

## Reduced Plasma RNA Integrity in Nasopharyngeal Carcinoma Patients

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**Abstract Purpose:** Recent research has shown the feasibility of detecting cell-free RNA markers in human subjects. As elevated RNase activity has previously been described in the circulation of cancer patients, we hypothesized that cancer patients may have reduced plasma RNA integrity. In this study, we used nasopharyngeal carcinoma (NPC) as a model system to test this hypothesis.

**Experimental Design:** Plasma RNA integrity was determined using the ratio of the concentrations of transcript sequences corresponding to the 3' to those from the 5' end of a housekeeping gene, *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*). Transcript concentrations were measured using real-time quantitative reverse transcription-PCR assays targeting the 5' and 3' regions. We analyzed the plasma RNA integrity in 49 untreated NPC patients and 53 healthy controls. We also assessed the plasma samples from 19 NPC patients before and after radiotherapy to further show the clinical potential of this marker.

**Results:** The 3' to 5' *GAPDH* ratio was significantly lower in the plasma of untreated NPC patients when compared with healthy controls (0.0252 versus 0.0485,  $P = 0.024$ ). Statistical analysis showed that plasma *GAPDH* ratio was correlated with tumor stage but not with sex and age. Moreover, 14 of 19 NPC patients (74%) showed significant increase in the plasma *GAPDH* ratio following radiotherapy ( $P = 0.003$ ). All of these patients were in clinical remission after treatment.

**Conclusions:** Our findings suggest that NPC is associated with disturbances in the integrity of cell-free circulating RNA, raising the possibility that measurement of plasma RNA integrity may serve as a useful marker for the diagnosis and monitoring of malignant diseases.

Plasma RNA analysis has emerged as a potential tool in cancer detection and monitoring (1). The clinical implication of cell-free circulating RNA was first shown by two independent groups in 1999. Kopreski et al. (2) detected tyrosinase mRNA in the serum of patients suffering from malignant melanoma. Concurrently, Lo et al. (3) showed the presence of EBV-encoded RNA in the plasma of nasopharyngeal carcinoma (NPC) patients. These findings have since prompted further investigation, linking circulating RNA with malignant diseases and opening up the possibility of finding new molecular tumor markers in blood. Moreover, the recent demonstration of the unexpected stability of circulating RNA has attested the practicality of this approach for future clinical use (4, 5).

Apart from potential diagnostic significance, the study of plasma nucleic acids has also yielded data with biological significance, concerning particularly the molecular characteristics of such species. In the area of plasma DNA, our group has previously shown that most of the EBV DNA molecules detected in the plasma of cancer patients were <180 bp long (6), and that fetal DNA observed in the plasma of pregnant women was relatively shorter than the maternal counterparts (7). Beyond plasma DNA analysis, plasma RNA was found to exhibit another remarkable feature in terms of integrity. It has been shown that fetal RNA in maternal plasma is associated with a preponderance of 5' mRNA fragments, and that a similar phenomenon could be observed in the plasma of nonpregnant subjects using the housekeeping gene, *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*; ref. 8). These findings not only provide new insights into the biology of cell-free circulating RNA but also have implications in facilitating the future development of new molecular markers based on RNA integrity measurement.

Previous studies have documented the elevation of RNase activity in the circulation of cancer patients (9, 10). Based on these observations, we hypothesized that plasma RNA integrity could be a potential marker for tumor detection and monitoring. As NPC is an important cancer in Southern China and Southeast Asia (11), we used this tumor type as a model system to test our hypothesis. We compared plasma RNA integrity in NPC patients with that of healthy controls. Plasma

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RNA integrity was measured using the ratio of the concentrations of transcript sequences corresponding to the 3' to those from the 5' end of a housekeeping gene, *GAPDH* (8). Furthermore, we studied the potential usefulness of such a marker by assessing the effect of radiotherapy on plasma RNA integrity in NPC patients.

