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ORIGINAL ARTICLE

Relative and Absolute Expression Analysis of MicroRNAs Associated with Luminal A Breast Cancer– A Comparison

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Abstract

MicroRNAs, as small non-coding regulatory RNAs, play crucial roles in various aspects of breast cancer biology. They have prognostic and diagnostic value, which makes them very interesting molecules to investigate. Reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) is the gold standard method to analyse miRNA expression in breast cancer patients. This study investigated two RT-qPCR methods (absolute and relative) to determine the expression of ten miRNAs in whole blood samples obtained from luminal A breast cancer patients compared to healthy controls. Whole blood samples were collected from 38 luminal A breast cancer patients and 20 healthy controls in Paxgene blood RNA tubes. Total RNA was extracted and analysed by relative and absolute RT-qPCR. For relative RT-qPCR, miR-16 was used as an endogenous control. For absolute RT-qPCR, standard curves were generated using synthetic miRNA oligonucleotides to determine the absolute copy number of each miRNA. Of the ten miRNAs that were analysed, the absolute RT-qPCR method identified six miRNAs (miR-16, miR-145, miR-155, miR-451a, miR-21 and miR-486) that were upregulated and one miRNA (miR-195) that was downregulated. ROC curve and AUC analysis of the data found that the combination of three miRNAs (miR-145, miR-195 and miR-486) had the best diagnostic value for luminal A breast cancer with an AUC of 0.875, with 76% sensitivity and 81% specificity. On the other hand, the relative RT-qPCR method identified two miRNAs (miR-155 and miR-486) that were upregulated and miR-195, which was downregulated. Using this approach, the combination of three miRNAs (miR-155, miR-195 and miR-486) was showed to have an AUC of 0.657 with 65% sensitivity and 69% specificity. We conclude that miR-16 is not a suitable normalizer for the relative expression profiling of miRNAs in luminal A breast cancer patients. Compared to relative quantification, absolute quantification assay is a better method to determine the expression level of circulating miRNAs in Luminal A breast cancer.

Keywords MicroRNAs · Luminal A breast cancer · Relative quantification · Absolute quantification

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Introduction

Human cancer is the among the leading causes of death globally, based on World Health Organization (WHO) estimates in 2015 [1]. Globally, there were 14.1 million new cancer cases and 8.2 million deaths from cancer reported in 2012 [2]. These include 3.45 million new cases and 1.75 million deaths within in the European Union during that same period [3]. In terms of its significance, breast cancer, as a heterogeneous disease, is one of the most common causes of death in women in developed countries with approximately 1.67 million new cases and 522,000 deaths from breast cancer recorded in 2012 [2].

Breast cancer can be classified into four subtypes based on hormone receptor status [4, 5]. These subtypes include; Luminal A (ER and/or PR positive, HER2/neu negative), Luminal B (ER, PR, and HER2/neu triple positive), Basal-

like or triple negative (ER, PR, and HER2/neu triple negative) and HER2 amplified (ER and PR negative, HER2/neu positive). Each of these subtypes has a different prognosis, biological behaviour and treatment regimen. Luminal A breast cancers, as the commonest subtype, have a good prognosis, and show satisfactory responses to hormone therapies [6, 7].

MicroRNAs (miRNAs) are small regulatory RNAs found in all tissues, including blood. It has been shown that miRNAs are linked to the etiology, progression and prognosis of cancer [8]. Furthermore, miRNAs expression profiles can be used to classify cancer types [9]. The abundance of particular miRNAs, including circulating miRNAs, has been shown to be dysregulated in certain cancers [10].

Dysregulation of miRNAs has also been observed in tumours and in the blood of breast cancer patients, suggesting that they may have diagnostic potential in breast cancer [11–14]. Iorio et al. (2005), identified 29 miRNAs that were differentially expressed in breast cancer tissue [10]. They found that the most consistently dysregulated miRNAs were miR-10b, miR-125b, miR145, miR-21, and miR-155. Of these, three (miR-10b, miR-125b and miR-145) were downregulated and two (miR-21 and miR-155) were upregulated [11]. Chan et al. (2013), examined the 20 most differentially expressed miRNAs in breast cancer tumours in a cohort (n = 32) of breast cancer patients [15]. They found several miRNAs that were dysregulated in these patients, including; miR-145, miR-21, miR-10b, miR-1, miR-92a, miR-133a, and miR-133b. In another study by McDermott et al. (2014), specific miRNA expression profiles were identified in whole blood of luminal A-like (ER + PR + HER2/neu-) breast cancer subtype using microarray and relative RT-qPCR assays [14]. This study found that miR-29, miR-181a and miR-652 were significantly downregulated in the blood of patients compared to healthy individuals.

Reverse transcriptase real-time quantitative PCR (RT-qPCR) is the gold standard technology for profiling the expression of miRNAs. Using RT-qPCR, miRNAs can be profiled in terms of their absolute copy number (absolute quantitation) or relative to a predefined reference biomarker (relative quantitation). In this study, we analysed and compared the expression level of ten selected miRNAs, identified in previous studies as dysregulated in breast cancer, with both absolute and relative RT-qPCR assays in whole blood from luminal A breast cancer patients.

Materials and Methods

Sample Collection

Whole blood from 38 luminal A breast cancer patients and 20 healthy females attending Galway University Hospital (GUH) was collected in PAXgene Blood RNA tubes (Qiagen, Germany). All patients have confirmed ER /PR positive and HER2/neu negative breast cancer. Control blood samples were collected from women without any history of malignancy, inflammatory or infectious diseases. Clinicopathological details of patient cohort are shown in Table S1.

Ethics Statement

The study was performed in accordance with the Clinical Research Ethics Committee, Galway University Hospital and written informed consent was obtained from all participants involved in this study.

Total RNA Extraction

Total RNA was extracted and purified using the PAXgene Blood RNA kit (Qiagen, Germany) according to the manufacturer's instructions. Following purification, total RNA was quantified by UV spectrophotometry (NanoDrop, ThermoFisher) and analysed by capillary electrophoresis (RNA 6000 NanoChip Kit Series II, Agilent Technologies, USA) to determine its integrity, (RNA Integrity Number > 8). Samples were then stored at −80 °C until required.

MicroRNA Reverse Transcription

Ten miRNAs (miR-195, miR-16, miR-21, miR-451a, miR-486, miR-181a, miR-145, miR-155, miR-10b and miR-205) were chosen for analysis (Table S2). Each miRNA was separately reverse transcribed using 100 nanograms of total RNA from each sample using the TaqMan miRNA reverse transcription kit (Cat. No. 4366596, Life Technologies, Paisley, UK) according to the manufacturer's instructions. Briefly, the reverse transcription reaction consisted of 7 μL master mix [4.16 μL nuclease-free water, 1.5 μL 10× RT buffer, 1 μL multiscribe RT enzyme (50 U/ μL), 0.19 μL RNase inhibitor (20 U/ μL) and 0.15 μL dNTP mix (100 mM total)], 3 μL miRNA specific primer and 5 μL (100 ng) RNA were added to the PCR microtube (total volume of 15 μL). Reverse transcription thermocycling conditions were 16 °C for 30 min, 42 °C for 30 min, 85 °C for 5 min. The resultant cDNA was stored at −20 °C until required.

Standard Curves and Quantitative Real-Time PCR

Complementary DNA (cDNA) was analysed by qPCR using specific miRNA TaqMan probes (Life technologies, Paisley, UK) and the Taqman Universal PCR master mix (Cat. No. 4440047, Life Technologies, Paisley, UK) according to the manufacturer's instructions (10 μL PCR master mix, 7.67 μL nuclease-free water, 1.33 μL cDNA and 1 μL 20× specific microRNA assay). QPCR reactions were performed on a LightCycler 480 real-time PCR instrument (Roche

Diagnostics, UK). The thermocycling conditions were initial heating to 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s/60 °C for 60 s. The threshold cycle (C_T) was determined by the *'Second Derivative Maximum* method' using the LightCycler instrument's software. All qPCR reactions were performed in triplicate.

