Robust global micro-RNA profiling with formalin-fixed paraffin-embedded breast cancer tissues

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Global micro-RNA (miR) profiling of human malignancies is increasingly performed, but to date, the majority of such analyses have used frozen tissues. However, formalin fixation is the standard and routine histological practice for optimal preservation of cellular morphology. To determine whether miR analysis of formalin-fixed tissues is feasible, quantitative real-time PCR (qRT-PCR) profiling of miR expression in 40 archival formalin-fixed paraffin-embedded (FFPE) breast lumpectomy specimens were performed. Taqman Low Density Arrays (TLDA) were used to assess the expression level of 365 miRs in 34 invasive ductal carcinomas and in 6 normal comparators derived from reduction mammoplasties. Its technical reproducibility was high, with intra-sample correlations above 0.9 and with 92.8% accuracy in differential expression comparisons, indicating such global profiling studies to be technically and biologically robust. The TLDA data were confirmed using conventional single-well qRT-PCR analysis, showing a strong and statistically significant concordance between these two methods. Paired frozen and FFPE breast cancer samples from the same patients showed a similar level of robust correlation of at least 0.94. Compared with normal breast samples, a panel of miRs was consistently dysregulated in breast cancer, including earlier-reported breast cancer-related miRs, such as upregulated miR-21, miR-155, miR-191, and miR-196a, and downregulated miR-125b and miR-221. Additional novel miR sequences of potential biological relevance were also uncovered. These results show the validity and utility of conducting global miR profiling using FFPE samples, thereby offering enormous opportunities to evaluate archival banks of such materials, linked to clinical databases, to rapidly acquire greater insight into the clinically relevant role for miRs in human malignancies.

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Micro-RNAs (miRs) are endogenous ~22-nt non-coding RNAs, which can alter protein levels in a sequence-specific manner.^{1,2} Since their initial identification in nematodes in 1993,³ miRs have been increasingly recognised as key regulators in many biological systems. Current estimates of the number of miRs in the human genome range from ~450 to 1000.^{1,4} These estimates represent 1–4% of all protein-coding genes, rendering them as one of the largest classes of regulatory genes.⁵ The breadth of their potential relates to their

imperfect complementarity, whereby a single miR may bind to as many as 200 target genes, with diverse functions ranging from transcription factors, proteins, and receptors, to transporters.² Bioinformatic analyses estimate that miRs could regulate the expression of up to one-third of all human mRNAs.⁶ Approximately half of the known miRs have been mapped to fragile sites in the genome,⁷ providing the first clue to their role in cancer. Since then, miRs have been associated with almost all varieties of human malignancies

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including haematological⁷ and solid cancers,⁸ such as lung,⁹ breast,^{10,11} prostate,¹² pancreas,¹³ and glioblastomas.¹⁴

The global alteration in the miR expression in cancer, compared with that in corresponding normal tissues, could be manifested by either overexpression or underexpression. *In silico* analyses show that among 18 commonly shared miRs in epithelial malignancies, 44% (115 of 263) of the predicted gene targets were well-known cancer-associated genes, such as Rb, TGF β R2, or Ras.⁸ Thus, depending on its targets, a miR could function as either a tumour suppressor or as an oncogene. For example, two early reports described the downregulation of miR-15a and miR-16-1 in chronic lymphocytic leukaemia (CLL),¹⁵ which were shown to target Bcl-2.¹⁶ The absence of these miRs thus leads to anti-apoptotic signalling. Conversely, miR-21 is often overexpressed in breast cancer and can inhibit pro-apoptotic genes^{14,17} and PTEN.¹⁸ Hence, the overexpression of miR-21 will result in the suppression of apoptosis and in the upregulation of PTEN targets, such as Akt, with both pathways converging on promoting survival.

Increasing data support the value of miR expression profiling¹⁹ in distinguishing one cancer subtype from another,⁸ such as luminal vs basal classifications for human breast cancer.²⁰ When the classification accuracy of miR profiles was directly compared with that of standard mRNA, miRs were shown to have superior performance for poorly differentiated tumours.^{8,21} Several recent reports have correlated miR expression with clinical outcome. Earlier ones have described a 13-miR signature in CLL, which correlated with clinical behaviour.¹⁶ Two recent lung studies have identified an 8-miR set for adenocarcinoma,⁹ and a second 5-miR signature that predicted outcome for patients with non-small-cell lung cancer.²²

All these aforementioned studies have used frozen tissues. However, routine histology processing uses formalin fixation to preserve the histological architecture of tissue specimens. Archival collections of formalin-fixed tissues, linked to clinical databases, provide a rich resource from which biological insights could be derived far more expeditiously than the prospective collection of frozen samples. In addition, any biomarker developed from formalin-fixed paraffin-embedded (FFPE) samples could be more readily translated into clinical practice. Unfortunately, RNA is degraded in tissues before, during, and after formalin fixation^{23,24} and can continue to deteriorate even during storage, leading to shortened fragments of RNA.²⁵ Interestingly, miRs appear to be better preserved, perhaps because of their intrinsically shorter lengths. Therefore, it should be possible to perform genome-wide screening for miRs using FFPE tissues.^{20,26–29} Indeed, this approach has been successful for colon and breast cancers;^{20,29,30} however, the technical robustness of these platforms has not been thoroughly investigated.

