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ORIGINAL ARTICLE

MicroRNA Expression in Laser Micro-dissected Breast Cancer Tissue Samples – a Pilot Study

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Abstract Breast cancer continues to represent a significant public health burden despite outstanding research advances regarding the molecular mechanisms of cancer biology, biomarkers for diagnostics and prognostic and therapeutic management of this disease. The studies of micro RNAs in breast cancer have underlined their potential as biomarkers and therapeutic targets; however most of these studies are still done on largely heterogeneous whole breast tissue samples. In this pilot study we have investigated the expression of four micro RNAs (miR-21, 145, 155, 92) known to be involved in breast cancer, in homogenous cell populations collected by laser capture microdissection from breast tissue section slides. Micro RNA expression was assessed by real time PCR, and associations with clinical and pathological characteristics were also explored. Our results have confirmed previous associations of miR-21 expression with poor prognosis characteristics of breast cancers such as high stage, large and highly proliferative tumors. No statistically significant associations were found with the other micro RNAs investigated, possibly due to the small sample size of our study. Our results also suggest that miR-484 could be a suitable endogenous control for data normalization in breast tissues, these results needing further confirmation by future studies. In summary, our pilot study showed the feasibility of detecting micro RNAs expression in homogenous laser captured microdissected invasive breast cancer samples, and confirmed some of the previously reported associations with poor prognostic characteristics of breast tumors.

Keywords Breast cancer · MicroRNA · Laser capture microdissection (LCM)

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Introduction

Estimated to affect 1 in 8 women in the USA, breast cancer also remains the oncological pathology with the highest incidence and mortality in women in Europe [8, 42]. Despite the tremendous research efforts into deciphering the molecular mechanisms underlying cancer biology, much is still left to be understood in order to develop sensitive biomarkers for diagnosis and prognostic, as well as more effective therapies and disease management of this heterogeneous disease [43].

Given the high incidence of breast cancer worldwide, basically all medical institutions possess rich archives of breast tumor tissue samples, which are usually not sufficiently and efficiently used for research, mainly due to technical burdens. The recent developments of new DNA, RNA, proteins and metabolites techniques overcame the difficulty of analyzing these molecules in difficult tissue samples such as archived tissues [20]. Of these molecules, micro RNAs (non-coding RNA molecules of 18–24 nucleotides in length) are of particular interest not only because of their potential as biomarkers and their involvement in carcinogenesis (including of breast cancer), but also due to their remarkable stability [4, 44]. The genes encoding miRNAs have been identified throughout the entire human genome, including the genomic regions

frequently altered in human cancers and thus can act both as tumor suppressor genes and oncogenes [51].

miRNAs expression is tissue specific and differ between normal and tumor tissues to such extent that similar tumor types cluster more consistently according to miRNA profiles than to protein-coding gene expression profiles [25]. Moreover, miRNA profiles in cancer tissues (breast cancer included) correlate with tumor characteristics and have been used to predict prognosis [18]. The first breast cancer specific miRNA signature was reported in 2005 and consisted of 29 differentially expressed miRNAs able to differentiate normal from cancer tissue with 100% accuracy [17]. Furthermore, breast cancer miRNA profiling revealed subsets of miRNAs capable of accurately reproducing the molecular classification of breast carcinomas; other miRNA subsets were associated with aggressiveness in lymph node negative, ER-positive breast cancers [3, 10]. Remarkably, several individual miRNAs have been associated with breast cancer characteristics; some are up-regulated while others are down-regulated in cancer tissue as compared to normal controls [15, 17, 26, 38].

It is well known that full-length messenger RNA has a short half-life, reflected in a high degree of pre-analytical variability in biological samples. By contrast, miRNAs are more stable in archival samples and can be reliably detected even in years old tissue samples [49]. However, breast cancer is heterogeneous, the same tissue slide containing several cell populations (epithelial, stromal, inflammatory infiltrate, etc.), and often even different tumor type (*in situ* or invasive), thus making the analysis of a certain biomolecule somewhat difficult. Laser capture microdissection (LCM) can overcome this limitation due to the potential of isolating specific cell populations from a tissue section, which can be further used for several downstream molecular applications, including miRNA analysis [23].