The absolute copy number of each miRNA was determined using a standard curve. For this purpose, a 10-fold serial dilution series of synthetic miRNA oligonucleotides (IDT, Iowa, USA) was used to generate standard curves at various concentrations (1×10^1–1×10^8 molecules per reaction).

Statistical Analysis

Using miR-16 as an endogenous control, the relative expression of each miRNA was determined using REST (Relative Expression Software Tool V2.0.13) [16, 17]. Absolute RT-qPCR (copy number) data were analysed using the software package SPSS 21.0 for windows and a one-way ANOVA test was performed to determine the p value. Receiver operating characteristic (ROC) curves were generated to evaluate the diagnostic specificity and sensitivity of each miRNA and for combined miRNAs (using binary logistic regression).

Results

All ten miRNAs were detected in all samples ($n = 58$). Two miRNAs (miR-10b and miR-205), were present at high C_T values (>38) were eliminated from further analysis.

Identification of Dysregulated miRNAs by the relative quantification method

Using miR-16 as a normaliser, the relative expression profiles of seven miRNAs (miR-21, miR-145, miR-155, miR-181a, miR-195, miR-451a and miR-486) were determined (Fig. 1 and summarised in Table 1). Two miRNAs (miR-486 and miR-155) were found to be upregulated ($P < 0.05$), whilst one miRNA (miR-195) was found to be downregulated ($P < 0.05$). MiR-21, miR-181a, miR-145 and miR-451a were not found to be differentially expressed in patients versus healthy controls ($P > 0.05$).

ROC curves and area under the ROC curves (AUCs) were generated for the miRNAs that were found to be dysregulated by relative quantification (Fig. 2). The AUCs for miR-155, miR-486 and miR-195 were 0.795, 0.895 and 0.746 respectively. When combined, the three dysregulated miRNAs (miR-155, miR-195 and miR-486) were determined to have an AUC of 0.657, with 65% diagnostic sensitivity and 69% specificity.

Identification of Dysregulated miRNAs by the absolute quantification method

Standard curves were generated using synthetic miRNA oligonucleotides. The curves were linear over the range of concentrations tested and amplification efficiencies varied between 1.839 and 2.021 (Fig. S1).

Figure 3 shows the absolute expression values of eight miRNAs (miR-16, miR-145, miR-155, miR-451a, miR-195, miR-21, miR-486 and miR-181a) in luminal A breast cancer patients ($n = 38$) compared to controls ($n = 20$). Of these eight miRNAs, six miRNAs (miR-16, miR-145, miR-155, miR-451a, miR-21 and miR-486) were found to be upregulated, and one miRNAs (miR-195) was found to be downregulated ($P < 0.05$). Mir-181a was not found to be differentially expressed ($P > 0.05$).

ROC curves and AUCs were established to evaluate the diagnostic value of these miRNAs for differentiating luminal A breast cancer patients from healthy controls. For the six miRNAs found to be upregulated at a statistically significant level (miR-16, miR-145, miR-155, miR-451a, miR-21 and miR-486), the AUCs were 0.612, 0.826, 0.775, 0.734, 0.652 and 0.893 respectively. For the one downregulated miRNA (miR-195), the AUC was 0.807. In addition, the three miRNAs showing the best diagnostic value were examined in combination. Combining miR-145, miR-195 and miR-486 resulted in an AUC of 0.875 with 76% diagnostic sensitivity and 81% specificity (Fig. 4).

Target genes prediction for upregulated miRNAs

To predict related target genes for the upregulated miRNAs, we used the TargetScan algorithm (http://www.targetscan.org/vert_72/) [18]. TargetScan computationally predicts targets of miRNAs by looking for the presence of a conserved site (8mer, 7mer, and 6mer sites) in the seed region of a given miRNA. The top fifty predicted target genes for six upregulated miRNAs (miRNAs (miR-16, miR-145, miR-155, miR-451a, miR-21 and miR-486) are shown in Table 2.

Discussion

Characterising the circulating miRNAs associated with diverse types of human cancer may increase our ability to diagnose and classify particular cancer types. Accordingly, miRNA expression profiles in breast cancer patients indicate that they may potentially be useful in diagnosing and classifying breast cancer into their different molecular subtypes.

Various techniques have been using to analyse microRNA expression profiles, such as microarray, next-generation sequencing, and reverse transcriptase quantitative PCR (RT-

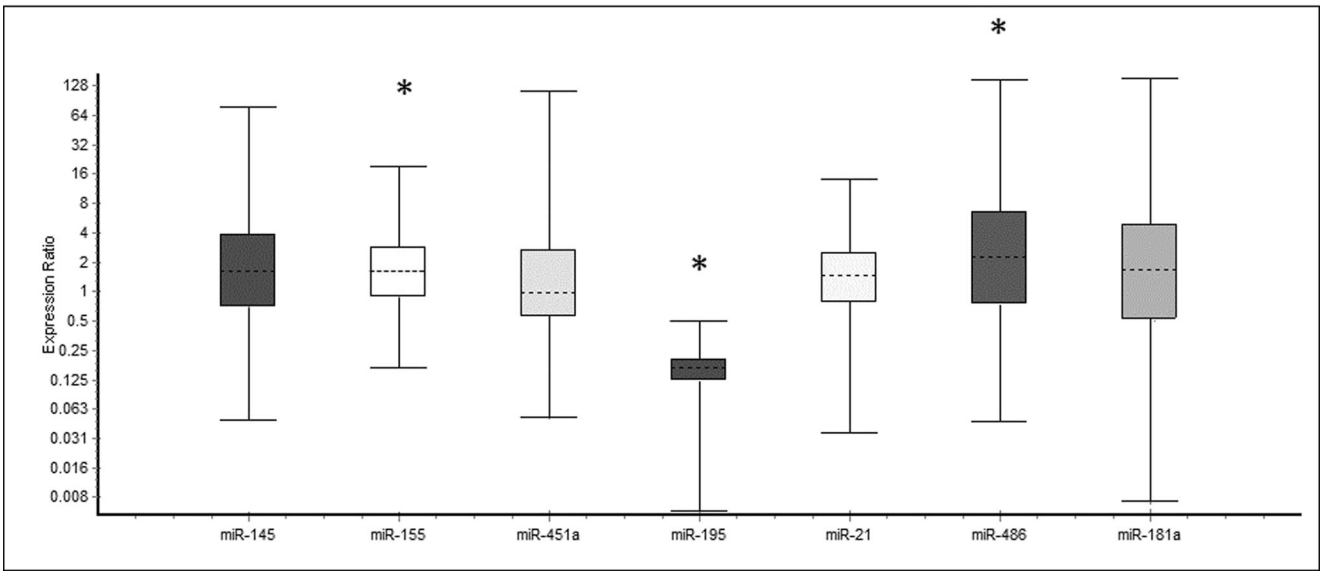

Fig. 1 Relative expression of seven circulating miRNAs in luminal A breast cancer patients by whisker-box plot (*$P < 0.05$)

qPCR). The most popular and powerful method is RT-qPCR, which is based on the amplification of a complementary DNA (cDNA) template after reverse transcription of the total RNA [19]. There are two methods of RT-qPCR; relative and absolute quantification RT-qPCR assays. In relative RT-qPCR, a reference RNA (mRNA, rRNA, or miRNA) is used to normalise the C_T values of the target transcripts between patient and control groups. Two mathematical models are applied to determine the relative expression ratio of the target genes, including the Pfaffl [17] and the Livak/ ΔΔCt method [20]. Several variations such as sample biological differences, RNA integrity, RT reaction efficiency and the amount of cDNA template can affect the expression level of miRNAs [21, 22]. Thus, choosing a stably expressed reference molecule as a normaliser is crucial to obtain accurate and reliable results in any relative quantification assay.

