analytical chemistry

Article

pubs.acs.org/ac

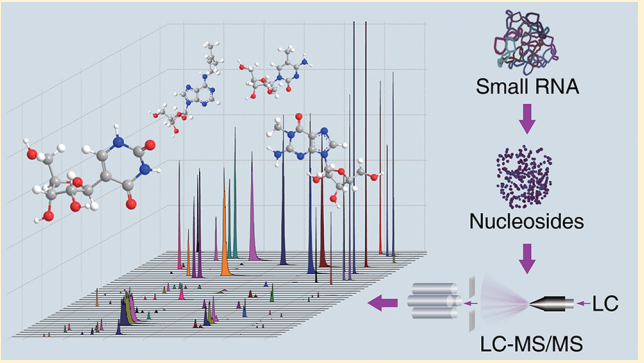
A High-Throughput Quantitative Approach Reveals More Small RNA Modifications in Mouse Liver and Their Correlation with Diabetes

Menghong Yan, Yuangao Wang, Yanan Hu, Yan Feng, Changgui Dai, Jingxia Wu, Dongmei Wu, Fang Zhang, and Qiwei Zhai*

Key Laboratory of Nutrition and Metabolism, Institute for Nutritional Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, 200031, China

Supporting Information

ABSTRACT: Studies of RNA modification are usually focused on tRNA. However the modification of other small RNAs, including 5.8S rRNA, 5S rRNA, and small RNA sized at 10−60 nt, is still largely unknown. In this study, we established an efficient method based on liquid chromatography−tandem mass spectrometry (LC−MS/MS) to simultaneously identify and quantify more than 40 different types of nucleosides in small RNAs. With this method, we revealed 23 modified nucleosides of tRNA from mouse liver, and 6 of them were observed for the first time in eukaryotic tRNA. Moreover, 5 and 4 modified nucleosides were detected for the first time in eukaryotic 5.8S and 5S rRNA, respectively, and 22 modified nucleosides were identified in the small RNAs sized at 30−60 or 10−30 nt. Interestingly, two groups of 5S rRNA peaks were observed when analyzed by HPLC, and the abundance of modified nucleosides is significantly different between the two groups of peaks. Further studies show that multiple modifications in small RNA from diabetic mouse liver are significantly increased or decreased. Taken together, our data revealed more modified nucleosides in various small RNAs and showed the correlation of small RNA modifications with diabetes. These results provide new insights to the role of modifications of small RNAs in their stability, biological functions, and correlation with diseases.



Modifications of RNA have been identified in many different types of small RNAs (<200 nt in length).[1,2] There are nearly 100 known modified nucleosides in tRNA, and about 50 nucleoside modifications have been identified in tRNA from diverse eukaryotic species.[1,2] In contrast with the chemical diversity of tRNA, the known modifications of other small RNAs such as 5.8S rRNA, 5S rRNA, microRNA (miRNA), and Piwi-interacting RNA (piRNA) have remained limited primarily to ribose methylations and pseudouridine.[1,3−5] For example, only four ribose methylations (Am, Um, Gm, and Cm) and pseudouridine were discovered in 5.8S rRNA from eukaryotes.[3] In 5S rRNA of eukaryotes, only pseudouridine has been found so far.[4] Recently, modifications of small RNAs such as miRNA and piRNA have gained considerable attention.[5] The conversion of adenosine to inosine within certain populations of miRNA precursor molecules by the ADAR (adenosine deaminases acting on RNA) family of enzymes was observed in mammals.[6] Methylation at the 2′-hydroxyl group on the ribose at the last nucleotide of miRNAs by a methyl transferase HEN1 has been reported in plants.[7,8] Methylation of miRNAs in animals is still unclear,[5,9] whereas piRNAs in animals such as *Drosophila*, mouse, and rat are 2′-O-methylated at the terminal ribose of 3′ ends by HEN1 homologues.[10−12] However, it remains unknown whether there are more small RNA modifications, especially in mammals.

RNA modifications play important roles in many aspects of biological functions.[13,14] For example, modified nucleosides in the anticodon region in tRNA species have significant effects on translational accuracy and efficiency.[15] In mammalian innate immune systems, nucleoside modifications suppress the potential of RNA to activate dendritic cells.[16] In yeast, dynamic control of tRNA modifications occurs in response to cellular stress[17] and deletion of *tuc1* and *elp3* genes leads to the removal of all modifications from the wobble uridine derivatives of some cytoplasmic tRNAs and is lethal to the cell.[18] Many diseases in human being such as cancer, dyskeratosis congenita, Alzheimer's disease, and diabetes are relevant to RNA modifications.[19−21] Small RNAs, such as miRNAs, have been reported to play important roles in the pathogenesis of diabetes.[22] However, the correlation between small RNA modifications and diabetes is still largely unknown.

Here, we report an efficient approach to identify and quantify the modifications in small RNAs based on enzymatic digestion

Received: November 7, 2013
Accepted: November 21, 2013
Published: November 21, 2013

and liquid chromatography−tandem mass spectrometry (LC−MS/MS) to simultaneously detect more than 40 different types of nucleosides. This quantitative systems approach reveals more small RNA modifications in mouse liver and provides the initial data for the correlation of various small RNA modifications with diabetes.

EXPERIMENTAL SECTION

Materials. Cytidine (C), adenosine (A), guanosine (G), uridine (U), 2′-*O*-methyladenosine (Am), 2′-*O*-methylcytidine (Cm), 2′-*O*-methyluridine (Um), 2′-*O*-methylguanosine (Gm), N^2-methylguanosine (m^2G), N^6-isopentenyladenosine (i^6A), N^4-acetylcytidine (ac^4C), 7-methylguanosine (m^7G), 5-methyl-2-thiouridine (m^5s^2U), inosine (I), 5-methoxyuridine (mo^5U), 1-methyladenosine (m^1A), 5-methylcytidine (m^5C), 5-methyluridine (m^5U), 4-thiouridine (s^4U), N^6-methyladenosine (m^6A), and 3′-*O*-methylurindine (3′-*O*MeU) were purchased from Sigma-Aldrich. Pseudouridine (ψ), 2-thiocytidine (s^2C), 2′-*O*-methylinosine (Im), 3-methyluridine (m^3U), 5,2′-*O*-dimethyluridine (m^5Um), 1-methylpseudouridine ($m^1\psi$), N^2-dimethylguanosine ($m^2{}_2G$), and 5-hydroxy-methylcytidine (hm^5C) were purchased from Berry and Associates Inc. 5,2′-*O*-dimethylcytidine (m^5Cm), 2-thiouridine (s^2U), 1-methylguanosine (m^1G), N^4-acetyl-2′-*O*-methylcytidine (ac^4Cm), N^2,N^2,7-trimethylguanosine ($m^{2,2,7}G$), 3-methylcytidine (m^3C), 5′-*O*-methylthymidine (5′-*O*MeT), 3′-*O*-methylcytidine (3′-*O*MeC), 3′-*O*-methyladenosine (3′-*O*MeA), 3′-*O*-methylinosine (3′-*O*MeI), and 3′-*O*-methylguanosine (3′-*O*MeG) were obtained from Carbosynth. Guanosine 5′-monophosphate ($^{13}C_{10}$, $^{15}N_5$) was from Cambridge Isotopes Laboratories.

