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Investigation of a Quality Check for Plasma Samples

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Abstract

Background: MicroRNA (miRNA) molecules have been detected in many body fluids and used as biomarkers. However, blood cell-derived miRNA molecules due to hemolysis have been shown to affect the amounts of plasma miRNAs. It is important to check the quality of plasma samples for clinical applications. In the present study, an objective method for quality checking plasma samples was investigated using permitted color tone level and the absorbance at 414 nm.

Material and method: An ROC analysis of the macroscopic color tone and the absorbance in 1213 clinical samples was performed. The optimal cut-off absorbance value was validated using the amount of plasma miRNA.

Results: AUC for detecting hemolyzed samples was very high (0.986) at a cut-off absorbance value of 1.664. A sensitivity and specificity were 99% and 92%, respectively. For validation study, 3 candidate miRNAs were selected by a miRNA microarray between fresh and hemolyzed plasma samples; the amounts of miR-16 and miR-19b increased in hemolyzed samples, whereas that of miR-223 remained unchanged. The amounts of plasma miR-16 and miR-19b were significantly increased in 5 clinical samples with higher absorbance values (p=0.0001 and p=0.0003, respectively), while the amounts of these miRNAs were not increased in samples with lower absorbance values.

Conclusions: A simple method for quality checking plasma samples using color tone and absorbance is very useful and significant.

Keywords: Plasma sample; Quality check; Color tone; Absorbance, miRNA

Introduction

MicroRNAs (miRNAs) are small non-coding RNA molecules that play important roles in a variety of normal and disease-related biological processes by post-transcriptionally regulating the expression of target mRNAs [1,2]. They have been detected in many body fluids, because circulating or extracellular miRNAs that bind to protein complexes or are contained within membranous particles such as exosomes or micro-vesicles are protected from degradation by RNase [3-7]. Therefore, plasma miRNA molecules are stable and a potential source of novel biomarker for a number of diseases, including cancer [8,9].

However, the amount of plasma miRNA molecules is known to be markedly affected by sample collection or storage methods. For example, hemolysis, which is caused by negative pressure during sample extraction, results in reddish color tone and marked increase in the amounts of plasma miRNA, whereas the freeze-thaw process decreases the amounts. Thus, although circulating miRNAs are useful biomarkers for many diseases, objective quality checks on plasma samples should be performed carefully.

In the present study, a potential objective method for quality checking plasma samples was examined using the macroscopic color tone and absorbance. And the optimal absorbance cut-off values for a quality check of plasma samples were validated using the amounts of plasma miRNA molecules.

Materials and Methods

Clinical samples

A total of 1213 plasma samples were collected from patients who had been diagnosed with digestive cancer and underwent surgery between January 2012 and April 2015 at Kyoto Prefectural University of Medicine. Written informed consent was obtained from all patients. Control samples were also collected from 10 adult healthy volunteers by standard cubital vein puncture.

Storage of plasma samples

Immediately after the collection of blood samples in sodium heparin tubes (BD Vacutainer, Franklin Lakes, NJ, USA), cell-free nucleic acids were isolated using a three-spin protocol (1500 rpm for 30 min, 3000 rpm for 5 min, and 4500 rpm for 5 min) in order to prevent contamination by cells or cellular nucleic acids. 120 µl of each plasma sample was added to the well of a 96-well plate and stored at -80°C until the absorbance was measured. The remainder of each plasma sample was stored at -80°C until further processing.

RNA extraction

Total RNA was extracted from 400 µl of plasma samples using a miRVana PARIS kit (Ambion, Austin, TX, USA) and eluted into 100 µl of pre-heated (95°C) elution solution according to the manufacturer's instructions. After plasma samples were thawed on ice and treated with Qiazol, synthetic *Caenorhabditis elegans* miRNA oligonucleotides, cel-miR-39 (a mixture of 25 fmol of each oligonucleotide in a total volume of 5 µl) were added to each plasma sample in order to normalize the efficacy of RNA extraction. RNA samples were stored at -80°C until further processing.