## Materials and Methods

**Patients.** Peripheral blood samples were collected from 49 untreated NPC patients under the care of the Department of Clinical Oncology at the Prince of Wales Hospital, Hong Kong. The patients had all undergone endoscopic examination of the nasopharynx and computed tomography and were staged according to the International Union Against Cancer Staging criteria (12). Fifty-three healthy individuals were recruited as controls. In another cohort, blood samples from 19 NPC patients were taken before the commencement of radiotherapy and 6 weeks after the completion of radiotherapy. The mean time interval between blood sampling at presentation and after radiotherapy was 3.4 months. All were in clinical remission at the time of the second blood sampling. Informed consent was obtained from all patients and healthy individuals. The study was approved by the Clinical Research Ethics Committee of The Chinese University of Hong Kong.

**Processing of blood samples.** Blood samples were collected into EDTA-containing tubes and centrifuged at  $1,600 \times g$  for 10 minutes at  $4^{\circ}\text{C}$  (Centrifuge 5810R; Eppendorf, Hamburg, Germany). Plasma was then carefully transferred to plain polypropylene tubes to be recentrifuged at  $16,000 \times g$  for 10 minutes at  $4^{\circ}\text{C}$  (Centrifuge 5415R; Eppendorf) for complete removal of residual cells (13). The supernatants were collected into fresh polypropylene tubes and mixed with TRIzol LS reagent (Invitrogen, Carlsbad, CA). All samples were stored at  $-80^{\circ}\text{C}$  until RNA extraction.

**RNA extraction.** Total RNA was extracted from 1.6 mL plasma using 2 mL TRIzol reagent (Invitrogen) and 0.4 mL chloroform, as previously described (5). Briefly, the mixture was centrifuged at  $12,000 \times g$  for 15 minutes at  $4^{\circ}\text{C}$  (Centrifuge 5417R; Eppendorf) for phase separation. The aqueous layer was then carefully transferred to fresh polypropylene tubes. For RNA precipitation, 1 volume of 700 mL/L ethanol was added to 1 volume of the aqueous layer. The mixture was applied to an RNeasy minicolumn (Qiagen, Hilden, Germany) and processed according to the protocols of the manufacturer. On-column DNase treatment was done using the RNase-Free DNase Set (Qiagen). Total RNA was then dissolved in 30  $\mu\text{L}$  RNase-free water and stored at  $-80^{\circ}\text{C}$  until use.

**Real-time quantitative reverse transcription-PCR.** Quantification of the 5' and 3' ends of the *GAPDH* transcript was done using one-step real-time reverse transcription-PCR assays (8). For the 5' assay, the amplification primers were 5'-GAAGGTGAAGTCGGAGT-3' (forward) and 5'-GAAGATGGTGATGGGATTTC-3' (reverse), and the dual-labeled fluorescent probe was 5'-(FAM)CAAGCTTCCCGTTCTCAGCC (TAMRA)-3', where FAM represents 6-carboxyfluorescein (reporter) and TAMRA represents 6-carboxytetramethylrhodamine (quencher). For the 3' assay, the amplification primers were 5'-AAACCTGCCAAATATGATGACAT-3' (forward) and 5'-ACCCTGTTGCTGTAGCCAAA-3' (reverse) and the dual-labeled fluorescent probe was 5'-(FAM)AGCTCATTTCCGTGATGACAACGAA(TAMRA)-3'.

Measurement of 5' and 3' *GAPDH* mRNA concentrations was based on a cloned calibrator (8). The *GAPDH* gene was amplified from human pooled organ cDNA (Clontech, Mountain View, CA) with the forward primer of the 5' assay and the reverse primer of the 3' assay using the Advantage cDNA Polymerase Mix (Clontech). This construct was then cloned into pGEM T-Easy Vector according to the protocols of the manufacturer (Promega, Madison, WI). Plasmid DNA was isolated with the Wizard Plus Minipreps DNA Purification kit (Promega),

confirmed by direct sequencing (BigDye Terminator v3.1, Applied Biosystems, Foster City, CA), and serially diluted from  $2.5 \times 10^6$  to 2.5 copies/ $\mu\text{L}$ .

