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Direct Assay of miR-16, miR-145 and miR-223 by a Novel Method of Mimic PCR in Serum of Breast Cancer Patients

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Abstract

The role of microRNAs (miRs) is shown as a biomarker whose expression level changes have been associated with cancer development and progression. Circulating miRNAs has been postulated as biomarkers for breast cancer. Detection and diagnosis of miRNA markers could provide an improved and sensitive method in clinical application. In this study, we applied and improved the effective approach by coupling a deoxyuridine-incorporated RT oligonucleotide with a secondary structure and a mimic PCR for assessment and direct detection of circulating miRs in serum. We designed mut miR-16, mut miR-145 and mut miR-223 as mimic miRs. These mimic miRs were used in RT-PCR for detection and quantification assay. As normalization of these mut miRs was performed with themselves, our mimic construct with 80 bp of these miRs shows the best normalization for this method in blood samples. This assay was tested in serum from 15 patients with different stages of breast cancer and 10 healthy female donors. According to our results, using a combination of miR-16 and miR-145 could represent as one of the best biomarker ($p < 0.001$) for sensitive and specific discrimination of breast cancer and yielded a ROC curve area of 96%. Applying the plasmid of multiple mut miR is suitable as a calibrator for clinical use without requiring prob. Using multivariate logistic regression analysis, the best combination ($p < 0.0001$) for breast cancer detection was miR-223 and miR-145.

Key words: Biomarker; Micro RNA; Mimic PCR; Breast Cancer

Introduction

Screening for breast cancer allows diagnosis at an early stage of malignancy which can reduce the mortality rate of patients. Regarding this, introducing new valuable biomarkers is necessary for early diagnosis and prognosis (Duffy 2012; Moon *et al.*, 2015; Schummer *et al.*, 2015; Shimomura *et al.*, 2016; Zhu, *et al.*, 2016). The cost and expertise required for mammography have reduced the broad application of mammography. In addition, the sensitivity of mammography is limited in young women with dense breasts. On the other hand,

sensitivity and specificity of the alternative methods such as ultrasound screening are very operator-dependent (Stout *et al.*, 2014; Loberg *et al.*, 2015; Sprague *et al.*, 2015). Therefore, developing a cost-effective and accurate screening method for breast cancer is on demand.

Regarding this, assessment of sensitive and non-invasive markers that represent tumor-associated changes in the peripheral blood might facilitate early detection of breast cancer as well as monitoring of tumor progression and treatment responses (Brooks 2009; Hossienzadeh Colagar

et al., 2015; Stearns 2016; Kazarian *et al.* 2017). miRNAs are small noncoding RNAs that serve as post-transcriptional regulators of eukaryotic gene expression. Furthermore, in tumor cells, miRNAs are released into the circulatory system. Pattern of miRNAs in plasma and serum have been shown to be altered in a variety of human solid tumors, including lung, breast, liver, thyroid and ovarian cancers (Iorio *et al.*, 2007; Iorio and Croce 2012; Berindan-Neagoe *et al.*, 2014; Lan *et al.*, 2015), which demonstrated that circulating miRNAs could be used as blood-based markers for molecular diagnosis. The circulating miRNAs could be a potential specific biomarker for breast cancer screening (Mathe *et al.*, 2015; van Schooneveld *et al.*, 2015). Differential expression of miR-16 has been reported in several cancers, including leukemia, pituitary adenomas, prostate carcinoma, lung cancer and cancers of the head and neck (Bottoni, *et al.*, 2005; Hui *et al.*, 2010; Patnaik *et al.*, 2012; Gu *et al.*, 2015; Renjie and Haiqian 2015; Bonci and De Maria 2016).

Ma *et al.* showed that miR-10b was highly expressed in metastatic breast cancer cells (Ma *et al.*, 2007; Ma, 2010). Some studies reported that miR-145 is a putative tumor suppressive gene that is downregulated in several types of tumors (Iorio, *et al.*, 2007; Iorio and Croce 2012; Berindan-Neagoe *et al.*, 2014; Lan *et al.*, 2015; Mathe *et al.*, 2015; Han *et al.*, 2016). A recent finding has shown that downregulation of miR-145 contributes to the progression of breast cancer (Zheng *et al.*, 2016). Increased level of miR-223 derived from platelets in modulating lung cancer is correlated with cell invasion (Liang *et al.*, 2015). An elevated level of miR-223 secreted by tumor-associated macrophages (TAMs) is significantly involved in invasiveness of breast cancer cells via *Mef2c*- β -catenin pathway (Yang *et al.*, 2011). Identification of miRNA markers which are specific to breast cancer would lead to a fast, easy, and optimal tool for assessment of the distribution and regulation of miRNAs in a clinical lab. For sensitivity and specificity of normalization, the result of this method (deoxyuridine-incorporated RT oligonucleotide with a secondary structure and a mimic) could be validated by using an external standard. Also, it could be used for the identification of biomarkers and potential

