpression group). These findings indicate that HuR does not preferentially influence the level of either allele.

To ascertain whether these effects relied on the association of AUF1 with the TS 3′-UTR, we employed expression vectors in

GAPDH mRNA, were calculated by using RT-qPCR. The resulting values are represented as percent of the levels of Luc(I) mRNA. B, the half-lives of Luc(D) and Luc(I) mRNAs were calculated after adding actinomycin D to block new transcription, collecting total RNA, assessing Luc(D) and Luc(I) mRNA levels by RT-qPCR, normalizing them to GAPDH mRNA, and calculating the time required for each Luc mRNA variant to reach 50% of its initial value (dashed lines). C, RKO cells were cotransfected as described in Fig. 3A along with pLuc(D) or pLuc(I), harvested 48 h later, and Luc(D) and Luc(I) mRNA levels assessed by RT-qPCR. Graphs represent the means and S.E. from three to six independent experiments. D, cells were cotransfected with pLuc(D) or pLuc(I) along with either control or HuR-expressing plasmids (pTAP and pHuR-TAP, respectively) as described in Fig. 3C. Left, the relative levels of Luc(D) and Luc(I) mRNAs were measured by RT-qPCR 48 h after transfection, as described in panel C, right, representative Western blot analysis of the levels of HuR (and loading control β-actin) in each transfection group.

FIGURE 4. A, schematic, plasmids used in transfections to express chimeric RNAs comprising the luciferase coding region and either the TS-D or the TS-I 3′-UTRs (pLuc(D) and pLuc(I), respectively). To measure the relative abundance of the Luc(D) and Luc(I) mRNAs in RKO cells, total RNA was collected and the levels of Luc(D) and Luc(I) mRNAs, as well as normalization control
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AUF1 silencing preferentially increases the expression of pLuc(D). A, 48 h following transfection of RKO cells with an empty vector (V) or a plasmid expressing AUF1 shRNA, the levels of AUF1 and loading control β-tubulin were analyzed by Western blotting. B, cells were cotransfected with the plasmids described in panel A along with either pLuc(D) or pLuc(I), harvested 48 h later, and Luc(D) and Luc(I) mRNA levels were assessed by RT-qPCR. C, 48 h after transfection of cells as described in panel B, the stability of Luc(D) and Luc(I) mRNAs was measured using actinomycin D (as described in the legend of Fig. 4C) in both control (V) and AUF1 shRNA transfection groups. The estimated half-lives are indicated. D, left, the levels of Luc(D) and Luc(I) mRNAs were measured by RT-qPCR in populations co-transfected with each plasmic vector along with either a control (Ctrl) siRNA or siRNA targeting HuR. Right, representative Western blot analysis of the levels of HuR (and loading control β-tubulin) in each transfection group. Graphs represent the means and S.E. from three–six independent experiments.

which the luciferase coding region was linked to the 3′-UTR of each allelic variant. Following transfection of RKO cells with pLuc(D) and pLuc(I) constructs (see “Experimental Procedures”), the basal expression levels of each chimeric transcript (Luc(D) and Luc(I), respectively) were monitored by RT-qPCR. As shown in Fig. 4A, Luc(D) RNA levels were ~60% of Luc(I) mRNA levels. The relative abundance of Luc(D) and Luc(I) mRNAs is in keeping with the levels of endogenous D and I allele mRNAs (Fig. 1C), supporting the view that, in addition to its natural promoter(s) (29), the 3′-UTR region contributes directly to the regulation of TS mRNA levels. To test whether the difference in chimeric transcript levels was due to alterations in transcript stability, the half-lives of Luc(D) and Luc(I) mRNAs were assessed. After inhibition of de novo transcription using actinomycin D, the rates of decay of the Luc(D) and Luc(I) mRNAs were monitored by using RT-qPCR, and the time periods required to achieve a reduction to 50% of the original transcript abundance (their half-lives) were calculated. As shown (Fig. 4B), Luc(I) mRNA had significantly longer half-life (~6.5 h) than Luc(D) mRNA (~2.3 h), indicating that Luc(D) mRNA was less stable.

To assess the effect of AUF1 on the levels of the chimeric transcripts, pLuc(D) and pLuc(I) were cotransfected with isoform-specific AUF1 expression vectors, and the levels of chimeric luciferase transcript were measured. As shown, these interventions modestly changed the levels of the I allele but reduced much more robustly the levels of D allele (Fig. 4C). Similar to what was observed with the endogenous D and I allele mRNAs, Luc(D) and Luc(I) mRNAs were elevated when HuR was overexpressed, but there was no selective increase of one allele relative to the other (Fig. 4D). Taken together, the results obtained after HuR overexpression indicate that HuR can influence TS mRNA expression levels, an effect that could be elicited directly, through binding of HuR to the TS mRNA. However, a more plausible explanation is that the effects of HuR on TS mRNA expression are indirect, because binding of HuR to the TS mRNA is modest (Fig. 2) and HuR overexpression globally increases cell proliferation, a situation in which TS expression can increase via enhanced transcription (29). These findings further support the notion that the 3′-UTR D allele polymorphism selectively decreases mRNA stability and AUF1 influences this process.