Quantitative reverse transcription-PCR was set-up in a reaction volume of 50  $\mu\text{L}$  using the EZ *rTth* RNA PCR Reagent kit (Applied Biosystems), as described by the manufacturer. For both the 5' and 3' assays, the primers and fluorescent probes were used at concentrations of 200 and 100 nmol/L, respectively. Three microliters of the extracted plasma RNA were applied to each reaction. Both assays could detect down to 7.5 copies of the calibrator in the reaction mixture and were run in parallel for each sample in duplicate analysis. Every batch of amplification included multiple water blanks as negative controls. All data were collected and analyzed with an ABI Prism 7700 Sequence Detector (Applied Biosystems).

An identical thermal profile was used for the 5' and 3' systems. Thermal cycling was initiated with an incubation at  $50^{\circ}\text{C}$  for 2 minutes to activate the uracil *N*-glycosylase, followed by reverse transcription at  $60^{\circ}\text{C}$  for 30 minutes, then a  $95^{\circ}\text{C}$  incubation for 5 minutes to activate the *rTth* polymerase, and finally 40 cycles of denaturation at  $94^{\circ}\text{C}$  for 20 seconds and annealing/extension at  $60^{\circ}\text{C}$  for 1 minute.

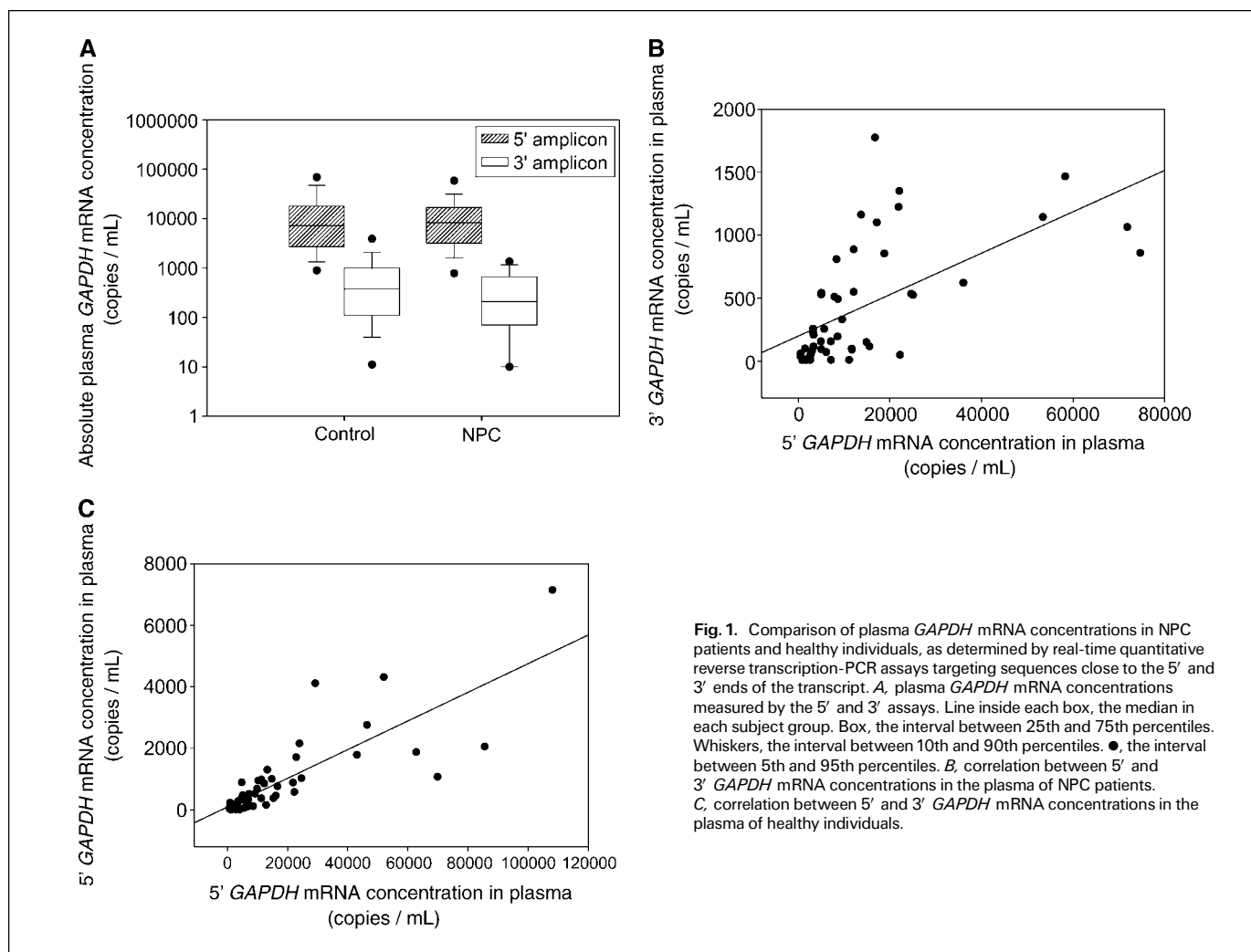
**Statistical analysis.** Statistical tests were done using the SigmaStat 2.03 software (SPSS, Inc., Chicago, IL).