therapeutic targets required by other methods such as real-time and Q-miR by using mutated miR as the calibrator plasmid. In this study, to assess the potential of serum miRs as biomarkers for breast cancer, we examined the transcript levels of miR-223, miR-16 and miR-145 in blood by a modified QC-PCR (coupling a deoxyuridine-incorporated RT oligonucleotide with a secondary structure and a mimic PCR) in serum of breast cancer patients. So, a mutated plasmid containing mutant miRs (miR-16, miR-145, miR-223) sequences were designed and a new RT-PCR applying this plasmid was developed. These assays were further extended for multiplexed PCR for miRNAs.

Materials and Methods

Patients and healthy controls

A total of 15 patients who were clinically diagnosed with breast cancer were recruited from Namazi Hospital in Shiraz and Shiraz Institute for Cancer Research (ICR) between September 2011 and July 2012. A total of 10 normal control subjects was applied, which pathologically confirmed not to have breast cancer and no history of other cancers.

Samples processing and RNA extraction

Total RNA containing small RNA was extracted from 5 ml of plasma using Trizol LS reagent (Invitrogen) and miRNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol with the following modifications [15]: 1 ml Trizol LS reagent was added to 5 ml plasma samples. After phase separation, 1.5 volume of 100% ethanol was added to the aqueous phase and the mixture was loaded into the miRNeasy column (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNase treatment was carried out to remove any contaminating DNA (RNase-Free DNase Set, Qiagen, Hilden, Germany). The final elution volume was 30 ml. The concentration of all RNA samples was quantified by NanoDrop 1000 (Nanodrop).

Primer design

Eight sets of primers were designed for this study using primer 3 online software: biotools.umassmed.edu/bioapps/primer3_www.cgi (Table 1). For the wild type form of each miR (16, 223 and 145), two sets of primers (miR F and miR R) were designed. For the mutated

form of these primers, two primers (mut miR F and mut miR R) containing an extra fragment at their 5' ends, were designed and used for production of two intermediate fragments with a few nucleotide overlaps (Table 1). These two large fragments were fused together by splicing overlapping extension PCR (SOE-PCR)

Table 1. The primer information

Target	Primer	Sequences (5'→3')
miR223	miR223R	TGGATCCGTGTCACCTCGGGCTTACCTG
	miR223F	CGAATTCGTAGACACAGCCAGGGCTGT
mut	mut miR223R	TGGATCCGTGTCACCTCGGGCTTACCTGCTGCAGCTATTGCATGAATCCGTACGTAACCGTCCATAGGCATACCAGATATAATACTATTACG
miR223	mut miR223F	CGAATTCGTAGACACAGCCAGGGCTGT GTCGGTTACGTACGGATTACGCACTATCTGCGAGACCAATTACATTAATCATACCAGGACATAT
miR145	miR145R	CGATCG CTCGAGTCTGGCACCTCTTCTCTGA
	miR145F	CGATCGCTCGAGTCTGGCACCTCTTCTCTGA
mut	mut miR145R	CGATCGCTCGAGTCTGGCACCTCTTCTCTGAGGTCGGTTACGTACGGATTACGCACTATCTGAATACCAGTAATTAAGATACATAATCAATA
miR145	mut miR145F	CGATCGCTCGAGTCTGGCACCTCTTCTCTGAAATGGTCGGTTACGTACGGATTACGCACTATCTG
miR16	miR16 R	CCACCGCTGGAGCCCTATAAAG
	miR16F	GGTCCAACAGATAATTTACCCAACAAGGC
mut miR16	mut miR16 R	CCACCGCTGGAGCCCTATAAAG ATGGTCGGTTACGTACGGATTACGCACTATCTG
	mut miR16F	GGTCCAACAGATAATTTACCCAACAAGGCATGGTCGGTTACGTACGGATTACGCACTATCTGAATACATACATAAGCATAACTAACCT

