Clinical Chemistry 48:8
1212–1217 (2002)

Cancer Diagnostics:
Discovery and Clinical
Applications

Presence of Filterable and Nonfilterable mRNA in the Plasma of Cancer Patients and Healthy Individuals

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Background: **As RNA is labile, we investigated whether circulating RNA in human plasma may be present in a particle-associated form.**
Methods: **Blood was collected from 27 healthy individuals and 16 hepatocellular carcinoma (HCC) patients. The plasma from each individual was processed by two means: filtration through filters with different pore sizes (from 5 μm to 0.22 μm) and ultracentrifugation. We assessed plasma RNA content by a real-time quantitative reverse transcription-PCR assay for *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) transcripts and plasma DNA by a real-time quantitative PCR assay for the *β-globin* gene.**
Results: **The plasma *GAPDH* mRNA concentrations in the healthy individuals were significantly different in every pair of these filter sizes ($P < 0.05$ for each pair). Overall, the plasma *GAPDH* mRNA concentration was higher by a median of 15-fold (interquartile range, 10- to 24-fold) in the paired unfiltered sample than in the sample filtered through a 0.22 μm filter. In contrast, no significant difference was seen in *β-globin* DNA concentrations among different pore-size-filtered plasma samples ($P = 0.455$). Similarly, a significant difference was observed for RNA, but not DNA, between unfiltered plasma and ultracentrifuged plasma ($P < 0.05$). No significant difference in *GAPDH* mRNA concentrations was seen between the 0.22-μm-filtered plasma samples and the ultracentrifuged plasma samples ($P > 0.05$). In HCC patients, filtration with a 0.22 μm filter produced a median 9.3-fold (interquartile range, 6.9- to 311-fold) reduction in *GAPDH* mRNA concentration in plasma. Plasma *GAPDH* mRNA concentrations in HCC patients were significantly higher than those in healthy individuals, both with or without filtration ($P < 0.0 5$ for filtered plasma samples; $P < 0.005$ for unfiltered plasma samples).**
Conclusions: **A substantial proportion of plasma mRNA species is particle-associated. In HCC patients, both circulating particle- and non-particle-associated plasma RNA are increased.**

Much interest has been focused on the diagnostic applications and biology of circulating nucleic acids *(1)*. A recent development in this field is the discovery of tumor-derived RNA in the plasma/serum of cancer patients *(2–6)*. Tumor-associated RNA targets that have been detected include mRNA of the tyrosinase gene *(2)*, telomerase components *(4, 6)*, and viral RNA *(3)*. The lability of RNA and the existence of ribonuclease in the plasma *(7)* make it surprising that circulating RNA should be detectable at all. Hasselmann et al. *(8)* recently made the interesting observation that in an in vitro model, mRNA within apoptotic bodies released by a melanoma cell line was protected from degradation when incubated in human serum. However, a conclusive demonstration that circulating RNA in human plasma is particle-associated is currently lacking. Such incomplete understanding of the fundamental biology of circulating RNA is compounded by the fact that all except one *(6)* of the recent publications on circulating RNA are qualitative studies. In this report, we address both of these issues, using a quantitative real-time reverse transcription-PCR (RT-

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Received March 15, 2002; accepted April 12, 2002.

PCR)[6] to measure circulating RNA in cancer patients and healthy individuals. In particular, we investigated the possibility that circulating RNA may be present in a particle-associated form by analyzing plasma samples that have been processed by filtration and ultracentrifugation.

Materials and Methods

PATIENTS

Sixteen hepatocellular carcinoma (HCC) patients undergoing investigation at the Department of Diagnostic Radiology and Imaging at the Prince of Wales Hospital were recruited with informed consent. The healthy control group consisted of 27 individuals. The study was approved by the Ethics Committee of The Chinese University of Hong Kong.

PROCESSING OF BLOOD SAMPLES

Our current protocol, based on our previous report that blood processing by different protocols would affect the results of plasma DNA quantification *(9)*, involves initial centrifugation of blood samples at 1600*g* followed by a second centrifugation at 16 000*g*. This protocol has been shown to minimize the contribution of DNA derived from residual cells in plasma *(9)*. Peripheral blood was collected from each participant into EDTA tubes. Blood samples were centrifuged at 1600*g* for 10 min at 4 °C, and plasma was carefully transferred into new tubes, followed by further centrifugation at 16 000*g* for 10 min at 4 °C.

