Biochemical and Biophysical Research Communications 454 (2014) 210–214

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

U6 is not a suitable endogenous control for the quantification of circulating microRNAs

Mengqin Xiang [a,1], Ying Zeng [b,1], Ruirui Yang [a], Haifan Xu [c,d], Zhuo Chen [c], Jing Zhong [e], Hailong Xie [a], Yinghui Xu [a], Xi Zeng [a,d,*]

[a] Key Laboratory of Tumor Cellular and Molecular Pathology of College of Hunan Province, Cancer Research Institute, University of South China, Hengyang 421001, China
[b] School of Nursing, University of South China, Hengyang 421001, China
[c] The Department of Surgical Oncology, The First Affiliated Hospital, University of South China, Hengyang 421001, China
[d] Center for Gastric Cancer Research of Hunan Province, University of South China, Hengyang 421001, China
[e] Institute of Clinical Medicine, The First Affiliated Hospital, University of South China, Hengyang 421001, China

ARTICLE INFO

Article history:
Received 13 October 2014
Available online 18 October 2014

Keywords:
Circulating microRNAs
Endogenous control
U6
MiR-16

ABSTRACT

Recently, microRNAs have been detected in serum and plasma, and circulating microRNA (miRNA) profiles have now been associated with many diseases such as cancers and heart disease, as well as altered physiological states. Because of their stability and disease resistance, circulation miRNAs appear to be an ideal material for biomarkers of diseases and physiological states in blood. However, the lack of a suitable internal reference gene (internal reference miRNA) has hampered research and application of circulating miRNAs. Currently, U6 and miR-16 are the most common endogenous controls in the research of miRNAs in tissues and cells. We performed microarray-based serum miRNA profiling on the serum of 20 nasopharyngeal carcinoma patients and 20 controls to detect the expressions of U6 and miRNAs. Profiling was followed by real-time quantitative Polymerase Chain Reaction (qPCR) in 80 patients (20 each with gastric cancer, nasopharyngeal carcinoma, colorectal cancer, and breast cancer) and 30 non-cancerous controls. qPCR was also performed to detect miRNAs in serum with repeated freezing and thawing. The results of microarray showed that with the exception of U6, Ct values of miR-16, miR-24, miR-142-3p, miR-19b and miR-192 in serum samples of nasopharyngeal carcinoma were greater than control samples. The results of 110 cases showed large fluctuations in U6 expression. The difference between the greatest and the least levels of expression was 3.29 for delta Ct values, and 1.23 for miR-16. The expressions of U6, miR-16 and miR-24 in serum subjected to different freeze–thaw cycles showed that U6 expression gradually decreased after 1, 2, and 4 cycles of freezing and thawing, while the expression of miR-16 and miR-24 remained relatively stable. Collectively, our results suggested that U6 is unsuitable as an internal reference gene in the research of circulating miRNAs.

1. Introduction

MicroRNAs (miRNAs) play a key role in the development of many diseases, which negatively regulate the expression of protein-coding genes at the post-transcriptional level [1,2]. Particularly in tumors, miRNAs play a role similar to that of oncogenes or tumor suppressor genes [1,3]. In 2008, several research groups have discovered miRNA in human serum and plasma [4,5]. These circulating miRNAs are very stable in the blood. Healthy individuals have a consistent level of circulating miRNAs expression, but the levels of circulating miRNAs in serum will significantly change in different diseases [5]. Therefore, circulating miRNAs can become potential biomarkers for the diagnosis, monitoring, and prognosis of diseases.

Although research on circulating miRNAs as biomarkers of disease is on the rise, researchers are encountering a practical problem, the lack of a recognized and reliable reference gene (housekeeping gene), which makes comparisons difficult between different samples and complicates the search for biomarkers in circulating miRNAs. Currently, researchers use U6 and miR-16 for

* Corresponding author at: Cancer Research Institute, University of South China, Changsheng West Road 28, Hengyang, Hunan Province 421001, China.
E-mail addresses: xiangmengqin@126.com (M. Xiang), zengying2003@126.com (Y. Zeng), 15200519639@163.com (R. Yang), xuhaifan0734@163.com (H. Xu), roychan158@163.com (Z. Chen), zhongjing2002@hotmail.com (J. Zhong), xhl0078@sina.com.cn (H. Xie), xyhh007@sina.cn (Y. Xu), zengxiying@163.com (X. Zeng).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.bbrc.2014.10.064
0006-291X/

