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Characterization of Amplifiable, Circulating RNA in Plasma and Its Potential as a Tool for Cancer Diagnostics

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Background: **Several recent reports have described the detection of circulating, cancer-related RNA molecules in serum or plasma from cancer patients, but little is known about the biology of this extracellular RNA. We aimed to determine how RNA is protected against degradation in serum, to optimize RNA isolation from large volumes of serum, and to test our optimized assays for serum-based cancer detection.**

Methods: **We used quantitative reverse transcription-PCR (QRT-PCR) analysis to investigate the isolation and biology of extracellular plasma RNA. We then examined the presence of amplifiable RNA transcripts in plasma and serum from controls and from patients with esophageal cancer and malignant melanoma.**

Results: **We found that extracellular RNA in plasma is highly degraded and can be isolated most efficiently by guanidinium–phenol extraction followed by precipitation. Extracellular RNA is stable in serum for up to 3 h but is destroyed immediately by addition of detergents. Extracellular RNA can be captured on 0.2 μm filters, allowing concentration of RNA from several milliliters of plasma. When we concentrated RNA from up to 4 mL of serum, detection of cancer-related transcripts in serum from cancer patients and controls was infrequent and inconsistent.**

Conclusions: **Extracellular RNA is most likely protected within protein or lipid vesicles, possibly apoptotic bodies, which can be disrupted by detergents. Despite optimizing many aspects of plasma RNA detection, we were unable to reproducibly detect cancer-related transcripts. Our data suggest that measurement of circulating RNA may not be a good approach to early cancer diagnosis.**

Despite substantial advances in cancer diagnostics, the search persists for simple and cost-effective diagnostic tests. Many studies have explored the possibility of using circulating tumor cells for detection or monitoring of cancer *(1–6)*. Although circulating tumor cells are frequently detectable in blood from patients with advanced stages of disease, this avenue of research has been unable to demonstrate reliable utility for early cancer detection or recurrence monitoring. Another approach, pioneered by Nawroz et al. *(7)* and also Chen et al. *(8)*, has focused on detection of cancer-specific alterations in extracellular DNA present in the serum or plasma. In tumors with certain DNA alterations, the serum approach has been able to detect those same DNA alterations in a significant percentage of cancer patients while maintaining good specificity in noncancer controls *(9–11)*. Although promising, major hurdles remain to be overcome before detection of serum DNA alterations can be considered a sensitive and cost-effective approach to cancer detection.

Although first reported decades ago *(12, 13)*, the possibility that extracellular RNA could survive in the blood was not widely accepted because plasma contains potent ribonucleases that should, in theory, destroy any free RNA *(14)*. Very recently, however, a few reports have documented the presence of circulating extracellular RNA in serum, and it has also been shown that this RNA is somehow protected from degradation by plasma ribonucleases *(15, 16)*. Furthermore, using nested reverse

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transcription-PCR (RT-PCR),[3] studies have demonstrated the ability to amplify tumor-related mRNAs from sera of patients with melanoma, breast cancer, and other malignancies *(17–21)*. Although provocative, the methods described in these reports achieved diagnostic sensitivities of only 25–78%, insufficient for clinical use in cancer detection. This is probably attributable in part to our poor understanding of the nature of the circulating RNA and the fact that RNA isolation from plasma is difficult and probably suboptimal with current methods. In addition, most studies isolated RNA from only small amounts of plasma/serum (50–500 μL), probably because of difficulties in scaling up the method for RNA isolation from larger volumes.

Very few reports have focused on the nature of circulating extracellular RNA and the possible mechanisms by which RNA is protected from plasma RNase activity. One mechanism that has been suggested is that extracellular RNA and DNA are bound to each other in the plasma *(22)*. Such a DNA–RNA hybrid would be resistant to both RNase and DNase activity. An alternative hypothesis is that the RNA is protected through binding to protein/lipoprotein complexes or that it is sequestered within lipid vesicles *(23, 24)*. The latter concept is supported by the demonstration that serum RNA content is greatly reduced (presumably trapped) when the serum is passed through 0.2 μm filters *(16)*. Furthermore, apoptotic bodies/vesicles containing RNA have been identified in cultured tumor cell lines *(25, 26)*, and the RNA in these vesicles was resistant to RNase digestion as long as the vesicles remained intact. It is possible that a similar RNA protection mechanism exists in vivo after either active release of RNA-containing vesicles or during programmed cell death. In either case, the protected RNA would enter the extracellular spaces and be drained into the lymph system and then into the blood.

In this study, we investigated the nature of circulating RNA and tested possible mechanisms for its protection against plasma RNase activity. In an attempt to improve sensitivity for RNA detection in plasma, we also compared RNA isolation efficiency, using many of the available RNA isolation protocols and extraction reagents. These methods were evaluated for RNA isolation efficiency and also for the ability to perform large-scale plasma RNA isolation. Finally, we applied this knowledge to detection of MART-1 and tyrosinase mRNAs in patients with melanoma and to detection of cytokeratins 19 and 20 in patients with esophageal cancer.

[3] Nonstandard abbreviations: RT-PCR, reverse transcription-PCR; GERD, gastro-esophageal reflux disease; GIT, guanidinium isothiocyanate; QRT-PCR, quantitative reverse transcription-PCR; β-gal, galactosidase; β-GUS, β-glucuronidase; SDS, sodium dodecyl sulfate; and CK, cytokeratin; FAM, 6-carboxyfluorescein; and TET, tetrachloro-6-carboxyfluorescein.