## Results

**Absolute *GAPDH* mRNA concentrations in the plasma of NPC patients.** Using one-step real-time quantitative reverse transcription-PCR, we measured the absolute concentrations of the 5' and 3' regions of the *GAPDH* transcript in the plasma of 49 untreated NPC patients (31 males and 18 females; median age, 49 years) and 53 healthy controls (28 males and 25 females; median age, 44 years). No significant difference was observed in the absolute *GAPDH* mRNA concentrations when the two groups were compared (Mann-Whitney test,  $P = 0.952$  for the 5' assay and  $P = 0.120$  for the 3' assay; Fig. 1A). Further statistical analysis showed that the overall variation of the 5' mRNA concentration was significantly correlated with that of the 3' mRNA concentration, for both NPC patients (Pearson correlation,  $r = 0.612$  and  $P < 0.001$ ; Fig. 1B) and healthy controls (Pearson correlation,  $r = 0.814$  and  $P < 0.001$ ; Fig. 1C).

**Reduced RNA integrity in the plasma of NPC patients.** We then investigated the integrity of plasma RNA in the same group of NPC patients and healthy controls. Plasma RNA integrity was measured by the ratio of 3' to 5' *GAPDH* mRNA concentrations (8). All patients and controls had ratios of  $<1$ , which is consistent with our previous observation that there is an overrepresentation of 5' mRNA fragments in the circulation of healthy individuals (8). The median 3' to 5' *GAPDH* ratios in the plasma of NPC patients and control subjects were 0.0252 (interquartile range, 0.0112-0.0646) and 0.0485 (interquartile range, 0.0257-0.0773), respectively (Fig. 2). Statistical analysis showed a significant difference between the two groups (Mann-Whitney test,  $P = 0.024$ ). These results suggest that NPC is associated with decreased RNA integrity in plasma.

**Correlation of plasma RNA integrity with tumor staging in NPC patients.** Of the 49 NPC cases, 18 had early-stage disease (stages I and II) and 31 had advanced-stage disease (stages III and IV). The median plasma RNA integrity in early-stage patients was significantly higher than that in advanced-stage



**Fig. 1.** Comparison of plasma *GAPDH* mRNA concentrations in NPC patients and healthy individuals, as determined by real-time quantitative reverse transcription-PCR assays targeting sequences close to the 5' and 3' ends of the transcript. **A**, plasma *GAPDH* mRNA concentrations measured by the 5' and 3' assays. Line inside each box, the median in each subject group. Box, the interval between 25th and 75th percentiles. Whiskers, the interval between 10th and 90th percentiles. ●, the interval between 5th and 95th percentiles. **B**, correlation between 5' and 3' *GAPDH* mRNA concentrations in the plasma of NPC patients. **C**, correlation between 5' and 3' *GAPDH* mRNA concentrations in the plasma of healthy individuals.

patients (0.0456 versus 0.0217, Mann-Whitney test,  $P = 0.034$ ; Fig. 3). In contrast, no correlation was observed with sex (Mann-Whitney test,  $P = 0.648$ ) and age (Mann-Whitney test,  $P = 0.647$ ).

**Change in plasma RNA integrity after radiotherapy.** To further show the clinical relevance of plasma RNA integrity, we studied plasma samples from 19 NPC patients (15 males and 4 females; median age, 44 years) before and after radiotherapy. All of them were in clinical remission at the time of posttreatment blood sampling. Plasma RNA integrity was found to increase significantly after the completion of radiotherapy in 14 of 19 (74%) patients (Wilcoxon test,  $P = 0.003$ ; Fig. 4). These data suggest that measurement of plasma RNA integrity may represent a new tool for monitoring treatment efficacy in NPC patients.