internal reference in tissue and cell miRNAs studies. Researchers use the following ways to solve the problem of internal reference in circulating miRNAs detection: (a) According to reports in the literature , researchers often consider stable RNA expression as an internal reference of circulating miRNAs detection: e.g. U1 [6], U6 [7–9], U43 [6], U44 [10,11], U48 [10,12,13], miR-16 [14–19], miR-19b [4], miR-24 [4,14], miR-30e [20], miR-142-3p [10], miR-192 [21], miR-638 [22], let-7a [14], 5S [23,24], 18S [25], etc. However, failure to undergo rigorous experimental verification has led to disagreement among researchers [26], thus some results have been questioned. Especially, there are much controversy about whether U6 and miR-16 are good internal references [5,7–9,14–19]. (b) Added the same amount of exogenous miRNAs (sample species does not contain) as internal reference into serum or plasma samples [27–32]. This method can eliminate some deviations of the experimental process and make the results reliable. But it also causes the experimental procedure cumbersome and clinical applications more inconvenient. (c) Use concentration of total RNA samples as the reference index [5]. In this method, the accuracy of RNA concentration measurement has strict requirements. But the low circulating miRNAs levels are difficult to measure. In addition, concentration of total RNA may not give a true reflection of the amount of miRNA samples. (d) some researchers directly compare the volume obtained by equal amounts of serum/plasma sample [33–35]. Because the internal reference is not set, the deviation between the samples cannot be corrected, reducing the credibility of the results [36]. Taken together, there is still no recognized and reliable reference gene in circulating miRNAs detection, which seriously interferes with investigators and limits the development of circulating miRNA study. Therefore, screening current miRNAs used as internal references and finding reliable internal references of circulating miRNAs is urgently needed.

In this study, we screened and compared the expression level of some miRNAs used as internal controls, and found that U6 expression is not stable in serum. It may be not suitable as an internal reference gene in the study of circulating miRNAs.

2. Materials and methods

2.1. Clinical samples

Following ethical approval and written informed consent, serum samples were collected from 150 individuals, including 100 patients (40 nasopharyngeal cancer, 20 gastric cancer, 20 colorectal cancer and 20 breast cancer, confirmed by pathology) and 50 non-cancerous volunteers. Among them, 80 patients and 30 non-cancerous volunteers were used for the validation (Table 1). All the patients had been diagnosed pathologically and their relevant demographic and clinical pathological details were obtained. The blood samples were collected prior to any therapeutic procedures. Control subjects were recruited from a large pool of individuals seeking a routine physical examination. The individuals with no evidence of cancer were selected as non-cancerous subjects.

2.2. Serum preparation and RNA isolation

For the serum collection, about 4 ml venous blood was collected in the morning before breakfast from non-cancerous control and tumor patients prior to any therapeutic procedures and held at room temperature for 1 h, centrifuged at 4 °C 1600 RCF for 10 min. Serum (upper phase) was collected gently for storage at −80 °C. In addition to the experiments related the number of freeze–thaw, the other experimental serum samples were after one freeze–thaw.

Table 1
Characteristics of subjects for qRT-PCR validation.

Variable		Cohort of qPCR $N = 110$
Non-cancer ($n = 30$)		
Age (year)	Mean ± SD	56 ± 8
Sex-No. (%)	Male	15(50)
	Female	15(50)
Gastric cancer ($n = 20$)		
Age (year)	Mean ± SD	58 ± 9
Sex-No. (%)	Male	12(60)
	Female	8(40)
TNM Stage-No. (%)	I	5(25)
	II	10(50)
	III	4(20)
	IV	1(5)
Nasopharyngeal cancer ($n = 20$)		
Age (year)	Mean ± SD	48 ± 8
Sex-No. (%)	Male	12(60)
	Female	8(40)
TNM Stage-No. (%)	I	2(10)
	II	5(25)
	III	8(40)
	IV	5(25)
Colorectal cancer ($n = 20$)		
Age (year)	Mean ± SD	55 ± 10
Sex-No. (%)	Male	13(65)
	Female	7(35)
Dukes Stage-No. (%)	A	2(10)
	B	9(45)
	C	8(40)
	D	1(5)
Breast cancer ($n = 20$)		
Age (year)	Mean ± SD	53 ± 7
Sex-No. (%)	Male	0(0)
	Female	20(100)
TNM Stage-No. (%)	I	5(25)
	II	10(50)
	III	5(25)

For RNA isolation, total RNA was extracted from 500 μl of each serum sample and eluted in 60 μl of RNase-free water using a mirVana PARIS kit (Ambion) following the manufacturer's protocol for liquid sample. During the RNA extraction, cel-miR-39 was spiked at a fixed concentration for data normalization. Samples should add the denaturation before the addition of cel-miR-39.