LCM has been extensively used for the analysis of proteins and DNA or RNA molecules in breast cancer tumors, and has been even proposed to replace the tests based on immunohistochemistry and/or fluorescent in situ hybridization [9, 30].

Similar to mRNA expression analysis, various analytical platforms can be used for miRNA analysis, the most widely used being solid-based hybridization arrays, liquid hybridization bead-based technology, and QRT-PCR profiling [22]. However, when analyzing biological specimens with low abundance of miRNA such as LCM captured samples, the preferred method is QRT-PCR due to its increased specificity and sensitivity [16, 40]. Moreover QRT-PCR is also generally used to validate the miRNA expression results obtained with other platforms [13].

In this pilot study we have investigated the expression in LC-micro-dissected breast cancer cells of four miRNA molecules known to be involved in breast cancer: miR-21, miR-92, miR-145, miR-155.

Materials and Methods

Patients and Tumor Samples

A detailed description of the study has been previously published [31]. Briefly, tumor slides from 31 patients with pathologically confirmed invasive ductal breast carcinoma undergoing surgical resection at the Department of Surgical Oncology of the Timisoara Municipal Hospital during 2009–2010 were evaluated in this study. All patients provided informed consent for the use of their tissue slides not needed for the clinical and pathological diagnostic and disease management procedures. The study was performed in accordance with the Declaration of Helsinki for Human Research and was approved by the Ethical Committee of our institution.

After surgical resection and pathological assessment, tissue samples were preserved in RNAlater solution (Ambion, Applied Biosystems, Germany) and stored at −80°C. Following the manufacturer's protocol, frozen tissues were embedded in TissueTek medium and cut at −30°C (Leica CM1850 cryostat, Leica Microsystems GmbH, Wetzlar, Germany).

Tissue Sample Processing

The cryosections were mounted on RNase free membrane slides (MMI MembranSlides, MMI, Glattburg, Switzerland), which were either immediately processed or stored at −80°C. The sections were stained with Hematoxylin–Eosin or cresyl violet before proceeding for LCM; a typical slide image is presented in Fig. 1. Consecutive cryosections from each specimen were mounted also on silanized glass slides and, after standard Hematoxylin–Eosin staining, the sections were evaluated by an experienced pathologist.

The mounted sections were covered with a clean RNase free glass slide to prevent contamination and loss of dissected tissues. The tissue regions of interest were cut using adequate power and focus for UV laser shots; the cut areas were then captured, pooled and placed in RNase free microcentrifuge tubes as described in the manufacturer's instructions.

RNA Extraction

Total RNA from laser-microdissected tumoral tissues were extracted using RNAqueous-Micro kit (Ambion, Applied Biosystems, Germany) according to the manufacturer's instructions. The RNA was eluted in 20 μL elution solution. RNA concentration and quality were spectrophotometrically

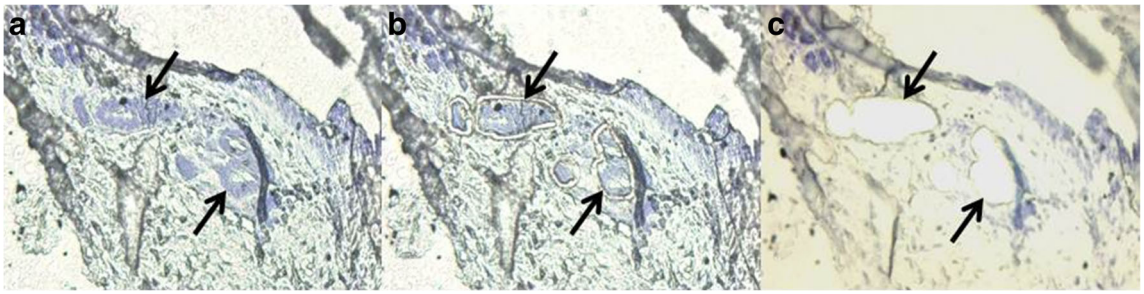

Fig. 1 Example of laser micro-dissected tissue of breast cancer samples (10 μm sections). a: before laser dissection; b: after laser dissection; c: after collection of dissected tissue (black arrows indicate the captured areas)

assessed (NanoDrop ND1000). Extracted RNA was stored at −80°C for downstream application.