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Study design

The study design is summarized in Figure 1. This study was divided into five steps: (1) Determination of the permitted color tone using intentionally hemolyzed plasma samples; i.e., to determine whether the samples were reddish or not, and (2) Examination of absorbance at 414 nm of the clinical plasma samples. (3) Decision of the optimal cut-off absorbance value for a quality check. (4) miRNA microarray analysis between fresh and intentionally hemolyzed plasma samples from a healthy volunteer, in order to select candidate miRNAs for validation. (5) Validation study of the optimal cut-off absorbance value in clinical plasma samples using the amounts of candidate miRNAs.

Measurement of absorbance in plasma samples

The absorbance values of plasma samples were measured using a fluorescent microplate reader with XFLUOR4 (TECAN, Mannedorf, Switzerland) at a wavelength of 414 nm (reference wavelength: 595 nm) according to the previous study [10].

MicroRNA microarray analysis

Plasma samples from healthy volunteers were analyzed by a microRNA microarray. The fresh sample and intentionally hemolyzed one due to negative pressure were examined [11-13]. Microarray analyses were performed using the 3D-Gene microRNA microarray platform (TORAY, Kamakura, Japan) [14,15], and RNA extraction was conducted according to the manufacturer's instructions. Only a very small amount of total RNA was present in the plasma samples; therefore 2 out of the 4 µl of total RNA extracted from 300 µl of plasma samples were used in microarray experiments. RNA was labeled with Hy5 and hybridized at 32°C for 16 h on the 3D-Gene chip. The 3D-Gene miRNA microarray contained more than 1000 miRNA molecules based on the Human miRNA Version 15 of MirBase (http://microrna.sanger.ac.uk/). We analyzed microarray analysis data and selected three candidate miRNA molecules, the levels of which were significantly higher in hemolyzed samples than in fresh samples or similar between both samples.

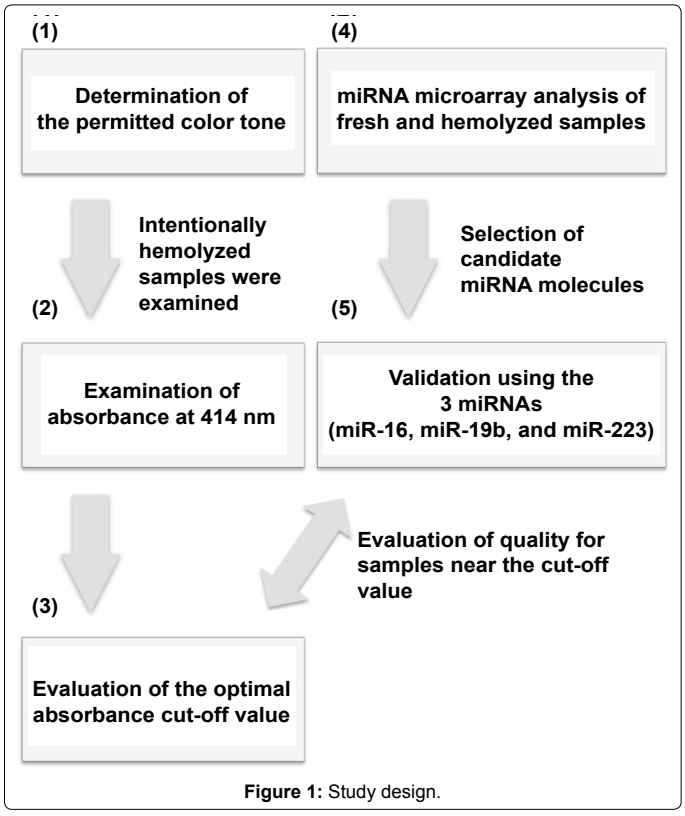

Figure 1: Study design.

MicroRNA detection protocol

The amounts of target miRNA molecules in plasma were measured by RT-PCR. A reverse transcription reaction was carried out using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA), and the amount of each miRNA molecule was quantified in duplicate by quantitative RT-PCR (qRT-PCR) with the human TaqMan MicroRNA Assay Kit (Applied Biosystems) in accordance with previously described protocols [16]. These assays were run on a Step One Plus PCR system v2.1 (Applied Biosystems). The primers for human miRNA (i.e., hsa-miR-16, hsa-miR-19b, and hsa-miR-223) and *C. elegans* miRNA (i.e., cel-miR 39) used in the TaqMan assays were obtained from Applied Biosystems. Changes in the plasma miRNA levels were calculated using the $2^{-\Delta\Delta Ct}$ method [17,18].