2.3. Circulating miRNA profiling assay

The serums were pooled from 20 age- and gender-matched NPC or non-cancerous volunteers. The serum miRNA profile was obtained by using TaqMan Low-Density Array. See our previous article published for more detail [37].

2.4. Quantitative PCR validation and data analysis

Serum samples from each 20 gastric cancer, nasopharyngeal cancer, colorectal cancer and breast cancer patients and 30 noncancerous volunteers respectively were used for the validation of interested miRNAs in serum. MiScript PCR System kit series (miScript Reverse Transcription Kit, miScript SYBR Green PCR Kit, Qiagen) were used to quantify specific miRNAs following the manufacturer's protocol. The changes of differentially expressed miRNAs level were calculated with Delta Ct (Delta Ct = $Ct_{test} - Ct_{cel\text{-}miR\text{-}39}$).

3. Results

3.1. Microarray analysis of serum miRNA

The serum miRNA profile in NPC patients or non-cancerous controls was obtained by using TaqMan Low-Density Array. To screen

endogenous control, six miRNAs was chosen, including U6, miR-16, miR-24, miR-142-3p, miR-19b and miR-192, which were reported in the literature as having stable expression in serum/plasma. Comparative analysis of microarray data was performed. The Ct values of these miRNAs in serum samples of nasopharyngeal carcinoma were greater than non-cancer serum samples, with the exception of U6. We found that the arithmetic mean and the geometric mean of Ct values of the serum in NPC patients are also higher than non-cancerous controls (Table 2), suggesting that the expression stability of U6 differs from the other five miRNAs.

Table 2
Related miRNA expression in TLDA miRNA array.

	Non-cancer samples Ct	NPC Ct	$Ct_{NPC} - Ct_{non\text{-}cancer}$
U6	26.230	24.742	-1.488
MiR-16	25.287	26.144	0.857
MiR-142-3p	32.929	33.633	0.704
MiR-19b	28.446	29.749	1.303
MiR-24	28.993	30.447	1.454
MiR-192	33.158	33.565	0.407
Arithmetic mean	32.98	33.41	0.43
Geometric mean	32.783	33.277	0.494

3.2. Validation of the miRNAs differentially expressed in larger sample with qPCR

To further verify the expression of U6 in different populations, we detected the U6 expression in serum samples from 30 healthy persons and 80 patients, including 20 each of gastric cancer, nasopharyngeal cancer, colorectal cancer and breast cancer, using qPCR detection. Exogenous cel-miR-39 was added as an internal reference. The results showed large fluctuations in U6. The difference of delta Ct values of U6 between the greatest and the least expression level is 3.29 (5.69 VS 9.98). But that of miR-16 is 1.23 (5.46 VS 6.69). The expression of miR-16 was relatively stable. (Figs. 1, 2 and Table 3).

3.3. Effects of the expression of U6 in serum with freeze–thaw cycles

Serum samples miRNAs may be unstable after repeated freezing and thawing in the process of research testing. On this basis, our group looked at the expression of U6, miR-16 and miR-24 in serum