qRT-PCR

cDNA synthesis for sensitive and specific miRNA detection were carried out using miScript II RT kit (Qiagen, Germany) starting from 20 ng of total RNA/20 μl reaction mix. PCR reactions were performed in triplicate, each starting from 2.5 μL RT-product diluted ten times in 25 μL reaction mix using QuantiTect SYBR Green PCR kit (Qiagen, Germany), with an initial 15 min polymerase activation step followed by 40 cycles (95 °C/15 s, 55 °C/30 s, 70 °C/30 s).

Statistical Analysis

The miRNA expression was calculated using the comparative ΔΔCt method relative to the selected controls, as previously described [37]. In order to establish the most suitable endogenous control for normalization, we used the following algorithms geNorm [46], NormFinder [2], BestKeeper [35], and the ΔCt method [41], and reference candidates were compared and ranked using the online analysis tool RefFinder (http://www.leonxie.com/referencegene.php).

Mann-Whitney U nonparametric tests were used to compare relative miRNA quantities among groups stratified according to pathological characteristics, with a cutoff value of $p < 0.05$ for statistical significance.

Results

Patients' characteristics are presented in Table 1. The majority of patients were post-menopausal (70.96%) and of normal BMI (77.41%). Among tumor characteristics, the vast majority was lymph node positive (90.32%), and hormone receptor positive (83.87% ER positive, 77.41% PR positive). Therefore, no statistical analysis was performed for these tumor characteristics due to low number of samples in each stratum.

Figure 2 presents the stability of each tested miRNA and the RNU-6 candidate control, and their ranking values provided by RefFinder. The best performing reference genes were proved to be miR-484 and RNU-6, as revealed by the tested algorithms and ranked according to the weighted geometric mean of all candidates. Therefore, we have used these two genes as normalizing controls when analyzing miRNA expression in our samples.

We were able to successfully extract sufficient quantity of good quality RNA from all samples, with a range of 44.99–354.00 ng/ul concentration and 1.75–1.98 purity as evaluated by the 260/280 absorbance ratio. Figure 1 presents an example of a typical tissue slide analyzed, before and after LCM.

The association of the relative expression of the investigated miRNAs with tumor characteristics is presented in Table 2. The only miRNA for which statistically significant fold change values were observed was miR-21, a well-established oncogene

Table 1 Patient's demographics and pathological characteristics of the tumors

Characteristics		N	%*
Age	<50	9	29.03
	>50	22	70.96
BMI	normal	24	77.41
	obese	7	22.58
Stage	I&II	13	41.93
	III	18	58.06
Tumor Size	<5 cm	19	61.29
	>5 cm	10	32.25
Lymph Node Involvement	No	2	6.45
	Yes	28	90.32
HER2	neg	11	35.48
	pos	12	38.70
Ki67	neg	7	22.58
	pos	18	58.06
ER	neg	0	0.00
	pos	26	83.87
PR	neg	2	6.45
	pos	24	77.41

*Percentages calculated based on the total of 31 samples; does not add up to 100% due to missing values