Therefore, in this study, we systematically investigated a comprehensive panel of 365 human miRs on 40 archival breast cancer FFPE samples. We used the Taqman Low

Density Array (TLDA), a 384-microfluidic card, designed to perform several hundred quantitative real-time PCR (qRT-PCR) reactions simultaneously. Three criteria were defined to evaluate the robustness of this platform: (a) technical reproducibility; (b) independent validation using an alternate experimental method, such as single-well qRT-PCR; and (c) the data probed biologically relevant processes. On the basis of these *a priori* defined criteria, the TLDA platform was shown to be technically and biologically robust for the analysis of FFPE samples.

MATERIALS AND METHODS

RNA Purification from FFPE Samples

Thirty-four archival FFPE lumpectomy blocks were evaluated by a breast cancer pathologist (NM). Regions of invasive ductal carcinoma were confirmed, thereby identifying areas containing >70% of malignant epithelial cells for macro-dissection. The clinical descriptors for these 34 breast cancer patients are provided in Table 1. Six reduction mammoplasty specimens were obtained; regions containing predominantly epithelial tissues were also macro-dissected.

Table 1 Clinical descriptors for the 34 breast cancer patients

<i>Age (years)</i>		
Median	64	
Range	51–86	
<i>T-category</i>	<i>Frequency</i>	<i>Percent</i>
T1	22	65%
T2	12	35%
<i>N-category</i>	<i>Frequency</i>	<i>Percent</i>
N0	29	85%
Nx	5	15%
<i>Grade</i>	<i>Frequency</i>	<i>Percent</i>
1	2	8%
2	15	58%
3	9	35%
<i>ER Status</i>	<i>Frequency</i>	<i>Percent</i>
Positive	27	79%
Negative	7	21%
<i>Her-2/Neu status</i>	<i>Frequency</i>	<i>Percent</i>
Positive	4	12%
Negative	25	73%
Indeterminate	5	15%

Total RNA enriched for small RNA species was isolated with Recoverall (Ambion, Austin, TX, USA), according to the manufacturer's instructions. Briefly, xylene was added to 4- μ m sections of FFPE samples to remove paraffin. The tissues were digested with protease at 50°C and treated with DNase. After washing, total RNA, including the small miR fraction, was eluted with distilled water. Recovered RNA concentrations and quality were measured using the Nanodrop 1000A spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA).

Real-Time Quantification of micro-RNAs

The quality of the 40 FFPE samples was assessed by RT-PCR analysis of the endogenous control RNU44 using Applied Biosystems TaqMan[®] Micro-RNA Assay. This assay includes a reverse transcription (RT) step using the High-Capacity cDNA Archive Kit (Applied Biosystems, CA, USA), wherein a stem-loop RT primer specifically hybridises with a miR molecule and is then reverse-transcribed with a MultiScribe reverse transcriptase.²⁷ Briefly, the RT mix includes 50-nM stem-loop RT primers, 1 $\times$ RT buffer, 0.25 mM each of dNTPs, 3.33 U/ μ l MultiScribe reverse transcriptase, and 0.25 U/ μ l RNase inhibitor. The 7.5- μ l reaction was then incubated in an Applied Biosystems 7900 Thermocycler for 30 min at 16°C, 30 min at 42°C, 5 min at 85°C, and then held at 4°C. The RT products were subsequently amplified with sequence-specific primers using the Applied Biosystems 7900 HT Real-Time PCR system. The 10- μ l PCR mix contains a 0.67- μ l RT product, the 1 $\times$ TaqMan[®] Universal PCR Master Mix, a 0.2- μ M TaqMan[®] probe, a 1.5- μ M forward primer, and a 0.7- μ M reverse primer. The reactions were incubated in a 384-well plate at 95°C for 10 min followed by 40 cycles of 95°C for 15 s and at 60°C for 1 min.

TaqMan Low Density Array

Global profiling of miR expression on 40 FFPE samples was performed using the TaqMan[®] Array Human Micro-RNA Panel v1.0. (Applied Biosystems, CA, USA), which runs the PCR in 1–2 μ l of reaction volume in a 384-well format. The TLDA contains 368 TaqMan[®] Micro-RNA Assays enabling the simultaneous quantitation of 365 human miRs plus 3 endogenous controls. Similar to the individual TaqMan[®] Micro-RNA Assay, total RNA was first reverse-transcribed with the Multiplex RT pool set, then quantitated using an Applied Biosystems 7900 HT Real-Time PCR system.