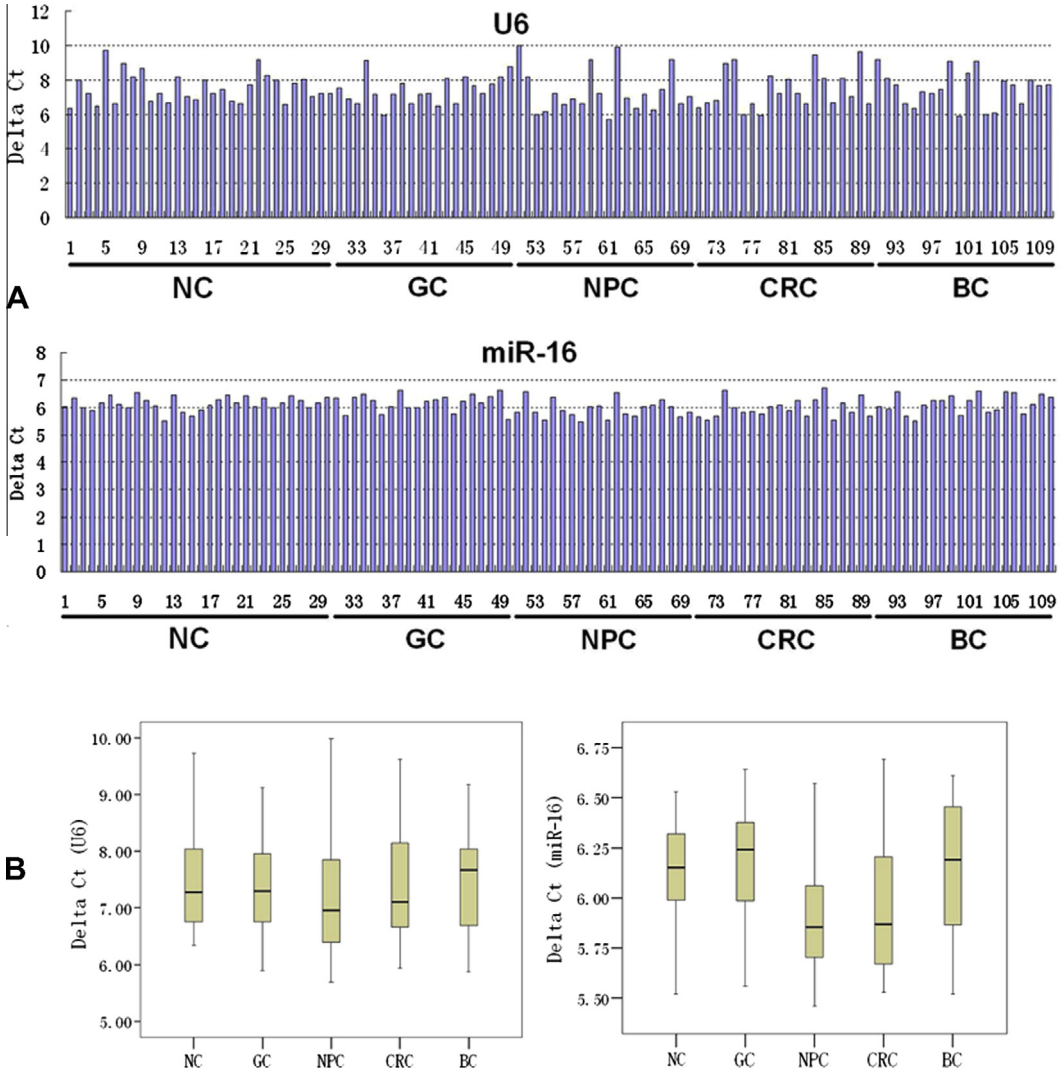

Fig. 1. The expression of U6 and miR-16 in different populations (A: Scatter; B: Box) 1–30: non-cancer (NC) 31–50: gastric cancer (GC) 51–70: nasopharyngeal cancer (NPC) 71–90: colorectal cancer (CRC) 91–110: breast cancer (BC).

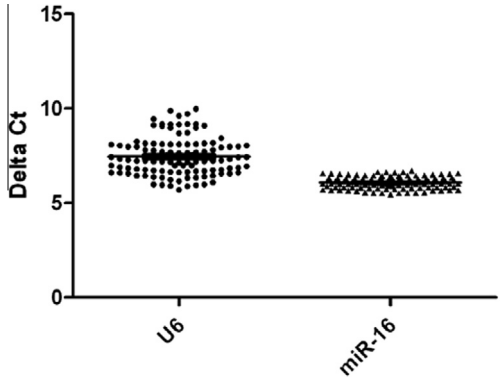

Fig. 2. Expression distribution of U6 and miR-16 expression in 110 subjects.

with different freeze–thaw cycles. The results showed that U6 expression gradually decreased after 1, 2, and 4 cycles of freezing and thawing, while the expressions of miR-16 and miR-24 were relatively stable (Fig. 3).

4. Discussions

Theoretically, serum and plasma have the same composition except for blood coagulation factors. Some research groups have found that miRNA expression in serum and plasma are consistent [5]. Thus, the internal reference of serum and plasma are likely to be in common. In this study, we used serum for related research.

We analyzed the miRNAs reported to have stable expression in serum and which could be used as internal references. We found that with the exception of U6, the values for miR-16, miR-24, miR-142-3p, miR-19b, and miR-192 Ct in NPC sera were higher than non-cancer sera. Accordingly, we speculated that miR-16, miR-24, miR-142-3p, miR-19b, miR-192 expression is stable and the U6 expression is relatively unstable. While there may be variation of individual serum miRNA in disease states, the total amount of miRNAs in the serum should remain constant. Therefore, it is necessary to use the concentration of total RNA as reference normalization [5,38]. The geometric mean and the arithmetic mean of Ct values of all miRNAs may be used to approximate the amount of total miRNA in every sample. In the microarray results, the geometric and the arithmetic means of all miRNAs in non-cancer serum samples were lower than those of nasopharyngeal carcinoma. This suggests that the amounts of total miRNA were greater in non-cancer serum samples than in nasopharyngeal samples. This phenomenon is not caused by the disease itself, but rather by the error and the bias in the experimental procedure. In the results of miRNA arrays, variation of miR-16, miR-24, miR-142-3p, miR-19b, miR-192 in serum of patients with nasopharyngeal carcinoma and the non-cancerous controls is consistent with the geometric and the arithmetic means. Therefore, because these

Table 3
Mean expression and CV value of U6 and miR-16 in different populations.