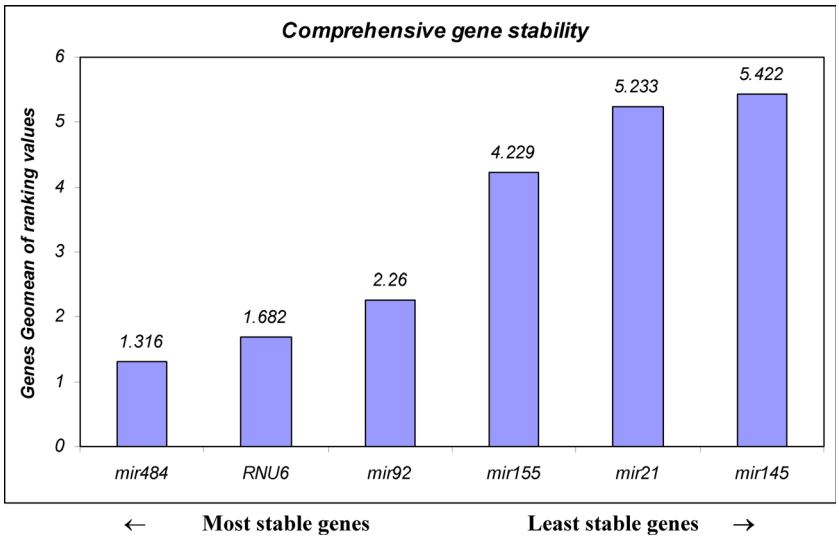

Fig. 2 Ranking of genes for the identification of normalization controls

miRNA in cancer. Figure 3 presents the significantly different relative expression of miR-21 in tumors stratified according to stage and ki-67 status. This miRNA was over four times more expressed in highly proliferative tumors with high ki-67 levels (FC = 4.443, p = 0.019) and over 6 times more expressed in higher stage tumors (FC = 6.284, p = 0.037). miR-21 also presented a 2.787 times higher expression in larger tumors, however without reaching statistical significance for this parameter (p = 0.265).

No statistically significant fold change differences were observed for miR-145, miR-155 and miR-92 between samples stratified according to stage, tumor size, ki-67 and HER2 status. However, miR-145 was 1.335 times more expressed in high proliferative tumors, although it did not reach statistical significance (p = 0.943). Similarly, for miR-155 the fold change was 2.107 for tumors expressing high levels of ki-67, 1.261 for tumors larger than 5 cm, and 1.196 for higher stage tumors, however they did not reach statistical significance (p = 0.151, p = 0.665, and p = 0.754, respectively).

Table 2 Relative expression of investigated miRNAs; association with tumor characteristics

		Stage III vs. I&II	Tumor size large vs. small	Ki67 high vs. low	HER2 pos vs. neg
miR-145	FC	0.786	0.469	1.335	0.924
	p	0.617	0.391	0.943	0.479
miR-155	FC	1.196	1.261	2.107	0.610
	p	0.754	0.665	0.151	0.312
miR-21	FC	6.284	2.787	4.443	0.788
	p	*0.037*	0.265	*0.019*	0.866
miR-92	FC	0.820	0.766	1.020	0.786
	p	0.562	0.785	0.879	0.284

FC - fold change, p - p value for the Mann-Whitney U test

Discussion

The involvement of miRNAs in breast cancer is well established; however most studies report miRNA levels in whole breast tumor tissues, the data on miRNAs expression in LCM collected samples being rather scarce [6, 12, 19]. LCM is a powerful tool since it allows the collection of homogenous cell populations from the widely heterogeneous breast cancer tissue sample. In this pilot study, we have investigated miRNA expression in LCM-collected invasive breast carcinoma cells, and explored their association with clinical and pathological characteristics of the breast tumors.

We chose to investigate four miRNAs previously described as being involved in breast cancer biology. One well known issue in measuring miRNA expression in biological samples is the difficulty of choosing a suitable endogenous control for data normalization. Although there is some data suggesting that certain miRNAs or small nuclear RNA molecules are suitable normalization controls due to limited expression variability in breast cancer samples, there is large heterogeneity among studies and there is no clear-cut evidence for choosing one over the other [7]. Most studies investigating miRNAs in tissue samples are using small nuclear RNA molecules such as U6 as normalizing controls, although there are some reports suggesting that this molecule also presents high expression variability that limits its suitability including in breast cancer [24]. Human miR-484 has been described as a possible endogenous control in breast cancer samples, especially in circulation [14, 36]. We wanted to investigate herein whether this miRNA is expressed at stable levels in LCM tissues as well. Therefore, we assessed the use of both U6 and miR-484 as endogenous controls in our samples, and as shown in Fig. 2, miR-484 appeared to have a higher stability even when compared to the established U6 endogenous normalization control. Given these results, we chose to use both U6 and miR-484 as endogenous controls for our data normalization.