In contrast, absolute RT-qPCR can be used to determine the exact copy number of a miRNA. Traditionally, this has been achieved by relating the signal in an unknown sample to a standard curve [23, 24].

More recently, digital RT-PCR has been applied to the absolute quantitation of miRNAs [25]. Digital PCR has the inherent advantage of not requiring external calibrators (standard curve) or normalisation to estimate the concentration of an unknown target [26].

Several groups have studied miRNA signatures in various matrices, including; breast tissue, whole blood, serum and plasma. Various normalisers have been used for the expression profiling of miRNAs associated with breast cancer, very often leading to conflicting results. For example, Mar-Aguilar et al. (2013) reported the upregulation of miR-145 in the serum of breast cancer patients using 18 s ribosomal RNA as the reference gene [27]. In another study, Kodahl et al. (2014) found that miR-145 was downregulated in the serum of breast cancer patients using miR-10b and miR-30a as reference genes [28]. The most common reference RNAs that have been used for miRNA profiling of breast cancer are RNU6B, RNU48, 18 s rRNA, Let-7a and cel-miR-39 [11, 13, 27, 29–35]. There are also a few studies that determined miRNA expression profiles in whole blood of breast cancer patients, which used miR-16 as the reference miRNA [14, 36].

Based on previously published studies, we chose ten circulating miRNAs previously identified as being dysregulated in breast cancer (miR-195, miR-16, miR-21, miR-451a, miR-486, miR-181a, miR-145, miR-155, miR-10b and miR-205) to investigate the luminal A breast cancer subtype [13–15, 35, 37–39]. Of these, miR-205 and miR-10b were eliminated from our analysis because of their high C_T values (>38). Mitchell et al. (2008) also reported that the miR-205 was not

Table 1 Relative expression of circulating miRNAs in luminal A breast cancer patients

MicroRNA	Relative expression	95% C. I.	P value	Result
miR-145	1.610	0.146–21.07	0.058	–
miR-155	1.633	0.303–10.316	0.009	Up
miR-451a	1.621	0.150–61.838	0.208	–
miR-195	0.144	0.025–0.335	0.0006	Down
miR-21	1.353	0.127–6.790	0.103	–
miR-486	2.246	0.104–44.785	0.014	Up
miR-181a	1.524	0.042–32.547	0.223	–

C. I. Confidence Interval, *Up* upregulation, *Down* downregulation, *P* value, $P < 0.05$ is considered statistically significant

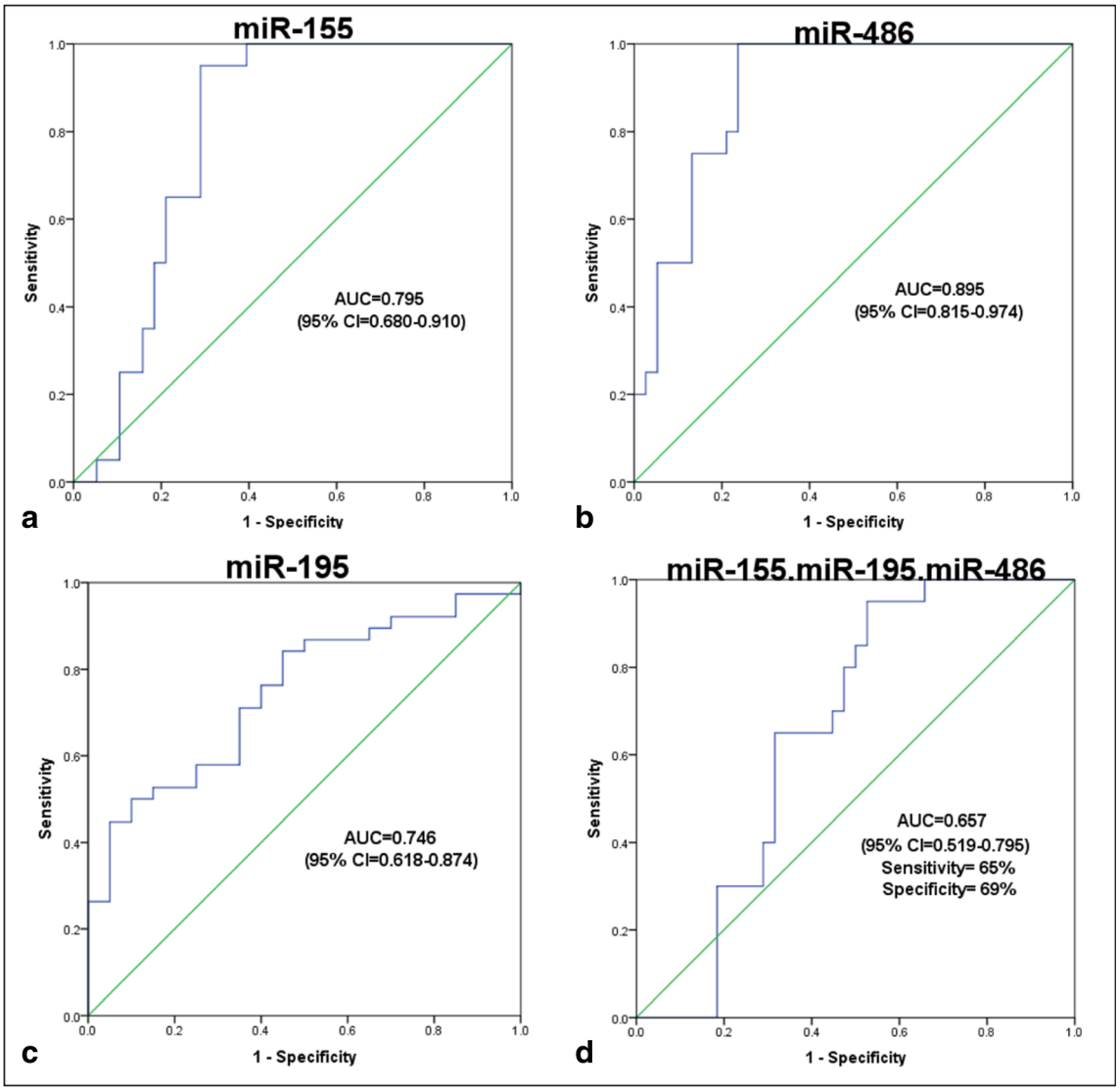

Fig. 2 ROC curve analysis for individually dysregulated (**a**–**c**) and a combination of three circulating miRNAs (**d**) in luminal A breast cancer patients by relative quantification assay

detectable in the serum of prostate cancer patients using an absolute RT-qPCR assay [40]. A few studies have reported that these two miRNAs were dysregulated in breast cancer patients. For instance, Liu et al. (2013) found that miR-205 was upregulated in the serum of breast cancer patients [41].

In another study, Filho and colleagues reported that miR-205 was downregulated in tissue samples obtained from luminal A and triple negative breast cancer patients [42]. Anfossi et al. (2014), observed the upregulation of miR-10b in the blood of breast cancer patients [43]. Furthermore, Mangolini and colleagues investigated the expression level of some miRNAs in the serum of two independent cohorts of breast cancer patients. They found that the upregulation of miR-10b is associated with poor prognosis in patients [44].

Of the seven miRNAs analysed by relative quantification, three miRNAs were found to be dysregulated in Luminal A breast cancer patients. Two miRNAs were upregulated (miR-155 and miR-486) and one (miR-195) was downregulated. None of the other miRNAs (miR-145, miR-451a, miR-21 and miR-181a) were found to be dysregulated by this method ($P > 0.05$).