Plasma samples from 17 healthy individuals were used in the filtration experiments. Each plasma sample was divided into four aliquots: three were individually passed through filters (Millex-GV; Millipore) with pore sizes of 0.22, 0.45, and 5 μm. The remaining aliquot was not subjected to filtration.

The plasma samples from 10 additional healthy individuals were used in the filtration and ultracentrifugation experiments. Each sample was divided into three aliquots: one-third of the plasma was filtered with a 0.22 μm filter, and one-third was subjected to ultracentrifugation (using a Beckman Optima™ TLX Ultracentrifuge) at 99 960*g* for 120 min at 4 °C. The remaining aliquot was not filtered or subjected to ultracentrifugation.

The plasma sample from each HCC patient was divided into two aliquots: one-half was filtered with a 0.22 μm filter, and the remaining aliquot remained unfiltered.

RNA EXTRACTION FROM PLASMA SAMPLES

RNA was extracted from 600 μL of plasma with an RNeasy Mini Kit (Qiagen). Briefly, 600 μL of plasma was mixed with 1.2 mL of Trizol LS reagent (Invitrogen) and 0.2 mL of chloroform. The mixture was centrifuged at 11 900*g* for 15 min at 4 °C, and the aqueous layer was transferred into new tubes. One volume of 700 mL/L ethanol was added to one volume of the aqueous layer. The mixture was then applied to the RNeasy minicolumn and was processed according to the manufacturer's recommendations. Total RNA was eluted with 15 μL of RNase-free water followed by DNase I (Invitrogen) treatment. RNA was stored at −80 °C until further processing.

DNA EXTRACTION FROM PLASMA SAMPLES

Plasma DNA was extracted with a QIAamp Blood Kit (Qiagen), using the "blood and body fluid protocol" as recommended by the manufacturer *(10)*. A final elution volume of 25 μL was used.

REAL-TIME QUANTITATIVE RT-PCR

One-step real-time quantitative RT-PCR was used for the measurement of mRNA concentration in plasma *(11)*. In this method, the r*Tth* DNA polymerase functioned both as a reverse transcriptase and a DNA polymerase. A real-time quantitative RT-PCR method was used for transcript quantification of the glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene. The amplification primers were GAPDHF (5′-GAAGGTGAAGGTCGGAGT-3′) and GAPDHR (5′-GAAGATGGTGATGGGATTTC-3′), and the dual-labeled fluorescent probe was GAPDHP [5′-(FAM)CAAGCTTCCCGTTCTCAGCC(TAMRA)-3′], where FAM is 6-carboxyfluorescein and TAMRA is 6-carboxytetramethylrhodamine.

RT-PCR was set up in a reaction volume of 50 μL using components supplied in an EZ r*Tth* RNA PCR reagent set and TaqMan® *GAPDH* Control Reagents (Human; PE Applied Biosystems). Each reaction contained 10 μL of 5× EZ buffer; 200 nM each of the primers; 100 nM fluorescent probe; 3 mM $Mn(OAc)_2$; 300 μM each of dATP, dCTP, and dGTP; 600 μM dUTP; 5 U of r*Tth* polymerase; and 0.5 U of uracil N-glycosylase. For amplification, we used 3 μL of extracted RNA. Amplification data were collected and analyzed with an ABI Prism 7700 Sequence Detector (PE Applied Biosystems). Each sample was analyzed in duplicate, and multiple negative water blanks were included in every analysis. A calibration curve for *GAPDH* quantification was prepared by subjecting serial dilutions of human control RNA (PE Applied Biosystems), with RNA concentrations ranging from 15 to 0.23 pg, to the RT-PCR assay. The manufacturer estimated that 1 pg of this control RNA contained ~100 copies of *GAPDH* transcript.

The thermal profile used for the *GAPDH* method was as follows: before reverse transcription, the reaction was initiated at 50 °C for 2 min in the presence of uracil N-glycosylase, followed by a reverse transcription step at 60 °C for 30 min. After a 5-min denaturation at 95 °C, PCR was carried out for 40 cycles using a denaturation step of 94 °C for 20 s and an annealing/extension step of 60 °C for 1 min.