Statistical analysis

The paired *t*-test was used to compare the results obtained for fresh and hemolyzed plasma samples. *P*-values of <0.05 were considered significant. ROC curves and the AUC were used to assess the most appropriate absorbance cut-off value for detecting hemolyzed plasma samples, which was determined with the Youden index [19]. Spearman's correlation co-efficient was used to investigate the relationship between absorbance and miRNA levels.

Results

Examination of the permitted color tone and absorbance in control sample

The color series of intentionally hemolyzed plasma samples were shown in Figure 2a. The macroscopic color tone gradually became dense, and the permitted color tone as the not-hemolyzed plasma sample was determined. A borderline of absorbance value at 414 nm, whether the samples were hemolyzed or not, was between 1.363 and 1.761.

Evaluation of the most appropriate absorbance cut-off value for detecting damaged plasma sample

Absorbance value at 414 nm and macroscopic color tone were assessed in 1213 plasma samples. The mean and standard deviation of absorbance value were 1.086 and 0.662. A receiver operating characteristic (ROC) analysis of absorbance value and color tone of each sample revealed the optimal absorbance cut-off value for detecting damaged plasma sample. The area under the ROC curve (AUC) was very high (AUC=0.986) at an absorbance cut-off value of 1.664 (Figure 2b).

Results of the miRNA microarray analysis and validation study

Table 1 shows a list of miRNA molecules whose levels were higher in hemolyzed samples than in fresh samples, or similar in both samples. The miRNAs that were not detectable in fresh samples were not listed in Table 1. Three miRNA molecules, miR-16, miR-19b, and miR-223, were selected for the validation study of this quality checking method, because these miRNAs were present at high amounts in plasma and markedly different between hemolyzed and normal samples (Table 1, squarish marks).

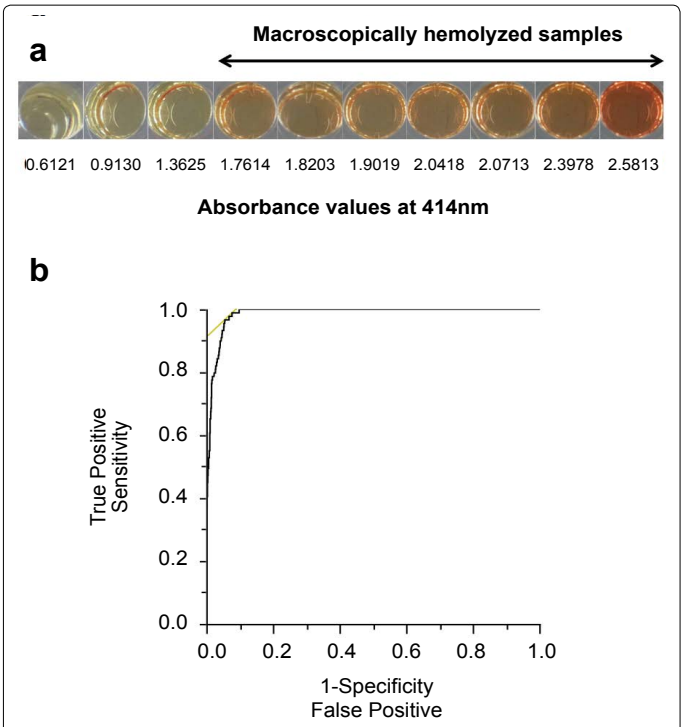

Figure 2: Evaluation of the most appropriate absorbance cut-off value for detecting damaged plasma sample. (a) Determination of the permitted color tone level using intentionally hemolyzed plasma samples. (b) Evaluation of the optimal plasma absorbance cut-off value in 1213 clinical plasma samples. Absorbance at 414 nm and the macroscopic color tone of the plasma samples were assessed. An analysis was performed based on these data, and the Youden index was used to determine the optimal absorbance cut-off value for detecting hemolyzed samples.