Preparation and Detection of Stable Isotope Labeled Guanosine. Guanosine 5′-monophosphate (^{13}C, ^{15}N) at 140 μg/mL was hydrolyzed to guanosine (^{13}C, ^{15}N) by calf intestine alkaline phosphatase (TakaRa) at a final concentration of 1 U/μL at 37 °C for 24 h. The products of enzymatic digestion were detected by HPLC using a ZORBAX SB-Aq reversed-phase HPLC column with a linear gradient (solvent A, 2 mM CH_3COONH_4; solvent B, CH_3OH; 0−5% solvent B, 0−5 min; 5%−15% solvent B, 5−45 min; 15%−100% solvent B, 45−70 min) at a flow rate of 0.6 mL/min and monitored by UV detector at 254 nm. The hydrolysates were also analyzed by electrospray mass spectrometry.

Preparation of Tissues. All animals were maintained and used in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Institute for Nutritional Sciences (protocol number 2010-AN-9). Male C57BL/6 mice and male diabetic *db/db* mice at the age of 8 weeks were obtained from the Model Animal Research Center of Nanjing University (Nanjing, China). After receiving, development of diabetes in *db/db* mice was confirmed by measurement of blood glucose as described previously.[23] Subsequently, the mice were fed chow (D12450B, Research diets) for 1 week. Then, the mice were sacrificed and the livers were harvested and frozen in liquid nitrogen and stored at −80 °C.

RNA Extraction, Purification, and Detection. The tissues were frozen in liquid nitrogen and ground to a powder. Total RNA was extracted with Trizol reagent (Invitrogen) according to the manufacturer's instruction. To extract tRNA, total RNA was separated by 7.5% PAGE (19:1 acrylamide:bisacrylamide for this and all the following polyacrylamide gels) containing 7 M urea (300 V, 30 min). The gel was stained with SYBR Green I solution (Invitrogen) and visualized by a gel documentation system (Wealtec). tRNA was excised from the gel and then extracted using 0.3 M NH_4Cl and precipitated with glycogen and ethanol as previously described.[24] Subsequently, the extracted tRNA was further purified on a second 7.5% PAGE containing 7 M urea and precipitated with glycogen and ethanol.

To obtain 5.8S and 5S rRNA, total RNA was fractionated with FPLC on a HiLoad superdex 200 (16/60) column (GE Healthcare) at 4 °C. After equilibrated in 10 mM phosphate buffer (pH 6.5) containing 100 mM NaCl, chromatography was performed at 1.5 mL/min, collecting 3 mL fractions. All the fractions were precipitated with glycogen and ethanol, and the fractions containing 5.8S or 5S RNA were combined. Subsequently, the combined RNAs were purified by 7.5% PAGE containing 7 M urea (300 V, 30 min) and further purified by HPLC two to three times using a proteomix SAX-NP column (4.6 mm × 150 mm, 5 μm; Sepax) with a linear gradient (solvent A, 25 mM Tris-Cl, pH 9.0; solvent B, 1 M LiCl in solvent A; 55−90% solvent B, 50 min) at a flow rate of 0.8 mL/min and monitored by UV detector at 260 nm. Finally, the 5.8S and 5S rRNA samples were purified by 15% native PAGE (100 V, 5h) and 7.5% PAGE containing 7 M urea (300 V, 30 min) respectively. 5.8S and 5S rRNA were excised from the gel and then extracted using 0.3 M NH_4Cl and precipitated with glycogen and ethanol as previously described.[24]

To extract small RNA sized less than 60 nt, the large RNAs were removed from total RNA by selective precipitation with 5% polyethylene glycol 8000 in 0.5 M NaCl (final concentration).[24] Small RNAs were precipitated from the supernatants by the addition of 3 volumes of absolute ethanol and 0.1 volume of 3 M sodium acetate (pH 5.2) with incubation overnight at −80 °C. Then the small RNAs were separated by 15% PAGE containing 7 M urea (300 V, 35 min). The gel was stained with SYBR Green I solution and visualized by gel documentation system (Wealtec). Small RNAs sized at 30−60 nt and 10−30 nt were excised, respectively, from the gel[25,26] and then extracted using 0.3 M NH_4Cl and precipitated with glycogen and ethanol.

The quality of the purified small RNAs were determined on an Agilent 2100 Bioanalyzer using a small RNA Kit (Agilent).

Northern Blot Analysis. Northern blot was performed as previously described[27] with minor modifications. Briefly, total RNA (1.2 μg), 5.8S rRNA (0.05 μg), 5S-1 rRNA (0.1 μg), 5S-2 rRNA (0.1 μg), and tRNA (0.8 μg) prepared from C57BL/6 mouse liver were electrophoresed through a 7.5% polyacrylamide gel containing 7 M urea (300 V, 30 min). Then nucleic acids were transferred onto positively charged nylon membranes (Roche) in 1× Tris-borate-EDTA buffer at 100 mA for 1 h. Subsequently, the membranes were air-dried and UV cross-linked. The hybridization procedure was carried out with the indicated 3′-digoxigenin (DIG) labeled probe at 8 nM in DIG Easy Hyb buffer (Roche) at 42 °C for 16 h. After hybridization, the blot was washed and detected using the DIG-High Prime DNA Labeling and Detection Starter Kit II (Roche). 5′-TTC TTC ATC GAC GCA CGA GC-DIG-3′, 5′-TTA GCT TCC GAG ATC AGA CGA-DIG-3′, and 5′-AGA TCT TCA GTC TAA CGC TCT CC-DIG-3′ were used as 5.8S rRNA, 5S rRNA, and tRNA probes, respectively.

RNA Cloning and Sequencing. The purified 5S-1 and 5S-2 rRNA from C57BL/6 mouse liver were cloned as previously described.[28,29] Briefly, 1 μg of denatured RNA was first ligated to a 3′ adapter (miRNA Cloning Linker-1, rAppCT GTA GGC ACC ATC AAT-3ddC) from IDT with T4 RNA ligase 2 (NEB) in the absence of ATP. Then the ligation product was purified by gel extraction (7.5% PAGE containing

