European Review for Medical and Pharmacological Sciences 2018; 22: 430-442

HuR, TTP, and miR-133b expression in NSCLC and their association with prognosis

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Abstract. – OBJECTIVE: This study sought to explore HuR, Thrombotic Thrombocytopenic Purpura (TTP), and microRNA 133b (miR-133b) expression levels in non-small cell lung cancer (NSCLC) patients and assess the relationship of expression with disease prognosis.

PATIENTS AND METHODS: One hundred and ten paraffin-embedded and 33 fresh flash-frozen NSCLC samples, together with matched tumor adjacent normal tissue controls, were collected from patients between January 2013 and July 2015 in Yidu Central Hospital of Weifang. Twenty-nine patients provided both paraffin-embedded and fresh frozen tissues. HuR and TTP protein expression levels were measured in paraffin-embedded tumors and matched controls using immunohistochemistry, while miR-133b levels were measured using Real-time fluorescent quantitative PCR.

RESULTS: Follow-up parameters included treatment response, relapse, post-relapse treatment, disease free survival (DFS), and overall survival (OS). HuR expression was significantly different between tumor and normal controls ($p < 0.0001$). Cytoplasmic expression levels of HuR and TTP correlated with pTNM staging ($p < 0.05$). A significant correlation was observed between HuR and TTP expression and other clinical pathological factors (gender, age, tumor size, pathological type, differentiation status, lymph node metastasis, distant metastasis, and tumor invasiveness). miR-133b expression correlated with tumor size ($p = 0.015$) and differentiation status ($p = 0.013$) in paraffin-embedded tissue sections, but was only correlated with pTNM ($p = 0.0$) in frozen tissue samples. No significant difference in DFS nor OS was observed between 68 HuR-positive and 42 HuR-negative patients (DFS, Log Rank $p = 0.712$; OS, Log Rank $p = 0.220$). However, DFS and OS were significantly different between miR-133b high-expression and low-expression patients (DFS, Log Rank $p = 0.048 < 0.05$; OS, Log Rank $p = 0.025 < 0.05$). This indicates that miR-133b may have prognostic value.

CONCLUSIONS: HuR expression was negatively correlated with TTP expression in NSCLC tissues. MiR-133b levels were downregulated in normal tissues compared to both paraffin and frozen tumor samples and correlated with both HuR and TTP expression, which may affect the prognosis of NSCLC patients.

Key Words:
NSCLC, RNA-binding proteins HuR, miR-133b, TTP.

Introduction

Lung cancer is one of the most common malignancies with non-small cell lung cancer (NSCLC) accounting for approximately 85%[1] of lung cancer cases. A comprehensive treatment strategy for NSCLC includes surgery, chemotherapy, radiation, and targeted therapies. However, due to heterogeneity and multidrug resistance, the therapeutic benefit to NSCLC patients has not significantly improved[2,3].

It has been reported that NSCLC development and progression is associated with multigene transcription and disrupted post-transcriptional gene modification[4]. RNA-binding proteins (RBPs) have been reported to play important roles in eukaryotic gene expression regulation, especially that of post-transcriptional modifications[5]. MiRNA, a highly conserved nucleic acid sequence, also regulates eukaryotic gene expression, and is a key factor of cell proliferation, apoptosis, and metastasis by post-transcriptional regulation of gene expression during tumor progression[6]. Both RBPs and miRNA act on 3'UTR, which suggests these two may share targeting pathways or interact with each other to facilitate oncogenesis[7]. Finally, it has been demonstrated in a variety of tumors that miR-133b overexpression inhibits tumor cell proliferation and induces apoptosis[8,9]. This implies that miR-133b functions as a classical miRNA, negatively regulating tar-

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get genes. However, the molecular mechanism of miR-133b in malignant tumors is still unclear. Moreover, the interaction between miR-133b and other post-transcriptional regulators such as HuR and TTP, also remains to be explored.

In this study, we assessed HuR, TTP, and miR-133b expression levels in 110 NSCLC samples. We then investigated their correlation with each other, clinical factors, and patient prognosis, with the aim of strengthening the foundation for future NSCLC diagnosis and treatment.

Patients and Methods

Patients

Paraffin-embedded NSCLC and matched control samples from 110 patients were collected in Yidu Central Hospital of Weifang between January 2013 and July 2015. Among all the patients, 78 were male and 32 were female. The median age was 59 years (range: 36-76 years). Forty-six patients were positive for lymph node metastasis and 64 years were negative. We also collected fresh frozen NSCLC tissues and normal controls. [illegible] were defined as being > 5 cm from th[illegible] margin, from 33 patients (25 males, 8 fe[illegible]s). The median age was 57 years (range: 41-74 y[illegible] Thirteen patients were positive for lymph n[illegible] metastasis. Twenty-nine patients [illegible]vided bo[illegible] frozen and paraffin-embedded [illegible]ne of the patients received any chemo[illegible]apy, ra[illegible]therapy, or other anti-tumor thera[illegible]before [illegible]. All patients were pathologica[illegible]firm[illegible] NSCLC after surgery[illegible]M [illegible] all patients was based on the [illegible]rican Jo[illegible]mmittee on Cancer (AJCC) a[illegible]on for Interna[illegible]Cancer Control (UICC[illegible] Among the [illegible] patients providing pa[illegible]-emb[illegible]samples, 47 patients were stage[illegible]A: 25; IB: 22[illegible]ere stage II (IIA: 19; IIB: [illegible]8 were stage III[illegible]A: 26; IIIB: 2), and 7[illegible]e stage[illegible]. Among the 33 patients with fres[illegible]zen sa[illegible]es, the number of stage I to IV pati[illegible]er[illegible](IA: 5[illegible]: 8), 10 (IIA: 3; IIB: 7), 9 (I[illegible]and 1[illegible]pectively. Eighty non-tu-[illegible]arafi[illegible]ed lung tissues were used as co[illegible]. All p[illegible]were informed of the study pur[illegible]and pro[illegible]ded written informed consent. Th[illegible]y was approved by the Ethical C[illegible]mittee o[illegible]Yidu Centre Hospital.