	U6		miR-16	
	Mean	CV (%)	Mean	CV (%)
Non-cancer	7.527	11.5	6.140	4.0
GC	7.393	11.0	6.180	5.0
NPC	7.328	17.6	5.932	5.3
CRC	7.488	15.3	5.971	5.8
BC	7.518	13.4	6.147	5.5
All	7.458	13.5	6.080	5.2

miRNAs show stable expression they could be used as internal reference in research on serum miRNAs. However, the expression of U6 was not stable, making it unsuitable for an internal reference. To further clarify the stability of the U6 expression in serum, we chose 20 cases each of gastric cancer, nasopharyngeal cancer, colorectal cancer and breast cancer samples for verification. Since human lack cel-miR-39 expression, exogenous cel-miR-39 was added as a standard of data normalization. Quantitative PCR data showed significant fluctuations in the expression levels of U6 compared with those of cel-miR-39 and miR-16. These results suggest that serum U6 is not suitable as an internal reference.

Next, we tested the stability of U6 in serum after repeated freezing and thawing while selecting a greater expression level of miR-16 and miR-24 in serum as a comparison. U6 expression decreased after repeated freezing and thawing. There was no significant change in the amount of miR-16 and miR-24 expression, consistent with the results found by Koberle [39]and Chen [5]. Thus, repeated freezing and thawing will result in degradation of U6. An internal reference expression must be stable under physiological and pathological conditions and unaffected by physical and chemical factors during the experiment. Because repeated freezing and thawing often occurred during the experiment, U6 is not suitable as an internal reference of circulating miRNA research in this case. The longer RNA is more easily degraded by RNase and U6 is significantly longer than miRNA. In addition, studies have shown that circulating miRNAs often combined with some of the carrier protein or are wrapped in exosomes making the circulating miRNAs more stable [40–43]. U6, however, contains a large amount of nucleotides and, after separating from the carrier protein during freezing and thawing, would be degraded by RNase in blood. According our study, U6 is not suitable as an endogenous control in circulating miRNA research.

In recent years, studies of circulating miRNAs and of cycling long non-coding RNA (long noncoding RNA, lncRNA) have proliferated. However, a lack of a suitable endogenous control has hampered researchers. Our group determined to find an internal control for circulating miRNA research. While some candidate miRNAs were screened out for the internal reference, a series of validation in different populations (Large sample validation) is needed. Although the number of samples and the population coverage

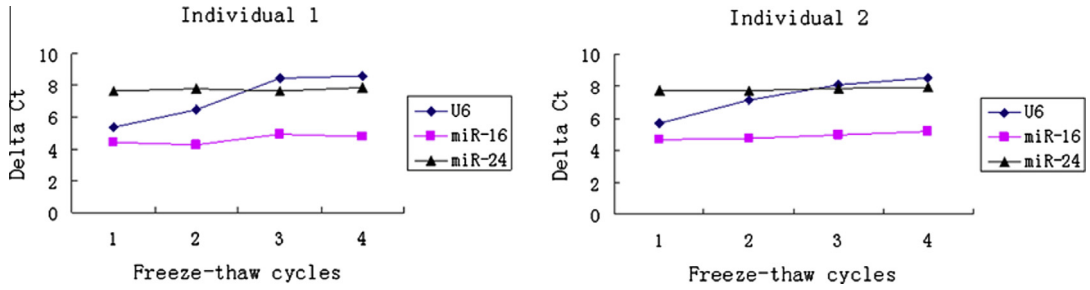

Fig. 3. The expression of U6, miR-16 and miR-24 after different freezing and thawing times.

are limited in our experiments, results confirm that U6 is not a suitable internal reference in serum miRNA research. Reliability of results reported in the literature using U6 as an internal reference will be questioned. Discovery of a reliable internal reference of circulating miRNA will advance research on circulating nucleic acids such as circulating miRNAs and lncRNAs.

Acknowledgments

This work is supported by the National Natural Science Foundation of China (81101643); The Foundation of the Construct Program of the Key Discipline in Hunan Province of China ((2011)76); The Foundation of the Department of Health of Hunan Province (B2012-044).