On the other hand, for absolute quantification of miRNAs, standard curves were generated using synthetic miRNAs to enable the absolute copy number of each miRNA to be determined. MiR-205 and miR-10b were eliminated from our analysis because of the high C_T values (>38).

Of the eight miRNAs that had their absolute copy number determined, seven were found to be dysregulated in Luminal A breast cancer. Six of these were upregulated (miR-16, miR-145, miR-155, miR-451a, miR-21 and miR-486) and one was downregulated (miR-195). MiR-181a was not found to be

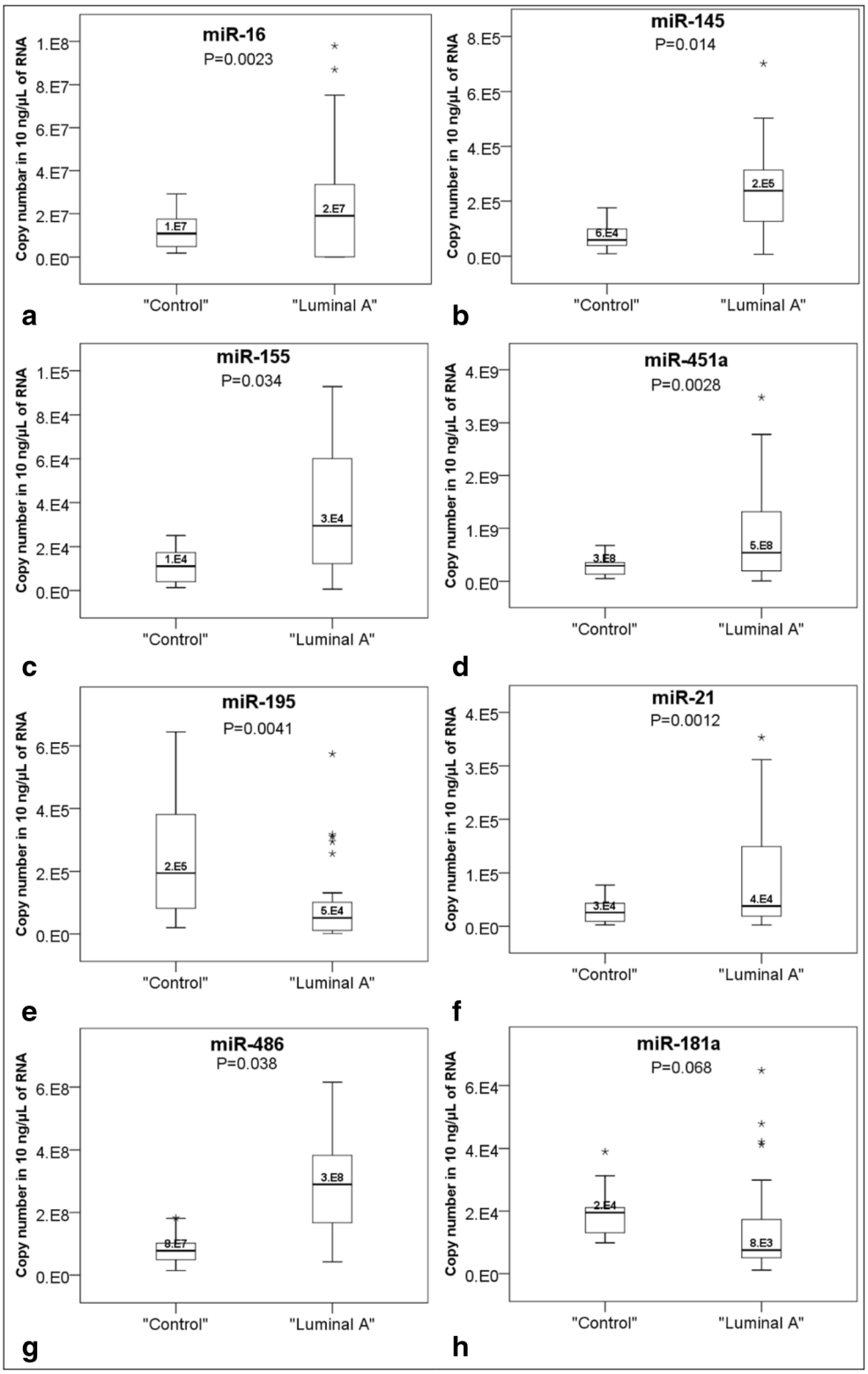

Fig. 3 Real-time PCR analysis for the absolute quantification of circulating miRNAs in luminal A breast cancer patients by whisker-box plot

differentially expressed between patients and controls ($P > 0.05$).

The expression levels of miR-155 and miR-486 were found to be significantly elevated in the patient group compared to

Fig. 4 ROC curve analysis for individually dysregulated (**a**–**g**) and a a ▶ combination of three circulating miRNAs (**h**) in luminal A breast cancer patients by absolute quantification assay

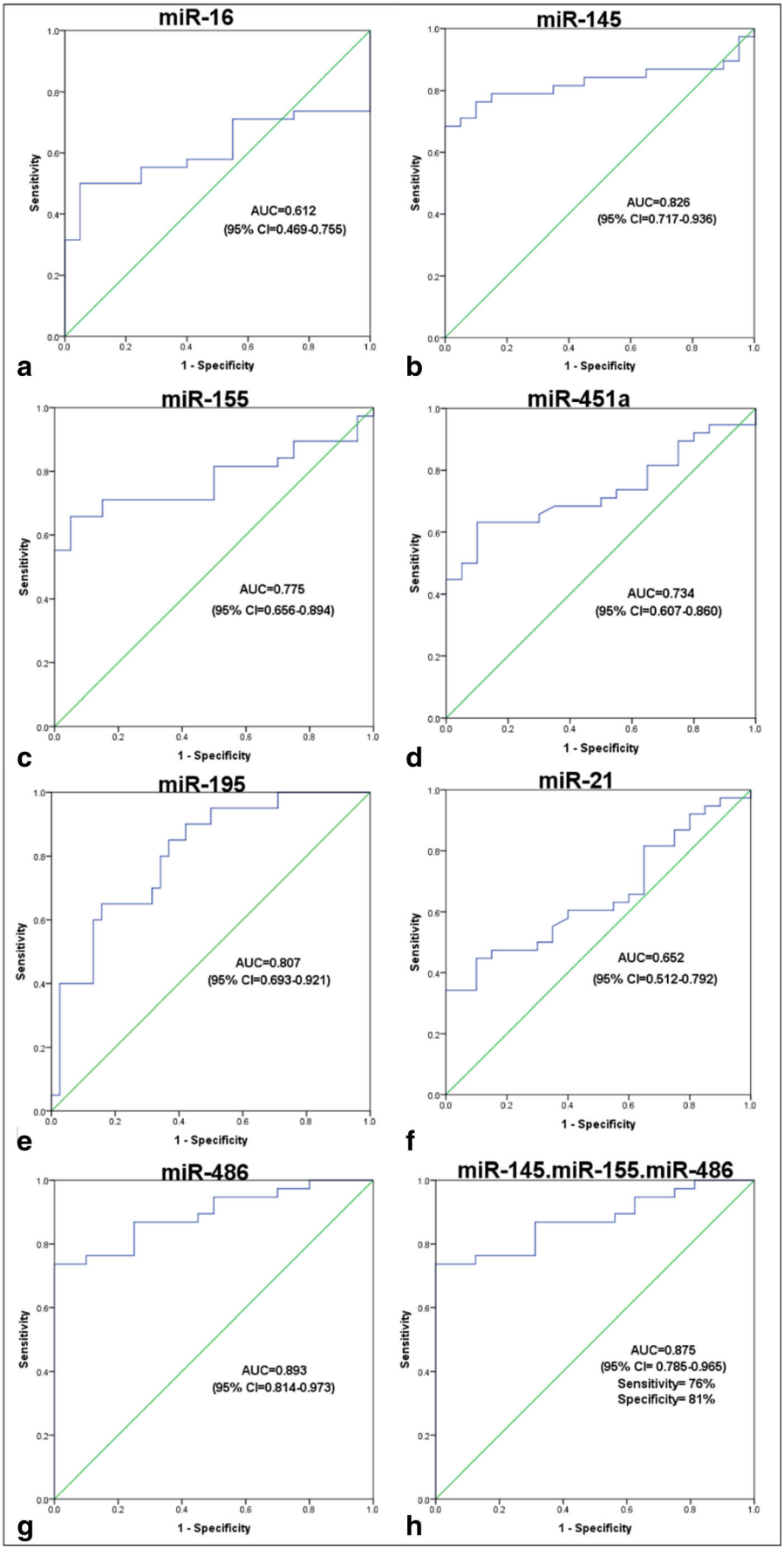
miR-16
Sensitivity
1 - Specificity
AUC=0.612
(95% CI=0.469-0.755)
a
miR-145
Sensitivity
1 - Specificity
AUC=0.826
(95% CI=0.717-0.936)
b
miR-155
Sensitivity
1 - Specificity
AUC=0.775
(95% CI=0.656-0.894)
c
miR-451a
Sensitivity
1 - Specificity
AUC=0.734
(95% CI=0.607-0.860)
d
miR-195
Sensitivity
1 - Specificity
AUC=0.807
(95% CI=0.693-0.921)
e
miR-21
Sensitivity
1 - Specificity
AUC=0.652
(95% CI=0.512-0.792)
f
miR-486
Sensitivity
1 - Specificity
AUC=0.893
(95% CI=0.814-0.973)
g
miR-145.miR-155.miR-486
Sensitivity
1 - Specificity
AUC=0.875
(95% CI= 0.785-0.965)
Sensitivity= 76%
Specificity= 81%
h