[6] Nonstandard abbreviations: RT-PCR, reverse transcription-PCR; HCC, hepatocellular carcinoma; and *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase.

REAL-TIME QUANTITATIVE DNA PCR

Plasma DNA was quantified by a real-time quantitative PCR for the *β-globin* gene as described previously *(10)*. For each reaction, 5 μL of the extracted plasma DNA was used and analyzed in duplicate. Thermal cycling conditions for the *β-globin* PCR were as described previously *(10)*. The precision of this method has been reported previously, with a CV for the threshold cycle of 1.1% *(10)*.

STATISTICAL ANALYSIS

Statistical analysis was performed using SigmaStat 2.03 software (SPSS).

Results

VALIDATION OF REAL-TIME QUANTITATIVE RT-PCR

To assess the linearity of the assay, we analyzed serial dilutions of the control RNA, using the *GAPDH* RT-PCR method. The calibration curve showed a correlation coefficient of 0.995. To assess the precision of both the RNA extraction and the RT-PCR steps, we prepared 10 replicate extracts from a plasma sample obtained from a healthy individual; these extracted RNA samples were then subjected to real-time quantitative RT-PCR analysis. The CV for the threshold cycle values of these replicate analyses was 1.7%. The lower limit of detection for this RT-PCR method was ~23 copies, the number of *GAPDH* transcripts present in 0.23 pg of the control RNA.

PARTICLE-ASSOCIATED NATURE OF CIRCULATING RNA IN THE PLASMA OF HEALTHY INDIVIDUALS

Plasma samples from 17 healthy individuals were analyzed for *GAPDH* mRNA and *β-globin* DNA concentrations. Aliquots from each sample were individually passed through filters with pore sizes of 5, 0.45, and 0.22 μm before quantitative analysis. The data in Fig. 1A show that filtration had a clearly observable effect on *GAPDH* mRNA concentrations in plasma samples (Friedman test, $P < 0.001$). Pairwise analysis further indicated that a statistically significant difference was detected in every pair of these filter sizes (Student-Newman-Keuls test, $P < 0.05$ for each pair). Overall, the plasma *GAPDH* mRNA concentration decreased by a median of 15-fold (interquartile range, 10- to 24-fold) in comparisons of paired samples from the unfiltered samples and samples filtered through the 0.22 μm filters. In contrast to these results, there was no statistically significant difference in *β-globin* DNA concentrations among plasma samples filtered through different-sized pores (Friedman test, $P = 0.455$; Fig. 1B). These results therefore indicate that a significant proportion of *GAPDH* mRNA in plasma is particle-associated. On the other hand, most of the circulating *β-globin* DNA is non-particle-associated.

To investigate whether any mRNA in plasma existed in a non-particle-associated form, we performed a second series of experiments using ultracentrifugation. Plasma samples were obtained from another group of 10 healthy individuals. Aliquots from each sample were subjected to either a 0.22 μm filtration step or ultracentrifugation at 99 960*g*. The data in Fig. 2 show a statistically significant difference among *GAPDH* mRNA concentrations in unfiltered plasma, plasma filtered through a 0.22 μm filter, and ultracentrifuged plasma (Friedman test, $P < 0.001$). Pairwise comparisons indicated a significant difference between unfiltered plasma and plasma filtered through 0.22 μm filters (Student-Newman-Keuls test, $P < 0.05$), and between unfiltered plasma and ultracentrifuged plasma (Student-Newman-Keuls test, $P < 0.05$). However, there was no significant difference in *GAPDH* mRNA concentrations between the plasma samples filtered through 0.22 μm filters and the ultracentrifuged plasma samples (Student-Newman-Keuls test, $P > 0.05$; Fig. 2A). The median