[illegible]icals and Antibodies

[illegible]e anti-human HuR (sc-365816) and anti-human TTP (sc-374305) monoclonal antibodies were provided by Santa Cruz Biotechnology (Santa Cruz, CA, USA). Immunohistoch[illegible]istry Kit (SP-9001), concentrated DAB kit[illegible] and neutral balsam were obtained[illegible]m ZSG[illegible] Bio (Beijing, China).

Instruments

The following equipm[illegible] was u[illegible]le tro-heating standing-[illegible]perature cu[illegible] (YLA-2000, Weifang[illegible]dical [illegible]rument [illegible]., Ltd., Shandong, Ch[illegible]micr[illegible]e (Lei[illegible] RM-2235, Wetzlar, G[illegible]rm[illegible]roscop[illegible]olym-pus BX51, To[illegible]Japa[illegible]rigera[illegible](Hisense203UN, Hi[illegible]e, Shangdo[illegible]; electromagnetic c[illegible]dia, Guangd[illegible]China); micropipett[illegible]Iso[illegible]diers-le-Bel, France). Other materials used in t[illegible]dy were provided by Yidu[illegible]al Hospital ([illegible]long, China).

[illegible]munohistochemistry

[illegible]After fixatio[illegible]d embedding, tissues were [illegible]into 4 μm [illegible]ions and dried in a 70°C i[illegible]tor for [illegible]. Tissues were then depar-affi[illegible]ylene for 5 min and hydrated using an [illegible]ohol gradient (100%, 95%, 80%, [illegible]70%, 2 min each). Following washing with [illegible]distilled water, antigen retrieval was [illegible]d by boiling for 2 min in citrate buffer solution (pH = 6.0, 0.01 mol/L), followed by 15 min incubation in room temperature. Sections were blocked with 3% peroxidase for 10 min, washed three times using PBS, and then incubated for 10 min with confining liquid. Tumor tissue was incubated overnight at 4°C with primary antibodies (HuR: 1:100; TTP 1:50). For control slides, phosphate-buffered saline (PBS) was used instead of antibody. After overnight incubation, each slide was washed 3 times for 3 min each, then incubated with biotin-labeled goat anti-mouse IgG second antibody at room temperature for 10 min. Following three washes using PBS, samples were incubated with peroxidase-labeled streptavidin at room temperature for 10 min, washed three times, and incubated with diaminobenzidine (DAB) solution. Staining efficacy was observed under light microscopy, and the slides were washed as necessary with tap water, then counterstained with hematoxylin for 1 min, and washed again with tap water until a blue background was obtained. Finally, slides were dehydrated using an alcohol gradient (75%, 85%, 95%, 100%, 1 min each), permeated with xylene (twice, 5 min each), and fixed with neutral balsam.

IHC Evaluation

Immunohistochemistry was evaluated at low and high magnification using an Olympus BX51 light microscope (Tokyo, Japan). Slide images were assessed with double-blind method and estimated with semi-quantitative integration. The staining intensity in both cytoplasm and nucleus was scored and stratified as follows: grade 0, no staining (negative); grade 1, light yellow (weak positive); grade 2, yellow (moderate positive); grade 3, yellow-brown (strong positive). The extent of staining was quantified by counting 100 cells at 5 typical 400x magnification areas of each slide. Staining extent was scored as follows: 0, < 5% positive cells; 1, 5-25% positive cells; 2, 26-50% positive cells; 3, 51-75% positive cells; 4, 76-100% positive cells. A final immunoreactivity score (IRS) was obtained for each case by adding the intensity grade to the stain score. Protein expression levels were defined as negative (IRS 0-1), positive (+, IRS 2-3), positive (++, IRS 4-5), and positive (+++, IRS 6-7). Finally, staining was further categorized based on localization expression patterns into the following 5 groups: nuclear expression only, cytoplasmic expression only, nuclear expression greater than cytoplasmic expression, cytoplasmic expression greater than nuclear expression and no expression.

RNA Extraction

Thirty mg deparaffinated fresh frozen tissues were ground in liquid nitrogen and placed in 1.5 ml RNAase-free Eppendorf (EP) tubes, suspended in 300 µl lysis buffer and 30 µl miRNA homogenate additive, vortexed, and left to stand on ice for 10 min. Following this, 300 µl mixture of phenol, chloroform, and isoamyl alcohol (25:24:1) were added and the samples were vortexed 30-60 s. The aqueous phase upper layer was collected, 375 µl ethanol was added, the solution was centrifuged, and the supernatant was discarded. The final product was washed 2-3 times, centrifuged again after addition of 100 µl preheated elution. Purity and concentration of total RNA were detected using an ultra-micro spectrophotometer.

Reverse Transcription

Total reaction volumes were 15 µl for the determinations of target genes and the reference gene U6. Reverse transcription was performed to generate cDNA, which was collected and stored at 4°C. PCR was performed on cDNA using a Taqman kit. Each PCR was performed in triplicate. RNase-free solution was used as a negative control. The reaction sequence was initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing at 60°C for 60 s.

Primer sequences were as follows: miR-133b upstream: 5'-UUUGGUCC UUC A GC UA-3'; miR-133b downstream: 5'-UAGCU UGAAGGGGACCA . U6 s A upstream: 5'-GTGCTCGCTT CAG ATAT CTA-AAATTGGAAC AT AA-3'; U6 downstream: ATT TGG CTGC-GCAAGGA CACGCA GAAGC-GTT CCA 3'.