Table 1. LC−MS/MS Parameters for Authentic Nucleosides

number	nucleoside	symbol	DP[a]	CE[b]	CXP[c]	Q1/Q3[d]	RT[e](min)
1	cytidine	C	39	16	8	244.1/112.1	13.93
2	adenosine	A	53	25	9	268.1/136.2	60.37
3	3′-*O*-methyladenosine	3′-OMeA	52	24	10	282.0/136.0	63.04
4	2′-*O*-methylcytidine	Cm	36	16	9	258.1/112.1	26.23
5	5-methylcytidine	m^5C	45	17	8	258.1/126.1	31.43
6	N^6-isopentenyladenosine	i^6A	35	23	12	336.2/204.2	67.64
7	5,2′-*O*-dimethylcytidine	m^5Cm	40	17	10	272.2/126.2	41.14
8	1-methyladenosine	m^1A	58	23	12	282.1/150.2	51.79
9	2-thiocytidine	s^2C	38	17	10	260.1/128.1	17.20
10	N^2,N^2,7-trimethylguanosine	$m^{2,2,7}G$	49	22	14	326.1/194.2	56.65
11	N^4-acetyl-2′-*O*-methylcytidine	ac^4Cm	35	15	13	300.1/154.1	59.21
12	N^6-methyladenosine	m^6A	50	27	12	282.1/150.1	62.65
13	3-methylcytidine	m^3C	40	18	10	258.0/126.0	22.47
14	3′-*O*-methylcytidine	3′-OMeC	35	16	7	258.0/112.0	24.85
15	2′-*O*-methyladenosine	Am	52	22	10	282.1/136.2	64.56
16	N^2,N^2-dimethylguanosine	$m^2{}_2G$	45	19	14	312.1/180.2	57.12
17	5′-*O*-methylthymidine	5′-OMeT	34	13	10	257.1/127.2	61.84
18	2′-*O*-methyluridine	Um	51	13	8	259.1/113.1	31.02
19	inosine	I	33	14	11	269.1/137.1	25.91
20	2′-*O*-methylguanosine	Gm	44	16	11	298.1/152.1	54.80
21	guanosine	G	43	17	12	284.1/152.2	32.17
22	1-methylguanosine	m^1G	43	19	14	298.1/166.1	49.23
23	7-methylguanosine	m^7G	46	19	12	298.2/166.2	28.90
24	N^2-methylguanosine	m^2G	40	17	12	298.1/166.1	50.03
25	3′-*O*-methylinosine	3′-OMeI	34	13	10	283.0/137.0	48.93
26	2-thiouridine	s^2U	35	14	10	261.1/129.1	23.96
27	4-thiouridine	s^4U	34	15	10	261.0/129.0	25.28
28	5-methyluridine	m^5U	42	13	9	259.1/127.1	24.91
29	uridine	U	54	14	9	245.0/113.1	15.17
30	N^4-acetylcytidine	ac^4C	35	16	12	286.1/154.1	46.64
31	3′-*O*-methyluridine	3′-OMeU	34	13	8	259.1/113.1	27.64
32	5-methyl-2-thiouridine	m^5s^2U	36	15	11	275.1/143.1	38.24
33	5-methoxyuridine	mo^5U	43	13	11	275.1/143.1	24.26
34	pseudouridine	Ψ	45	24	10	245.2/125.1	10.49
35	2′-*O*-methylinosine	Im	33	14	11	283.3/137.2	47.76
36	3-methyluridine	m^3U	40	15	10	259.1/127.1	31.49
37	3′-*O*-methylguanosine	3′-OMeG	41	17	7	298.1/152.1	55.15
38	1-methylpseudouridine	$m^1Ψ$	45	15	5	259.0/223.1	15.72
39	5-hydroxymethylcytidine	hm^5C	45	17	10	274.3/142.3	13.39
40	5,2′-*O*-dimethyluridine	m^5Um	40	15	10	273.1/127.1	49.67
41	guanosine (^{13}C,^{15}N)	G (^{13}C,^{15}N)	85	17	13	299.1/162.1	32.04

[a]DP: declustering potential. [b]CE: collision energy. [c]CXP: collision cell exit potential. [d]Q1/Q3: the mass of the precursor ion and the mass of the product ion. [e]RT: retention time. Retention time was measured using a 4.6 mm × 250 mm reversed-phase column in positive mode.

7 M urea, 300 V, 30 min) and subsequently dephosphorylated and phosphorylated with calf intestinal alkaline phosphatase (NEB) and T4 Polynucleotide Kinase (NEB), respectively. A second ligation was performed using a 5′ adapter (5′-TGG AAT TCT CGG GCA CCA AGG T-3′) with T4 RNA ligase 1 (NEB) in the presence of ATP, and then the ligation product was purified by gel extraction (7.5% PAGE containing 7 M urea, 300 V, 30 min) again. The purified ligation product was reverse transcribed with 5′-GAT TGA TGG TGC CTA CAG-3′, amplified by PCR with 5′-GAT TGA TGG TGC CTA CAG-3′ and 5′-TGG AAT TCT CGG GCA CCA AGG T-3′, and was ligated to pGEM-T easy vector (Promega) for sequencing.

Enzymatic Digestion of RNAs. RNA samples (1 μg) were digested with 0.2 U nuclease P1 (Sigma-Aldrich) in 60 μL of 50 mM NH_4OAc, pH 5.3 at 50 °C for 3 h and then treated with 0.04 U phosphodiesterase I (USB) for 2 h at 37 °C. Subsequently, the RNA samples were treated with 2 U alkaline phosphatase (Sigma-Aldrich) for 2 h at 37 °C.[30] Proteins were removed by centrifugation through Nanosep 3K device with Omega membrane (Pall) and the small-molecule fraction was retained. Hydrolysates were lyophilized and stored at −80 °C and were resuspended with 175 ng/mL G (^{13}C, ^{15}N) in 100 μL of 2 mM ammonium acetate for LC−MS/MS analysis.

LC−MS/MS Analysis. Analysis of nucleoside mixtures was performed on an API 4000 Q-TRAP mass spectrometer (Applied Biosystems) with an Agilent 1200 HPLC system and a diode array UV detector (190−400 nm) and equipped with an electrospray ionization source. ESI-MS was conducted in a positive ion mode and the nebulizer gas, auxiliary gas, curtain gas, turbo gas temperature, entrance potential, and ionspray voltage were set at 60 psi, 65 psi, 15 psi, 550 °C, 10 and 5500 V,

dx.doi.org/10.1021/ac4036026 | Anal. Chem. 2013, 85, 12173−12181

respectively. Multiple reaction monitoring (MRM) mode was performed because of its high selectivity and sensitivity attained working with parent-to-product ion transitions. The symbol or abbreviation, the optimum voltage settings for the declustering potential (DP), collision energy (CE), collision cell exit potential (CXP), m/z of the transmitted parent ion (Q1), m/z of the monitored product ion (Q3), and retention time (RT) of each nucleoside are summarized in Table 1.

Chromatographic separation of nucleosides was performed using an Agilent 1200 HPLC system with a ZORBAX SB-Aq reversed-phase column (4.6 mm × 250 mm, 5 μm; Agilent) with a ZORBAX SB-Aq guard column (4.6 mm × 12.5 mm, 5 μm; Agilent). Elution solvents consisted of 2 mM ammonium acetate (solvent A) and methanol (solvent B). The mobile phase gradient was 0−5 min, 0%−5% solvent B; 5−45 min, 5%−15% solvent B; 45−70 min, 15%−100% solvent B. The mobile phase composition was held at 100% solvent B for 5 min prior to re-equilibrating the column for 15 min under initial mobile phase conditions before the next injection. The flow rate was 0.6 mL/min at ambient temperature, and the sample injection volume was 60 μL. One complete mobile phase gradient with a 60 μL solvent A injection was run prior to the analysis of each RNA sample to confirm no memory effect.

Calibration curves were constructed by linear fitting. The ratio of peak area of each nucleoside to G (^{13}C, ^{15}N) was linearly related to the ratio of concentrations of each nucleoside to G (^{13}C, ^{15}N). The indicated nucleoside was measured at the concentrations from 0.005 to 100 ng/mL (0.005, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50, and 100). Calibration curves of 18 nucleosides were established with five of the concentrations, which are the closest to the concentrations in samples. The concentration of G (^{13}C, ^{15}N) was 175 ng/mL.

The intraday and interday test were performed for the four nucleosides Ψ, I, m^{1}A, and Am. Five replicate samples for each nucleoside were measured in the same day to assess the intraday variation of the assay. The interday variation was assessed by injection of five replicate samples of each nucleoside in three different days.

LC−MS/MS data were acquired and processed using Analyst 1.6 software. The nucleoside in each sample with a signal-to-noise ratio no less than 3 and 10 was considered as a detectable nucleoside and a quantifiable nucleoside, respectively.

Statistical Analysis. Data are expressed as mean ± SD of at least three independent experiments, and statistical significance was assessed by Student's t test. Differences were considered statistically significant at $p < 0.05$.