Increased				
Name	**ID**	**Hemolyzed (A)**	**Fresh (B)**	**Ratio (A/B)**
has-miR-4484	MIMAT0019018	4612.381617	231.3811	19.93413
has-miR-20b	MIMAT0001413	328.401954	17.35625	18.92125
has-miR-16	MIMAT0000069	2716.468946	144.752	18.76636
has-miR-15a	MIMAT0000068	373.143256	45.38332	8.222035
has-miR-19b	MIMAT0000074	767.425979	93.79371	8.182062
has-miR-20a	MIMAT0000075	371.74509	47.93124	7.7558
has-miR-4417	MIMAT0018929	1816.050244	236.477	7.679607
has-let-7b	MIMAT0000063	230.530356	30.09583	7.659877
has-miR-3135b	MIMAT0018985	129.862426	17.35625	7.482169
has-miR-26b	MIMAT0000083	71.139468	9.712508	7.324521
Not changed				
Name	**ID**	**Hemolyzed (A)**	**Fresh (B)**	**Ratio (A/B)**
has-miR-223	MIMAT0000280	451.440534	379.1602	1.190663
has-miR-4745-5p	MIMAT0019878	4397.064101	3693.998	1.190327
has-miR-4513	MIMAT0019050	163.418403	149.8479	1.090562
has-miR-3187-5p	MIMAT0019216	79.528462	81.05414	0.981177
has-miR-3925-5p	MIMAT0018200	82.324793	73.41039	1.121432
has-miR-4496	MIMAT0019031	59.954142	60.67082	0.988188
has-miR-3137	MIMAT0015005	55.759645	55.57498	1.003323
has-miR-4299	MIMAT0016851	52.963314	53.02707	0.998798
has-miR-513a-5p	MIMAT0002877	51.565148	47.93124	1.075815
has-miR-4514	MIMAT0019051	43.176154	40.28749	1.071701

Table 1: Results of the miRNA microarray analysis. MicroRNA molecules whose concentrations were increased or unchanged in hemolyzed plasma samples.

The amounts of miR-16 and miR-19b were significantly increased with approximately 100- and 60-times in hemolyzed plasma samples, respectively ($p<0.0001$ in both cases; paired t-test; Figures 3a and 3b), whereas that of miR-223 remained unchanged in fresh and hemolyzed samples ($p=0.169$, Figure 3c). The absorbance of these plasma samples at 414 nm was also examined, and it was found that the absorbance value correlated with the amounts of miR-16 or miR-19b ($p<0.001$; Spearman's correlation co-efficient; Figure 3d, data for only miR-16 was shown).

Absorbance values and miRNA amounts in clinical plasma samples

The absorbance values and amounts of plasma miR-16 and miR-19b were examined in 10 clinical plasma samples obtained from gastric or pancreatic cancer patients (Table 2). Tumor progression of these 10 randomly selected patients was various degree. The samples obtained from 5 patients were macroscopically reddish, and the others were not. The absorbance values at 414 nm of 5 reddish samples were more than 1.664, while those of the others were less than 1.664. The amounts of miR-16 and miR-19b were also increased in the samples with higher absorbance values regardless of their tumor progression ($p=0.0001$ and $p=0.0003$ for miR-16 and miR-19b, respectively; Figures 4a and 4b), however no significant difference was observed in the amounts of miR-223 between both groups ($p=0.241$, data was not shown).

Discussion

The quality of clinical sample is extremely important when researching new plasma biomarkers. Although many studies identified plasma miRNAs as disease biomarker and previous studies reported a relationship between hemolysis and the increase of plasma free nucleic acid [10], only a few studies have examined the effects of hemolysis and other damaging processes on clinical plasma researches. We also previously identified some plasma miRNA biomarkers, however only macroscopic color tone was mainly checked for the quality of clinical plasma samples. In the present study, we confirmed a more objective method for quality checking plasma samples based on their color tone and absorbance values.