Table 2 The top 50 target genes for upregulated miRNAs in luminal A breast cancer patients

hsa-miR-16	hsa-miR-145	hsa- miR-155	hsa-miR-451a	hsa-miR-21	hsa-miR-486
SIX6	MUC19	ZNF385D	CMTM6	C1orf143	ZNF331
ARL2	CSRNP2	AC023632.1	MTRNR2L13	BRWD1	CCDC38
CCNE1	AC099552.4	C2orf80	OSR1	ZNF367	ZNF544
SLC17A9	FSCN1	CPA3	CDKN2B	KHDC1L	C19orf73
SPRED1	ABHD17C	ZC2HC1B	CXCL16	ABHD12B	AC011366.3
PLSCR4	TMEM9B	TMPRSS11BNL	CXorf21	KHDC1	LCE3E
DNAJB4	MYO5A	RP11-382 J12.1	TBC1D9B	HTN1	CTB-186H2.3
WNT3A	FLI1	ZNF860	BATF	KRIT1	TRAPPC6B
TNFSF13B	ABRACL	OR5H14	SPC25	IL12A	MTERFD1
ZBTB34	MBTD1	MARCH1	NEDD9	FASLG	FGF7
LSM11	BLOC1S6	TPRKB	EVL	FGF18	ZNF268
CYB561D1	SWAP70	GALT	ATF2	CCL1	AC005609.1
ANO3	TEX37	AKR1C1	HELLS	CALCB	GPX8
FP15737	ATP6V0B	VAV3	FAM9C	GPR64	CSPG5
TMEM74B	TPT1	VSIG1	CBLN4	AIM1L	EHHADH
EDA	HHEX	ETS1	SEC23IP	MTPN	CRLS1
PHF19	PTGR2	ACTA1	MIF	PLEKHA1	ANXA13
WEE1	KCNA4	ARID2	PSMB8	RIOK1	SRSF3
BCL2L2	CCBL2	H3F3A	AP000350.10	RSAD2	XXbac-BPG32J3.20
WNT7A	RP11-861 L17.3	C12orf39	KIF24	TRAPPC2	GPR78
PTH	GLIS1	MTRNR2L1	POU3F2	ATXN10	CDKN2B
CD80	ITGB8	RBP2	CYB561D1	LUM	AP003774.4
AQP11	GMFB	ZNF652	CTDSP1	RPL36A	ASZ1
MAMSTR	RP11-94B19.4	SPIN2B	AC114546.1	ALDH1A1	KIAA0226L
CDCA4	DAW1	ZNF724P	FAM91A1	YOD1	PYCR2
HTR2A	ACBD5	VMA21	MSC	PELI1	TM4SF20
CCDC19	PPP3CA	ACTL7A	TMEM170B	ANGPTL5	TRNT1
APLN	FKBP3	TM6SF1	TSC1	TGFBI	C6orf100
OOEP	YTHDF2	GABARAPL1	C21orf91	ARMCX1	LRRC25
SLC9A6	SKP1	CCDC126	CRIP2	B3GAT2	AP000889.3
SLC9A8	FLG2	CHST9	CPNE3	MATN2	BCAS2
SNCG	C13orf35	CEBPB	MCCC2	HSD17B4	NEK2
CYB561A3	DIRC1	SLCO5A1	ATP5E	SKP2	BTAF1
NEK10	ITLN2	FOS	KIAA1217	CDK2AP1	CD247
MYB	ACTG1	WWC1	FNTA	NTF3	C19orf12
DESI1	CHAC2	RP11-67H2.1	PLSCR2	MRPL9	ST5
BTLA	PGM3	SERPINA10	S1PR2	SPDYA	PPP1R16A
KRTAP4–4	TMEM139	CSRP2	GDI1	TIMP3	SLC10A7
TMEM221	SNX24	TAOK1	WDFY2	BEST3	CYB5R4
SPRYD3	IFNGR2	ZNF260	CERK	SMAD7	FOXO1
ZNF622	DDC	UBQLN1	C12orf5	MCMDC2	TOB1
UBE2V1	DST	POTEC	GRSF1	MSH2	SLMO1
RBM6	SCGN	TMEM202	NHSL1	SC5D	HAT1
OMG	TFRC	TDRD9	GALK2	EIF1AX	CNPY3
VAPB	RNF170	ZNF254	C11orf30	SEPT10	DHFRL1
FGF7	RP11-147C23.1	TSHZ3	AEBP2	RNFT1	CTD-2140B24.4
TMEM100	AKAP9	ZIC3	ACADSB	SATB1	CTSK
EMC4	RINT1	PTN	UNC93A	PHF14	CCDC91
CDC42SE2	IFI30	PRKCI	GK	FAM13A	EMP1

healthy controls, using both absolute and relative quantification methods.

Chan et al. (2013) reported that miR-486 was upregulated in the serum of breast cancer patients using miR-103 and miR-191 as endogenous controls [15]. However, in contrast, a few studies have found that miR-486 is downregulated in breast tissue, using small nuclear RNAs as normaliser [30, 45, 46].

MiR-155 is known as a multifunctional miRNA which is over-expressed in a number of different cancers, including; Acute Myeloid Leukaemia [47], Hodgkin's Lymphoma [48], Colon cancer [49], Cervical cancer [50] and Lung cancer [51]. Several studies have also described the function and upregulation of miR-155 in breast cancer tumours and serum [27, 31, 38, 52].

MiR-195 was found to be downregulated in the whole blood of patients by both methods in our study. Zhao et al. (2014) reported the downregulation of miR-195 in plasma of breast cancer patients using miR-16 as an endogenous control [53]. Several groups have also found that miR-195 is downregulated in breast tumours compared to healthy controls [13,

38, 54, 55]. However, Heneghan et al. showed that miR-195 is upregulated in a blood sample of breast cancer patients [34].