Calculation of Re

A CT values ple replicates and c were calculated or tumor tissue and rol groups. These were then used to determine group rage CT values, using the lation aver $CT_{target\ gene}$ – average CT_{U6}. T pression l of the tumor group relative to c based on the average ΔCT of each group ($2^{-\Delta\Delta CT}=2^{-(\Delta CTtumor-l\Delta CTcontrol)}$). Relative expression levels for each patient were calculated compared with group averages in order de mine individual expression profile. The difference in expression between paraffin-embedded and fresh frozen samples was compared using $2^{-\Delta CT}$.

Follow-up

The follow-up endpoint was March 2014. Disease-free survival (DFS) was defined as the period between treatment to relapse or death occurring due to any reason. Overall survival (OS) was defined as the period from surgically confirmed NSCLC to death. Losing follow-up and living patients were defined as censors.

Statistical Analysis

SPSS 13.0 (SPSS Inc., Chicago, IL, USA) was used to conduct statistical analysis. Experimental data are expressed as mean ± standard deviation. Student's *t*-test was used to compare the mean between two groups. A nonparametric statistical test, the Wilcoxon rank sum test, was used to test data with unknown distribution. χ^2-test was used for count data. Kaplan-Meier curve, log rank test, and Cox regression model were applied for survival analysis. $p < 0.05$ was considered statistically significant.

Results

HuR Protein Expression in NSCLC and Tumor-Adjacent Normal Tissues

Immunohistochemistry showed that HuR protein was expressed in NSCLC tissues, and that the pattern varied among different cell types. We observed that HuR was mainly expressed by tumor cells and mesenchymal cells, with low expression by macrophages (Figure 1). There were 61 adenocarcinoma, 42 squamous, and 7 other pathological NSCLC subtype cases. The HuR expression positive rate in NSCLC cytoplasm was 61.82% (68/110), while it was 100% (110/110) in nuclei. In contrast, cytoplasmic HuR expression was only found in 3.64% (4/110) of control cases, while nuclear expression was found in 98.18% (108/110) of cases. Therefore, cytoplasmic HuR expression was significantly different in NSCLC tissues *vs.* control (p=0.000), but no difference was observed in nuclear expression (Table I).

TTP Protein Expression in NSCLC and Tumor-Adjacent Normal Tissues

We observed TTP protein expression tumor and normal tissues (Figure 2). TTP e[illegible]s-sion was found in 35.45% (39/110) and 35[illegible] (39/110) of cytoplasmic and nuclear NSCLC c[illegible]es, respectively. In controls, the c[illegible]plasmic an[illegible] nuclear expression rates were 4[illegible]/110) and 29.09% (32/110), respectivel[illegible]herefo[illegible] while a significant difference in c[illegible]asmic T[illegible]pres-sion was observed betwe[illegible]SCL[illegible] tissue, no difference[illegible]s o[illegible]n terms of nuclear expression[illegible]e I).

Correlation [illegible]d TTP Exp[illegible]ssion with NSCL[illegible]inica[illegible]racteristics

In 110 N[illegible]LC patients, [illegible]nd that the cytoplasmic [illegible]ession of both Hu[illegible]d TTP significantly[illegible]elated[illegible]ith pTNM stage ($p < 0.05$). No sign[illegible]nt cor[illegible]ions with other clinical parameters[illegible]e[illegible], age, t[illegible] size, pathology type, were ob[illegible]in this[illegible]dy (Table II).

[illegible]3b Ex[illegible]ion in Frozen NSCLC and [illegible]umor-Adj[illegible]cent Normal Tissues

[illegible]ozen tumor and matched normal t[illegible]es, the [illegible]xponential transformed miR-133b [illegible]ression level was 0.0155 ± 0.06616 in tumor [illegible] and 0.0397 ± 0.13634 in normal tissues (W[illegible]on rank-sum test, p = 0.033). The average CT value for miR133b was 30.82 ± 3.50 and that of U6 was 19.25 ± 3.96 (ΔCT: 11.57 ± 4.38) in tumor tissues. The corresponding v[illegible] in normal tissues were 29.40 ± 3.16 and [illegible] (ΔCT: 9.00 ± 3.51). The expression [illegible]l of miR-133b in tumor tissues was only [illegible]t 1/6 of that in matched normal tissues ($2^{-\Delta\Delta CT} = 2^{-(11.57-9.00)} \approx 0.168$). Based on whether the expre[illegible]level of miR133b was above 0.618[illegible] in the c[illegible]nd ing normal tissue, 16 p[illegible]ts were classi[illegible] having high miR-133[illegible]pressio[illegible]ith 17 c[illegible]-sified as having lo[illegible]ressio[illegible]imilar [illegible] HuR and TTP, miR133[illegible] ex[illegible]evel al[illegible]gnifi-cantly correlate[illegible]th p[illegible]agin[illegible]sher ex-act test, p = 0[illegible], with no si[illegible]orrelation observed b[illegible]iR133b exp[illegible]n and other clinical f[illegible]rs ([illegible]III).

MiR[illegible]Expressio[illegible]araffin-Embedded N[illegible]nd Normal Tiss[illegible]es

[illegible]o further validate the expression pattern of [illegible]R-133b in NS[illegible]C, we expanded the number [illegible]e specimen[illegible]d measured the relative ex-p[illegible]n level o[illegible]R-133b in 110 NSCLC tissues and [illegible]ng tissues using RT-PCR. The exponential transformed delta CT value ($2^{-\Delta CT}$) [illegible]as 0.0041 ± 0.0184 in tumor tissues *vs.* 0.0100 ± [illegible] control tissues (Mann-Whitney U test, [illegible] = 0[illegible]or the expression of miR-133b, the ΔCT value was 12.93 ± 3.76 (miR-133b: 32.05 ± 2.81; U6: 19.12 ± 3.46) in tumor tissues and 10.17 ± 3.49 (miR-133b: 32.38 ± 2.94; U6: 22.21 ± 3.63) in normal tissues. Similar to fresh frozen tissues, the expression level of miR-133b in tumor tissues was only 0.127 fold of that in matched normal tissue ($2^{-\Delta\Delta CT} = 2^{-(12.93-10.17)} \approx 0.127$), which indicates a significant downregulation. Therefore, patients were classified based on this value, and 54 patients were noted to have high miR-133b expression, with another 56 having low expression. The miR133b expression level significantly correlated with pTNM staging (Fisher exact test, p = 0.015) and tumor size (p = 0.013). No significant correlation was observed between miR133b expression and other clinical factors (Table IV).