Data Pre-Processing

The TLDA data were pre-processed using the same strategy used earlier for mRNA data,³¹ with some modifications. For each sample, a normalisation factor was calculated as the mean of the intensity of two endogenous controls, RNU44 and RNU48. A ΔC_t was calculated for each miR species as $C_t(\text{gene}) - \text{Normalisation Factor}$. The normalised expression level was then calculated as $\log_2[2^{-\Delta C_t}]$. No scaling was used, so that the normalised expression values represent the levels

of miR species relative to the levels of the two housekeeping control genes. Normalisation was performed in the R statistical environment (v2.6.1).

Assessment of TLDA and PCR Technical Reproducibility in FFPE Samples

To assess the technical reproducibility of the TLDA platform, replication of three samples was performed multiple times. One normal sample was assessed in duplicate (N1R1 and N1R2), another normal sample in triplicate (N2R1, N2R2, N2R3), and a tumour sample in triplicate (T1R1, T1R2, T1T3). We first performed a clustering analysis by calculating Spearman's correlation coefficient between each pair of samples, then subjecting the correlation data to agglomerative hierarchical clustering using Euclidean distance as the distance metric and complete linkage. The results were then visualised using a heatmap. Clustering was implemented in the R statistical environment (v2.6.1), and visualisation was carried out using the lattice (v0.17-2) and latticeExtra (v0.3-1) packages. We also assessed the distributional similarity of the technical replicates using box and scatter plots. We further tested the accuracy of the technical replicates by determining the fraction of tumour–normal differences that were replicable when using different technical replicates at a fold change threshold of two.

Comparison of miRNA Profile in FFPE and Frozen Samples

Corresponding pairs of frozen (F1 and F2) and FFPE (R1 and R2) breast cancer samples were derived from two different patients and subjected to global miR profiling as described above. We performed a clustering analysis by calculating Spearman's correlation coefficient between each pair of samples, then subjecting the correlation data to agglomerative hierarchical clustering using Euclidean distance as the distance metric and complete linkage. The results were then visualised using a heatmap. Clustering was implemented in the R statistical environment (v2.7.1) and visualisation was carried out using the lattice (v0.17-14) and latticeExtra (v0.5-2) packages.

PCR Validation

Single-well PCR analysis was conducted to validate 18 miRs (let-7d, miR-21, miR-24, miR-29c, miR-32, miR-125b, miR-145, miR-155, miR-184, miR-191, miR-194, miR-221, miR-328, miR-497, miR-518b, miR-572, miR-601, and miR-622) in 6 normal breast samples and at least 18 breast cancer samples. The analysis was performed using the TaqMan[®] Micro-RNA Assay as described above. Pearson's correlation was calculated across all 18 validations to compare the tumour–normal fold changes measured by TLDA with those measured by single-well PCR. Each experiment was conducted in duplicate or triplicate, depending on RNA availability.

Tumour–Normal Comparison

To compare the miR expression profiles of normal and tumour breast tissues, we first used clustering to visualise the profiles. We used the DIANA divisive hierarchical clustering algorithm with Pearson's correlation as the distance metric, as implemented in the cluster package (v1.11.9) for the R statistical environment (v2.6.1) on the pre-processed expression values. Only genes that showed detectable expression in all samples were included. The resulting expression profiles were visualised with a heatmap using the lattice (v0.17-2) and latticeExtra (v0.3-1) packages.

To identify specific miRs that are differentially expressed between tumour and normal samples, we performed gene-wise *t*-tests with Welch's correction for heteroscedasticity between tumour and normal samples. To account for multiple testing, we performed a false-discovery rate adjustment on the resultant vector of *P*-values. We also calculated the fold changes (in log₂ space) for each gene. Genes detected in fewer than 10 samples were excluded from this analysis. All calculations were performed in the R statistical environment (v2.6.1).

Finally, to determine whether miR expression signatures could predict if a given sample were normal or a tumour, we used a machine-learning approach. We used leave-one-out cross-validation and *k*-nearest neighbours,³² and set *k* = 5 because the number of normal samples was 6. *k*-nearest neighbours was implemented in the class package (v7.2-38) of the R statistical environment (v2.6.1).

RESULTS

Global miRNA Profiling Using FFPE Samples is Highly Reproducible

The primary objective of this study was to show the reliability of high-throughput TLDA platform for miR quantitation using archival FFPE samples. To achieve this goal, three criteria were established: technical reproducibility, independent validation, and the generation of biologically relevant results. We evaluated these criteria in sequence, focussing on 34 breast cancer samples obtained from archival FFPE lumpectomy blocks (Table 1) and from six reduction mammaplasty specimens.