Using absolute quantification, we found that miR-451a was increased in the whole blood of luminal A patients compared to the control group. Hu et al. (2012) and Ng et al. (2013) reported that miR-451a was upregulated in the serum and plasma of breast cancer patients [29, 56]. However, using relative quantification, we did not find that miR-451a was differentially expressed.

MiR-21 is a well-known oncomir and various studies have found that it is upregulated not only in breast cancer [11, 29, 42, 43] but also in a wide range of other cancers [49, 57–59]. Here, we observed upregulation of miR-21 in whole blood of luminal A breast cancer patients by the absolute quantification method.

We showed that miR-145 was upregulated in patients versus healthy controls using the absolute qPCR method. In two separate studies, Heneghan et al. (2010) and Park et al. (2014) found that miR-145 transcription was increased in the blood of breast cancer patients [34, 35].

McDermott et al. (2014) found that miR-181a was downregulated in luminal A breast cancer patients [14]. Here, we observed that miR-181a does not change in luminal A breast cancer, using either relative and absolute quantitation methods.

Our absolute RT-qPCR assay showed that miR-16 was upregulated in patients versus healthy controls. Stuckrath and colleagues (2015) reported that miR-16 was upregulated in the plasma of different subtypes of invasive breast cancer before and after chemotherapy [60]. In another study by Shin et al. (2015), they found that the expression level of miR-16 is higher in plasma of none triple negative breast cancer (none TNBC) patients compare to healthy controls [61]. Ng et al. (2013) and Chan et al. (2013) also found that miR-16 was upregulated in plasma and serum of breast cancer patients using RNU6B and miR-103/miR-191 as reference genes [15, 29]. In another study, which was carried out by Filho et al. (2014), miR-16 was found to be upregulated in tumour samples from triple negative and luminal A breast cancer patients [42].

In this study, we used miR-16 as an internal normalisation control for relative quantification qPCR, based on previously published reports [35–37, 53, 62]. To date, only miR-16 has been evaluated as a stable control in whole blood of breast cancer patients and healthy controls. Two separate studies have found miR-16 to be stably expressed in whole blood [34, 37]. In another study, McDermott et al. (2013) carried out a more in-depth study specifically concerned with the identification of appropriate miRNA endogenous controls for use in whole blood miRNA quantification [63]. Of 377 human miRNAs, they concluded that miR-16 and miR-425 were the most stably expressed miRNAs in whole blood samples from breast cancer patients and healthy controls. All these studies used microarray and relative RT-qPCR assays to evaluate miRNA expression profiles. In our study, we found that miR-16 was increased ($P = 0.0023$) in the whole blood of luminal A breast cancer patients compare to healthy controls using our absolute quantification assay. Absolute quantification permits the generation of highly reproducible and sensitive data for the expression profiling of miRNAs [23, 24]. As the standard curve is generated, the exact copy number of target genes is determined which allows a comparison of target gene expression between patient and control groups directly without the requirement to know the cDNA synthesis efficiency.

Furthermore, we used an online algorithm (TargetScan) to predict target genes for the upregulated miRNAs. It has been shown that each individual miRNA may have thousands of mRNA targets, with estimates that between 30 and 80% of human genes may be regulated by one or more miRNA [64].

miRNAs regulate gene expression by targeting the 3′ untranslated region (3′ UTR) of mRNA. However, some studies have shown target sites for miRNAs in open reading frames (ORF) and 5′ UTR [18]. Here we listed the top fifty target genes for miRNAs identified as upregulated, predicted by TargetScan algorithm.

To identify the direct and functionally related target genes for these upregulated miRNAs in luminal A breast cancer, further experiments will need to be carried out including the global gene expression profiling (e.g., whole genome RNA sequencing) and gain/loss- of- function studies.

Conclusions

Using relative quantification, we observed the upregulation of two miRNAs (miR-155 and miR-486) and downregulation of one miRNA (miR-195) in the luminal A breast cancer versus healthy controls. When combined, these three miRNAs were found to have an AUC of 0.657 with diagnostic sensitivity of 65% and specificity of 69%.

We found that miR-16 is upregulated in patients compared to the control group by the absolute quantification method. Consequently, miR-16 is not a suitable normaliser for the relative expression profiling of miRNAs in luminal A breast cancer patients. in addition to miR-16, another five miRNAs (miR-145, miR-155, miR-451a, miR-21 and miR-486) were found to be upregulated and one miRNA (miR-195) was found to be downregulated when analysed using absolute quantification. The combination of miR-145, miR-195 and miR-486 resulted in the best diagnostic value (AUC = 0.875, sensitivity = 76% and specificity = 81%) for luminal A breast cancer.

The availability of a rapid, robust, and reliable molecular diagnostics test that detects clinically relevant miRNAs from blood would represent an invaluable diagnostic tool to support

and enhance mammography as a diagnostic instrument. While our investigation identifies a three-miRNA combination that shows potential as a complementary diagnostic test to mammography, clearly further detailed clinical validation studies are required, including large scale prospective studies, to elucidate the potential of circulating miRNAs as breast cancer sub-type diagnostic markers.

Authors Contributions Conceived and designed the experiments: TJS VA. Performed the experiments: VA. Analysed the data: VA EH OK JN. Contributed reagents/materials/analysis tools: TJS RMD MJK. Wrote the paper: VA EC TJS.