Construction of mutated miR plasmid as an internal standard (IS)

For amplification of a mutated form of each miR, PCR reaction was carried out in a 25 µl volume containing 1X PCR buffer, 1.5 mM MgCl₂, 2 mM dNTP mixture, 0.5 unit *Taq* DNA polymerase, 20 ng template DNA, and 1 pmol of each primer (miR F/ mut miR R primers were used in PCR I and mut miR F/ miR R primers were used in PCR II). Thermocycling conditions started with an initial denaturation step at 95°C for 5 min, followed by 35 additional cycles at 95°C for 1 min, annealing temperature of 70°C for 1 min, and extension at 72°C for 1 min. The PCR products were electrophoresed on a 1.5% agarose gel, and the DNA was purified using the DNA Extraction Kit (Bioneer). The two intermediate fragments were fused together by a two-step PCR; step 1 of the SOE-PCR involved in 15 cycles with annealing temperature of 56°C; in step 2, SOE-PCR was continued by adding 1.5 pmol of miR F and R primers, which were annealed at 63.4°C for another 30 cycles. The PCR reaction was set up with 0.2 mM dNTP, 1 mM MgCl₂, 1X PCR buffer, and 1 unit *Taq* DNA polymerase. After separation of Mut miR -16, -145 and -223 with an additional fragment at the 5' end, PCR was performed using miR-

145F and miR-223R primers, respectively. Subsequently, the PCR products were purified and inserted into the pGEM-T assay vector (Promega), which was then sequenced and used as an internal standard in QC-PCR. At the end, the DNA plasmid was digested by *ScaI* restriction enzyme. The copy number of the mutant plasmid was calculated based on the concentration of plasmid using the *ds copy number analyzer* online software which can be found at www.uri.edu/research/gsc/resources/cndna.html. The estimated copy number was 5/6 x 10⁷ copies/µl. The plasmid was serially diluted and stored at -70°C.

Quantifying expression levels of miRs (miR-16, -145 and -223) using quantitative-competitive (QC)- PCR

Deoxyuridine-incorporated RT oligonucleotide with a mimic PCR was use for assessment and direct detection of miR-16, miR-145, miR-223 in serum. Uracil-DNA Glycosylase (UDG) treatment used as carry-over contamination can be controlled by the following two steps in mimic PCR. Treating all subsequent fully preassembled starting reactions with uracil DNA glycosylase (UDG), followed by thermal

inactivation of UDG. UDG cleaves the uracil base from the phosphodiester backbone of uracil-containing DNA, but has no effect on natural (i.e., thymine-containing) DNA. So The RT oligonucleotides with deoxyuridine (dU) incorporation were synthesized by Integrated DNA (mutation segments of miRs) Technologies. The cDNA samples were then treated with 5 U UDG (New England Biolabs) at 37°C for 10 min. The reaction was inactivated at 95°C for 10 min and subjected to QC-PCR. Equal volumes (4 µl) of target cDNA and serial dilutions of the internal standard plasmid (9 x 10⁶, 8.0 x 10⁵, 7.97 x 10⁴, 6.86 x 10³, 5.0 x 10³, 4.56 x 10², 3.11 x 10¹ copies/µl) were used in each QC-RT-PCR reaction in order to quantify the unknown mRNA expression level. PCR reactions were conducted in each QC-RT-PCR series. Each QC-RT-PCR reaction mixture contained 1 unit of *Taq* DNA polymerase, 1 mM MgCl₂, and 1 pmol of each primer (miR F and R). Nuclease-free water (Sigma) was added to bring the final volume to 50 µl. PCR cycling conditions were one cycle of 1 min at 94°C, followed by 30 cycles of 30 sec. at 94°C, 60 sec. at 67.3°C, 60 sec. at 72°C, and finally, a 300 sec. extension at 72°C. The amplified products were separated by gel electrophoresis, and the images were digitally recorded. The intensity of amplified wild type and mutated products was directly measured using an Image program (Java version of NIH image developed at the US National Institute of Health) and available on the internet at <http://rsb.info.nih.gov/ig>. And total lab and SAS software (statistical analysis system, version 7.12; SAS, Cary, N.C.) Validation of experiments was biased on MCAS (multiple comparative assay system). This method was accomplished by the normalization

of the expression level of each miR-223, and miR-145 beside as CF (control foreign: miR-16) relative to CI (internal control: standard plasmid)

Statistical analysis

The relevance of plasma miRNA levels was determined by, the Mann-Whitney test, Wilcoxon test, κ^2 test or Kruskal-Wallis test where appropriate. The sensitivity and specificity were calculated according to the standard formulas. A multivariate logistic regression model was established and leave-one-out cross validation to find the best logistic model. ROC curves were established for discriminating patients with or without breast cancer.