During hemolysis, the cell membranes of red corpuscles are damaged physically, chemically, or biologically by various factors, and their protoplasm leaks into the bloodstream. Thus, when clinical blood samples are extracted, clinicians need to be aware that surplus negative pressure may cause hemolysis. Michaela et al. reported that plasma miRNA concentration was affected by hemolysis, and the degree of hemolysis in a particular plasma sample was able to be determined from its macroscopic color tone or absorbance at 414 nm [10]. Colin CP et al. also showed that blood cells were a major contributing factor to circulating miRNA levels and that blood cell count or hemolysis markedly altered plasma miRNA levels. Therefore, plasma miRNA-based data should be carefully interpreted, because the majority of the reported plasma miRNA biomarkers of cancer are strongly expressed in blood cells. The change of a particular miRNA biomarker may reflect the blood cell-based changes rather than cancer-specific ones [11,12]. Thus, clinical samples need to be handled very carefully when blood cell-derived miRNA molecules are examined as plasma biomarkers [13].

Some difficulties were associated with quality checking plasma samples. Chyle-containing samples were sometimes found, and the absorbance of these samples was slightly higher than that without hemolysis or chyle (data not shown). The chyle contained in plasma samples originates from various sources including chylomicrons,

neutral fats, very low-density lipids, and intravenously administered fat tablets. Further studies are needed to confirm whether these samples are suitable for biomarker-based experiments. On the other hand, cancer and other diseases may affect the amounts of plasma miRNAs. If cancer-specific miRNAs are used to quality check of plasma samples, we may inappropriately exclude suitable samples and miss the cancer-specific changes. Therefore, the candidate miRNA molecules used for quality checking should be selected carefully, and some miRNAs should be examined repeatedly.

In the present study, the samples which showed higher absorbance more than the cut-off value contained significantly larger amounts of miR-16 and miR-19b than those showed lower absorbance value. These results may indicate that the amounts of these miRNAs are influenced by hemolysis rather than tumor progression, and support the accuracy and reproducibility of this cut-off absorbance value. The measurement of absorbance is an easy and objective method for quality checking plasma samples. Although plasma samples are very useful for a range of biomarker analyses, it may be better to discard samples with higher absorbance.

Cases	Age	Gender	Stage	Absorbance*	M acroscopic color
PK1	86	Female	Pancreas cancer, Stage III	0.998	Not reddish
PK2	72	Female	Pancreas cancer, Stage IA	1.112	Not reddish
MK1	72	Male	Gastric cancer, Stage III	1.227	Not reddish
MK2	70	Male	Gastric cancer, Stage IA	1.035	Not reddish
MK3	55	Male	Gastric cancer, Stage IA	1.297	Not reddish
PK3	39	Male	Pancreas cancer, Stage III	2.259	Reddish
PK4	80	Male	Pancreas cancer, Stage IA	1.916	Reddish
MK4	73	Male	Gastric cancer, Stage IB	1.67	Reddish
MK5	63	Male	Gastric cancer, Stage IA	1.861	Reddish
MK6	51	Male	Gastric cancer, Stage IB	1.9	Reddish

*414 nm absorbance

Table 2: Clinical features and absorbance values of clinical plasma samples. These ten random samples exhibited various degree of tumor progression. Five samples were macroscopically reddish, and the other five were not.