Materials and Methods

SAMPLE COLLECTION AND PATIENT POPULATION

All samples were collected from consenting individuals under protocols approved by the University of Pittsburgh Institutional Review Board. Plasma samples were collected from 18 esophageal cancer patients and 12 patients with gastro-esophageal reflux disease (GERD) during clinical visits or at the time of surgery. Additional plasma samples were obtained from healthy volunteers and used for characterization and optimization of RNA isolation methods. The blood samples for plasma separation were collected into EDTA-containing Vacutainer Tubes, and the blood was centrifuged for 10 min at 1800*g*. Seventy-five percent of the supernatant was removed and centrifuged for a second time at 1300*g* for 10 min to eliminate any remaining cells. To evaluate the possibility that RNA-containing vesicles may be lost after the two-spin protocol, we also processed aliquots of plasma from the same blood samples (17 esophageal cancer and 12 GERD patients) without a second centrifugation. Plasma aliquots were either processed immediately for RNA isolation or stored at −70 °C.

In addition to plasma, serum samples were collected from 16 patients with advanced melanoma and from 12 controls (6 healthy volunteers and 6 patients with GERD). For this purpose, blood was drawn into empty (red-top) Vacutainer Tubes and left to coagulate before it was centrifuged for 10 min at 1200*g*. The isolated serum was centrifuged again at 1300*g* to eliminate contaminating cells and then stored at −70 °C.

RNA ISOLATION PROTOCOLS

A total of nine RNA isolation protocols were tested. The commercial RNA isolation reagent sets tested were as follows: RNeasy Mini-Kit (QIAGEN), QIAamp viral RNA Mini-Kit (QIAGEN), SV Total RNA Isolation System (Promega), Eppendorf Perfect RNA Eukaryotic mini reagent set (Brinkman Instruments Inc.), MagnaZorb DNA Mini-Prep Kit (CORTEX Biochem), and TriBD reagent (Sigma). All RNA isolations were performed according to the manufacturers' instructions. RNA extraction using the RNeasy mini reagent set was also tested with a previously published, modified protocol adapted to isolation of RNA from plasma samples *(21)*.

In addition to the commercial reagent sets, a modified guanidinium isothiocyanate (GIT)–phenol extraction protocol was developed based on the original method of Chomczynski and Sacchi *(27)*. In brief, 4 mol/L GIT solution was premixed with acid phenol (1:3 ratio) and Triton-X (1 mL/L). After the addition of GIT (equal volume to that of plasma), acetic acid was added to a final concentration of 125 mmol/L. The aqueous phase was then separated by addition of 1-bromo-3-chloropropane (1:10 by volume), and RNA was precipitated from the aqueous phase by addition of ammonium acetate and isopropanol (final concentrations, 0.5 mol/L and 500 mL/L, respectively) followed by centrifugation.

All RNA isolation protocols were compared for their ability to isolate endogenous, plasma 18S ribosomal RNA (18S rRNA). In addition, we tested their ability to isolate short RNA fragments in the form of an exogenous, 81-nucleotide, in vitro RNA transcript of the bacterial β-galactosidase (β-gal) gene. The isolated RNA was resuspended/eluted in small volumes of RNase-free water, treated with DNase (DNA-Free; Ambion Inc.), and stored at −70 °C before analysis.

inhibition of plasma RNase activity

Bentonite (50 g/L; Sigma), cadmium chloride (0.15 mol/L in saline; Sigma), SUPERase. In (50 units; Ambion), and Prime RNase inhibitor (50 units; Eppendorf AG) were added to plasma samples to test their ability to inhibit plasma RNase activity. After addition of the RNase inhibitors, 6 μg of mouse liver RNA and/or 100 pg of the in vitro bacterial β-galactosidase transcript were added to the plasma sample, and RNA was isolated by our modified GIT–phenol protocol. The integrity of the recovered mouse RNA was evaluated by agarose gel electrophoresis.

rna stability in plasma samples

Because of the high RNase content in plasma, we tested the mechanism of circulating RNA resistance to nuclease activity. Separated plasma or whole blood samples were incubated at room temperature for 0–3 h before RNA isolation and quantitative RT-PCR (QRT-PCR). The amount of recoverable RNA at each time point was determined from calibration curves constructed from QRT-PCRs for the 18S rRNA gene. To test the theory that RNA may be protected through binding to serum DNA, we used excess RNase-H (230 U/mL of plasma; Takara), DNase (50 U/mL; Ambion), or RNase-A/T1 (2.5 and 100 U/mL; Ambion) with the plasma samples and incubated the reaction for 30 min at 37 °C before RNA isolation and subsequent QRT-PCR. Triton-X and sodium dodecyl sulfate (SDS; 1 mL/L) were also added to other plasma samples to disrupt possible RNA–protein or RNA–lipid complexes, another suggested mechanism for protection of circulating RNA. These detergents were added either before or after inhibition of the plasma RNase activity by addition of the RNA isolation lysis reagent.

concentration of plasma rna by filtration

We attempted to concentrate plasma RNA by two different protocols: The first approach involved concentrating the GIT–phenol-extracted plasma RNA with spin filters (Nanosep 1K, 3K, and 10K; Pall Life Sciences) designed to retain molecules of certain molecular weight. The water phase (after GIT–phenol plasma extraction) was loaded on the filter and centrifuged in a microcentrifuge according to the manufacturer's guidelines. To determine the efficiency of the centrifugal devices to retain plasma RNA, the RNA yield was compared with that after precipitation of RNA from an aliquot of the same plasma sample. In addition, β-galactosidase in vitro transcript (100 pg) and human spleen RNA (10 ng) were added to some samples to determine the efficacy of the centrifugation devices to retain both long and short RNA fragments.