Results

Patient characteristics

In this study, 15 breast cancer patients and 10 healthy donors as normal control were participated. Additionally, 15 colorectal cancer, 15 gastric cancer, 15 lung cancer, and 15 hematopoietic cancer patients were recruited. There were no significant differences in mean age between breast cancer patients and healthy controls.

Quantifying the expression of the mut miRs by QC-RT PCR

The specificity of the primers was examined with different type of cancers for these miR-16, miR-145 and miR-223 (Table 2).

Table 2. Quantification of miRs levels in different cancers by QC-RT-PC

Different type of cancers	Quantified by qRT- PCR	Regression equations from QC-RT-PCR and R ²	R ²
HCC	1.14	Y=-1.10X + 5. 41	0.98
LC	1.69	Y=-0.96X + 6.66	0.98
CRC	2.76	Y=-0.98X + 3.47	0.94
GC	1.98	Y=-0. 95X + 6.28	0. 63
ER	0.009	Y=-0. 94X + 5.87	0.98

PCR amplicons of each miR were observed for all types of cancers. For sensitivity tests, the detection limit was first evaluated using a

dilution series of cDNA used in at least 1 pg for miR-145, so, the detection level was 1 pg on agarose gel (Fig. 1).

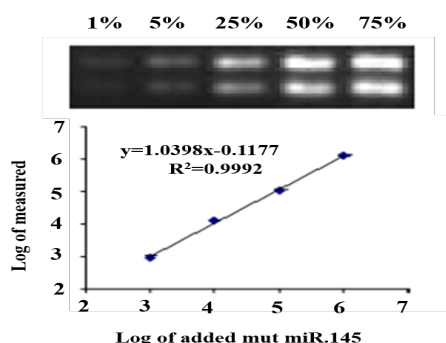


Fig. 1. Normalization of miR-145 relative to standard for 1% detection: The MCAS PCR products of the co-amplified serially diluted competitor and the fixed amount of target DNA separated on the 2% agarose gel. lane1: containing 1%; lane 2: 5%; lane 3: 25%; lane 4: 50% and lane 5: 75%.

We could detect 250 copies of mut miR with this pair of primers. The most consistent result was obtained by spiking a dilution series of cloned miRs into 10 ng of each miR DNA. Specific amplification was observed from 1 pg of plasmid DNA, which was equivalent to 250 copies of each miR-145. The regression equations from QC-RT-PCR and R2 equation for the miR-145 primers as the case of breast biomarker was examined with different type of cancers for these (Table 2).

Foreign control of miR-16 for miR-223 and miR-145

The validation amplification efficiency was examined by co-amplifying equal molar quantities of miR-16 and the target miR-145 fragment. Similar amplification efficiencies were obtained with the template over 4 orders of magnitude that were examined. The PCR reactions reached plateau periods after 30, 32, 34 and 38 cycle, respectively, when 10^5 , 10^6 and 10^7 copies of miR-223, miR-16 and miR-145 were used as templates. To obtain accurate quantitation, competitive PCR should be performed within the exponential phase (Wiesner *et al.*, 1993). Thus, competitive PCR for quantitation of 10^7 , 10^6 , 10^5 to 10^3 copies of each miR should be performed for less than 32, 34, 36 and 41 cycles, respectively (Fig. 2 A, B, C).