The authors wish to thank Dr. Wang Chun of Southern Illinois University for modification to language of this article.

References

[1] W.P. Kloosterman, R.H. Plasterk, The diverse functions of microRNAs in animal development and disease, Dev. Cell 11 (2006) 441–450.
[2] G. Stefani, F.J. Slack, Small non-coding RNAs in animal development, Nat. Rev. Mol. Cell Biol. 9 (2008) 219–230.
[3] Y.S. Lee, A. Dutta, MicroRNAs in cancer, Annu. Rev. Pathol. 4 (2009) 199–227.
[4] P.S. Mitchell, R.K. Parkin, E.M. Kroh, B.R. Fritz, S.K. Wyman, E.L. Pogosova-Agadjanyan, A. Peterson, J. Noteboom, K.C. O'Briant, A. Allen, D.W. Lin, N. Urban, C.W. Drescher, B.S. Knudsen, D.L. Stirewalt, R. Gentleman, R.L. Vessella, P.S. Nelson, D.B. Martin, M. Tewari, Circulating microRNAs as stable blood-based markers for cancer detection, Proc. Natl. Acad. Sci. U.S.A. 105 (2008) 10513–10518.
[5] X. Chen, Y. Ba, L. Ma, X. Cai, Y. Yin, K. Wang, J. Guo, Y. Zhang, J. Chen, X. Guo, Q. Li, X. Li, W. Wang, Y. Zhang, J. Wang, X. Jiang, Y. Xiang, C. Xu, P. Zheng, J. Zhang, R. Li, H. Zhang, X. Shang, T. Gong, G. Ning, J. Wang, K. Zen, J. Zhang, C.Y. Zhang, Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases, Cell Res. 18 (2008) 997–1006.
[6] I. Sanders, S. Holdenrieder, G. Walgenbach-Brunagel, R.A. Von, G. Kristiansen, S.C. Muller, J. Ellinger, Evaluation of reference genes for the analysis of serum miRNA in patients with prostate cancer, bladder cancer and renal cell carcinoma, Int. J. Urol. 19 (2012) 1017–1025.
[7] Z.H. Chen, G.L. Zhang, H.R. Li, J.D. Luo, Z.X. Li, G.M. Chen, J. Yang, A panel of five circulating microRNAs as potential biomarkers for prostate cancer, Prostate 72 (2012) 1443–1452.
[8] L. Adam, M.F. Wszolek, C.G. Liu, W. Jing, L. Diao, A. Zien, J.D. Zhang, D. Jackson, C.P. Dinney, Plasma microRNA profiles for bladder cancer detection, Urol. Oncol. 31 (2013) 1701–1708.
[9] M. Tsujiura, D. Ichikawa, S. Komatsu, A. Shiozaki, H. Takeshita, T. Kosuga, H. Konishi, R. Morimura, K. Deguchi, H. Fujiwara, K. Okamoto, E. Otsuji, Circulating microRNAs in plasma of patients with gastric cancers, Br. J. Cancer 102 (2010) 1174–1179.
[10] K.E. Resnick, H. Alder, J.P. Hagan, D.L. Richardson, C.M. Croce, D.E. Cohn, The detection of differentially expressed microRNAs from the serum of ovarian cancer patients using a novel real-time PCR platform, Gynecol. Oncol. 112 (2009) 55–59.
[11] R.J. Bryant, T. Pawlowski, J.W. Catto, G. Marsden, R.L. Vessella, B. Rhees, C. Kuslich, T. Visakorpi, F.C. Hamdy, Changes in circulating microRNA levels associated with prostate cancer, Br. J. Cancer 106 (2012) 768–774.
[12] S. Miah, E. Dudziec, R.M. Drayton, A.R. Zlotta, S.L. Morgan, D.J. Rosario, F.C. Hamdy, J.W. Catto, An evaluation of urinary microRNA reveals a high sensitivity for bladder cancer, Br. J. Cancer 107 (2012) 123–128.
[13] G. Wang, E.S. Chan, B.C. Kwan, P.K. Li, S.K. Yip, C.C. Szeto, C.F. Ng, Expression of microRNAs in the urine of patients with bladder cancer, Clin. Genitourin. Cancer 10 (2012) 106–113.
[14] C.H. Lawrie, S. Gal, H.M. Dunlop, B. Pushkaran, A.P. Liggins, K. Pulford, A.H. Banham, F. Pezzella, J. Boultwood, J.S. Wainscoat, C.S. Hatton, A.L. Harris, Detection of elevated levels of tumour-associated microRNAs in serum of patients with diffuse large B-cell lymphoma, Br. J. Haematol. 141 (2008) 672–675.