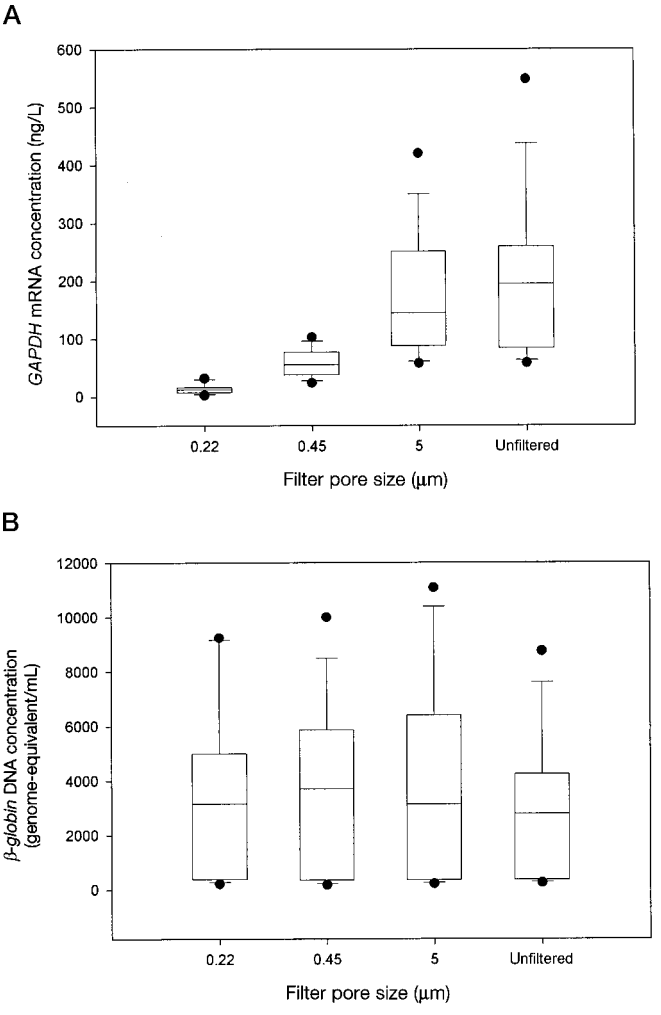

Fig. 1. Plasma mRNA (*A*) and DNA (*B*) concentrations in healthy individuals after filtration through different pore sizes.

(*A*), plasma *GAPDH* mRNA concentrations (ng/L), as determined by real-time quantitative RT-PCR (*y axis*), plotted against filter pore size (*x axis*). (*B*), plasma *β-globin* DNA concentrations (genome-equivalents/mL), as determined by real-time quantitative PCR (*y axis*), plotted against filter pore size (*x axis*). The *lines inside the boxes* denote medians. The *boxes* mark the interval between the 25th and 75th percentiles. The *whiskers* denote the interval between the 10th and 90th percentiles. ● indicate data points outside the 10th and 90th percentiles.