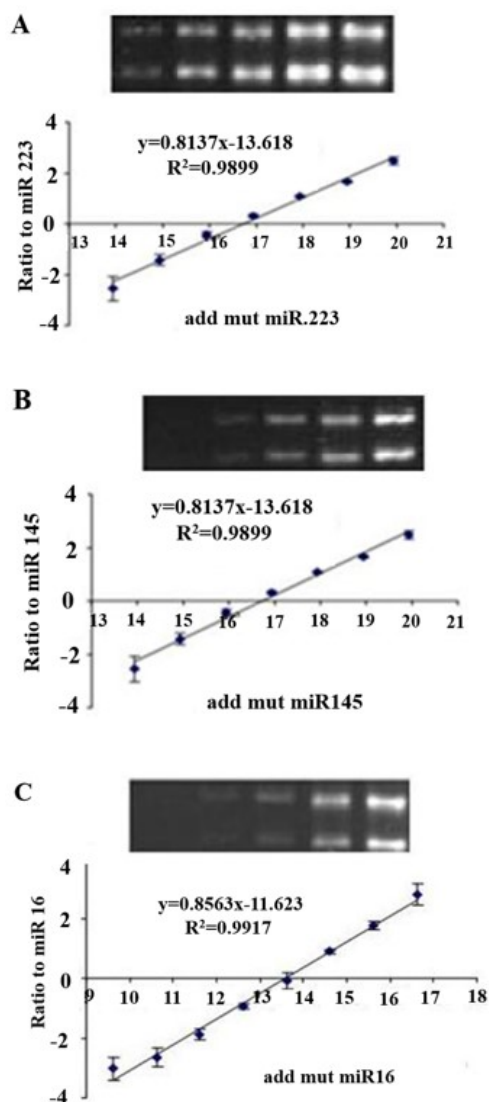


Fig. 2. Co-amplification of miRs and standard plasmid: (A) 10^5 copies of miR-223 and standard plasmid; (B) 10^7 copies of miR-145 and standard plasmid; (C): 10^6 copies of miR-16 and standard plasmid. Similar plot patterns were observed in a replicate experiments.

Accuracy and quantitation limit

The accuracy of quantitative competitive PCR was evaluated by using a known amount of cloned miR-145 fragments, ranging from 10^3 to 10^7 copies. To evaluate how heterogeneous templates will affect the accuracy of quantification, all reactions included 10 ng of cDNA.

cDNA from miRs to maintain the amplification within the exponential phase, a total of 30, 32, 34 and 38 cycles were used for quantitation of 10^7 , 10^6 , 10^5 and 10^4 copies of miR-145 and miR-223 fragments, consecutively. Validation of combined plasma miRs (miR-145 and miR-223) by multiple comparative assay system was done for participants, including 15 breast cancer patients, 10 normal control subjects, 15 colorectal cancer, 15 gastric cancer, 15 lung cancer, and 15 hematopoietic cancer patients. The Real accuracy of this method was checked for every normal and other cancers relative to breast cancer participants. The results showed that there is no significant correlation for other types of cancer by this method. A two-fold dilution series of standard plasmid were used to co-amplify with a constant amount of each miRs. Linear regression of ratios of internal standard to each of the miRs (\log^2) and added to mut miRs (\log^2) were obtained for all assays with an R2 of 0.9867, and for 10^7 copies of miR-145, 0.9917, and for 10^7 copies of miR-145, 0.9899, and for 10^5 copies of miR-223 and 0.9846, and for 10^6 copies of miR-16. The correlation between measured and added values was extremely high (slope=1.0398), which indicated that a precise quantitation was over a wide range. When there were less than 10^3 copies of miR-145 fragments in the competitive PCR reaction, no acceptable linear regression was achieved ($R^2=0.9$); thus, we were unable to quantify less than 10^3 using this system. To further verify the discriminating power of the miRNAs identified for breast cancer diagnosis, plasma levels of miR-16, miR-145, miR-223 were assessed in plasma of an independent group of 90 cancer patients. Efficiency and specificity of the competitive RT-PCR were determined as described. In brief, 10-fold dilutions of mine were subjected to the quantitative competitive RT-PCR. Changes in the plasma levels of miR-145 and miR-223 in breast cancer patients ($n = 15$) relative to the normal participants were obvious. Expression levels of the miRNAs were normalized to mut miR-16 in quantitative competitive RT-PCR. The standard curve of the miRNA was obtained

by plotting Ct versus Log (copies) of the standard plasmid dilutions. The assay efficiency was calculated by $(101/S-1) \times 100\%$, where S is the slope of the standard curve (Fig. 3A, B).

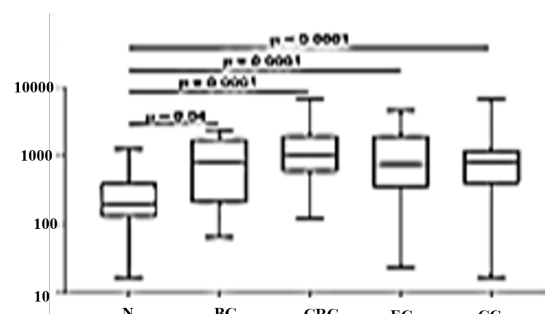


Fig. 3. Validation of combined plasmid mut miR-145 and miR-223 by multiple comparative assay system: Accuracy of this RT method was checked for normal and other cancers relative to breast cancer.