The second RNA concentrating approach was based on the hypothesis that plasma RNA is present in circulating apoptotic bodies/lipoprotein vesicles. Aliquots of plasma or serum (1–4 mL) were passed through syringe-fitted 0.2 μm filters (Osmonics Inc.). After this step, 1 mL of 4 mol/L GIT solution was used to lyse the vesicles and flush the RNA off the filter. RNA was then extracted with phenol–chloroform and precipitated with isopropanol and ammonium acetate. In addition, the filtrate was also extracted (using our modified GIT–phenol method) to determine whether any RNA had passed through the filter before flushing with GIT. RNA yields were compared with those from processing aliquots of the same plasma sample without a filtration step. As a second control, GIT solution was added directly to the plasma sample before filtration to exclude the possibility that the 0.2 μm filter was trapping the free RNA in addition to RNA vesicles. All isolated RNA was treated with DNase and was either stored at −70 °C or processed immediately for QRT-PCR.

real-time qrt-pcr

QRT-PCR was performed on the ABI PRISM 7700 Sequence detection system (Perkin-Elmer Applied Biosystems) in a two-stage, single-tube reaction as described by us previously *(28)*. The sequence information of the gene-specific reverse transcription primers, PCR primers, and the labeled probes are given in Table 1. The primer designs for β-GUS, cytokeratin 20 (CK20), tyrosinase, and MART-1 span exon junctions to provide cDNA specificity. However, the presence of pseudogenes and a lack of introns prohibit a cDNA-specific assay for cytokeratin 19 (CK19) and 18S rRNA, respectively. The quantitative PCRs for 18S, β-GUS, and CK19 were carried out with the following conditions: 12 min of activation of the *Taq* enzyme (AmpliTaq Gold; Roche Molecular Systems Inc.) at 95 °C, followed by 45 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min.

Alternatively, CK19, CK20, and tyrosinase were amplified by a seminested RT-PCR protocol. For the reverse transcription step and the first round of PCR, an external reverse primer (R ext; see Table 1) was used. The first round of PCR amplification was performed for 30 cycles without fluorescent probes. An aliquot of the PCR product (diluted 1:1000) was used as a template for a second round of PCR amplification (45 cycles) using the forward primer (F), a nested reverse primer (R), and the corresponding fluorescent probe. The PCR conditions for the seminested PCR were identical to those of the quantitative PCR component of the one-tube assay (described above) with the following difference: the anneal/extend temper-

Table 1. PCR primers and fluorescently labeled probes.

Primer/Probe	Sequence, 5′–3′
β-GUS F	CTCATTTGGAATTTTGCCGATT
β-GUS R	CCGAGTGAAGATCCCCTTTTTA
β-GUS FA	TGAACAGTCACCGACGAGAGTGCTGG
18S F	CCCTGTAATTGGAATGAGTCCAC
18S R	GCTGGAATTACCGCGGCT
18S FAM	TGCTGGCACCAGACTTGCCCTC
β-Actin F	CCACACTGTGCCCATCTACG
β-Actin 129R	GTGGTGGTGAAGCTGTAGCC
β-Actin TET	ATGCCCTCCCCCATGCCATCCTGCGT
CK19 F	AGATCGACAACGCCCGT
CK19 R	AGAGCCTGTTCCGTCTCAAA
CK19 R Ext[a]	CGTTGATGTCGGCCTCCA
CK19 FAM	TGGCTGCAGATGACTTCCGAACCA
CK20 F	CACCTCCCAGAGCCTTGAGAT
CK20 R	GGGCCTTGGTCTCCTCTAGAG
CK20 R Ext[a]	GGCTAACTGGCTGCTGTAACG
CK20 FAM	CCATCTCAGCATGAAAGAGTCTTTGGAGCA
Tyr F	CTAACTTACTCAGCCCAGCATCATTC
Tyr R	ACTGATGGCTGTTGTACTCCACCAA
Tyr R Ext[a]	GGGCGTTCCATTGCATAAAG
Tyr FAM	TCTCCTCTTGGCAGATTGTCTGTAGCCGA
MART-1 F	GATGCTCACTTCATCTATGGTTACC
MART-1 R	ACTGTCAGGATGCCGATCC
MART-1 TET	AGCGGCCTCTTCAGCCGTGGTGT

[a] The external reverse primers (R Ext) were used to reverse-transcribe as well as amplify the corresponding genes in the first PCR reaction of a seminested RT-PCR analysis.

ature for the second round of PCR in the tyrosinase assay was 64 °C. The 18S and β-GUS concentrations in plasma were quantified against a calibration curve of human spleen total RNA (1–1000 pg). Additionally, RNA from colon cancer (for CK19) and malignant melanoma (for tyrosinase) were used as positive controls for the RT-PCR.

Serum RNA was also analyzed for the presence of MART-1 and tyrosinase in a mutiplexed rapid QRT reactions on the SmartCycler instrument (Cepheid) according to previously published protocols *(29)*. Total RNA (from 3 mL of serum) was reverse-transcribed by gene-specific reverse transcription primers at 48 °C for 20 min. PCR primers and corresponding florescent probes were added to the reaction tube after the reverse transcription stage was completed. Two different florescent labels were used [6-carboxyfluorescein (FAM) and tetrachloro-6-carboxyfluorescein (TET); see Table 1] for both tyrosinase and MART-1, respectively, enabling the detection and differentiation of both transcripts simultaneously. After *Taq* activation at 95 °C for 30 s, the multiplexed quantitative PCR was performed for 45 cycles using 95 °C and 64 °C for 1 and 10 s, respectively. Serial dilutions of RNA from malignant melanoma were used to establish the detection limit of the assay, and the lowest detectable concentration was used as the positive control for the multiplexed assay.

Results

PRESENCE OF AMPLIFIABLE RNA IN NORMAL PLASMA SAMPLES

Using real-time QRT-PCR, we verified the presence of amplifiable cell-free circulating RNA in every tested cell-free plasma sample from individuals without disease. The plasma RNA concentrations were in the range of 1–10 μg/L, as determined by quantifying the amounts of 18S rRNA, β-actin, and β-GUS mRNA transcripts against human spleen RNA calibration curve (1–1000 pg). Contaminating DNA in the extracted RNA samples was excluded from being the primary template for the PCR because no significant amplification was detectable in reactions performed in the absence of the reverse transcription enzyme (−RT controls), thus confirming the effectiveness of the DNase treatment step (Fig. 1). In addition, RNase A/T treatment prevented any subsequent amplification, thus verifying the RNA nature of the amplification signal (data not shown).