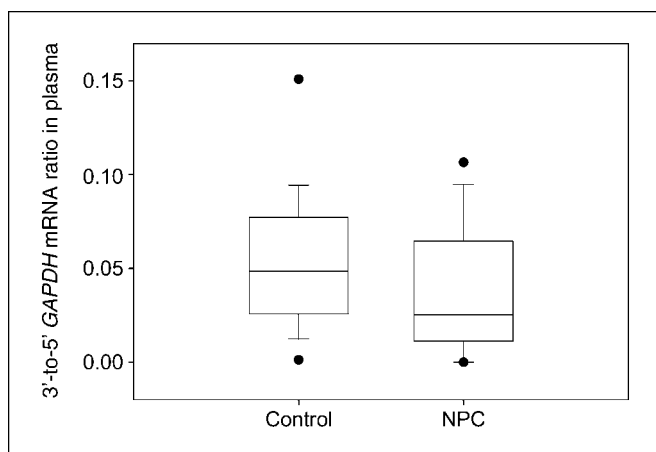
## Discussion

Analysis of cell-free circulating RNA has offered new promises for noninvasive cancer diagnostics. The development of quantitative assays for tumor RNA detection in plasma has further enhanced the potential application of such markers (14–16). We have recently studied a new variable, plasma RNA integrity,

and have shown that transcript fragments corresponding to the 5' end of the *GAPDH* gene are predominant when compared with those derived from the 3' end in the plasma of healthy individuals (8). As RNase activity has been shown to be increased in the circulation of cancer patients, we sought to investigate whether plasma RNA integrity could be used as a tumor-associated marker.

In this study, we used NPC as a model system for plasma RNA integrity analysis. Our results showed that untreated NPC patients had decreased plasma RNA integrity when compared with healthy controls. The median 3' to 5' *GAPDH* mRNA ratio was found to decline by ~2-fold in the plasma of NPC patients. On the other hand, no significant difference was observed in the absolute *GAPDH* mRNA concentrations (for both the 5' and 3' assays) between the two groups. Further analysis showed that plasma RNA integrity in early-stage patients (stages I and II) was 2-fold higher than that in advanced-stage patients (stages III and IV).

To further evaluate the clinical potential of plasma RNA integrity, we also analyzed plasma samples from NPC patients before and at 6 weeks after radiotherapy. It was shown that 14 of 19 patients (74%) had increased 3' to 5' *GAPDH* ratios in the posttreatment plasma samples. These data provide evidence that plasma RNA integrity may serve as a tumor marker not

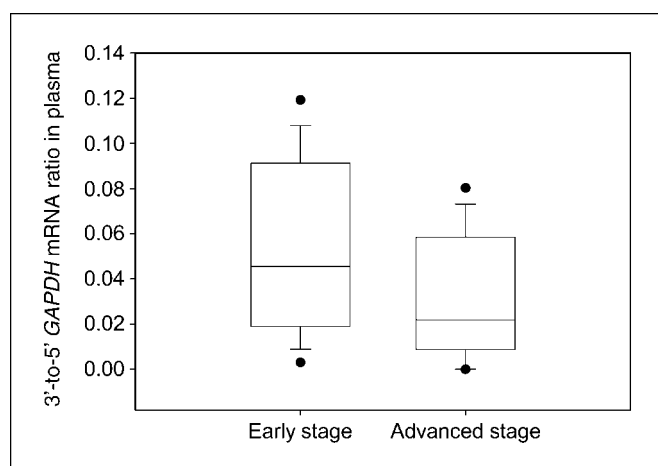


**Fig. 2.** Comparison of plasma RNA integrity in NPC patients and healthy individuals. RNA integrity was defined as the ratio of 3' to 5' *GAPDH* mRNA concentrations, as determined by real-time quantitative reverse transcription-PCR assays targeting the 5' and 3' ends of the transcript. Line inside each box, the median in each subject group. Box, the interval between 25th and 75th percentiles. Whiskers, the interval between 10th and 90th percentiles. ●, the interval between 5th and 95th percentiles.