For the calculation of specificity, we used identical amounts (10^7 copies per RT) of miR-145 and miR-223. This alone generated a satisfactory ROC value, an ideal combination for diagnosis by this method. Using multivariate logistic regression analysis, the combination ($p<0.001$) of miR-223 and miR-145 could provide the ideal marker for breast cancer detection. We demonstrated that the sensitivity of this combination by this method, such as combined markers of miR-451 and miR-145 plasma levels in breast cancer patients, were significantly discriminated not only in normal control subjects, but also in all other types of cancers included in this study (Fig. 3A). Furthermore, such combined markers yielded a ROC curve area of 0.956 (95% CI 0.936; Fig. 4A). The optimal sensitivity and specificity were 90.2% (95% CI, 84.1%, 294.5%) and 89% (95% CI, 82.5 %, 292.9%) in discriminating breast cancer including benign tumors from normal control plus all other types of cancers (Fig. 4B). Using this type of plasma marker, the positive predictive value was 90% (153/170) and the negative predictive value was 91.3% (174/195). The odds ratio for cases with combined miRNAs being associated with cancer was 36.8 (95% CI 19.32101.1).

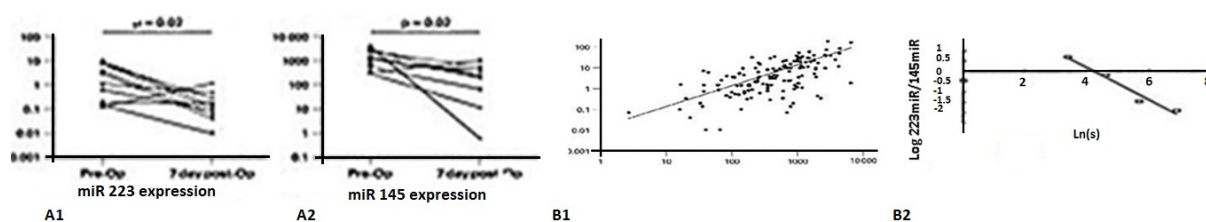


Fig. 4. The differential expression of microRNAs in patients with breast cancer. Changes of miR-223(A1) and miR-145(A2) plasma levels in breast cancer patients (n=15) relative to normal. Correlation between miR223 and miR145 in these patients (B1). Logarithmic value of miR-223 expression level relative to miR-145 (B2). Statistically significant differences were determined using Wilcoxon tests.

Discussion

For miRNAs detection, direct methods including fluorescent, colorimetric, and electrical-based methods or indirect methods including northern blotting, microarray which are semi-quantitative, as well as RT-PCR can be applied. The sensitivity and discrimination power of direct methods are low (Hunt *et al.*, 2009). Otherwise, miRNA assessment with isothermal methods is labor exhaustive (Cheng *et al.*, 2009; Yao *et al.*, 2009). At present, the broadly used and capable, accurate method for RNA measurement is real-time RT-PCR, but it is not cost effective and may also accompany with technical problems (Benes and Castoldi, 2010; Marabita *et al.*, 2016). Therefore, improving the other reliable and sensitive miRNA detection has been more considered.

In the current study, we applied and improved the rapid and effective approach by coupling a deoxyuridine-incorporated RT oligonucleotide with a secondary structure and a mimic PCR for miRNAs quantification from serum which represented high efficiency and sensitivity for microRNA detection.

We have identified a significant alteration of the miR-16, miR-145, and miR-223 by this novel method of mimicking PCR for breast cancer prediction which yield in a ROC curve area of 96% (Fig. 5). The odds ratio for the cases with combined miR-145 and miR-223 level being associated with breast cancer was 44.2. The combination of plasma miR-16 and miR-145 levels provided the best markers for breast cancer prediction and yielded a ROC curve area of 96% previously, qRT-PCR was applied for miR-16 and miR-145 separately. This

demonstrates that a reduction of plasma miR-16 level occurred in breast cancer patients alone. But normalization miR-16 relative to external genes (mut miR-16) can be the best optimization.