RESULTS

Developing an LC−MS/MS Method for Analysis of 41 Nucleosides. As shown in Figure 1, we developed an LC−MS/MS method for the analysis of 41 authentic nucleosides, including ^{13}C and ^{15}N labeled guanosine used as an internal control (Figure S1, Supporting Information), within 70 min in MRM mode. This mode can be used for simultaneous multi-nucleoside analysis and also provides a high degree of selectivity. LC−MS/MS parameters for all mentioned nucleosides in Figure 1 including collision energy, retention time, m/z of the monitored parent ion and product ion, and so on are summarized in Table 1. Nucleosides are analyzed in a positive ion mode by ESI-MS. All the analyzed modified nucleosides underwent the loss of the sugar group leaving the protonated nucleobase generated by collision-induced dissociation, except Ψ and m^{1}Ψ. The stable C−C glycosidic bond of Ψ and m^{1}Ψ

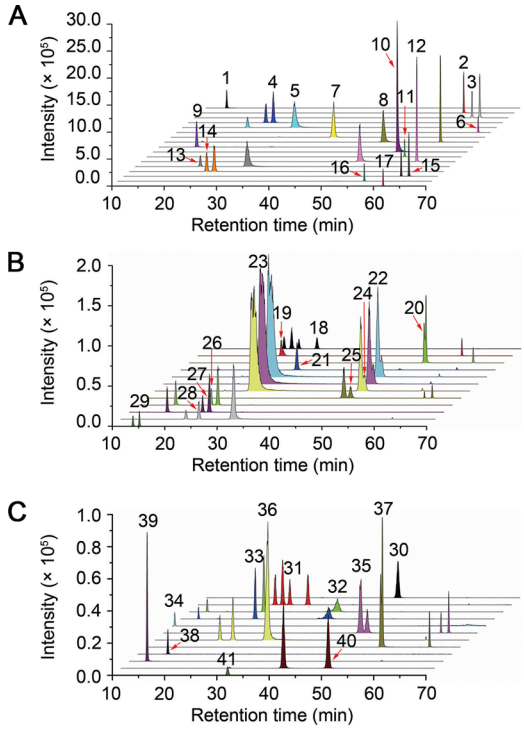


Figure 1. LC−MS/MS chromatogram in MRM mode for the indicated authentic nucleosides. The concentration of each ribonucleoside is 0.03 μg/mL except for isotope labeled guanosine (^{13}C, ^{15}N) (0.35 μg/mL). Numbered peaks are 1, C; 2, A; 3, 3′-*O*MeA; 4, Cm; 5, m^{5}C; 6, i^{6}A; 7, m^{5}Cm; 8, m^{1}A; 9, s^{2}C; 10, m^{2,2,7}G; 11, ac^{4}Cm; 12, m^{6}A; 13, m^{3}C; 14, 3′-*O*MeC; 15, Am; 16, m$^2_2$G; 17, 5′-*O*MeT; 18, Um; 19, I; 20, Gm; 21, G; 22, m^{1}G; 23, m^{7}G; 24, m^{2}G; 25, 3′-*O*MeI; 26, s^{2}U; 27, s^{4}U; 28, m^{5}U; 29, U; 30, ac^{4}C; 31, 3′-*O*MeU; 32, m^{5}s^{2}U; 33, mo^{5}U; 34, ψ; 35, Im; 36, m^{3}U; 37, 3′-*O*MeG; 38, m^{1}ψ; 39, hm^{5}C; 40, m^{5}Um; 41, G (^{13}C, ^{15}N). The MRM transition (Q1/Q3) settings for different nucleosides are given in Table 1

led to fragmentation of the ribose ring, so we used the m/z 245.2/125.1 and 259.0/223.1 for identification and quantification of Ψ and m^{1}Ψ similar as described previously.[17,31] The structures and mass spectra of the 41 nucleosides are shown in Figures S1 and S2 (Supporting Information). The identification of an individual nucleoside was established by comparison of its retention time and mass spectrum with commercially available authentic nucleosides (Table 1 and Figure S2, Supporting Information).[32]

Method Validation. On the basis of the developed method, we performed a series of experiments to validate it. As shown in Table S1 in the Supporting Information, the detection linearity of this method was investigated using 175 ng/mL G (^{13}C, ^{15}N) standard spiked with 18 nucleosides at different concentrations ranging from 0.005 to 100 ng/mL. The calibration curves were constructed by linear fitting. The ratio of peak area of each nucleoside to G (^{13}C, ^{15}N) was linearly related to the ratio of concentrations of each nucleoside to G (^{13}C, ^{15}N). The calibration curve had a good correlation coefficient (R^2) from 0.980 to 0.999. The molar concentrations of nucleosides were calculated from the obtained area ratios and the linear equations of the calibration curves (Table S1, Supporting Information).

The intraday and interday test were performed for the four nucleosides Ψ, I, m^{1}A, and Am. Five replicate samples for each nucleoside were measured in the same day to assess the intraday variation of this assay. The interday variation was assessed by