[15] H.M. Heneghan, N. Miller, A.J. Lowery, K.J. Sweeney, J. Newell, M.J. Kerin, Circulating microRNAs as novel minimally invasive biomarkers for breast cancer, Ann. Surg. 251 (2010) 499–505.
[16] Z. Huang, D. Huang, S. Ni, Z. Peng, W. Sheng, X. Du, Plasma microRNAs are promising novel biomarkers for early detection of colorectal cancer, Int. J. Cancer 127 (2010) 118–126.
[17] J. Wang, J. Chen, P. Chang, A. LeBlanc, D. Li, J.L. Abbruzzesse, M.L. Frazier, A.M. Killary, S. Sen, MicroRNAs in plasma of pancreatic ductal adenocarcinoma patients as novel blood-based biomarkers of disease, Cancer Prev. Res. (Phila) 2 (2009) 807–813.
[18] T.S. Wong, X.B. Liu, B.Y. Wong, R.W. Ng, A.P. Yuen, W.I. Wei, Mature miR-184 as potential oncogenic microRNA of squamous cell carcinoma of tongue, Clin. Cancer Res. 14 (2008) 2588–2592.
[19] M. Redova, A. Poprach, J. Nekvindova, R. Iliev, L. Radova, R. Lakomy, M. Svoboda, R. Vyzula, O. Slaby, Circulating miR-378 and miR-451 in serum are potential biomarkers for renal cell carcinoma, J. Transl. Med. 10 (2012) 55.
[20] A. Watahiki, R.J. Macfarlane, M.E. Gleave, F. Crea, Y. Wang, C.D. Helgason, K.N. Chi, Plasma miRNAs as biomarkers to identify patients with castration-resistant metastatic prostate cancer, Int. J. Mol. Sci. 14 (2013) 7757–7770.
[21] C. Vasilescu, S. Rossi, M. Shimizu, S. Tudor, A. Veronese, M. Ferracin, M.S. Nicoloso, E. Barbarotto, M. Popa, O. Stanciulea, M.H. Fernandez, D. Tulbure, C.E. Bueso-Ramos, M. Negrini, G.A. Calin, MicroRNA fingerprints identify miR-150 as a plasma prognostic marker in patients with sepsis, PLoS ONE 4 (2009) e7405.
[22] M. Tanaka, K. Oikawa, M. Takanashi, M. Kudo, J. Ohyashiki, K. Ohyashiki, M. Kuroda, Down-regulation of miR-92 in human plasma is a novel marker for acute leukemia patients, PLoS ONE 4 (2009) e5532.
[23] B.M. Von, J.J. Pandarakalam, L. Kroon, H. Loeser, J. Herden, G. Braun, K. Wendland, H.P. Dienes, U. Engelmann, J.W. Fries, MicroRNA 15a, inversely correlated to PKCalpha, is a potential marker to differentiate between benign and malignant renal tumors in biopsy and urine samples, Am. J. Pathol. 180 (2012) 1787–1797.
[24] A. Zhao, G. Li, M. Peoc'h, C. Genin, M. Gigante, Serum miR-210 as a novel biomarker for molecular diagnosis of clear cell renal cell carcinoma, Exp. Mol. Pathol. 94 (2013) 115–120.
[25] W. Zhu, W. Qin, U. Atasoy, E.R. Sauter, Circulating microRNAs in breast cancer and healthy subjects, BMC Res. Notes 2 (2009) 89.
[26] G. Reid, M.B. Kirschner, N. van Zandwijk, Circulating microRNAs: association with disease and potential use as biomarkers, Crit. Rev. Oncol. Hematol. 80 (2011) 193–208.
[27] A.S. Ho, X. Huang, H. Cao, C. Christman-Skieller, K. Bennewith, Q.T. Le, A.C. Koong, Circulating miR-210 as a novel hypoxia marker in pancreatic cancer, Transl. Oncol. 3 (2010) 109–113.
[28] J.F. Wang, M.L. Yu, G. Yu, J.J. Bian, X.M. Deng, X.J. Wan, K.M. Zhu, Serum miR-146a and miR-223 as potential new biomarkers for sepsis, Biochem. Biophys. Res. Commun. 394 (2010) 184–188.
[29] J.C. Brase, M. Johannes, T. Schlomm, M. Falth, A. Haese, T. Steuber, T. Beissbarth, R. Kuner, H. Sultmann, Circulating miRNAs are correlated with tumor progression in prostate cancer, Int. J. Cancer 128 (2011) 608–616.