AUF1 Silencing Preferentially Increases Allele D mRNA Levels and Stability—To further investigate the influence of AUF1 on the expression of each TS allele, AUF1 levels were knocked down by using a vector that expressed a hairpin RNA (shRNA) targeting all AUF1 isoforms (Fig. 5A and “Experimental Procedures”). The shRNA-mediated knock down of AUF1 caused a reduction of cell proliferation, and consequently the endogenous TS mRNA levels (both alleles) decreased significantly (not shown). Because the down-regulation of endogenous TS mRNA in the growth-inhibited cells likely had a significant transcriptional component, we focused instead on the influence of silencing AUF1 on the ectopically expressed Luc(D) and Luc(I) alleles, which continued to be transcribed despite the growth inhibition. Co-transfection experiments carried out with plasmid pLuc(D) or pLuc(I) vectors revealed a striking 30-fold increase in Luc(D) expression after AUF1 silencing, compared with only a 5-fold increase for Luc(I) (Fig. 5B). To test the hypothesis that mRNA stability contributed to these differences, the half-lives of Luc(D) and Luc(I) mRNAs were measured in cells expressing normal AUF1 levels (V) or after silencing (AUF1 shRNA). Using actinomycin D treatment to measure half-life (as described in Fig. 4B), the short half-life of Luc(D) mRNA in control cells was found to increase ~3-fold.
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after AUF1 silencing (from 2 to ~7 h), as shown in Fig. 5C. By contrast, the half-life of I allele mRNA increased ~2-fold, from 5.1 to ~10 h (Fig. 5C). These results strengthen the view that AUF1 selectively promotes the degradation of the D allele mRNA.

The specificity of AUF1 silencing upon the stability of each TS allele mRNA was assessed by silencing a different RBP. HuR levels were reduced by RNA interference, and the relative abundance of the D and I alleles was measured. Silencing HuR (Fig. 5D) reduced the abundance of both D and I allelic variants, but their relative levels remained constant (Luc(D) was 69% of Luc(I) in the control siRNA group; Luc(D) was 74% of Luc(I) in the HuR siRNA group), indicating no preferential effect on either allele variant. As mentioned above, HuR may form longer half-life. The usefulness of TS genetic variants is emerg-

Implications—Our data reveal that a 6-bp D/I polymorphic region within the TS 3’-UTR influences its association with the decay-promoting RBP AUF1. Consequently, the preferred target, D allele mRNA, is less stable, whereas I allele mRNA has a longer half-life. The usefulness of TS genetic variants is emerging as an effective means of identifying individuals at risk for a particular disease, its course, complications, and outcome. In addition, it provides valuable information on the most effective and safest treatment protocols. Thus far, the mechanisms of action of three such TS genetic variants have been elucidated: TS gene amplification, a 28-bp tandem repeat polymorphism, and a G→C promoter region single-nucleotide polymorphism (31, 32). In addition to the aforementioned association with inflammatory pathologies and developmental malformations, the 6-bp D/I polymorphism studied here has been linked to the etiology of various carcinomas and with non-Hodgkin lymphoma (19–22).

Elevated AUF1 levels were found to correlate with neoplasia and hyperplasia in mouse lung, while p37-overexpressing transgenic mice developed tumors associated with the deregulated expression of AUF1 target mRNAs (33, 34). Several AUF1-regulated target genes (c-myc, cyclin D1, c-fos, granulocyte macrophage colony-stimulating factor, tumor necrosis factor-α, and as shown here, TS) are likely to have a direct influence on the onset, maintenance, and progression of the neoplastic phenotype (26, 33, 35). Accordingly, it will be of interest to investigate correlations between AUF1 and TS allelic expression in cancers of various origins. Although the D allele mRNA was previously shown to be less stable (23), this is the first study on its underlying mechanism of action and to identify the decay-promoting AUF1 as a regulatory factor for TS. Along with the transcriptional and translational regulation of TS expression (15, 29) and the influence of an antisense transcript (16), the control of TS allele levels by AUF1 described here adds an important level of complexity to the mechanisms of TS expression. Numerous reports have described polymorphisms, either single-nucleotide polymorphisms or deletion/insertions, within 3’-UTR ARES of other genes (e.g. the 1784 TGTG deletion/insertion polymorphism in NRAMP1, the 1188 A→C single-nucleotide polymorphism of IL-12β, the 8473 T→C single-nucleotide polymorphism of COX2, etc.) (36–38). To our knowledge, this report provides the first example of an RNP involving a non-coding RNA polymorphism that differentially influences mRNA expression levels. Our findings further underscore the critical roles of RBPs as post-transcriptional genetic determinants in health and disease.

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