dx.doi.org/10.1021/ac4036026 | Anal. Chem. 2013, 85, 12173−12181

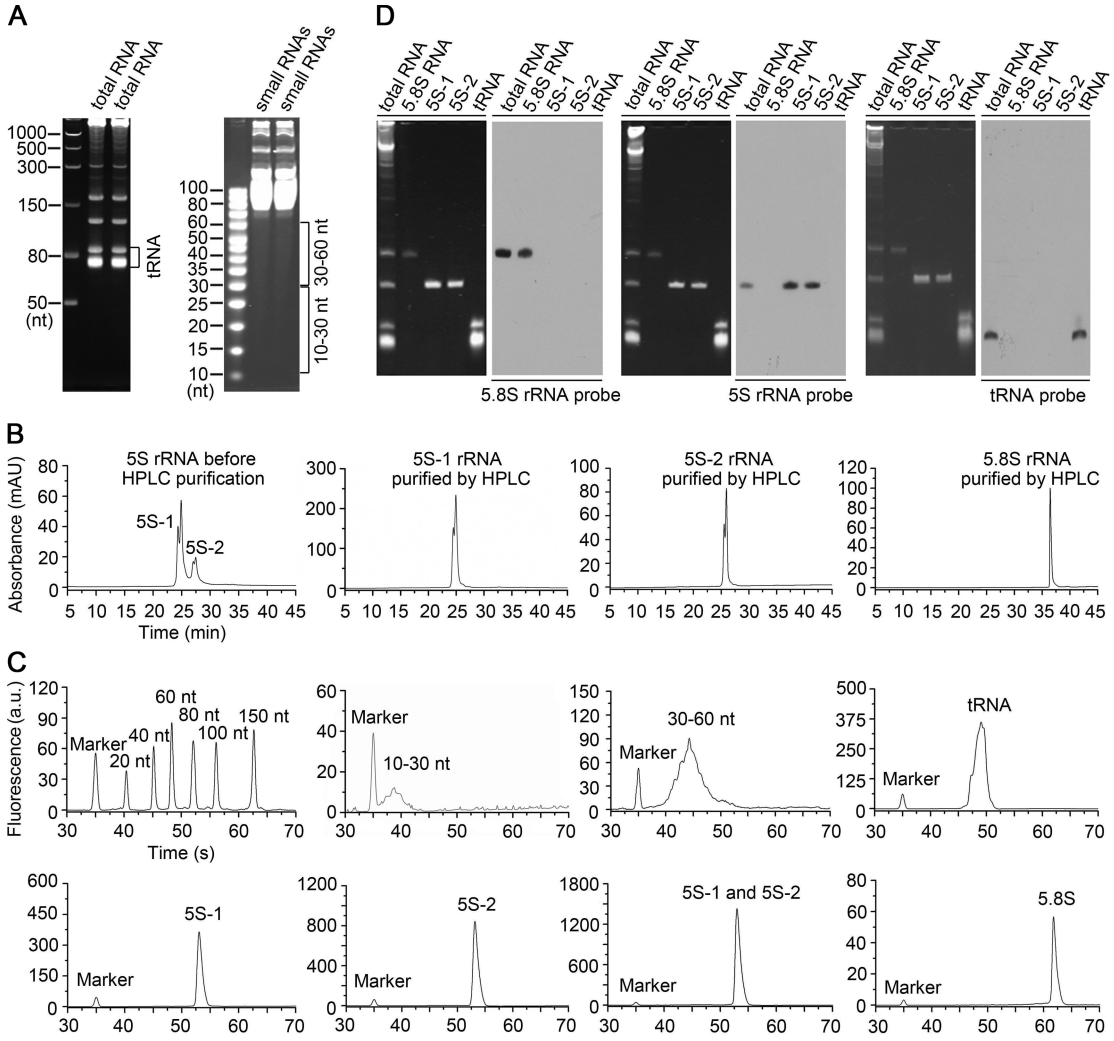


Figure 2. Analysis of purified small RNAs from mouse liver. (A) The indicated small RNAs were excised and purified from 7.5% (left panel) and 15% (right panel) denaturing PAGEs and visualized by SYBR green I staining. (B) HPLC analysis of 5S or 5.8S rRNA before and/or after HPLC purification. Notably, two groups of 5S rRNA peaks were observed and purified. (C) Agilent 2100 Bioanalyzer electrophoretic profiles of small RNA ladder, the purified small RNA sized at 10−30 and 30−60 nt, tRNA, 5S-1, 5S-2, 5S rRNA (mixed 5S-1 and 5S-2 rRNA) and 5.8S rRNA. (D) Northern blot analysis of the purified tRNA, 5S-1, 5S-2, and 5.8S rRNA. Total RNA and the indicated purified small RNAs were visualized with SYBR green I in 7.5% denaturing PAGE and detected by Northern blot analysis with the indicated probes.

injection of five replicate samples of each nucleoside in three different days. As shown in Table S2 (Supporting Information), the relative standard deviations are less than 14% and the relative errors are less than 12%, respectively.

Purification of Small RNAs. tRNA, 5.8S, and 5S rRNA and small RNAs sized at 30−60 nt and 10−30 nt in mouse liver were isolated and purified by PAGE, FPLC, and/or HPLC as described in the Experimental Section. As shown in Figure 2A, tRNA and small RNAs sized at 30−60 nt and 10−30 nt were purified by denatured PAGE. To obtain 5.8S and 5S rRNA, total RNA was first isolated and purified by FPLC and denatured PAGE. Interestingly, when subsequently analyzed by HPLC, two groups of peaks for 5S rRNA were observed and were designated as 5S-1 and 5S-2 rRNA, respectively (the left panel of Figure 2B). Then the 5S-1, 5S-2, and 5.8S rRNA were purified by HPLC (the right three panels of Figure 2B). Moreover, 5S-1, 5S-2, and 5.8S rRNA further purified by PAGE and other purified small RNAs were analyzed with an Agilent 2100 Bioanalyzer (Figure 2C). In addition, the purified tRNA, 5S-1, 5S-2, and 5.8S rRNA were confirmed by Northern blot (Figure 2D), and both 5S-1 and 5S-2 rRNA were cloned and confirmed by sequencing.

Analysis of Modified Nucleosides in Mouse Small RNAs by LC−MS/MS. For the purified small RNAs, we first analyzed the modified nucleoside compositions of tRNA molecules, which are abundant and contain a wide variety of modifications.[33,34] The bulk tRNA samples isolated from mouse liver were fully hydrolyzed to nucleosides using nuclease P1, phosphodiesterase I, and alkaline phosphatase, then mixed with stable isotope-labeled guanosine (^{13}C, ^{15}N), and analyzed by LC−MS/MS. With this method, we revealed 23 modified nucleosides (ψ, Am, Cm, Um, Gm, I, m^7G, m^2G, m^5U, m^5C, m^1A, $m^2{}_2G$, m^1G, $m^{2,2,7}G$, m^3C, ac^4C, i^6A, hm^5C, m^5Cm, m^5Um, m^3U, Im, and m^6A) in the tRNA from mouse liver (Figure 3A). Compared to the reported RNA modification database,[1] six modifications, including $m^{2,2,7}G$, m^3U, hm^5C, Im, m^6A, and m^5Cm, were observed for the first time in eukaryotic tRNA (Figure 3A), and 12 modifications were observed for the first time in mouse liver tRNA (Figure 3A and Table S3 in the Supporting Information) compared to previous findings in mouse liver.[35]

dx.doi.org/10.1021/ac4036026 | Anal. Chem. 2013, 85, 12173−12181

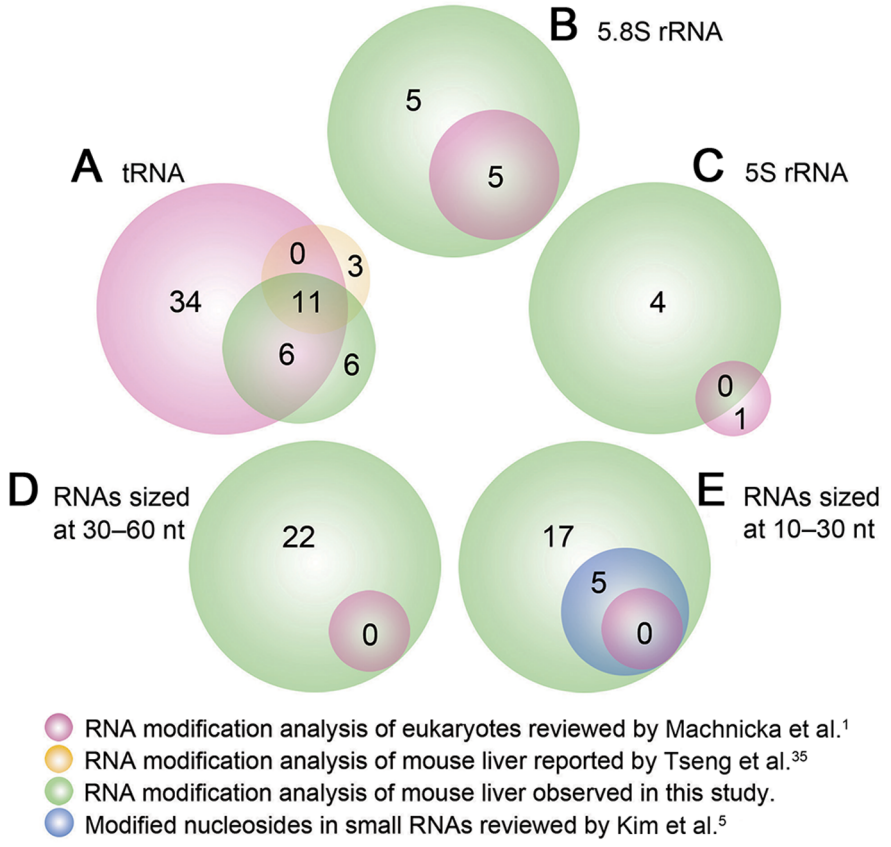


Figure 3. Venn diagrams depicting the number of nucleoside modifications detected in small RNAs from different studies.

A representative LC−MS/MS chromatogram of modified nucleosides in the tRNA sample was shown in Figure S3 (Supporting Information).