NSCLC miR-133b Expression in Frozen and Paraffin-Embedded Tissues

To explore the effect of tissue processing on miR133b expression, we examined 29 patients who provided both paraffin-embedded and frozen tissue samples. The average miR133b expression level in frozen tumor tissue was 0.0160 ± 0.07041, whereas paraffin-embedded tumor tissue showed expression of 0.0103 ± 0.03385. Since the expres-

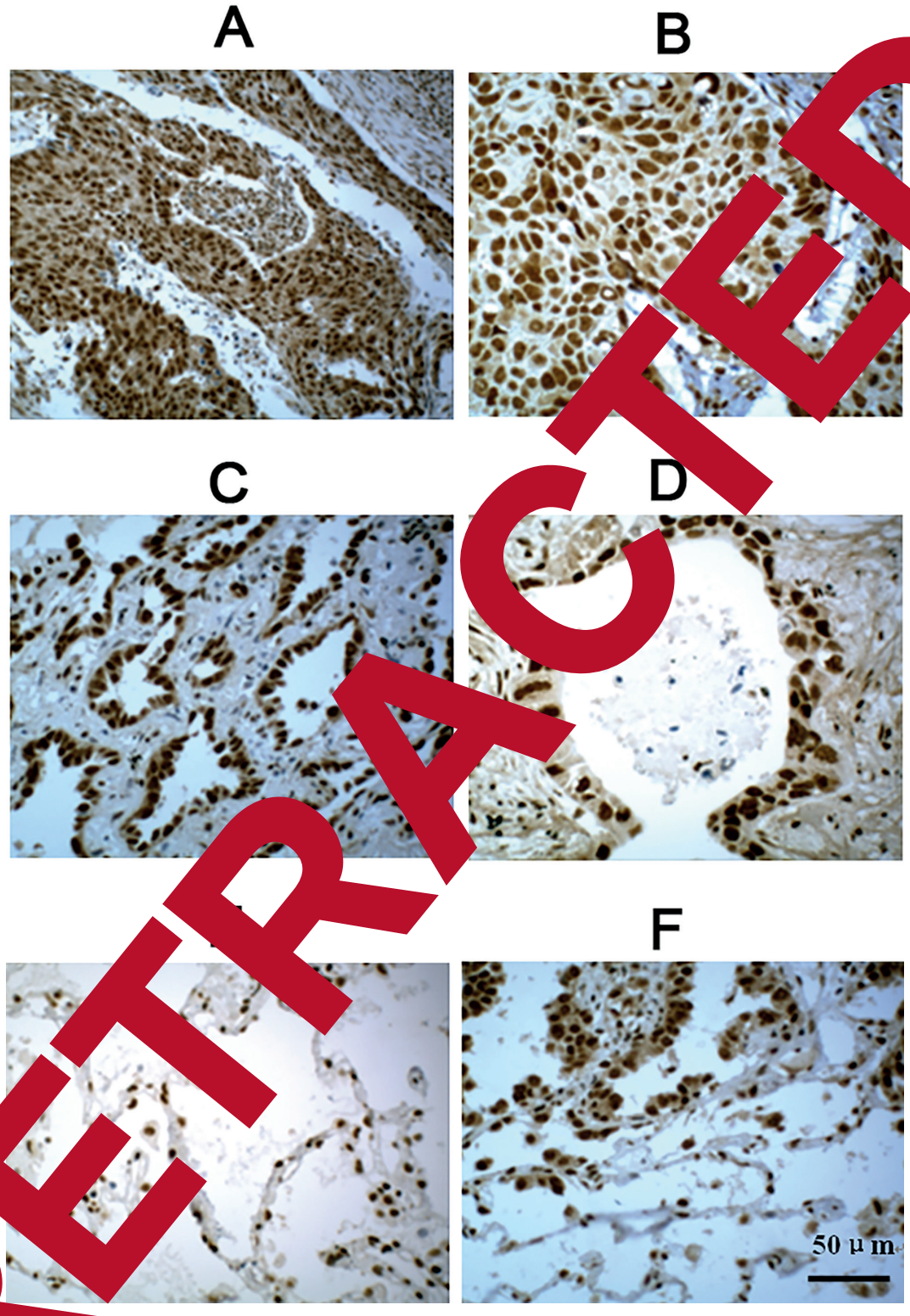

Figure 1. ...pression in NSCLC tissues. Immunohistochemical staining for HuR; a positive result is marked by yellow-...wn coloration. All images are 400× magnification. ***A,*** Cytoplasmic/nuclear double-positive HuR expression in lung ...mous cell carcinoma. ***B,*** Cytoplasmic positive/nuclear negative HuR expression in lung squamous cell carcinoma. ***C,*** C...mic/nuclear double-positive HuR expression in lung adenocarcinoma. ***D,*** Cytoplasmic negative/nuclear positive HuR expr...n in lung adenocarcinoma. ***E,*** Cytoplasmic negative/nuclear positive HuR expression in normal alveolar epithelial cells. ***F,*** Cytoplasmic/nuclear double-positive HuR expression in tumor-adjacent normal tissues.

Table I. Comparison of HuR and TTP expression in NSCLC and tumor-adjacent normal tissues.