EFFECT OF DIFFERENT CENTRIFUGATION PROTOCOLS FOR SEPARATION OF PLASMA RNA

Plasma samples were separated by either single or double centrifugation protocols as described in the *Materials and Methods*. The second centrifugation was intended to remove any remaining cells, and we found that there was indeed a pellet at the bottom of the tube after the second centrifugation. The RNA yield (as quantified by 18S rRNA and β-actin QRT-PCR) from the samples processed by the single centrifugation protocol was >20-fold higher than that observed after the double-centrifugation protocol. As expected, analysis of the pellet from the second centrifugation showed high RNA content, but microscopic analysis of the pellet failed to identify a significant number of lymphocytes. Instead, this pellet was composed of numerous particles/bodies that we presume to be mostly platelets. However, using our standard cytologic examination, we were unable to determine whether the pellet consisted entirely of platelets or if it may have also contained apoptotic bodies or other RNA-containing vesicles.

CIRCULATING RNA IS PROTECTED AGAINST POTENT PLASMA RNase ACTIVITY

Plasma RNA was stable for at least 3 h at room temperature in either whole blood or plasma before RNA extraction steps (Fig. 2A). However, the inherent plasma RNase activity immediately degraded both full-length RNA (20 μg of total mouse spleen RNA, as shown in Fig. 2B), as well as short (81-nucleotide) in vitro transcripts of the β-gal gene (data not shown). In addition, known chemical (cadmium chloride), physical adsorption (bentonite), and commercial RNase inhibitors (SUPERase.In and prime RNase inhibitor) failed to protect the added RNA from degradation apparently attributable to the high concentrations of RNase in plasma (Fig. 2B). Because it has been suggested that plasma RNA may be protected by hybridization with DNA, we examined the effect of RNase-H on

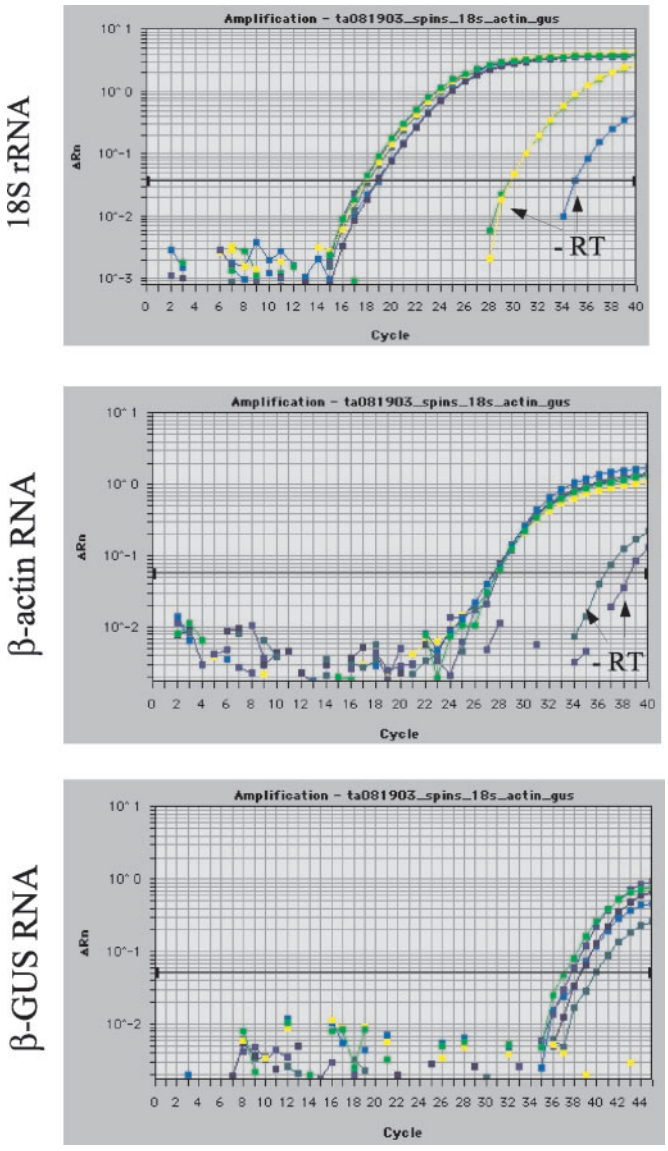

Fig. 1. Detection of plasma 18S rRNA, β-actin, and β-GUS RNAs on the ABI 7700 instrument.

The three *graphs* show examples of the QRT-PCR amplification plots using plasma RNA from three healthy controls. RNA samples were analyzed in duplicate except in the case of "−RT" controls, where samples were assayed in single reactions. The plasma samples used in these experiments were extracted by the modified GIT–phenol extraction method. The amount of RNA in each RT-PCR was equivalent to 30 μL of plasma separated by the double-spin protocol. For 18S rRNA and actin RNA, weak amplification signals were detected in the −RT controls, indicating the presence of some remaining DNA. Because the GUS primers are cDNA specific, no amplification signal was seen in the absence of the reverse transcription enzyme.