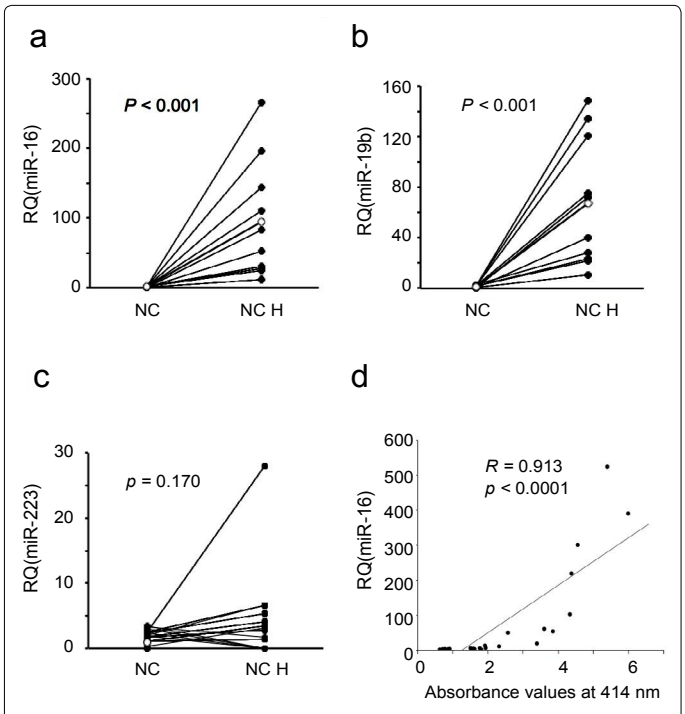

Figure 3: Validation study of paired fresh and hemolyzed plasma samples. The amounts of plasma miR-16, miR-19b, and miR-223 were examined by RT-PCR (a, b, and c). Differences between fresh and hemolyzed samples were analyzed using the paired *t*-test. NC: fresh samples, NC H: hemolyzed samples. (d) The relationship between 414 nm absorbance value and the amount of plasma miR-16 was analyzed in intentionally hemolyzed samples obtained from healthy volunteers.

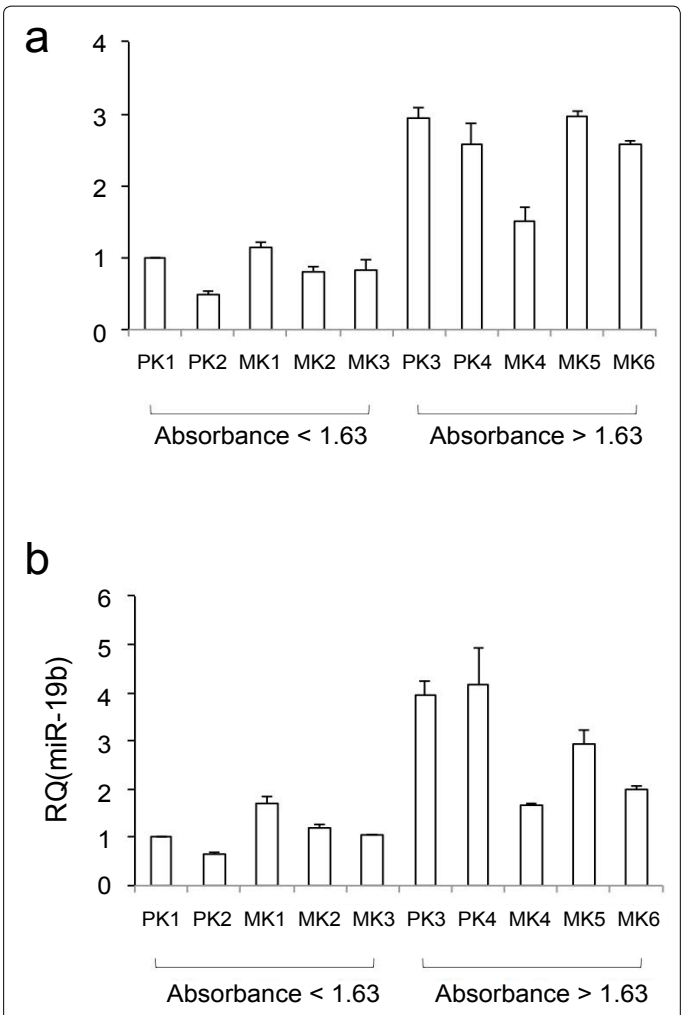

Figure 4: Comparison of plasma miRNA levels in cancer patients according to absorbance value. Plasma miR-16 (a) and miR-19b (b) levels of cancer patients are shown relative to the level of PK1. PK1, PK2, MK1, MK2, and MK3 exhibited absorbance values of less than 1.664 at 414 nm. PK3, PK4, MK4, MK5, and MK6 exhibited absorbance values of more than 1.664 at 414 nm.

Conclusion

A simple method for quality checking plasma samples using color tone and absorbance is very useful and significant.

Competing Interests

None of the authors have any conflict of interest to disclose.

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