HuR/TTP expression	NSCLC (n = 110)	Cancer-adjacent normal tissues (n = 80)	χ^2-value	
Cytoplasmic Hur			82.198	0.000
Negative	42 (0.38)	106 (0.96)		
Positive	68 (0.62)	4 (0.04)		
Nuclear Hur				
Negative	0 (0)	2 (0.02)		
Positive	110 (1)	108 (0.98)		
Cytoplasmic TTP			4.172	0.029
Negative	71 (0.65)	56 (0.51)		
Positive	39 (0.35)	54 (0.49)		
Nuclear TTP				
Negative	71 (0.65)	78 (0.71)		
Positive	39 (0.35)	32		

sion level did not follow a normal distribution, the rank sum test was used, with no significance being observed ($p = 0.443 > 0.05$).

The Correlation Between HuR, TTP, and miR-133b in NSCLC

Among 39 cytoplasmic TTP positive tissue samples, only 14 showed positive cytop HuR, and 4 showed downregulated m levels. In 71 cytoplasm TTP negative sa 52 were positive for cytoplasmic HuR an showed miR133b downregulation. Cytoplas HuR expression showed inverse c lations wi TTP ($p = 0.027$) and miR-133 on levels ($p = 0.034$). The positive ex ion o oplasm TTP showed a negative elation miR-133b downregulation (p 0). H was no significant elat d between nuclear expression luR, T miR-133b (Table V).

Effect of Cy plasm R, TTP, and miR-133b xpression o CLC Prognosis

The an follow-up ti as 20 months (range 9 mo s). No significant differences in D or OS e observed between 68 cytoplas itive ti s and 42 cytoplasmic HuR ne issues S: Log Rank $p = 0.712$, og R 220). However, the prog- f the p group was slightly worse than at of the egative group, suggesting that cy R expression may be an adverse tor for NSCLC patients (Figure B). Similarly, we did not find any significant ation in DFS (Log Rank $p = 0.060$) or OS (L ank $p = 0.094$) between 39 cytoplasmic TTP positive tissues and 71 cytoplasmic TTP negati sues. The s l curve of cytoplasm positive patients was higher than that e negative group, indicating TTP might be anti-tumor f r (Figure 3C-D). Among 110 LC paraffin bedded tissues, 56 showed m 3b dow ulation while 54 showed upregu was significant difference in both DFS (Log Rank $p = 0.048 < 0.05$) and OS (Log Rank $p = 0.025 < 0.05$) between the diff- iR133b groups. This dysregulation of iR- b in NSCLC exerted an effect on patient survival (Figure 3E-F).

Discussion

Lung cancer has the highest worldwide mortality among all types of malignant tumors. In China, more than 80% NSCLC patients are already in the advanced stage at the time of diagnosis. Unfortunately, even for early stage NSCLCs, the 5 year survival rate is only 65 to 80% after standard clinical management. The reason for this poor prognosis is the highly aggressive and heterogeneous nature of NSCLC. Therefore, the identification of prognostic biomarkers is of great significance.

Human antigen R (HuR) is an RNA-binding protein belonging to the embryonic lethal abnormal vision (ELAV) family. Its main function is regulating eukaryotic post-transcriptional gene expression modification. Evidence has shown that HuR participates in regulating various biological progresses such as proliferation, differentiation, invasion, apoptosis, angiogenesis, and lymphangiogenesis in multiple cancer types[10]. HuR exerts its biological function via interacting with