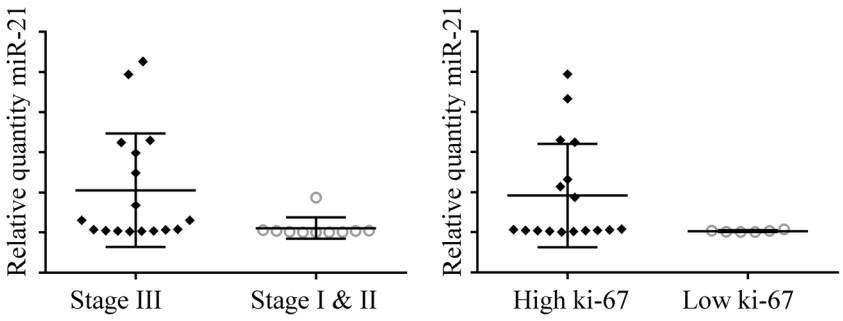

Fig. 3 Relative expression of miR-21 in samples stratified according to stage and ki-67 status

The human miR-21 has been extensively studied in the context of cancer and has been shown to be highly expressed in several cancer types, including breast cancer [5, 34]. In our study miR-21 was significantly associated with higher stage and highly proliferative breast tumors. High expression of this miRNA is associated with increased cell growth, migration, invasion, and decreased apoptosis, all of these being a hallmark of cancer aggression [34]. In a recent meta-analysis, increased miR-21 in breast cancer has been associated with ER/PR negative, HER2 positive, lymph node metastasis positive, and high-grade breast tumors [34]. Moreover, it was suggested that miR-21 could be a marker of poor prognostic for breast cancer, being linked to poor survival. However, all the studies included in this meta-analysis have investigated the expression of miR-21 by real time PCR in the breast tumor tissue as a whole, and, although carefully trimmed to contain mostly tumor cells, one cannot exclude the possible bias due to collecting normal tissue adjacent to the tumor.

The other miRNAs investigated in the present study, miR-145, miR-155 and miR-92 did not present any statistically significant associations, a fact that could be attributed to the rather limited number of samples analyzed. However, the discrepancy between our data and the previously published ones has an alternative explanation, stemming from the possibility that the latter include cellular sources other than tumor cells.

Human miR-145 has been shown to have a tumor suppressor effect in breast cancer models by negatively regulating the *ERBB3* gene and thus reducing proliferation and invasion of breast tumors [50]. It has been also reported that miR-145 has an inhibitory role of angiogenesis and tumor growth through VEGF and N-RAS in breast cancer [52]. Indeed, this miR was found down-regulated in human invasive breast carcinoma compared to normal tissue, measured in whole tumors and adjacent normal tissue samples [29, 45]. Interestingly enough, there are conflicting reports regarding the circulating expression of miR-145 in serum or plasma of breast cancer patients and its use as a possible biomarker; some studies found it down-regulated [21, 32], thus in accordance with tissue data, while others reported it overexpressed [27]. These discrepancies could stem from the differences in methodologies or study designs, or could simply be due to the large heterogeneity of breast tumors and selective release of miRs in the circulation. In our study, miR-145 was slightly down-regulated according to tumor size and stage, and slightly up-regulated in ki-67 expressing tumors, although these findings did not reach statistical significance (Table 2).

Human miR-155 has been identified as an oncogenic miR in several cancers including breast cancer, being involved in the regulation of multiple important signaling pathways in cancer biology such as TGF-β, JAK-STAT, and FOXO3a pathways among others [28, 47]. This miR was found up-regulated in breast cancer tissues [17] and in sera and plasma of breast cancer patients compared to controls [48]. One recent study reported that miR-155 was up-regulated in breast cancer patients and its expression was associated with lymph node and distant metastasis [11]. In our study, miR-155 was more than twice more expressed in highly proliferative tumors as indicated by ki-67 status and slightly overexpressed according to stage and tumor size, although the findings did not reach statistical significance (Table 2).