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References

1. WHO. World Health Organization (2015) Global health observatory data repository. 2015. Number of deaths (World) by cause. http://wwww.hoint/mediacentre/factsheets/fs310/en/
2. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer Journal international du cancer. 2015;136(5):E359–E386. https://doi.org/10.1002/ijc.29210
3. Ferlay J, Steliarova-Foucher E, Lortet-Tieulent J, Rosso S, Coebergh JW, Comber H et al (2013) Cancer incidence and mortality patterns in Europe: estimates for 40 countries in 2012. Eur J Cancer 49(6):1374–1403. https://doi.org/10.1016/j.ejca.2012.12.027
4. Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA et al (2000) Molecular portraits of human breast tumours. Nature. 406(6797):747–752. https://doi.org/10.1038/35021093
5. Sorlie T, Wang Y, Xiao C, Johnsen H, Naume B, Samaha RR et al (2006) Distinct molecular mechanisms underlying clinically relevant subtypes of breast cancer: gene expression analyses across three different platforms. BMC Genomics 7:127. https://doi.org/10.1186/1471-2164-7-127
6. Goldhirsch A, Wood WC, Coates AS, Gelber RD, Thurlimann B, Senn HJ (2011) Strategies for subtypes–dealing with the diversity of breast cancer: highlights of the St. Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2011. Ann Oncol 22(8):1736–1747. https://doi.org/10.1093/annonc/mdr304
7. Rouzier R, Perou CM, Symmans WF, Ibrahim N, Cristofanilli M, Anderson K et al (2005) Breast cancer molecular subtypes respond differently to preoperative chemotherapy. Clin Cancer Res 11(16):5678–5685. https://doi.org/10.1158/1078-0432.ccr-04-2421
8. Kumar MS, Lu J, Mercer KL, Golub TR, Jacks T (2007) Impaired microRNA processing enhances cellular transformation and tumorigenesis. Nat Genet 39(5):673–677. https://doi.org/10.1038/ng2003
9. Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, Sweet-Cordero A, Ebert BL, Mak RH, Ferrando AA, Downing JR, Jacks T, Horvitz HR, Golub TR (2005) MicroRNA expression profiles classify human cancers. Nature. 435(7043):834–838. https://doi.org/10.1038/nature03702
10. Zen K, Zhang C-Y (2012) Circulating MicroRNAs: a novel class of biomarkers to diagnose and monitor human cancers. Med Res Rev 32(2):326–348. https://doi.org/10.1002/med.20215
11. Iorio MV, Ferracin M, Liu CG, Veronese A, Spizzo R, Sabbioni S, Magri E, Pedriali M, Fabbri M, Campiglio M, Ménard S, Palazzo JP, Rosenberg A, Musiani P, Volinia S, Nenci I, Calin GA, Querzoli P, Negrini M, Croce CM (2005) MicroRNA gene expression deregulation in human breast cancer. Cancer Res 65(16):7065–7070. https://doi.org/10.1158/0008-5472.CAN-05-1783
12. Heneghan HM, Miller N, Lowery AJ, Sweeney KJ, Kerin MJ (2009, 2009) MicroRNAs as Novel Biomarkers for Breast Cancer. J Oncol:950201. https://doi.org/10.1155/2010/950201
13. Tahiri A, Leivonen SK, Luders T, Steinfeld I, Ragle Aure M, Geisler J et al (2014) Deregulation of cancer-related miRNAs is a common event in both benign and malignant human breast tumors. Carcinogenesis. 35(1):76–85. https://doi.org/10.1093/carcin/bgt333
14. McDermott AM, Miller N, Wall D, Martyn LM, Ball G, Sweeney KJ et al (2014) Identification and validation of oncologic miRNA biomarkers for luminal A-like breast cancer. PLoS One 9(1):e87032. https://doi.org/10.1371/journal.pone.0087032
15. Chan M, Liaw CS, Ji SM, Tan HH, Wong CY, Thike AA et al (2013) Identification of circulating microRNA signatures for breast cancer detection. Clin Cancer Res 19(16):4477–4487. https://doi.org/10.1158/1078-0432.CCR-12-3401
16. Pfaffl MW, Horgan GW, Dempfle L (2002) Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res 30(9):e36
17. Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29(9):e45
18. Agarwal V, Bell GW, Nam JW, Bartel DP (2015) Predicting effective microRNA target sites in mammalian mRNAs. Elife 4. https://doi.org/10.7554/eLife.05005
19. Klein D (2002) Quantification using real-time PCR technology: applications and limitations. Trends Mol Med 8(6):257–260
20. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) Method. Methods 25(4):402–408. https://doi.org/10.1006/meth.2001.1262
21. Mannhalter C, Koizar D, Mitterbauer G (2000) Evaluation of RNA isolation methods and reference genes for RT-PCR analyses of rare target RNA. Clin Chem Lab Med 38(2):171–177. https://doi.org/10.1515/cclm.2000.026
22. Karge WH 3rd, Schaefer EJ, Ordovas JM (1998) Quantification of mRNA by polymerase chain reaction (PCR) using an internal standard and a nonradioactive detection method. Methods Mol Biol 110:43–61. https://doi.org/10.1385/1-59259-582-0:43
23. Bustin SA (2000) Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J Mol Endocrinol 25(2):169–193
24. Pfaffl MW, Hageleit M (2001) Validities of mRNA quantification using recombinant RNA and recombinant DNA external calibration curves in real-time RT-PCR. Biotechnol Lett 23(4):275–282. https://doi.org/10.1023/a:1005658330108
25. Hindson CM, Chevillet JR, Briggs HA, Gallichotte EN, Ruf IK, Hindson BJ, Vessella RL, Tewari M (2013) Absolute quantification by droplet digital PCR versus analog real-time PCR. Nat Methods 10(10):1003–1005. https://doi.org/10.1038/nmeth.2633
26. Pohl G, Shih IM (2004) Principle and applications of digital PCR. Expert Rev Mol Diagn 4(1):41–47
27. Mar-Aguilar F, Mendoza-Ramirez JA, Malagon-Santiago I, Espino-Silva PK, Santuario-Facio SK, Ruiz-Flores P et al (2013) Serum circulating microRNA profiling for identification of potential breast cancer biomarkers. Dis Markers 34(3):163–169
28. Kodahl AR, Lyng MB, Binder H, Cold S, Gravgaard K, Knoop AS, Ditzel HJ (2014) Novel circulating microRNA signature as a potential non-invasive multi-marker test in ER-positive early-stage