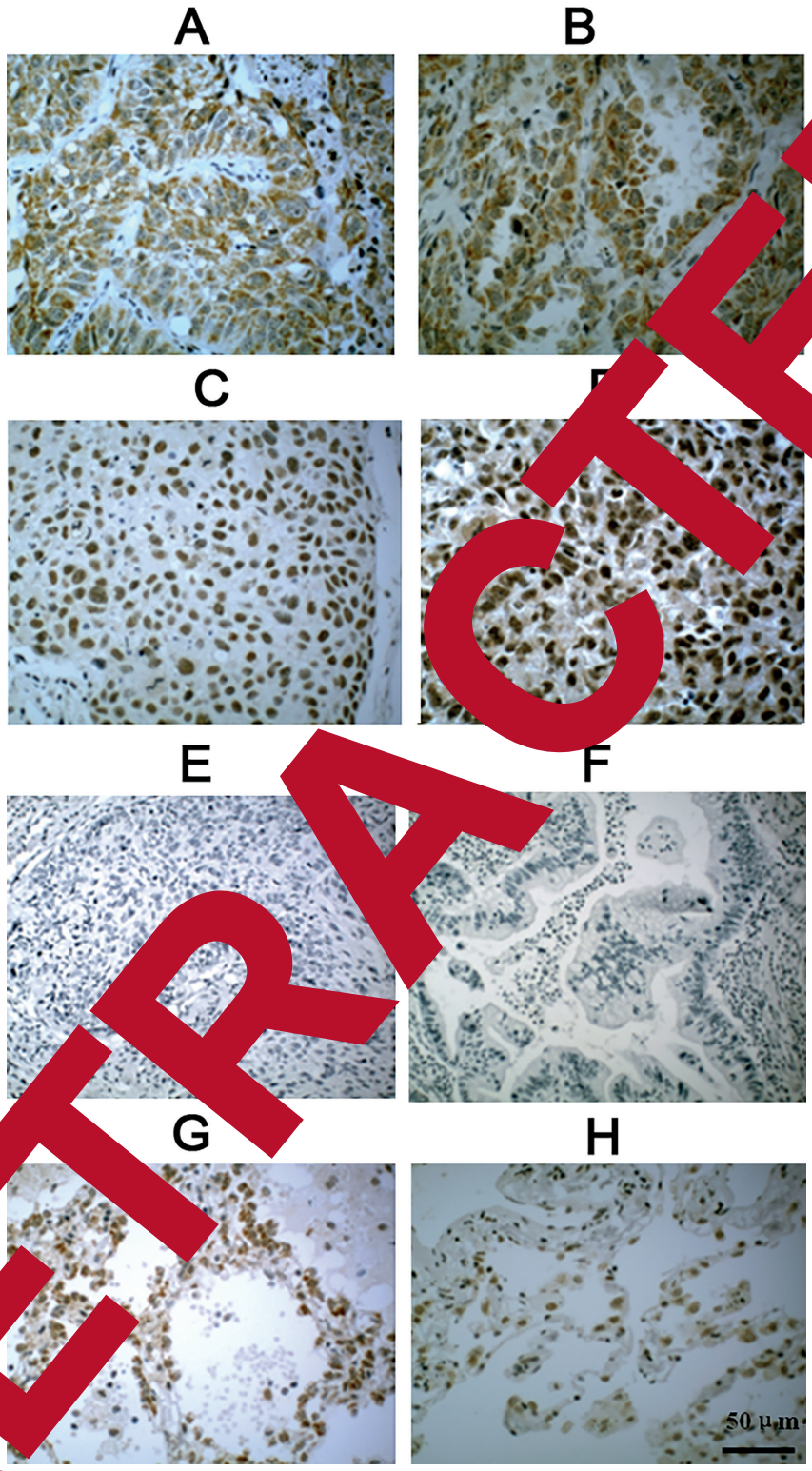

Figure 2. TTP expression in NSCLC tissues. Immunohistochemical staining for HuR; a positive result is marked by yellow-brown coloration. All images are 400× magnification. ***A,*** Cytoplasmic negative/nuclear positive TTP expression in lung squamous cell carcinoma. ***B,*** Cytoplasmic positive/nuclear negative TTP expression in lung squamous cell carcinoma. ***C,*** Cytoplasmic negative/nuclear positive TTP expression in lung squamous cell carcinoma. ***D,*** Cytoplasmic/nuclear double-positive TTP expression in lung squamous cell carcinoma. ***E,*** Negative TTP expression in lung squamous cell carcinoma. ***F,-G,*** Cytoplasmic positive/nuclear negative TTP expression in alveolar epithelial cells. ***H,*** Cytoplasmic negative/nuclear positive TTP expression in alveolar epithelial cells.

Table II. The relationship between NSCLC cytoplasmic expression of HuR and TTP and clinical pathology.

Clinical factor	Number	HuR			TTP		
		Positive	χ^2 value	*p*-value	Positive	χ^2-value	*p*-value
Gender			0.287	0.592		1.2	0.265
Male	78	50			24		
Female	32	18			15		
Age			0.112	0.732		0.046	
≥ 59	57	33			21		
< 59	53	34			18		
Tumor size			0.562	0.473			0.181
≥ 5 cm	35	25			8		
< 5 cm	75	42					
Pathology subtype			0.208	0.917		23	0.318
Adenocarcinoma	61	35			27		
Squamous carcinoma	42	26			10		
Others	7	6					
Differentiation			0.519	0.433		2.293	0.317
Low	21	11			6		
Middle-high	89	56			33		
pTNM stage			1.932	.018		0.265	0.012
I	46	21			25		
II-IV	64	46			4		
Lymphatic metastasis			0.167	0.712		3.273	0.072
Yes	46	31			0		
No	64	36			29		
Distant metastasis			0.183			1.281	0.292
Yes	8	3			5		
No	102	64			34		
Invasive depth			0.1	.758		0.736	0.382
T1+T2	86	51			33		
T3+T4	24	16			6		

the 3'UTR sequence, whic rich adenine and uracil (AU-rich eleme AREs) geted genes to stabilize transc pro normal conditions, H is in the nucleus. After certai muli, Hu transported from the nucleu he cytoplas several mechanisms, i inding with UTR, thus escaping Ase ation.

Elevate toplasmic H ression has been reported oth atypical duc yperplasia and ductal cinom *in situ* (DCIS), and correlates wit gh dif tiation and progesterone receptor expressi Accordingly, Zhu et al[13] fou high c lasmic HuR expression iates l nucleus differentiation, an mone positive expression. Zhang et al ave show at cytoplasm HuR expression in ncer was elevated and correlated pathological characteristics including lymtic metastasis, tumor invasion degree, and stage. Moreover, they proved that the cytopla HuR expression rate was an independent prognosis factor on patient 5-year survival rates. Indeed, cytoplasmic overexpression of HuR also associated with high nuclear expression, and correlated with DFS as an independent adverse prognostic factor. The overexpression of nuclear HuR was associated with disease-related and progression-free survival[15].

In this research, HuR was expressed in both cytoplasm and nucleus, with nuclear expression higher in both NSCLC and tumor-adjacent normal tissues. Indeed, while cytoplasmic HuR expression was hardly observed in normal tissues, it was upregulated in NSCLC. Thus, HuR may promote oncogenesis and tumor invasion. We propose that the transportation of nuclear HuR to the cytoplasm by certain shuttle mechanisms leads to changes in various mRNAs and may play a key role in accelerating carcinogenesis and NSCLC progression, whereas high nuclear HuR expression in NSCLC and tumor-adjacent normal tissues can be recycled by the nucleus after its function. In contrast with previous studies, no correlation was found between cytoplasmic HuR expression and the clinical characteristics. Given

Table III. The relationship between miR-133b expression in frozen NSCLC tissues and clinical characteristics.

Clinical characteristics	Number	miR-133b		-value
		Low-expression	High-expression	
Gender				0.683
Male	25	13	12	
Female	8	3	5	
Age				
≥ 59	15	8	7	
< 59	18	8		
Tumor size				0.156
≥ 5 cm	12	8		
< 5 cm	21	8		
Pathological subtype				
Adenocarcinoma	23	11	12	
Squamous carcinoma	9	5	4	
Others	1	0	1	
Differentiation				1
Low	8	3		
Middle-high	25	13		
pTNM stage				0.032
I	13	3	10	
II-IV	20		7	
Lymphatic metastasis				0.295
Yes	13	8	5	
No	20	8	12	
Distant metastasis				0.473
Yes	1	1	0	
No	32		17	
Invasive depth				0.438
T1+T2	25		14	
T3+T4	8		3	

Table IV. Correlation between miR ion in para SCLC specimens and clinical characteristics.