Human miR-92 was previously reported as being up-regulated in breast cancer tissues compared to normal tissue samples, and its expression was negatively correlated with estrogen receptor beta expression [1]. However, other studies provide a more complex involvement of this miR in breast carcinogenesis, the expression varying among different breast cancer samples, a lower expression being associated with higher stage breast cancers and poor survival in one study [33], and with lymph node metastasis and tumor size in another [39]. We did not observe any significant association of miR-92 with pathological tumor characteristics in our study, although it was slightly down-regulated in larger tumors.

In our pilot study we have confirmed previous findings on whole tumor tissue samples, that overexpressed miR-21 is associated with higher stage, larger and highly proliferative breast tumors as revealed by the Ki-67 status, although the association did not reach statistical significance for tumor size. Correlated with the results on miR-155 and miR-92, this underlines the usefulness of LCM as a method for fine tuning the expression analysis of specific cells from a tumor sample. It is therefore plausible that, in the case of miR-155 and miR-92, the previously published studies have included other significant sources than tumor cells to a such extent that the excess of tumor cells in the trimmed whole breast tissue samples was

not sufficient to compensate. Our data also suggest that out of the four microRNAs analyzed, miR-21 is specifically up-regulated in the tumor cells. Taking into consideration several other studies reporting the presence of miR-21 in the serum and plasma of breast cancer patients, one could propose miR-21 as a *bona fide* diagnostic an prognostic biomarker, with promising clinical applications in the management of breast cancer pathology [5].

Conclusions

In summary, our pilot study showed the miR-484 could be a suitable endogenous control for data normalization in breast tissues. Furthermore, the analysis of miR-21, miR-155 and miR-92 compared to previously published data underlines the importance of detecting miRs in homogenous laser captured microdissected invasive breast cancer samples. In addition, we have confirmed previous associations of miR-21 expression with poor prognosis characteristics of breast cancers. These results need further confirmation by future studies, especially on dissecting the contribution of various tumor and non-tumor cells to miR expression during the different stages of breast cancer.