Similar analyses were performed with other kinds of small RNAs including 5.8S rRNA, 5S rRNA, small RNAs sized at 30−60 nt and 10−30 nt from mouse liver. To our surprise, the 5.8S rRNA sample contained not only the five reported modified nucleosides (ψ, Am, Cm, Um, Gm)[2,3] but also five other modified nucleosides (m^1A, m^3C, $m^2{}_2G$, m^6A, $m^{2,2,7}G$) (Figure 3B and Table S3 in the Supporting Information). Analysis of the 5S-1 and 5S-2 rRNA samples revealed four modified nucleosides including Am, Cm, m^3C, and m^6A in 5S rRNA (Figure 3C and Table S3 in the Supporting Information).[2,4] Notably, the most abundant modified nucleoside ψ in mouse liver tRNA was not detected in 5S rRNA. Representative LC−MS/MS chromatograms of modified nucleosides in 5.8S, 5S-1, and 5S-2 rRNA were shown in Figures S4 and S5 (Supporting Information). Furthermore, we found many modifications (ψ, Am, Cm, Um, Gm, I, m^7G, m^2G, m^5U, m^5C, m^1A, $m^2{}_2G$, m^1G, $m^{2,2,7}G$, m^3C, ac^4C, i^6A, hm^5C, m^5Cm, m^5Um, m^3U, and m^6A) in small RNAs sized at 30−60 nt and 10−30 nt (Figure 3D,E and Table S3 in the Supporting Information). The modifications of RNAs sized at 30−60 nt such as half-tRNA molecules are still largely unknown.[36] Our data implicate that half-tRNA molecules or other small RNAs sized at 30−60 nt might contain some modifications as described above. A representative LC−MS/MS chromatogram of modified nucleosides in small RNAs sized at 30−60 nt was shown in Figure S6 (Supporting Information). Recent evidence from *Arabidopsis*, *Drosophila*, mouse, and rat has demonstrated that small RNAs sized at 10−30 nt such as miRNA, siRNA, and piRNA contain methylation modifications including Am, Cm, Um, and Gm.[11,37] Our results implicate that miRNA or other small RNAs sized at 10−30 nt might contain much more modifications than previously reported. A representative LC−MS/MS chromatogram of modified nucleosides in small RNAs sized at 10−30 nt was shown in Figure S7 (Supporting Information). Our results suggest that small RNAs sized at 10−60 nt contain much more modifications than previously reported.[1]

To assess the relative abundance of modified nucleosides in different types of small RNAs, we further quantified about 18 modifications according to their calibration curves using stable isotope labeled guanosine (^{13}C, ^{15}N) as an internal standard (Table S1, Supporting Information). For comparison, the modified nucleosides in total RNA were also quantified. The results of the quantification are depicted in Figure 4 and Table S4 (Supporting Information). The most abundant modified nucleoside in tRNA is Ψ. This result is consistent with the previous report that Ψ is the most abundant modification in tRNA molecules from mouse liver.[35] The abudance of modified nucleosides in 5.8S, 5S-1, and 5S-2 rRNA was also shown in Figure S8 (Supporting Information). In general, the abundance of many modified nucleosides in different types of small RNA are significantly different, suggesting specific modification patterns in different types of small RNA. The abundance of all the quantified modifications in small RNA sized at 10−30 nt is about 41% to that of G, suggesting that various modifications might have occurred in mammal miRNA, siRNA, piRNA, and other small RNAs sized at 10−30 nt.

Comparison of Modified Nucleoside Levels in Small RNAs from Normal and Diabetic Mouse Liver. To investigate the correlation between small RNA modifications

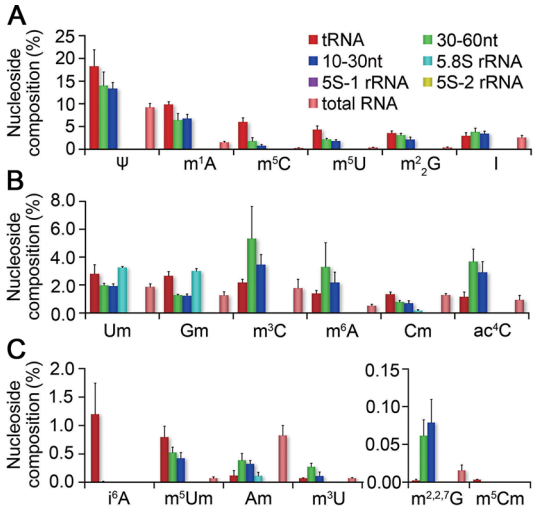


Figure 4. Comparison of modified nucleoside levels in the indicated small RNAs and total RNA. The modified nucleoside compositions (mol %) were normalized to guanosine. Error bars represent standard deviations. n = 7 for tRNA, small RNAs sized at 30−60 nt or 10−30 nt, 5S-2 RNA, and total RNA from normal mice; n = 6 for 5.8S and 5S-1 RNAs from normal mice.

and diabetes, small RNAs from diabetic *db*/*db* mouse liver were also analyzed. The levels of Gm and m^5Cm in tRNA were significantly decreased and increased, respectively, in diabetic mouse liver when compared with those in normal mouse liver (Figure 5A). The levels of Cm and Am in small RNAs sized

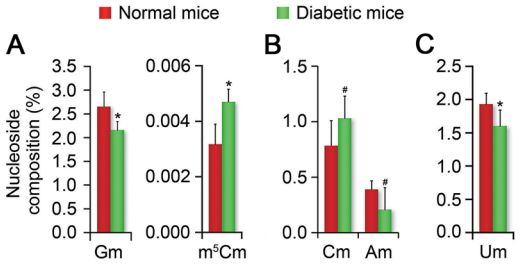


Figure 5. Comparison of modified nucleoside levels in small RNAs from the liver of normal and diabetic mice. Significant differences were observed in tRNA (A) and small RNAs sized at 30−60 nt (B) and 10−30 nt (C). The nucleoside compositions (mol %) were normalized to guanosine. Error bars represent standard deviations. n = 7 for small RNAs from normal mice; n = 3 for small RNAs sized at 30−60 nt or 10−30 from diabetic mice; n = 6 for tRNA from diabetic mice. $^{\#}p < 0.05$, $^{*}p < 0.01$ compared with corresponding nucleoside from normal mice.

at 30−60 nt were increased and decreased, respectively, in diabetic mouse liver (Figure 5B). Um in small RNAs sized at 10−30 nt was significantly decreased in diabetic mouse liver (Figure 5C). These data show that modifications of small RNAs are correlated with diabetes and suggest that modifications of small RNAs might be involved in the development of diabetes.

DISCUSSION

Although there is a long history of investigation of tRNA modification, the modification in other small RNAs such as 5.8S rRNA, 5S rRNA, miRNA, and piRNA is still largely unknown. Our present study is the first one to systematically analyze the modifications of several small RNAs including tRNA, 5.8S rRNA, 5S rRNA, small RNAs sized at 30−60 nt and 10−30 nt using LC−MS/MS. This method offers good sensitivity, selectivity, precision, and accuracy for the identification and quantification of modified nucleosides in a high-throughout manner. With this method, we found more modifications in these small RNAs than previously reported.[11,36,37] The discovery of diverse modifications in different small RNAs and their correlation with diabetes suggests that modifications in these small RNAs are important for their biological functions. Future studies to identify the genes and enzymes responsible for these modifications, such as using a reverse genetic approach combined with mass spectrometry,[38] will help to reveal the biological functions of these modifications.

It has been reported that 5S rRNA has different conformations, which is evidenced by various methods including solution X-ray scattering, NMR, and biochemical methods.[39−41] Similarly, we also found that 5S rRNA showed two groups of peaks when analyzed by HPLC with an ion exchange column. Each group of peaks should be 5S rRNA, according to the results of Northern blot, reverse transcription, cloning, and sequencing. They have no significant difference when analyzed by 7.5% urea-PAGE or Agilent 2100 Bioanalyzer. However, the abundance of some detected modified nucleosides in each group of peaks is significantly different, which implicates that modified nucleosides have some contribution for the formation of two groups of 5S rRNA peaks.