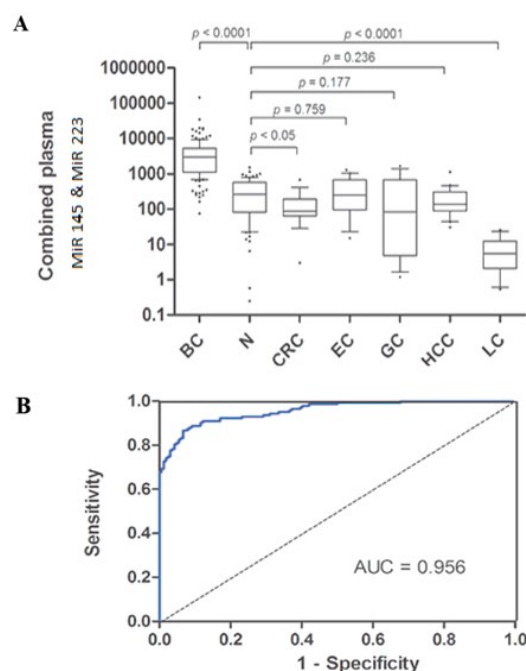


Fig. 5. Validation of combined plasma miR-145 and miR-223. (A) Box plot of combined plasma miR-145 and miR-223 in patients with breast cancer (BC), healthy, normal (N), colorectal cancer (CRC), esophagus cancer (EC), gastric cancer (GC), hepatocellular carcinoma (HCC) and lung cancer (LC). B) ROC analysis using combined plasma miR-223 and miR-145 for discriminating breast cancer from normal subjects and other cancers.

Combination of miR-16 and miR-145 plasma levels is very specific to the breast cancer. Aberrant expression of plasma miR-16 in breast cancer patients supports the notion that miR-16 is not suitable for normalization (Davoren *et al.*, 2008). Our study could detect miR-16 clearly as a constant standard. It also could detect normalization with mut miR-16 by mut plasmid, and because of this identification, our study may be unique. Thus, miR-16 can be used as an internal normalization control for miRNA quantitation by mimicking RT-PCR. In other studies of miRNAs in breast cancer diagnosis (Murakami *et al.*, 2006; Kodahl *et al.*, 2014; Zhao *et al.*, 2014; Vilquin *et al.*, 2015; Shimomura *et al.*, 2016), a combination of miRNA markers were applied which were very specific only to breast cancer, but not other cancers such as gastric, lung and HCC. Interestingly, these plasma levels were significantly decreased in lung cancer when compared with breast cancer and even the normal control. When the sample size is small, validations in large cohort studies or in different ethnic groups is recommended.

It is uncertain whether this method of assessment is specific for certain subtypes of breast cancer. It is desirable to examine whether such plasma miRNAs would change in patients undertaking various treatments. In addition, plasmid calibration can greatly be changed (intention CV plasmid=0.9)

In conclusion, change of miR-16, miR-223, and miR-145 in plasma of breast cancer patients has been reported in this study for detecting acute metastasis of breast cancer. Noticeably, we showed that the plasma miR-145 level is reduced in breast cancer patients. The combination of miR-223 and miR-145 in our plasma quantitative assay could provide a very promising and specific breast cancer screening test. Although the sensitivity and specificity of these markers for breast cancer prediction is 90%, the positive predictive value was determined to be approximately 90% and the negative predictive value was approximately 92%. A combination of other potential markers using this plasma quantitation may further enhance the assessment discriminating ability in the future. We developed a simple technique for

miRNA analysis in blood serum. This method can be parallelized to quantitatively detect multiple miRNA-based biomarkers in different biological samples. In some countries, real-time PCR is used for screening and quantification. In Iran and developing countries, it could be used for screening and detection by miR-145 and miR-223 levels.

Because this plasmid is calibrated and normalized by real-time PCR, it can be used as an appropriate standard. The reference materials for analysis are not easily obtained. Several RMs have been developed by standard plasmid, and a series of these can be purchased from Fluka. However, the cost of these standard plasmids is very high, and the samples may degrade during use, so, this construct can be used in Qc-RT analysis instead of RM.

Real-time PCR is the most reliable technique, but it is an expensive method and additionally, needs qualified staff to set up the protocol. So, improved miR-based methods with optimized multi-elements and reference genes (housekeeping) can be used in order to detect multi-miRNA. Application of the methods introduced in this project can be used for diagnosis other novel miRNA markers.

The greatest advantage of this method is the greater sensitivity of these micro-RNA normalization relative to themselves, especially to housekeeping miR. Moreover, this method was able of detecting miRNAs as little as 1 pg.

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