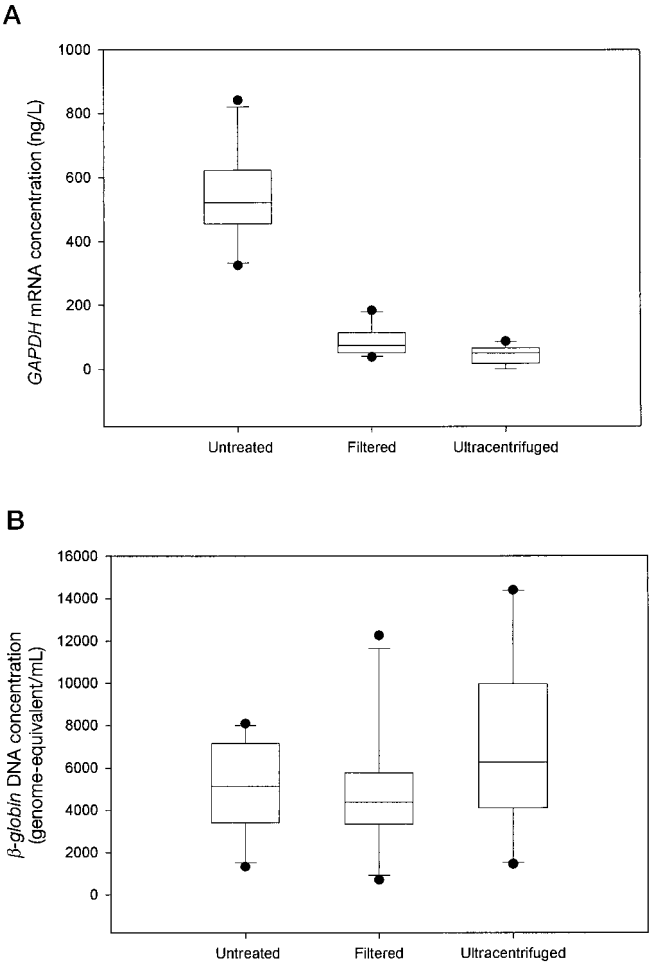

Fig. 2. Plasma mRNA (*A*) and DNA (*B*) concentrations in healthy individuals after filtration and ultracentrifugation treatments.

(*A*), plasma *GAPDH* mRNA concentrations (ng/L), as determined by real-time quantitative RT-PCR (*y axis*), plotted against the processing categories (*x axis*). (*B*), plasma *β-globin* DNA concentrations (genome-equivalents/mL), as determined by real-time quantitative PCR (*y axis*), plotted against the processing categories (*x axis*). *Untreated*, no treatment; *Filtered*, filtered through 0.22 μm filter; *Ultracentrifuged*, ultracentrifugation at 99 960*g*. The *lines inside the boxes* denote the medians. The *boxes* mark the interval between the 25th and 75th percentiles. The *whiskers* denote the interval between the 10th and 90th percentiles. ● indicate data points outside the 10th and 90th percentiles.

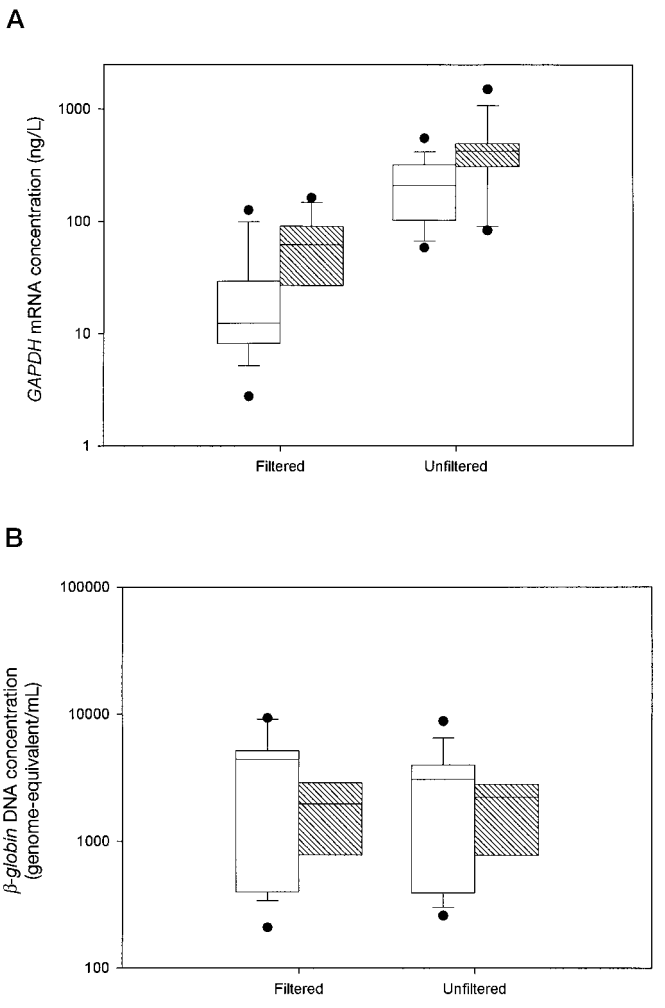

Fig. 3. Comparison of plasma mRNA and DNA concentrations in healthy individuals (□) and HCC patients (▨) with or without filtration through 0.22 μm filters.

The processing categories are shown on the *x axis*. (*A*), plasma *GAPDH* mRNA concentrations (ng/L), as determined by real-time quantitative RT-PCR, are plotted on the *y axis* (common logarithmic scale). (*B*), plasma *β-globin* DNA concentrations (genome-equivalents/mL), as determined by real-time quantitative PCR, are plotted on the *y axis* (common logarithmic scale). The *lines inside the boxes* denote the medians. The *boxes* mark the interval between the 25th and 75th percentiles. The *whiskers* denote the interval between the 10th and 90th percentiles. ● indicate data points outside the 10th and 90th percentiles.

fractional concentration of *GAPDH* mRNA in the supernatants of the ultracentrifuged plasma samples was 7.4% (interquartile range, 4.3–13%) that of the unfiltered samples. On the other hand, the differences in *β-globin* DNA concentrations among the samples subjected to different treatments did not reach statistical significance (Friedman test, $P = 0.122$).