It has been shown that miRNAs are present in serum or plasma in a remarkable stable form and they are resistant to RNase A digestion and other harsh conditions such as boiling, low or high pH, extended storage and freeze−thaw cycles.[42−44] However, the mechanisms of the serum miRNA stability are still unclear. One possibile explanation is the modifications of miRNAs, which make them resistant to RNase activity.[43] It was reported that nucleoside modifications in some RNAs such as tRNA and rRNA may protect RNA molecules from unspecific degradation by bases and nucleases.[45−48] In plants, the 3′ end methylation of miRNAs and siRNAs is crucial for their stability.[49] These reports suggest that modifications in miRNAs, siRNAs, and piRNAs of mammals may also contribute to their stability similarly. Our observation of more than 20 modifications occurred in small RNA sized 10−30 nt provides further support that modifications may contribute to their stability. The stability of small RNA is very important for their potential therapeutic application.[50,51] Presently, the potential of small RNA drugs is still limited, because of their very low stability in biological fluids.[49,52] Various chemical modifications have been tried to increase the stability of small RNA; however, the modified small RNAs are still not stable enough or have reduced specificity or other side effects.[49,52,53] Therefore, using different or multiple native modifications to stabilize small RNA should be applaudable. Our findings provide the possibility to increase small RNA stability with the abundant detected modified nucleosides in small RNA sized at 10−30 nt. Future studies to further elucidate the modifications in some specific stable small RNAs will be beneficial for the improvement of small RNA stability and the development of small RNA drugs.

It is well-known that protein modification is closely related with many diseases, but the knowledge of the correlation between RNA modification and diseases is limited. It has been reported that impaired rRNA pseudouridylation is associated with dyskeratosis congenita and cancer.[19] 8-Hydroxyguanosine, a marker of oxidative damage to RNA, is correlated with Alzheimer's disease,[20,54] and tRNA anticodon base modification

dx.doi.org/10.1021/ac4036026 | Anal. Chem. 2013, 85, 12173−12181

defect is involved in a mitochondrial disease.[55] Defect in biosynthesis of ms^2t^6A in tRNA of pancreatic β cells results in a problem in the quality control of protein translation and is relevant to type 2 diabetes.[21] These reports suggest that RNA modifications are associated with diseases. However, whether the modifications of small RNA sized at 10−30 nt, such as miRNA, siRNA, and piRNA, are also correlated with diseases are yet to be elucidated. In this study, we systematically investigate the correlation of small RNA modifications with diabetes using the mouse model and found a few modified nucleosides are significantly changed in tRNA, small RNAs sized at 30−60 nt and 10−30 nt from diabetic mouse liver (Figure 5). Especially, we found that Um was downregulated in small RNA sized at 10−30 nt from diabetic mouse liver, which suggests that Um in these hepatic small RNAs is correlated with diabetes. Further studies focused on RNA modification, especially small RNA modification, in different diseases will shed new light on the pathogenesis of different diseases.

CONCLUSION

A high-performance liquid chromatography−electrospray ionization tandem mass spectrometry method was developed for simultaneously detecting 41 nucleosides in small RNA samples. This method offers good sensitivity, selectivity, precision, and accuracy for the identification and quantification of modified nucleosides in a high-throughput manner. To our knowledge, this study is the first one to systematically analyze the modifications of various small RNAs including tRNA, 5.8S rRNA, 5S rRNA, small RNAs sized at 30−60 nt and 10−30 nt using an LC−MS/MS method. With this method, we found more modifications in these small RNAs than previously reported.[11,36,37] Especially, we revealed many modifications in small RNA sized at 10−30 nt, which provides new clues for the potential role of various modifications in noncoding small RNAs, such as miRNA, siRNA, and piRNA. Moreover, we demonstrate the correlation of various small RNA modifications with diabetes, which provides new insights for understanding the pathogenesis of diabetes.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*Phone: +86 21 5492 0903. Fax: +86 21 5492 0291. E-mail: qwzhai@sibs.ac.cn.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We gratefully acknowledge Zi Li for her help in the LC−MS/MS analysis. This research was supported by grants from National Natural Science Foundation of China (Grants 30825009, 31030022, 81321062, 30900250, and 31200591), the National Basic Research Program of China (Grants 2014CB542300 and 2009CB918403), China Postdoctoral Science Foundation (Grant 20080440658), Program of Shanghai Subject Chief Scientist (Grant 11XD1405800), and Shanghai Xuhui Central Hospital.

REFERENCES

(1) Machnicka, M. A.; Milanowska, K.; Osman Oglou, O.; Purta, E.; Kurkowska, M.; Olchowik, A.; Januszewski, W.; Kalinowski, S.; Dunin-Horkawicz, S.; Rother, K. M.; Helm, M.; Bujnicki, J. M.; Grosjean, H. *Nucleic Acids Res.* **2013**, *41*, D262−D267.
(2) Meng, Z.; Limbach, P. A. *Brief. Funct. Genomics Proteomics* **2006**, *5*, 87−95.
(3) MacKay, R. M.; Doolittle, W. F. *Nucleic Acids Res.* **1981**, *9*, 3321−3334.
(4) Miyazaki, M. *J. Biol. Chem.* **1974**, *75*, 1407−1410.
(5) Kim, Y. K.; Heo, I.; Kim, V. N. *Cell* **2010**, *143*, 703−709.
(6) Luciano, D. J.; Mirsky, H.; Vendetti, N. J.; Maas, S. *RNA* **2004**, *10*, 1174−1177.
(7) Yu, B.; Yang, Z.; Li, J.; Minakhina, S.; Yang, M.; Padgett, R. W.; Steward, R.; Chen, X. *Science* **2005**, *307*, 932−935.
(8) Mui Chan, C.; Zhou, C.; Brunzelle, J. S.; Huang, R. H. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 17699−17704.
(9) Chen, X. *Nat. Struct. Mol. Biol.* **2007**, *14*, 259−260.
(10) Ohara, T.; Sakaguchi, Y.; Suzuki, T.; Ueda, H.; Miyauchi, K.; Suzuki, T. *Nat. Struct. Mol. Biol.* **2007**, *14*, 349−350.
(11) Saito, K.; Sakaguchi, Y.; Suzuki, T.; Siomi, H.; Siomi, M. C. *Genes Dev.* **2007**, *21*, 1603−1608.
(12) Houwing, S.; Kamminga, L. M.; Berezikov, E.; Cronembold, D.; Girard, A.; van den Elst, H.; Filippov, D. V.; Blaser, H.; Raz, E.; Moens, C. B.; Plasterk, R. H.; Hannon, G. J.; Draper, B. W.; Ketting, R. F. *Cell* **2007**, *129*, 69−82.
(13) Karijolich, J.; Kantartzis, A.; Yu, Y. T. *Methods Mol. Biol.* **2010**, *629*, 1−19.
(14) Gustilo, E. M.; Vendeix, F. A.; Agris, P. F. *Curr. Opin. Microbiol.* **2008**, *11*, 134−140.
(15) Yarian, C.; Townsend, H.; Czestkowski, W.; Sochacka, E.; Malkiewicz, A. J.; Guenther, R.; Miskiewicz, A.; Agris, P. F. *J. Biol. Chem.* **2002**, *277*, 16391−16395.
(16) Karikó, K.; Buckstein, M.; Ni, H.; Weissman, D. *Immunity* **2005**, *23*, 165−175.
(17) Chan, C. T.; Dyavaiah, M.; DeMott, M. S.; Taghizadeh, K.; Dedon, P. C.; Begley, T. J. *PLoS Genetics* **2010**, *6*, e1001247.
(18) Björk, G. R.; Huang, B.; Persson, O. P.; Byström, A. S. *RNA* **2007**, *13*, 1245−1255.
(19) Ruggero, D.; Grisendi, S.; Piazza, F.; Rego, E.; Mari, F.; Rao, P. H.; Cordon-Cardo, C.; Pandolfi, P. P. *Science* **2003**, *299*, 259−262.
(20) Nunomura, A.; Perry, G.; Pappolla, M. A.; Wade, R.; Hirai, K.; Chiba, S.; Smith, M. A. *J. Neurosci.* **1999**, *19*, 1959−1964.
(21) Wei, F. Y.; Suzuki, T.; Watanabe, S.; Kimura, S.; Kaitsuka, T.; Fujimura, A.; Matsui, H.; Atta, M.; Michiue, H.; Fontecave, M.; Yamagata, K.; Suzuki, T.; Tomizawa, K. *J. Clin. Invest.* **2011**, *21*, 3598−3608.
(22) Poy, M. N.; Hausser, J.; Trajkovski, M.; Braun, M.; Collins, S.; Rorsman, P.; Zavolan, M.; Stoffel, M. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 5813−5818.
(23) Sun, C.; Zhang, F.; Ge, X.; Yan, T.; Chen, X.; Shi, X.; Zhai, Q. *Cell Metab.* **2007**, *6*, 307−319.
(24) Nashimoto, M. *RNA* **1996**, *2*, 523−534.
(25) Ciechanover, A.; Wolin, S. L.; Steitz, J. A.; Lodish, H. F. *Proc. Natl. Acad. Sci.* **1985**, *82*, 1341−1345.
(26) Bullerwell, C. E.; Schnare, M. N.; Gray, M. W. *RNA* **2003**, *9*, 287−292.
(27) Kim, S. W.; Li, Z.; Moore, P. S.; Monaghan, A. P.; Chang, Y.; Nichols, M.; John, B. *Nucleic Acids Res.* **2010**, *38*, e98.
(28) Zhang, X. H.; Chiang, V. L. *Nucleic Acids Res.* **1996**, *24*, 990−991.
(29) Viollet, S.; Fuchs, R. T.; Munafo, D. B.; Zhuang, F.; Robb, G. B. *BMC Biotechnol.* **2011**, *11*, 72.
(30) Crain, P. F. *Methods Enzymol.* **1990**, *193*, 782−790.
(31) Dudley, E.; Tuytten, R.; Bond, A.; Lemière, F.; Brenton, A. G.; Esmans, E. L.; Newton, R. P. *Rapid Commun. Mass Spectrom.* **2005**, *19*, 3075−3085.
(32) Pomerantz, S. C.; McCloskey, J. A. *Methods Enzymol.* **1990**, *193*, 796−824.