Clinical factors		miR-133b		χ^2 value	*p*-value
		ession	High-expression		
Tumor size				6.492	0.015
≥ 5 cm	35	24	11		
< 5 cm	75	32	43		
Pathological s				5.617	0.062
Adenocarcin		25	36		
Squamous noma		26	16		
Others		5	2		
Differ ion				0.022	0.013
Low	21	11	10		
M igh	89	45	44		
pTN				2.506	0.114
I	46	17	29		
	64	39	25		
atic me				0.13	0.245
Yes	46	27	19		
No	64	29	35		
				1.334	0.718
	8	2	6		
	102	54	48		
ive depth				0.013	0.438
	86	43	43		
T3+	24	13	11		

Table V. Correlation of cytoplasm HuR, TTP, and miR-133b expression.

	Number	Cytoplasmic HuR positive			miR-133b expres		
		Positive	χ^2 value	*p*-value	Positive	χ^2-val	*p*-value
Cytoplasmic TTP			4.443	0.027		15.	0.000
Positive	39	14			4		
Negative	71	54			52		
Cytoplasmic HuR			2.837	0.108		0.792	
Negative	42	25			16		
Positive	68	43			40		
miR-133b expression			4.492	0.034			
Low-expression	56	46					
High-expression	54	22					

the limited sample size employed (110 patients) and interpatient variability, further investigations where specimen number and statistical analysis are amplified, are required. TTP is usually expressed at low levels in the nucleus. However, upon environmental stimulation, TTP also shuttles from the nucleus to the cytoplasm with the involvement of the nuclear export sequence (NES), a two zinc finger structure in the amino acid terminus. In malignant cancer, TT[illegible] pression is downregulated, leading to i[illegible] transcription stability and matrix metallop[illegible]n-ase 9 (MMP9), MMP2, and interleukin-6 ([illegible]) levels, and thus promoting tumor invasiver[illegible] and metastasis[16]. The proviral i[illegible]gration si[illegible] for Moloney murine leukemia[illegible]Pim-1), a serine-threonine kinase wi[illegible]ncoge[illegible]effects, is upregulated in a varie[illegible]human[illegible]ors. It has been shown that T[illegible]ys [illegible] in modulating the s[illegible]lity[illegible]-1 mRNA. Cell proliferation w[illegible]nhibited[illegible]TP-induced degradation of P[illegible] which in tu[illegible]fers an anti-tumor eff[illegible]is work, a [illegible]gnificant correlation be[illegible]een cy[illegible]mic TTP expression and clinic[illegible]athological c[illegible]teristics was not observe[illegible]mong the 39 T[illegible]ositive NSCLC sampl[illegible]5 were[illegible]age I, while only 14 were stages I[illegible]. This i[illegible]ated that TTP expression was high[illegible]in[illegible]rly st[illegible]of NSCLC, suggesting the p[illegible]ive ef[illegible]of TTP against tumors. [illegible]133b [illegible]at[illegible]n the sixth chromosome (or[illegible]) and [illegible]st found in skeletal muscle. It w[illegible]considered a muscle specific miRNA that pa[illegible]keletal muscle development. In [illegible]ion, it p[illegible]ticipates in the development of car-[illegible]c muscle, and its ectopic expression was ob-[illegible]d during myocardial hypertrophy and heart fai[illegible]. Moreover, it can affect the nervous system, such as the growth of astrocyte and axons, causing [illegible]ous [illegible]n diseases[5]. miR-133b, like many microRNAs, [illegible]en observed to be ectopically[illegible]essed in a g[illegible]iversity of cancers. It[illegible]een shown to serve as a pro-tumor factor [illegible]ervical cancer, promoting tumor development [illegible]metastasis [illegible]ugh the AKT and ERK signaling pathway[6]. [illegible]addition, down regulation of m[illegible]3b was o[illegible]ved in rectal cancer, head and neck[illegible]l carcinoma (HNSCC), gastrointestinal[illegible]omal tumor (GIST), gastric cancer, [illegible]rostate cancer, bladder cancer, osteosarcoma, [illegible]er, and other malignant tumors. Based [illegible]t[illegible] studies, miR-133b is likely a tumor suppressor, but the mechanism is still unknown. There are limited studies regarding miR-133b in lung cancer. We showed that the expression of miR-133b in NSCLC is lower than in healthy controls, which also indicates that miR-133b is a tumor suppressor. We found that the expression of miR-133b was associated with pTNM stage, suggesting miR-133b was involved in NSCLC metastasis. However, inconsistent results were observed in paraffin-embedded specimens. Since xylene and other dyes are capable of damaging nucleic acid structures, miR-133b may have been partially degraded in paraffin-embedded samples. We concluded that the effective detection miR-133b in paraffin-embedded specimens was time-dependent, and that miR-133b may affect the oncogenesis, progression, and metastasis of NSCLC.