QUANTITATIVE ANALYSIS OF *GAPDH* mRNA IN THE PLASMA OF HCC PATIENTS

To determine whether similar results could be obtained for cancer patients, we analyzed plasma samples from 16 HCC patients. As shown in Fig. 3, filterable *GAPDH* mRNA was present in the plasma of HCC patients. Indeed, filtration with a 0.22 μm filter produced a median 9.3-fold (interquartile range, 6.9- to 311-fold) decrease in *GAPDH* mRNA concentration in plasma. The data in Fig. 3A also indicate that plasma *GAPDH* mRNA concentrations in HCC patients were significantly higher than those in healthy individuals, both with or without filtration (Mann–Whitney rank-sum test, $P < 0.05$ for filtered plasma samples and $P < 0.005$ for unfiltered plasma samples). In 7 of the 16 HCC cases, sufficient plasma samples had been collected for an additional experiment involving plasma *β-globin* DNA quantification. No statistically significant difference was observed for plasma *β-globin* DNA concentrations between HCC patients and healthy individuals, both with or without filtration (Mann–Whitney rank-sum test, $P = 0.525$ for filtered

plasma samples and $P = 0.418$ for unfiltered plasma samples; Fig. 3B).

Discussion

The discovery of circulating RNA in the plasma/serum of healthy individuals and cancer patients has opened up new possibilities for noninvasive cancer detection and monitoring *(2–6, 12, 13)*. Currently, little is known about the characteristics and biological origin of circulating RNA. Cell death, including apoptosis, has been postulated to be one of the mechanisms responsible for the release of nucleic acids into the plasma *(14)*. Because cells undergoing apoptosis dispose nucleic acids into apoptotic bodies *(15)*, it is possible that at least part of the circulating RNA is particle-bound and, thus, potentially protected from nuclease degradation *(8)*. This hypothesis is particularly likely in view of the recent demonstration of apoptotic cells in human plasma *(16)*.

In this study, we investigated the particle-associated nature of circulating RNA by subjecting plasma samples to filtration through filters with different pore sizes. Our data clearly indicate that filterable *GAPDH* mRNA species are present in human plasma. Thus, the median concentration of plasma *GAPDH* mRNA was decreased 1.4-fold in samples passed through a 5 μm filter. An additional 2.4-fold reduction was observed when the data for the samples filtered through the 5 μm filters were compared with the results obtained for samples filtered through a 0.45 μm filter. The greatest reduction in *GAPDH* signal was observed after filtration through a 0.22 μm filter; the median concentration after filtration through the 0.22 μm filters was 5.1-fold lower than in samples filtered through 0.45 μm filters.

The existence of particle-associated circulating mRNA species raises the question as to whether all circulating mRNA species exist in this fashion. This question is relevant to the *GAPDH* mRNA signal, which was still detectable after filtration through a 0.22 μm filter (Fig. 1A). To address this question, we performed a series of experiments in which we measured the concentrations of *GAPDH* mRNA in plasma that had been subjected to ultracentrifugation. In a previous study, Yamamoto et al. *(17)* used a centrifugal force of 70 000*g* to pellet viral particles. The higher centrifugal force (99 960*g*) used in our study would therefore be expected to pellet virtually all particulate matter. It is interesting to note, therefore, that even after this ultracentrifugation step, a small amount of *GAPDH* mRNA was still detectable in the supernatant. This signal represented a median of 7.4% of the original *GAPDH* mRNA concentration present in the nonultracentrifuged plasma sample and was likely to represent non-particle-associated circulating mRNA species. In the present study, we did not determine whether these non-particle-associated mRNA species might be protected from degradation by complexing with protein or lipid moieties.