References

1. Al-Nakhle H, Burns PA, Cummings M, Hanby AM, Hughes TA, Satheesha S et al (2010) Estrogen receptor {beta}1 expression is regulated by miR-92 in breast cancer. Cancer Res 70:4778–4784
2. Andersen CL, Jensen JL, Orntoft TF (2004) Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res 64:5245–5250
3. Blenkiron C, Goldstein LD, Thorne NP, Spiteri I, Chin SF, Dunning MJ et al (2007) MicroRNA expression profiling of human breast cancer identifies new markers of tumor subtype. Genome Bio 8: R214
4. Calin GA, Croce CM (2006) MicroRNA signatures in human cancers. Nat Rev Cancer 6:857–866
5. Chen J, Wang X (2014) MicroRNA-21 in breast cancer: diagnostic and prognostic potential. Clin Transl Oncol 16:225–233
6. Chen L, Li Y, Fu Y, Peng J, Mo MH, Stamatakos M et al (2013) Role of deregulated microRNAs in breast cancer progression using FFPE tissue. PLoS One 8:e54213
7. Davoren PA, McNeill RE, Lowery AJ, Kerin MJ, Miller N (2008) Identification of suitable endogenous control genes for microRNA gene expression analysis in human breast cancer. BMC Mol Biol 9:76
8. 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 49:1374–1403
9. Fetica B, Balacescu O, Balacescu L, Rus M, Berindan-Neagoe I (2014) An alternative and sensitive method based on LCM and Q-PCR for HER2 testing in breast cancer. Cancer Biomark 14:129–135
10. Foekens JA, Sieuwerts AM, Smid M, Look MP, de Weerd V, Boersma AW, et al (2008) Four miRNAs associated with aggressiveness of lymph node-negative, estrogen receptor-positive human breast cancer. Proc Natl Acad Sci U S A 105:13021–13026
11. Hagrass HA, Sharaf S, Pasha HF, Tantawy EA, Mohamed RH, Kassem R (2015) Circulating microRNAs - a new horizon in molecular diagnosis of breast cancer. Genes Cancer 6:281–287
12. Hannafon BN, Sebastiani P, De Las Morenas A, Lu J, Rosenberg CL (2011) Expression of microRNA and their gene targets are dysregulated in preinvasive breast cancer. Breast Cancer Res 13: R24
13. Hasemeier B, Christgen M, Kreipe H, Lehmann U (2008) Reliable microRNA profiling in routinely processed formalin-fixed paraffin-embedded breast cancer specimens using fluorescence labelled bead technology. BMC Biotechnol 8:90
14. Hu Z, Dong J, Wang LE, Ma H, Liu J, Zhao Y et al (2012) Serum microRNA profiling and breast cancer risk: the use of miR-484/191 as endogenous controls. Carcinogenesis 33:828–834
15. Huang Q, Gumireddy K, Schrier M, le Sage C, Nagel R, Nair S, et al. (2008) The microRNAs miR-373 and miR-520c promote tumour invasion and metastasis. Nat Cell Biol 10:202–210
16. Hui AB, Shi W, Boutros PC, Miller N, Pintilie M, Fyles T et al (2009) Robust global micro-RNA profiling with formalin-fixed paraffin-embedded breast cancer tissues. Lab Investig 89:597–606
17. Iorio MV, Ferracin M, Liu CG, Veronese A, Spizzo R, Sabbioni S et al (2005) MicroRNA gene expression deregulation in human breast cancer. Cancer Res 65:7065–7070
18. Iorio MV, Casalini P, Tagliabue E, Menard S, Croce CM (2008) MicroRNA profiling as a tool to understand prognosis, therapy response and resistance in breast cancer. Eur J Cancer 44:2753–2759
19. Khoshnaw SM, Powe DG, Ellis IO, Green AR (2011) Detection and quantification of microRNAs in laser-microdissected formalin-fixed paraffin-embedded breast cancer tissues. Methods Mol Biol 755:119–142
20. Klopfleisch R, Weiss AT, Gruber AD (2011) Excavation of a buried treasure DNA, mRNA, miRNA and protein analysis in formalin fixed, paraffin embedded tissues. Histol Histopathol 26:797–810
21. Kodahl AR, Lyng MB, Binder H, Cold S, Gravgaard K, Knoop AS et al (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 8:874–883
22. Kong W, Zhao JJ, He L, Cheng JQ (2009) Strategies for profiling microRNA expression. J Cell Physiol 218:22–25
23. Liu H, McDowell TL, Hanson NE, Tang X, Fujimoto J, Rodriguez-Canales J (2014) Laser capture microdissection for the investigative pathologist. Vet Pathol 51:257–269
24. Lou G, Ma N, Xu Y, Jiang L, Yang J, Wang C et al (2015) Differential distribution of U6 (RNU6-1) expression in human carcinoma tissues demonstrates the requirement for caution in the internal control gene selection for microRNA quantification. Int J Mol Med 36(5):1400–1408
25. Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D et al (2005) MicroRNA expression profiles classify human cancers. Nature 435:834–838
26. Ma L, Teruya-Feldstein J, Weinberg RA (2007) Tumour invasion and metastasis initiated by microRNA-10b in breast cancer. Nature 449:682–688
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 34:163–169
28. Mattiske S, Suetani RJ, Neilsen PM, Callen DF (2012) The oncogenic role of miR-155 in breast cancer. Cancer Epidemiol Biomark Prev 21:1236–1243