Multiple studies have indicated that HuR and TTP target the same gene with opposing effects. Al-Ahmadi et al[18] have shown that HuR overexpression increases gene stability two-fold greater than TTP degradation. In tumor tissues, the relative ratio of TTP and HuR mRNA is significantly different compared to normal tissues. Both RNA binding proteins and microR-

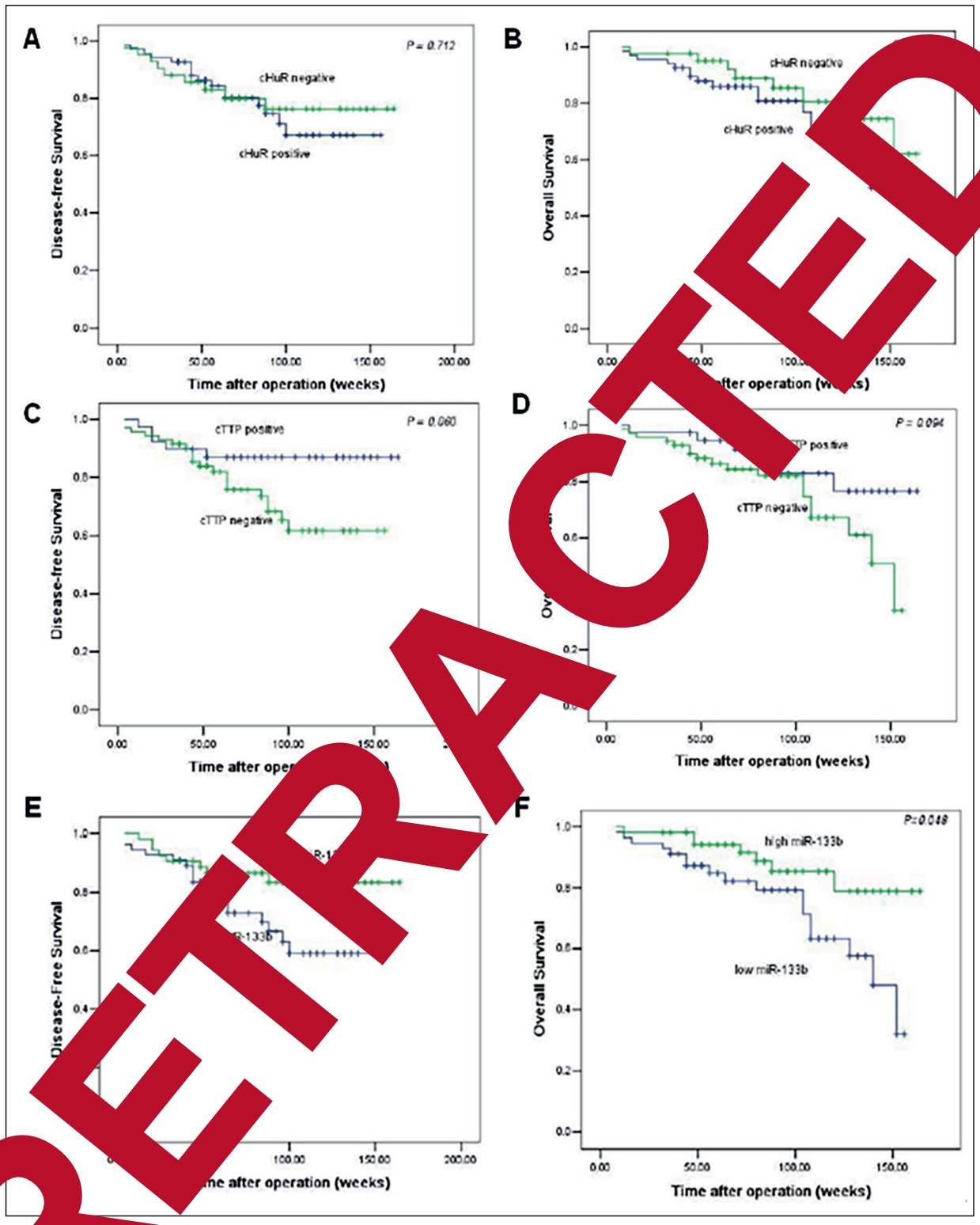

Figure 3. Correlation between miR-133b, cytoplasmic HuR, and cytoplasmic TTP with prognosis. ***A,-B,*** No significant correlation of DFS (Log Rank $p = 0.712$) and OS (Log Rank $p = 0.220$) was found between 68 cytoplasmic HuR positive and negative tissues. ***C,-D,*** No significant correlation of DFS (Log Rank $p = 0.060$) and OS (Log Rank $p = 0.094$) was found between 39 cytoplasmic TTP positive and 71 negative tissues. ***E,-F,*** There were significant correlations between miR-133b expression and DFC (Log Rank $p = 0.048 < 0.05$) and OS (Log Rank $p = 0.025 < 0.05$), based on the analysis of 56 miR-133b downregulated and 54 upregulated tissues.

NAs can co-regulate mRNA by acting on the 3'UTR of the target mRNA, while the effect of HuR can be increased by interacting with other miRNAs[19]. Many studies have shown that HuR, TTP, and microRNAs have a close relationship, and can influence diverse malignant tumor phenotypes via synergy or antagonism. Similarly, there is a significant correlation between cytoplasmic HuR, TTP, and miR-133b expression levels. Low miR-133b, high cytoplasmic HuR, and low cytoplasmic TTP levels all play key roles in the progression of NSCLC. However, the specific mechanism still needs further investigation. Based on our findings, DFC and OS were affected by the expression of miR-133b. The downregulation of miR-133b shortened patient DFC and OS. No significant association between HuR and TTP with prognosis was observed. This may be due to a relatively short follow-up period and many excluded cases in our study population. Our results match our hypothesis that miR-133b expression in NSCLC was downregulated, and that most patients presented high cytoplasm HuR and low or absent cytoplasmic TTP levels. There was a significant corre n between miR-133b, HuR, and TTP leve cytoplasmic HuR expression negatively co t-ed with miR-133b, and TTP levels and miR- expression, which positively correlated cy plasmic TTP levels.

Conclu s

We suggest miR-1 pr ncogenesis, development, and m stasis by ctions with HuR and TTP. I future, we w duct *in vitro* experime stigate the b avior of miR-133b, Hu and T d explore their interactions an ir underlin chanisms.

Co f I rest

The Aut re that t ave no conflict of interests.

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