With particular relevance to cancer detection and monitoring, we also demonstrated that the phenomenon of particle-associated plasma mRNA species can also be observed in cancer patients. Thus, filtration with a 0.22 μm filter produced a marked decrease in plasma *GAPDH* mRNA concentrations in HCC patients (Fig. 3A). Interestingly, we also found that with or without filtration, *GAPDH* mRNA concentrations in the plasma of HCC patients were significantly higher than those in healthy individuals (Fig. 3A). These results suggest that the plasma of HCC patients contains increased concentrations of both particle-associated and non-particle-associated mRNA species. It is tempting to speculate that such mRNA species are released by the tumor cells, although confirmation of this hypothesis requires future experimentation. With regard to the particle-associated mRNA species, one possibility is that apoptosis of neoplastic cells in cancer patients might release apoptotic bodies packaged with RNA into plasma.

Our demonstration of particle-associated mRNA in plasma indicates that this component should be taken into account in future studies involving circulating mRNA. Apart from HCC, it is also important that similar experiments be carried out for other cancer types. It would also be of interest to investigate the relationship between the relative amount of particle-associated and non-particle-associated mRNA species and clinical variables such as tumor stage and treatment response. The phenomenon of particle-associated plasma RNA should also be studied in other scenarios in which circulating mRNA has been detected, such as fetal RNA in maternal plasma *(18)*.

Future research is needed to elucidate the nature of such particle-associated mRNA in plasma. Techniques such as flow cytometry and conventional and electron microscopy might provide further physical information on such particles. Another important issue concerns the cellular origin of such particle-associated mRNA. In this regard, our recent demonstration in a bone marrow transplantation model that plasma DNA is predominantly hematopoietic in origin *(19)* would suggest that one should perhaps start by studying mRNA species of hematopoietic origin.

In addition to our *GAPDH* mRNA findings, we also determined that with or without filtration, there is no significant difference in plasma *β-globin* DNA concentrations between cancer patients and healthy individuals. In contrast to mRNA, most *β-globin* DNA in plasma is nonfilterable, which is consistent with our previous study that the double centrifugation protocol used in this study (1600*g* followed by 16 000*g*) would produce no significant difference in *β-globin* DNA concentrations between filtered and unfiltered plasma samples *(9)*.

There are potentially many possible explanations for the apparent difference in the particle- and non-particle-associated nature of circulating RNA and DNA. One possible hypothesis is that both DNA and RNA are initially released into the plasma as both particle- and

non-particle-associated forms. We would further hypothesize that the non-particle-associated forms are present in much higher concentrations than the particle-associated forms. By extension of the work by Hasselmann et al. *(8)*, we would propose that the particle-associated forms of DNA and RNA are protected from degradation. If, as we hypothesize, the non-particle-associated form of DNA is released in substantially higher concentrations than the particle-associated forms, any quantitative removal of the latter by filtration will be "masked" by the overwhelming concentration of the former. However, because of the lability of RNA, the situation for this type of nucleic acid would be different. Thus, we would suggest that, because of its labile nature, most of the non-particle-associated RNA is degraded after release. The RNA species that are left would therefore be predominantly the relatively protected particle-associated forms. The predominance of such particle-associated RNA would thus explain the results of the filtration and ultracentrifugation experiments described in this report. Obtaining conclusive evidence to support this hypothetical model would require further experimentation.

To our knowledge, this study represents the second study of plasma RNA using quantitative techniques. The only previous quantitative study was based on the measurement of telomerase reverse transcriptase mRNA in the plasma of cancer patients *(6)*. It is important to note that in the previous study, the results were expressed as a ratio of telomerase reverse transcriptase mRNA to *GAPDH* mRNA. Our demonstration of increased particle-associated and non-particle-associated *GAPDH* mRNA concentrations in the plasma of HCC patients indicates that caution should be exercised when expressing or interpreting plasma RNA data as a ratio. Indeed, we believe that until the precise elucidation of the distribution of particle-associated and non-particle-associated mRNA species of different genes in the plasma of cancer patients is accomplished, it would be prudent to express quantitative mRNA measurements in plasma as an absolute concentration.

The work is supported by the Innovation and Technology Fund (AF/90/99). We thank Lisa Chan, Allen Chan, Katherine Chow, and Blenda Wong for help and advice during this study.

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