[30] L.A. Selth, S. Townley, J.L. Gillis, A.M. Ochnik, K. Murti, R.J. Macfarlane, K.N. Chi, V.R. Marshall, W.D. Tilley, L.M. Butler, Discovery of circulating microRNAs associated with human prostate cancer using a mouse model of disease, Int. J. Cancer 131 (2012) 652–661.
[31] H.C. Nguyen, W. Xie, M. Yang, C.L. Hsieh, S. Drouin, G.S. Lee, P.W. Kantoff, Expression differences of circulating microRNAs in metastatic castration resistant prostate cancer and low-risk, localized prostate cancer, Prostate 73 (2013) 346–354.
[32] S. Hauser, L.M. Wulfken, S. Holdenrieder, R. Moritz, C.H. Ohlmann, V. Jung, F. Becker, E. Herrmann, G. Walgenbach-Brunagel, R.A. Von, S.C. Muller, J. Ellinger, Analysis of serum microRNAs (miR-26a-2*, miR-191, miR-337-3p and miR-378) as potential biomarkers in renal cell carcinoma, Cancer Epidemiol. 36 (2012) 391–394.
[33] Z. Hu, X. Chen, Y. Zhao, T. Tian, G. Jin, Y. Shu, Y. Chen, L. Xu, K. Zen, C. Zhang, H. Shen, Serum microRNA signatures identified in a genome-wide serum microRNA expression profiling predict survival of non-small-cell lung cancer, J. Clin. Oncol. 28 (2010) 1721–1726.
[34] R. Liu, C. Zhang, Z. Hu, G. Li, C. Wang, C. Yang, D. Huang, X. Chen, H. Zhang, R. Zhuang, T. Deng, H. Liu, J. Yin, S. Wang, K. Zen, Y. Ba, C.Y. Zhang, A five-microRNA signature identified from genome-wide serum microRNA expression profiling serves as a fingerprint for gastric cancer diagnosis, Eur. J. Cancer 47 (2011) 784–791.
[35] S.S. Chim, T.K. Shing, E.C. Hung, T.Y. Leung, T.K. Lau, R.W. Chiu, Y.M. Lo, Detection and characterization of placental microRNAs in maternal plasma, Clin. Chem. 54 (2008) 482–490.
[36] H.M. Heneghan, N. Miller, M.J. Kerin, Circulating miRNA signatures: promising prognostic tools for cancer, J. Clin. Oncol. 28 (2010) e573–574 (author reply e575–576).
[37] X. Zeng, J. Xiang, M. Wu, W. Xiong, H. Tang, M. Deng, X. Li, Q. Liao, B. Su, Z. Luo, Y. Zhou, M. Zhou, Z. Zeng, X. Li, S. Shen, C. Shuai, G. Li, J. Fang, S. Peng, Circulating miR-17, miR-20a, miR-29c, and miR-223 combined as non-invasive biomarkers in nasopharyngeal carcinoma, PLoS ONE 7 (2012) e46367.
[38] D. Wylie, J. Shelton, A. Choudhary, A.T. Adai, A novel mean-centering method for normalizing microRNA expression from high-throughput RT-qPCR data, BMC Res. Notes 4 (2011) 555.
[39] V. Koberle, T. Pleli, C. Schmithals, A.E. Augusto, J. Haupenthal, H. Bonig, J. Peveling-Oberhag, R.M. Biondi, S. Zeuzem, B. Kronenberger, O. Waidmann, A. Piiper, Differential stability of cell-free circulating microRNAs: implications for their utilization as biomarkers, PLoS ONE 8 (2013) e75184.
[40] M.L. Squadrito, C. Baer, F. Burdet, C. Maderna, G.D. Gilfillan, R. Lyle, M. Ibberson, M. De Palma, Endogenous RNAs modulate MicroRNA sorting to exosomes and transfer to acceptor cells, Cell Rep. 8 (2014) 1432–1446.
[41] D.M. Pegtel, L. Peferoen, S. Amor, Extracellular vesicles as modulators of cell-to-cell communication in the healthy and diseased brain, Philos. Trans. R. Soc. Lond. B Biol. Sci. 369 (2014).
[42] A. Suetsugu, K. Honma, S. Saji, H. Moriwaki, T. Ochiya, R.M. Hoffman, Imaging exosome transfer from breast cancer cells to stroma at metastatic sites in orthotopic nude-mouse models, Adv. Drug Deliv. Rev. 65 (2013) 383–390.
[43] N.P. Hessvik, K. Sandvig, A. Llorente, Exosomal miRNAs as biomarkers for prostate cancer, Front. Genet. 4 (2013) 36.