breast cancer: a case control study. Mol Oncol 8(5):874–883. https://doi.org/10.1016/j.molonc.2014.03.002
29. Ng EK, Li R, Shin VY, Jin HC, Leung CP, Ma ES et al (2013) Circulating microRNAs as specific biomarkers for breast cancer detection. PLoS One 8(1):e53141. https://doi.org/10.1371/journal.pone.0053141
30. Rask L, Balslev E, Sokilde R, Hogdall E, Flyger H, Eriksen J et al (2014) Differential expression of miR-139, miR-486 and miR-21 in breast cancer patients sub-classified according to lymph node status. Cell Oncol (Dordr) 37(3):215–227. https://doi.org/10.1007/s13402-014-0176-6
31. Sochor M, Basova P, Pesta M, Dusilkova N, Bartos J, Burda P, Pospisil V, Stopka T (2014) Oncogenic microRNAs: miR-155, miR-19a, miR-181b, and miR-24 enable monitoring of early breast cancer in serum. BMC Cancer 14:448. https://doi.org/10.1186/1471-2407-14-448
32. Madhavan D, Zucknick M, Wallwiener M, Cuk K, Modugno C, Scharpff M et al (2012) Circulating miRNAs as surrogate markers for circulating tumor cells and prognostic markers in metastatic breast cancer. Clin Cancer Res 18(21):5972–5982. https://doi.org/10.1158/1078-0432.ccr-12-1407
33. Godfrey AC, Xu Z, Weinberg CR, Getts RC, Wade PA, LA DR et al (2013) Serum microRNA expression as an early marker for breast cancer risk in prospectively collected samples from the Sister Study cohort. Breast Cancer Res 15(3):R42. https://doi.org/10.1186/bcr3428
34. Heneghan HM, Miller N, Lowery AJ, Sweeney KJ, Newell J, Kerin MJ (2010) Circulating microRNAs as novel minimally invasive biomarkers for breast cancer. Ann Surg 251(3):499–505. https://doi.org/10.1097/SLA.0b013e3181cc939f
35. Park IH, Kang JH, Lee KS, Nam S, Ro J, Kim JH (2014) Identification and clinical implications of circulating microRNAs for estrogen receptor-positive breast cancer. Tumour Biol 35:12173–12180. https://doi.org/10.1007/s13277-014-2525-5
36. Schrauder MG, Strick R, Schulz-Wendtland R, Strissel PL, Kahmann L, Loehberg CR, Lux MP, Jud SM, Hartmann A, Hein A, Bayer CM, Bani MR, Richter S, Adamietz BR, Wenkel E, Rauh C, Beckmann MW, Fasching PA (2012) Circulating micro-RNAs as potential blood-based markers for early stage breast cancer detection. PLoS One 7(1):e29770. https://doi.org/10.1371/journal.pone.0029770
37. Heneghan HM, Miller N, Kelly R, Newell J, Kerin MJ (2010) Systemic miRNA-195 differentiates breast cancer from other malignancies and is a potential biomarker for detecting noninvasive and early stage disease. Oncologist 15(7):673–682. https://doi.org/10.1634/theoncologist.2010-0103
38. Ouyang M, Li Y, Ye S, Ma J, Lu L, Lv W, Chang G, Li X, Li Q, Wang S, Wang W (2014) MicroRNA profiling implies new markers of chemoresistance of triple-negative breast cancer. PLoS One 9(5):e96228. https://doi.org/10.1371/journal.pone.0096228
39. Ng EK, Chong WW, Jin H, Lam EK, Shin VY, Yu J et al (2009) Differential expression of microRNAs in plasma of patients with colorectal cancer: a potential marker for colorectal cancer screening. Gut. 58(10):1375–1381. https://doi.org/10.1136/gut.2008.167817
40. Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, Peterson A, Noteboom J, O'Briant KC, Allen A, Lin DW, Urban N, Drescher CW, Knudsen BS, Stirewalt DL, Gentleman R, Vessella RL, Nelson PS, Martin DB, Tewari M (2008) Circulating microRNAs as stable blood-based markers for cancer detection. Proc Natl Acad Sci 105(30):10513–10518. https://doi.org/10.1073/pnas.0804549105
41. Liu J, Mao Q, Liu Y, Hao X, Zhang S, Zhang J (2013) Analysis of miR-205 and miR-155 expression in the blood of breast cancer patients. Chin J Cancer Res 25(1):46–54. https://doi.org/10.3978/j.issn.1000-9604.2012.11.04
42. Calvano Filho CM, Calvano-Mendes DC, Carvalho KC, Maciel GA, Ricci MD, Torres AP et al (2014) Triple-negative and luminal A breast tumors: differential expression of miR-18a-5p, miR-17-5p, and miR-20a-5p. Tumour Biol 35(8):7733–7741. https://doi.org/10.1007/s13277-014-2025-7
43. Anfossi S, Giordano A, Gao H, Cohen EN, Tin S, Wu Q, Garza RJ, Debeb BG, Alvarez RH, Valero V, Hortobagyi GN, Calin GA, Ueno NT, Woodward WA, Reuben JM (2014) High serum miR-19a levels are associated with inflammatory breast cancer and are predictive of favorable clinical outcome in patients with metastatic HER2+ inflammatory breast cancer. PLoS One 9(1):e83113. https://doi.org/10.1371/journal.pone.0083113
44. Mangolini A, Ferracin M, Zanzi MV, Saccenti E, Ebnaof SO, Poma VV, Sanz JM, Passaro A, Pedriali M, Frassoldati A, Querzoli P, Sabbioni S, Carcoforo P, Hollingsworth A, Negrini M (2015) Diagnostic and prognostic microRNAs in the serum of breast cancer patients measured by droplet digital PCR. Biomarker Res 3:12. https://doi.org/10.1186/s40364-015-0037-0
45. Zhang G, Liu Z, Cui G, Wang X, Yang Z (2014) MicroRNA-486-5p targeting PIM-1 suppresses cell proliferation in breast cancer cells. Tumour Biol 35(11):11137–11145. https://doi.org/10.1007/s13277-014-2412-0
46. Lerebours F, Cizeron-Clairac G, Susini A, Vacher S, Mouret-Fourme E, Belichard C, et al. miRNA expression profiling of inflammatory breast cancer identifies a 5-miRNA signature predictive of breast tumor aggressiveness. Int J Cancer Journal international du cancer. 2013;133(7):1614–1623. https://doi.org/10.1002/ijc.28171
47. O'Connell RM, Rao DS, Chaudhuri AA, Boldin MP, Taganov KD, Nicoll J, Paquette RL, Baltimore D (2008) Sustained expression of microRNA-155 in hematopoietic stem cells causes a myeloproliferative disorder. J Exp Med 205(3):585–594. https://doi.org/10.1084/jem.20072108
48. Kluiver J, Poppema S, de Jong D, Blokzijl T, Harms G, Jacobs S, Kroesen BJ, van den Berg A (2005) BIC and miR-155 are highly expressed in Hodgkin, primary mediastinal and diffuse large B cell lymphomas. J Pathol 207(2):243–249. https://doi.org/10.1002/path.1825
49. Volinia S, Calin GA, Liu C-G, Ambs S, Cimmino A, Petrocca F et al (2006) A microRNA expression signature of human solid tumors defines cancer gene targets. Proc Natl Acad Sci U S A 103(7):2257–2261
50. Wang X, Tang S, Le SY, Lu R, Rader JS, Meyers C et al (2008) Aberrant expression of oncogenic and tumor-suppressive microRNAs in cervical cancer is required for cancer cell growth. PLoS One 3(7):e2557. https://doi.org/10.1371/journal.pone.0002557
51. Yanaihara N, Caplen N, Bowman E, Seike M, Kumamoto K, Yi M, Stephens RM, Okamoto A, Yokota J, Tanaka T, Calin GA, Liu CG, Croce CM, Harris CC (2006) Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. Cancer Cell 9(3):189–198. https://doi.org/10.1016/j.ccr.2006.01.025
52. Kong W, He L, Coppola M, Guo J, Esposito NN, Coppola D et al (2016) MicroRNA-155 regulates cell survival, growth, and chemosensitivity by targeting FOXO3a in breast cancer. J Biol Chem 291(43):22855. https://doi.org/10.1074/jbc.A110.101055
53. Zhao FL, Dou YC, Wang XF, Han DC, Lv ZG, Ge SL et al (2014) Serum microRNA-195 is down-regulated in breast cancer: a potential marker for the diagnosis of breast cancer. Mol Biol Rep 41(9):5913–5922. https://doi.org/10.1007/s11033-014-3466-1
54. Li D, Zhao Y, Liu C, Chen X, Qi Y, Jiang Y et al (2011) Analysis of MiR-195 and MiR-497 expression, regulation and role in breast cancer. Clin Cancer Res 17(7):1722–1730. https://doi.org/10.1158/1078-0432.ccr-10-1800
55. Luo Q, Wei C, Li X, Li J, Chen L, Huang Y et al (2014) MicroRNA-195-5p is a potential diagnostic and therapeutic target for breast

cancer. Oncol Rep 31(3):1096–1102. https://doi.org/10.3892/or.2014.2971

56. Hu Z, Dong J, Wang LE, Ma H, Liu J, Zhao Y, Tang J, Chen X, Dai J, Wei Q, Zhang C, Shen H (2012) Serum microRNA profiling and breast cancer risk: the use of miR-484/191 as endogenous controls. Carcinogenesis. 33(4):828–834. https://doi.org/10.1093/carcin/bgs030
57. Iorio MV, Visone R, Di Leva G, Donati V, Petrocca F, Casalini P et al (2007) MicroRNA signatures in human ovarian cancer. Cancer Res 67(18):8699–8707. https://doi.org/10.1158/0008-5472.can-07-1936
58. Asangani IA, Rasheed SA, Nikolova DA, Leupold JH, Colburn NH, Post S et al (2008) MicroRNA-21 (miR-21) post-transcriptionally downregulates tumor suppressor Pdcd4 and stimulates invasion, intravasation and metastasis in colorectal cancer. Oncogene 27(15):2128–2136. https://doi.org/10.1038/sj.onc.1210856
59. Chan JA, Krichevsky AM, Kosik KS (2005) MicroRNA-21 is an antiapoptotic factor in human glioblastoma cells. Cancer Res 65(14):6029–6033. https://doi.org/10.1158/0008-5472.can-05-0137
60. Stuckrath I, Rack B, Janni W, Jager B, Pantel K, Schwarzenbach H (2015) Aberrant plasma levels of circulating miR-16, miR-107, miR-130a and miR-146a are associated with lymph node metastasis and receptor status of breast cancer patients. Oncotarget. 6(15):13387–13401. https://doi.org/10.18632/oncotarget.3874
61. Shin VY, Siu JM, Cheuk I, Ng EK, Kwong A (2015) Circulating cell-free miRNAs as biomarker for triple-negative breast cancer. Br J Cancer 112(11):1751–1759. https://doi.org/10.1038/bjc.2015.143
62. Guo LJ, Zhang QY (2012) Decreased serum miR-181a is a potential new tool for breast cancer screening. Int J Mol Med 30(3):680–686. https://doi.org/10.3892/ijmm.2012.1021
63. McDermott AM, Kerin MJ, Miller N (2013) Identification and validation of miRNAs as endogenous controls for RQ-PCR in blood specimens for breast cancer studies. PLoS One 8(12):e83718
64. Lu J, Clark AG (2012) Impact of microRNA regulation on variation in human gene expression. Genome Res 22(7):1243–1254. https://doi.org/10.1101/gr.132514.111