(33) Brückl, T.; Globisch, D.; Wagner, M.; Müller, M.; Carell, T. *Angew. Chem., Int. Ed.* **2009**, *48*, 7932−7934.
(34) Kowalak, J. A.; Pomerantz, S. C.; Crain, P. F.; McCloskey, J. A. *Nucleic Acids Res.* **1993**, *21*, 4577−4585.
(35) Tseng, W. C.; Medina, D.; Randerath, K. *Cancer Res.* **1978**, *38*, 1250−1257.
(36) Horwich, M. D.; Li, C.; Matranga, C.; Vagin, V.; Farley, G.; Wang, P.; Zamore, P. D. *Curr. Biol.* **2007**, *17*, 1265−1272.
(37) Kirino, Y.; Mourelatos, Z. *Nat. Struct. Mol. Biol.* **2007**, *14*, 347−348.
(38) Suzuki, T.; Ikeuchi, Y.; Noma, A.; Suzuki, T.; Sakaguchi, Y. *Methods Enzymol.* **2007**, *425*, 211−229.
(39) Kime, M. J.; Moore, P. B. *Nucleic Acids Res.* **1982**, *10*, 4973−4983.
(40) Müller, J. J.; Misselwitz, R.; Zirwer, D.; Damaschun, G.; Welfle, H. *Eur. J. Biochem.* **1985**, *148*, 89−95.
(41) Göringer, H. U.; Szymkowiak, C.; Wagner, R. *Eur. J. Biochem.* **1984**, *144*, 25−34.
(42) Chen, X.; Ba, Y.; Ma, L.; Cai, X.; Yin, Y.; Wang, K.; Guo, J.; Zhang, Y.; Chen, J.; Guo, X.; Li, Q.; Li, X.; Wang, W.; Zhang, Y.; Wang, J.; Jiang, X.; Xiang, Y.; Xu, C.; Zheng, P.; Zhang, J.; Li, R.; Zhang, H.; Shang, X.; Gong, T.; Ning, G.; Wang, J.; Zen, K.; Zhang, J.; Zhang, C. Y. *Cell Res.* **2008**, *18*, 997−1006.
(43) Mitchell, P. S.; Parkin, R. K.; Kroh, E. M.; Fritz, B. R.; Wyman, S. K.; Pogosova-Agadjanyan, E. L.; Peterson, A.; Noteboom, J.; O'Briant, K. C.; Allen, A.; Lin, D. W.; Urban, N.; Drescher, C. W.; Knudsen, B. S.; Stirewalt, D. L.; Gentleman, R.; Vessella, R. L.; Nelson, P. S.; Martin, D. B.; Tewari, M. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 10513−10518.
(44) Keller, A.; Leidinger, P.; Gislefoss, R.; Haugen, A.; Langseth, H.; Staehler, P.; Lenhof, H. P.; Meese, E. *RNA Biol.* **2011**, *8*, 506−516.
(45) Steinberg, S.; Cedergren, R. *RNA* **1995**, *1*, 886−891.
(46) Metodiev, M. D.; Lesko, N.; Park, C. B.; Cámara, Y.; Shi, Y.; Wibom, R.; Hultenby, K.; Gustafsson, C. M.; Larsson, N. G. *Cell Metab.* **2009**, *9*, 386−397.
(47) Motorin, Y.; Helm, M. *Biochemistry* **2010**, *49*, 4934−4944.
(48) Kumar, R. K.; Davis, D. R. *Nucleic Acids Res.* **1997**, *25*, 1272−1280.
(49) Ghildiyal, M.; Zamore, P. D. *Nat. Rev. Genet.* **2009**, *10*, 94−108.
(50) Heidenreich, O.; Pieken, W.; Eckstein, F. *FASEB J.* **1993**, *7*, 90−96.
(51) Morrissey, D. V.; Lockridge, J. A.; Shaw, L.; Blanchard, K.; Jensen, K.; Breen, W.; Hartsough, K.; Machemer, L.; Radka, S.; Jadhav, V.; Vaish, N.; Zinnen, S.; Vargeese, C.; Bowman, K.; Shaffer, C. S.; Jeffs, L. B.; Judge, A.; MacLachlan, I.; Polisky, B. *Nat. Biotechnol.* **2005**, *23*, 1002−1007.
(52) Musacchio, T.; Vaze, O.; D'Souza, G.; Torchilin, V. P. *Bioconjugate Chem.* **2010**, *21*, 1530−1536.
(53) Corey, D. R. *J. Clin. Invest.* **2007**, *117*, 3615−3622.
(54) Honda, K.; Smith, M. A.; Zhu, X.; Baus, D.; Merrick, W. C.; Tartakoff, A. M.; Hattier, T.; Harris, P. L.; Siedlak, S. L.; Fujioka, H.; Liu, Q.; Moreira, P. I.; Miller, F. P.; Nunomura, A.; Shimohama, S.; Perry, G. *J. Biol. Chem.* **2005**, *280*, 20978−20986.
(55) Yasukawa, T.; Suzuki, T.; Ishii, N.; Ohta, S.; Watanabe, K. *EMBO J.* **2001**, *20*, 4794−4802.