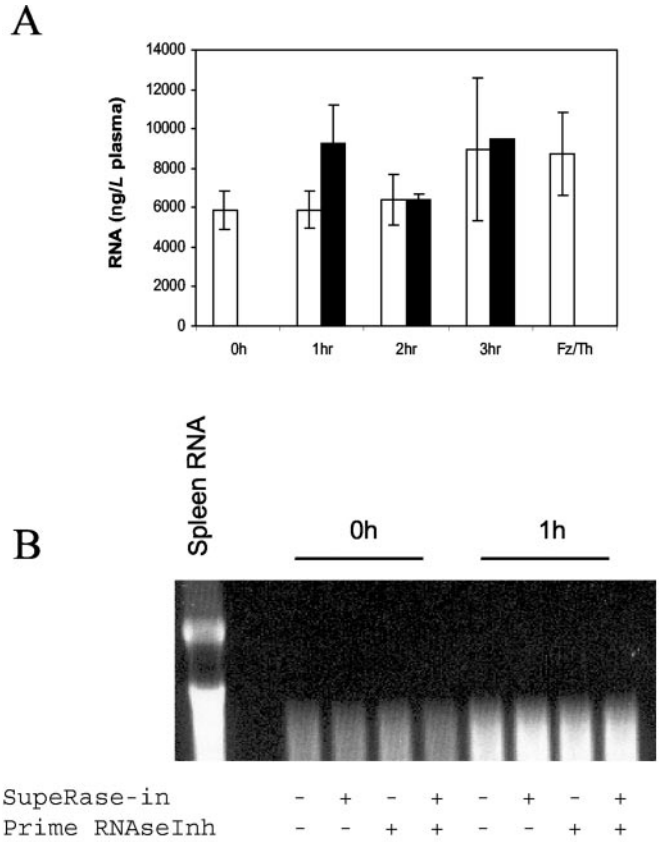

Fig. 2. Stability of plasma RNA (*A*) and added mouse spleen RNA (*B*) in plasma and unprocessed blood samples.

In *A*, the samples were incubated at room temperature for 0–3 h before (open bars) or after (black bars) plasma separation. The effect of one cycle of freezing and thawing (*Fz/Th*) on plasma RNA was tested and found to have no significant effect on RNA integrity. Each *column* represents the mean of three to six independent samples (*error bars*, SD). (*B*), example of an agarose gel electrophoresis for added mouse spleen RNA (6 μg) in plasma. The degradation of the added RNA (immediately or after 1 h) was also tested in the presence (+) or absence (−) of commercial RNase inhibitors.

the integrity of plasma RNA (before and after extraction). We also tested the ability of high concentrations of RNase A/T or DNase to degrade the plasma RNA before the extraction steps. All tested nucleases displayed no adverse effect on plasma RNA concentrations (data not shown), indicating that DNA binding is probably not the mechanism for RNA protection against degradation. We then evaluated the hypothesis that circulating RNA is sequestered within vesicles/apoptotic bodies that may be actively secreted or released during cell death. The addition of detergent (Triton-X or SDS at a final concentration of 3–10 mL/L and 1–10 g/L) to the plasma samples completely destroyed all amplifiable RNA as observed by 18S rRNA QRT-PCR (Fig. 3). This was not attributable to interference in the isolation process itself or in the RT-PCRs because the same amount of detergent had no effect when added after protection of RNA by addition of the lysis reagent.

EFFICIENCY OF DIFFERENT RNA ISOLATION PROTOCOLS

We evaluated several RNA isolation protocols for RNA recovery and their ability to extract RNA from several milliliters of plasma. Because most plasma RNA is probably present as short fragments, we tested the efficiency of the isolation methods using a short synthetic bacterial β-galactosidase transcript (β-gal; 81 nucleotides) as well as endogenous plasma 18S rRNA. As illustrated in Table

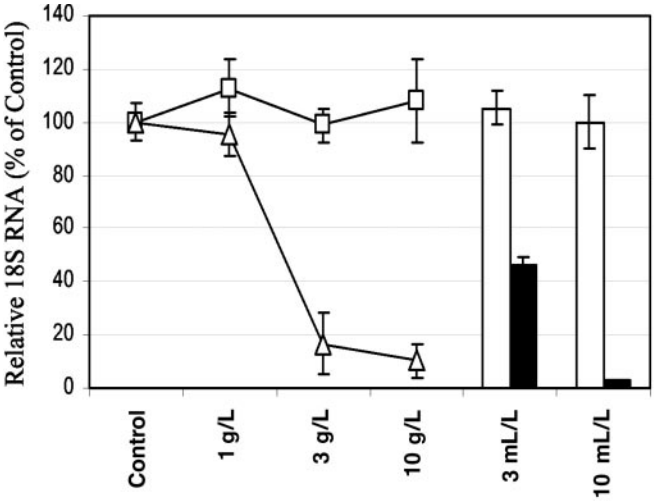

Fig. 3. Effects of SDS and Triton-X on the integrity of plasma RNA.

The detergents were added before or after protection of the RNA by the lysis reagent to destroy inherent plasma RNase activity. Data are presented as a percentage of the control RNA. Each *point* represents the mean (*error bars*, SD) from six independent samples. □, SDS added after protection; △, SDS added before protection; open column, Triton added after protection; filled column, Triton added before protection.

2, the best recovery was obtained with the precipitation-based RNA isolation methods: modified GIT–phenol extraction and Tri-BD reagent. The recovery of short RNA transcripts by nonspecific binding of RNA to resins/columns or magnetic beads was much lower, although it was better than that of a recently published modification of the RNeasy plasma RNA isolation protocol *(21)*.

Table 2. Comparison for RNA extraction based on 18S RNA and β-GUS transcript recovery between different column/matrix binding- and precipitation-based protocols.

	Plasma 18S rRNA, μg/L	β-GUS transcript, % recovery
Precipitation methods		
Modified GIT–phenol extraction	5–10	35–40
Sigma Tri-BD	6–7.5	31–41
Gentra RNA isolation	10	Not tested
Matrix-binding methods		
Qiagen Viral RNA isolation reagent set	5–8	5–7
Cortex MagnaZorb	0.3–6	0.2–0.4
SV total RNA isolation reagent set (Promega; breast cancer)[b]	3–4	Undetectable
Qiagen RNA easy reagent set (breast cancer)[c]	Not tested	0.2–0.4
Eppendorf perfect RNA (melanoma)[d]	0.02	0.23

[a] Precipitation-based protocols (values in bold; GIT–phenol and Tri-BD) were superior to all other methods, particularly in recovering short RNA transcripts. Examples of studies that utilized certain isolation methods for detection of tumor-related circulating RNA are also indicated.