29. Min W, Wang B, Li J, Han J, Zhao Y, Su W et al (2014) The expression and significance of five types of miRNAs in breast cancer. Med Sci Monit Basic Res 20:97–104
30. Murray GI (2011) Laser Capture Microdissection: Methods and Protocols. Methods in molecular biology. Springer
31. Narita D, Anghel A, Seclaman E, Ilina R, Cireap N, Ursoniu S (2010) Molecular profiling of ADAM12 gene in breast cancers. Romanian J Morphol Embryol 51:669–676
32. 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:e53141
33. Nilsson S, Moller C, Jirstrom K, Lee A, Busch S, Lamb R et al (2012) Downregulation of miR-92a is associated with aggressive breast cancer features and increased tumour macrophage infiltration. PLoS One 7:e36051
34. Pan F, Mao H, Deng L, Li G, Geng P (2014) Prognostic and clinicopathological significance of microRNA-21 overexpression in breast cancer: a meta-analysis. Int J Clin Exp Pathol 7:5622–5633
35. Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP (2004) Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper–Excel-based tool using pair-wise correlations. Biotechnol Lett 26:509–515
36. Rice J, Roberts H, Rai SN, Galandiuk S (2015) Housekeeping genes for studies of plasma microRNA: A need for more precise standardization. Surgery 158(5):1345–1351
37. Schmittgen TD, Lee EJ, Jiang J, Sarkar A, Yang L, Elton TS et al (2008) Real-time PCR quantification of precursor and mature microRNA. Methods 44:31–38
38. Si ML, Zhu S, Wu H, Lu Z, Wu F, Mo YY (2007) miR-21-mediated tumor growth. Oncogene 26:2799–2803
39. Si H, Sun X, Chen Y, Cao Y, Chen S, Wang H et al (2013) Circulating microRNA-92a and microRNA-21 as novel minimally invasive biomarkers for primary breast cancer. J Cancer Res Clin Oncol 139:223–229
40. Siebolts U, Varnholt H, Drebber U, Dienes HP, Wickenhauser C, Odenthal M (2009) Tissues from routine pathology archives are suitable for microRNA analyses by quantitative PCR. J Clin Pathol 62:84–88
41. Silver N, Best S, Jiang J, Thein SL (2006) Selection of housekeeping genes for gene expression studies in human reticulocytes using real-time PCR. BMC Mol Biol 7:33
42. Society AC. Cancer Facts & Figures 2015 (2015) Atlanta: American Cancer Society
43. Stahel R, Bogaerts J, Ciardiello F, de Ruysscher D, Dubsky P, Ducreux M, et al (2015) Optimising translational oncology in clinical practice: strategies to accelerate progress in drug development. Cancer Treat Rev 41:129–135
44. Stefani G, Slack FJ (2008) Small non-coding RNAs in animal development. Nat Rev Mol Cell Biol 9:219–230
45. Sun EH, Zhou Q, Liu KS, Wei W, Wang CM, Liu XF et al (2014) Screening miRNAs related to different subtypes of breast cancer with miRNAs microarray. Eur Rev Med Pharmacol Sci 18:2783–2788
46. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, et al (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3:RESEARCH0034
47. Wang J, Wu J (2012) Role of miR-155 in breast cancer. Front Biosci (Landmark Ed) 17:2350–2355
48. Wang F, Hou J, Jin W, Li J, Yue Y, Jin H et al (2014) Increased circulating microRNA-155 as a potential biomarker for breast cancer screening: a meta-analysis. Molecules 19:6282–6293
49. Xi Y, Nakajima G, Gavin E, Morris CG, Kudo K, Hayashi K et al (2007) Systematic analysis of microRNA expression of RNA extracted from fresh frozen and formalin-fixed paraffin-embedded samples. RNA 13:1668–1674
50. Yan X, Chen X, Liang H, Deng T, Chen W, Zhang S et al (2014) miR-143 and miR-145 synergistically regulate ERBB3 to suppress cell proliferation and invasion in breast cancer. Mol Cancer 13:220
51. Zhang L, Huang J, Yang N, Greshock J, Megraw MS, Giannakakis A et al (2006) microRNAs exhibit high frequency genomic alterations in human cancer. Proc Natl Acad Sci U S A 103:9136–9141
52. Zou C, Xu Q, Mao F, Li D, Bian C, Liu LZ et al (2012) MiR-145 inhibits tumor angiogenesis and growth by N-RAS and VEGF. Cell Cycle 11:2137–2145