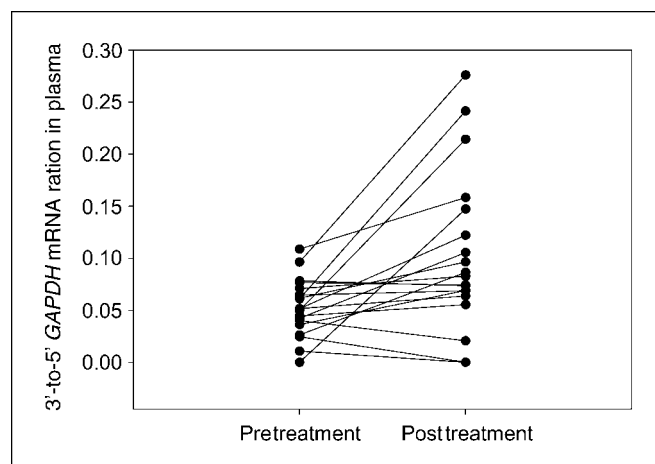
only for NPC detection but also for the monitoring of treatment response. It would be of interest to perform a follow-up study on these patients, particularly those with "unnormalized" ratios, to investigate the relationship between plasma RNA integrity and patient survival.

In our previous studies (17–19), we have shown that plasma EBV DNA is a good molecular marker for NPC. Whether plasma EBV DNA and plasma RNA integrity may be used synergistically for NPC diagnostics will require further investigation. However, we have to emphasize that NPC is used as a model system for this first study of plasma RNA integrity in cancer. With the current set of promising results, we and other investigators could potentially launch similar studies for other tumor types.

For accurate interpretation of our data, we have considered the possibility of aberrant alternative splicing (20, 21) and of



**Fig. 3.** Correlation of plasma RNA integrity with tumor staging in NPC patients. These patients were classified into the early-stage (stages I and II) and advanced-stage (stages III and IV) groups. Line inside each box, the median in each subject group. Box, the interval between 25th and 75th percentiles. Whiskers, the interval between 10th and 90th percentiles. ●, the interval between 5th and 95th percentiles.



**Fig. 4.** Measurement of plasma RNA integrity in NPC patients before and after radiotherapy. Line inside each box, the median in each subject group. Box, the interval between 25th and 75th percentiles. Whiskers, the interval between 10th and 90th percentiles. ●, the interval between 5th and 95th percentiles.

aberrant transcription of pseudogenes (22, 23) that may cause the difference in plasma RNA integrity between NPC patients and healthy controls. We have used the Alternative Splicing Annotation Project database (24, 25) and have found no evidence of alternative splicing for the human *GAPDH* gene. Identification of *GAPDH* pseudogenes, however, has been previously documented (26, 27). In this study, we conducted DNase I treatment on the plasma RNA samples to prevent contamination with genomic DNA. Also, based on a previous study by Harrison et al. (28), who have identified a list of transcribed pseudogenes using a data-mining strategy, *GAPDH* pseudogenes are not shown to be transcribed in the human genome.

The mechanism by which plasma RNA integrity is reduced is most likely to be associated with increased activities of circulating RNases. High levels of RNases have previously been observed in the serum of pancreatic cancer patients (9) and in the plasma of ovarian cancer patients (10). The origin of such elevations, however, is still unknown. It has been shown that RNases are expressed in different types of human tissues (29). For example, Peracaula et al. (30) reported that the patterns of RNase expression observed in pancreatic adenocarcinoma were distinct from those in normal pancreatic tissues. It is speculated that tumor tissues may contribute to the increase of RNases in the circulation. Furthermore, these findings raise the possibility that decreased plasma RNA integrity may also be associated with other types of malignant diseases. This would have potential effect in the diagnosis of many cancer types that are currently without a reliable biochemical or molecular marker.

In conclusion, this study is the first to show the potential of plasma RNA integrity as a noninvasive tumor-associated marker. Apart from their clinical implications, our findings have also provided new insights into the molecular nature of cell-free RNA circulating in the plasma of cancer patients.

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