[b] Chen et al. *(17)*.

[c] Silva et al. *(40)*.

[d] Kopreski et al. *(19)*.

CONCENTRATION OF EXTRACTED PLASMA RNA BY SPIN COLUMNS

Because of the low RNA concentration in plasma, we believe that the ability to isolate RNA from larger volumes may increase sensitivity for cancer detection. Unfortunately, however, the precipitation-based RNA isolation techniques are not practical for scaling up because of difficulties in precipitating low concentrations of RNA from excessively large aqueous phase volumes. We therefore investigated the possibility of reducing the final volume of the extracted RNA, thus making precipitation more efficient. For this purpose we used spin columns with small pore sizes in an attempt to concentrate plasma RNA before the precipitation step. We tested three different pore size filters with various centrifugation speeds and durations. Both 10K and 3K spin columns were not efficient in retaining plasma RNA above the porous membrane (data not shown). Technically, the centrifugation column with the smallest pore size (1K) was not effective because it did not allow the aqueous phase to pass through even after lengthy centrifugation at high speed, possibly because or high salt content or other components in the phenol-extracted plasma samples. Interestingly, however, both the 10K and 3K columns were efficient in retaining full-length spleen RNA, suggesting that endogenous plasma RNA is fragmented (data not shown).

CONCENTRATION OF RNA-CONTAINING VESICLES BY 0.2 μM FILTERS

Recent studies have demonstrated that after serum filtration through 0.2 μm filters, RNA concentrations are greatly diminished (but not completely absent) in the filtrate. Consistent with these observations, we found that the RNA content in the filtrate was greatly reduced (0.3 ng of total RNA from 3 mL of filtered serum vs ~5 ng of total RNA from 3 mL of unfiltered plasma). We also found that this was not attributable to nonspecific binding of plasma RNA to the filter membrane because all of the RNA passed through in the filtrate when GIT was added to disrupt the vesicle–protein association before the filtration step (data not shown). We then tested whether we could isolate RNA from the trapped vesicles on the filter membrane, which would potentially enable us to extract RNA from large plasma volumes (several milliliters). The amount of RNA isolated from filter-trapped vesicles (from 3 mL of serum) was approximately six- to eightfold higher than the RNA recovered by our modified GIT–phenol RNA extraction from 300 μL of plasma (Fig. 4). The fact that RNA recovery was not 10-fold higher after filtration of 3 mL of plasma may be attributable to some disruption of the RNA-containing particles by the pressure in the syringe during the filtration step and subsequent degradation of the unprotected RNA. Alternatively, some of the RNA could be contained in vesicles smaller than 0.2 μm.

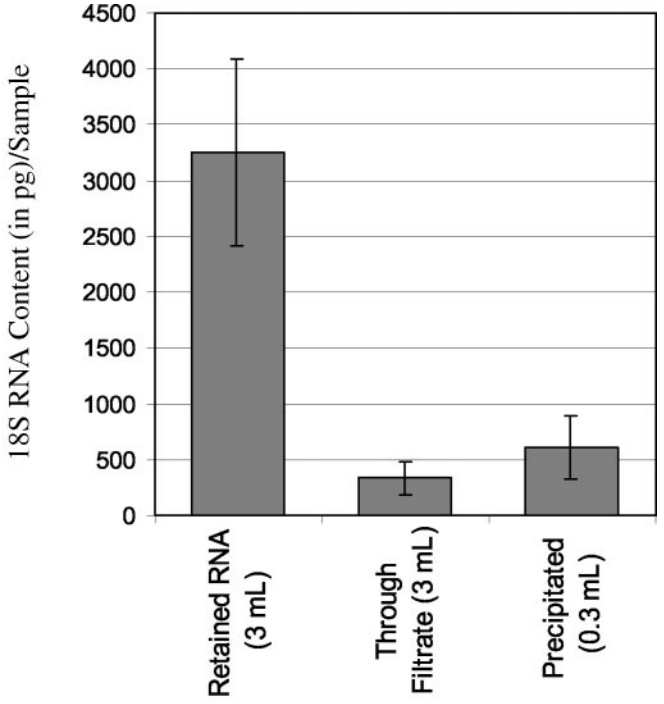

Fig. 4. Trapping of RNA particles/vesicles on 0.2 μm filter membrane after filtration of serum through syringe-fitted filter.

The amount of extracted RNA represents the relative quantity of 18S rRNA as determined on the ABI prism 7700. The filter-retained RNA was extracted by passing 1 mL of GIT solution through the filter. The recovery of RNA (from 3 mL of serum) after the filtration step was compared with that from 0.3 mL of serum extracted with GIT–phenol without a prefiltration step. In addition, the amount of 18S RNA in the pass-through of serum samples was quantified. Each *column* represents the mean (*error bars*, SD) from six samples.

DETECTION OF CIRCULATING TUMOR-RELATED RNA FROM PATIENTS WITH MELANOMA

The presence of melanoma-related transcripts was examined in total RNA isolated from 1–3 mL of serum after concentration of the circulating RNA vesicles on 0.2 μm filters. The RNA was analyzed by nested RT-PCR on the ABI 7700 instrument for tyrosinase alone or by a single round of multiplex QRT-PCR on the SmartCycler for tyrosinase and MART-1. Serum was analyzed from 16 patients with advanced-stage melanoma and 12 nonmelanoma controls (6 healthy volunteers and 6 GERD patients). No tyrosinase or MART-1 signals were detectable in any of the samples obtained with either technique despite the fact that amplification of β-GUS indicated the presence of RNA (data not shown). As expected, the positive control melanoma tumor RNA always gave positive results, even at very low RNA input, indicating adequate assay sensitivity.

DETECTION OF CIRCULATING TUMOR-RELATED RNA FROM PATIENTS WITH ESOPHAGEAL CANCER

Single-round RT-PCR failed to detect the presence of mRNA for the epithelial markers CK19 and CK20 in eight plasma samples from patients with esophageal cancer (Table 3). To increase the detection sensitivity, we also tested for the presence of CK19 and CK20 transcripts in plasma samples from 17 esophageal cancer patients, 12 patients with GERD, and 3 healthy individuals by seminested RT-PCR. The RNA amount used for each reverse transcription reaction corresponded to 100 μL of plasma. In all, CK19 and CK20 failed to significantly and consistently distinguish esophageal cancer patients from the control groups regardless of the centrifugation protocol used to separate the plasma samples (see Table 3).

Table 3. Detection of tumor-related RNA transcripts (CK19, CK20) in plasma samples from healthy controls or from patients with GERD or esophageal cancer.[a]

			n	
	Sample	Amplification	CK19	CK20
Healthy controls	Pl.[b] 2 spins	Single-round RT-PCR	0/3	NT
GERD	Pl. 2 spins	Single-round RT-PCR	1/6	NT
Esophageal cancer	Pl. 2 spins	Single-round RT-PCR	0/8	NT
Healthy controls	Pl. 1 spin	Seminested RT-PCR	0/3	1/3
GERD	Pl. 1 spin	Seminested RT-PCR	1/11	1/11
Esophageal cancer	Pl. 1 spin	Seminested RT-PCR	3/16	1/16
GERD	Pl. 2 spin	Seminested RT-PCR	5/10	NT
Esophageal cancer	Pl. 2 spin	Seminested RT-PCR	4/16	NT

[a] RNA was extracted by a modified GIT–phenol extraction. Several spin protocols for plasma separation were also tested to determine the sensitivity/specificity of the assay when performed on differentially processed plasma samples from the same individuals. The plasma volume corresponding to the RNA input per reverse transcription reaction was equivalent to 100 mL. Detection of each marker is indicated per total number of samples used.

[b] Pl, plasma; NT, presence of a particular RNA marker was not tested.

Discussion

Early detection and treatment of cancer offers much promise for improving overall survival rates. Unfortunately, however, current approaches to cancer screening tend to lack sensitivity and/or specificity for detection of early-stage disease. Furthermore, current methods are often invasive (e.g., colonoscopy, digital rectal exam, PAP smear) and expensive. For this reason, many research groups are seeking to develop sensitive and cost-effective methods for early cancer detection and for monitoring of disease recurrence. Because of the low cost and relatively noninvasive nature of phlebotomy, one very appealing approach to this problem is the development of a simple, blood-based test for cancer. Unfortunately, the most obvious technique, detection of circulating cancer cells, has not been demonstrated as useful, and many researchers are now turning to plasma or serum-based methods for detection of cancer-related proteins or nucleic acids.

The detection of nucleic acids in plasma dates back to the 1970s *(12)*, but it was not until recently, with the help of major advances in molecular biology, that this approach began to emerge as a possible tool for cancer diagnostics. There are now numerous reports describing the use of PCR-based techniques to detect cancer-specific mutations in circulating DNA from plasma or serum *(30)*. For example, mutations in the K-*ras* gene were detected in

32% of plasma samples from patients with colorectal cancer *(31, 32)*, loss of heterozygosity has been demonstrated in 21% of serum samples from patients with early-stage breast cancer *(33, 34)*, and mitochondrial DNA somatic mutations were detected in 33% of patients with hepatocellular carcinoma *(35)*. Although these data are promising, it is becoming clear that DNA-based cancer diagnostics will be somewhat limited by the paucity of mutations that occur at sufficiently high frequency in a given tumor type and the technical complexity of the assays involved for mutation detection. One possible exception to this is the detection of hypermethylation at specific gene loci in many cancer cells, although this method also faces major technical hurdles. More recently, the detection of circulating RNA has been reported as a potential cancer diagnostic approach, and if successful, this may offer several advantages over DNA-based assays. One advantage is that RNA transcripts can be both tumor and tissue-type specific, thus potentially indicating not only the presence of a tumor but also its location. Another advantage is that RNA markers can be chosen that are expressed in all cells (or a very high percentage of cells) of a given tumor type. Finally, there are multiple copies of mRNA in each cell compared with only two copies of DNA. This could provide better sensitivity for cancer detection, although the instability of RNA compared with DNA may eliminate this potential advantage.

The presence of extracellular RNA in serum/plasma was suggested as early as 1988 *(36)*, but RNA is an extremely labile molecule, and the notion that cell-free RNA could survive in RNase-rich plasma was not widely accepted. This has recently changed with several reports clearly demonstrating the presence of amplifiable RNA in plasma/serum. Since the pioneering studies by Kopreski et al. *(19)* and Lo et al. *(37)*, several groups have investigated the presence of tumor-related circulating RNA in serum from patients with malignant melanoma or breast, lung, or colon cancer *(17, 20, 38–40)*. Although these reports are innovative and promising, none of the methods has achieved a sensitivity for cancer detection that would be acceptable for clinical application. We hypothesized that this may be attributable in part to the small volumes of serum used per assay (in some studies as little as 50 μL was used), difficulties in efficiently isolating RNA from serum/plasma, and the poor understanding of the nature of RNA in plasma/serum. In addition, we hoped that the use of QRT-PCR would improve specificity, particularly when more RNA was analyzed and background expression of marker RNAs could become a problem. In this study we therefore attempted to gain a better understanding of the RNA in serum, to optimize RNA isolation methods, and then to analyze candidate marker RNAs from several milliliters of serum from cancer patients to improve sensitivity of cancer detection.

Our experiments showed that exogenous RNA added to plasma or blood is immediately degraded, whereas endogenous plasma RNA is stable for at least 3 h. This indicates the existence of a protective mechanism through which circulating plasma RNA is rendered insensitive to the potent RNase activity in serum and plasma. The formation of a RNA–DNA hybrid has been suggested to be one mechanism for RNA protection. Such a hybrid would, in theory, be resistant to any nuclease but RNase-H. Our results, however, did not support this RNA–DNA hybrid hypothesis: the addition of RNase-H to plasma samples had no effect on RNA recovery. A second hypothesis for RNA protection is that it exists within lipoprotein vesicles that are either actively secreted or released during programmed cell death, possibly as apoptotic bodies *(26)*. We tested this theory by adding either SDS or Triton-X to plasma in an attempt to disrupt any RNA–protein or RNA–lipid complexes. The degradation of plasma RNA after SDS or Triton-X was added to plasma does indeed favor the idea that RNA is protected in a lipid-containing vesicle such as apoptotic bodies. Furthermore, although we were unable to concentrate RNA by use of molecular weight-based filters, we were able to concentrate the RNA directly from serum by use of 0.2 μm filters. Because free RNA, or RNA in a RNA–DNA hybrid, would pass through a 0.2 μm filter, these results also favor the theory that serum RNA is in some kind of protein or lipid complex larger than 0.2 μm *(41)*. Interestingly, separate packaging of DNA and RNA appears to take place during the formation of apoptotic bodies *(25, 26)*. This phenomenon could explain the findings of Ng et al. *(15)*, who showed that >95% of serum RNA did not pass through 0.2 μm filters, whereas almost all serum DNA was in the filtrate.

Because previous studies have examined the presence of tumor-related RNA in plasma as well as in serum, we found it necessary to optimize the RNA extraction methods for both fluids. The mean amounts of plasma RNA did not differ dramatically from those obtained with serum. This optimization was also important because our prebanked samples from melanoma patients were sera, whereas our samples from patients with esophageal cancer were plasma. Although this liberal choice of sample type may adversely affect the results, the overall finding was our inability to diagnose cancer in either sample type with the current technology despite optimization of RNA extraction and amplification methods.

RNA isolation from plasma or serum is made difficult by the high protein content of these matrices and their buffering capacities. In addition, the RNA in serum is fragmented to the point that commonly used binding matrices (such as silica or cellulose) are not effective for RNA capture. Our data show that although RNA isolation methods based on these binding matrices are efficient at isolation of full-length RNA from plasma, recovery of the 81-nucleotide in vitro transcript was very poor. By comparison, RNA extraction with organic solvents followed by precipitation was at least equivalent for intact RNA and much better for the short RNA fragments. In this study, we found that a classic phenol–chloroform extrac-

tion protocol (with minor modifications) provided the best overall isolation of endogenous circulating RNA from plasma or serum.

Having improved our understanding of the nature of circulating RNA and determined the most efficient methods for RNA extraction, we hoped to obtain better sensitivity for detecting tumor-related RNA from serum/plasma of cancer patients. Initially we chose to analyze sera from melanoma patients because these sera were available in a tissue bank at the University of Pittsburgh Cancer Institute and because we had already developed very sensitive QRT-PCR assays for the melanoma markers MART-1 and tyrosinase as part of another study. In addition, Kopreski et al. *(19)* had previously reported the ability to detect tyrosinase mRNA in as little as 50 μL of serum from melanoma patients. In our analysis, we used prefiltration of RNA from 1–3 mL of serum followed by RNA isolation by the modified phenol–chloroform RNA extraction protocol. Despite the fact that this gave our method the advantage over previous studies of having 20–60 times more input RNA (total of 5–10 ng/reverse transcription reaction as measured by 18S rRNA concentrations) in the RT-PCR, we were unable to detect tyrosinase or MART-1 mRNA in sera from patients with advanced melanoma.

Despite the disappointing results on our melanoma samples, we decided to attempt detection of CK19 and CK20 mRNA in plasma from patients with esophageal cancer. Although we were able to detect cytokeratin mRNA in some samples, the results were inconsistent and failed to differentiate between cancer patients and controls (Table 3). The presence of circulating cytokeratins in extremely low concentrations in the plasma samples may have made this a hit-or-miss phenomenon. This could explain the lack of consistency in the detection sensitivity/specificity. It is also possible that because GERD is usually associated with an abnormal pathologic condition of the esophagus (Barrett metaplasia), this may account for the presence of cytokeratins in the plasma samples from control patients.

The RNA content of the plasma samples varied greatly with the one- vs two-spin protocols for plasma separation, and the two-spin protocol successfully removed any cellular/platelet contamination. Although after the second centrifugation we observed a large decrease in plasma RNA content, we cannot state with absolute certainty that the RNA content in the cell-free plasma did not in fact come from platelets that were not separated by the second centrifugation because platelets are known to contain RNA *(42, 43)*. On the other hand, we also thought that we could actually be losing the potentially crucial, tumor-related apoptotic bodies with the more vigorous centrifugation protocol. For this reason we examined the presence of cytokeratin mRNAs in plasma that was separated by both spin protocols. Regardless of the protocol used, however, we were unable to consistently distinguish between cancer and control patients.

In conclusion, we have shown that cell-free RNA present in plasma is protected from degradation not by binding to DNA, as has been suggested, but probably by inclusion in lipid or lipoprotein complexes. This finding supports the hypothesis that the RNA may be present in apoptotic vesicles, as has been shown in other in vitro studies. In addition, however, our data seem to indicate that much of the cell-free RNA in plasma is contained within platelets and is therefore not derived from tumor cells. We have also demonstrated that plasma RNA can be concentrated by filtration, thus facilitating isolation from larger volumes of plasma. However, despite optimization of RNA isolation procedures, concentration of plasma RNA, and use of sensitive, QRT-PCR assays, our data suggest that plasma RNA may not be a useful tool for detection of cancer.

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