To investigate the technical replicability of the TLDA platform, we performed global miR profiling in triplicate for one normal and one breast cancer sample and in duplicate for a second normal breast sample. These eight expression profiles were normalised to a panel of two endogenous controls (RNU44 and RNU48), as established earlier.³¹ The distributional similarity of the technical replicates was first verified using scatter plots (Figure 1a). Unsupervised machine learning (clustering) was then used to visualise the similarity of the technical replicates (Figure 1b). The two normal samples clustered separately from the single tumour sample, whereas the technical replicates for each biological sample clustered closely together. The expression profiles correlated well between the technical replicates, with

Spearman's rho above 0.90 in all cases. In addition, the two normal samples correlated strongly with each other (Spearman's rho above 0.85), but less strongly with the tumour sample (Spearman's rho between 0.75 and 0.85).

We also evaluated the technical reproducibility of this data set in terms of the reported tumour–normal differences. The tumour–normal differences were identified in these eight profiles using a two-fold threshold. Predicted tumour–normal differences between technical replicates were again very similar, with a 92.8 ± 1.6% agreement between different technical replicates. Taken together, these data show that the TLDA profiles of miR expression in samples extracted from FFPE blocks were highly reproducible.

Consistent miRNA Profiling Between Frozen and FFPE Samples

To further corroborate the validity of global miR profiling of FFPE samples, the same experiments were repeated, using pairs of frozen and FFPE blocks from the same patients. The miR profiles of these samples were generated and normalised to the two endogenous controls (RNU44 and RNU48). Analysis with unsupervised machine learning (clustering) showed the similarity of the two types of samples (Figure 1c). miRNA profiles from FFPE and frozen samples from the same tumour were highly correlated, with Spearman's rho of at least 0.94. By contrast, miR profiles from different patients were less correlated, with Spearman's rho between 0.86 and 0.92.

PCR Validation

A robust experimental system should not only be technically replicable but should also yield similar results to alternative experimental methodologies. Accordingly, we directly compared the TLDA-derived expression profiles with single-well PCR. Eighteen miRs were selected and validated in six normal and up to 24 tumour samples. These included 11 miRs that were predicted by TLDA to be differentially expressed between tumour and normal samples, and seven miRs predicted not to be differentially expressed. The tumour–normal differential expression identified by these two techniques was concordant in 14/18 miRs (78%), with similar trends observed for the remaining four miRs (Table 2 and Supplementary Figure 1). Furthermore, the fold changes predicted for these 18 miRs by the two methods were highly correlated ($R = 0.79$, $P = 9.5 \times 10^{-5}$). Hence, these analyses show that the TLDA results can be confirmed using an independent experimental approach.

Tumour–Normal Comparison

The ultimate test for a novel experimental system is to probe new and biologically important questions. The TLDA system was used to assess the expression levels of 6 normal breast samples and 34 breast cancer samples. This data set was then examined in three different ways: (a) global patterns of miR expression; (b) specific miR species exhibiting differential

Figure 1 Replicability of Taqman TLDA analysis. To determine the relationship between technical and biological replicates, we assessed global miR expression in two normal breast samples (N1 and N2) and in one invasive ductal carcinoma sample (T1). Two technical replicates were performed on one normal sample (N1.R1 and N1.R2); three were performed for the other normal sample (N2.R1, N2.R2, and N2.R3), and for the carcinoma sample (T1.R1, T1.R2, and T1.R3). **(a)** Scatter plot analysis of TLDA analyses showing similar pattern of expression in the replicate experiments. **(b)** We calculated Spearman's correlation between all pairs of samples and performed agglomerative hierarchical clustering using complete linkage. The resulting heatmap shows that the technical replicates from the two normal samples (blue and green bars) clustered together, separate from the tumour sample (red bars). The correlations range from 0.75 between normal and tumour, to >0.95 for technical replicates of a single sample. **(c)** Comparison of microRNA expression of two pairs of frozen and FFPE samples from two invasive breast carcinoma patients. Spearman's correlation between the two pairs of samples was calculated and agglomerative hierarchical clustering was performed. The resulting heatmap shows that the paired frozen and FFPE samples of the same patient has correlations of at least 0.94.

Table 2 PCR and TLDA validation

miR	P		M		Classification
	PCR	TLDA	PCR	TLDA	
miR-155	1.6×10^{-3}	1.9×10^{-4}	2.4	3.0	TP
miR-125b	8.2×10^{-3}	2.6×10^{-4}	-2.3	-3.6	TP
miR-145	5.8×10^{-2}	1.4×10^{-1}	-1.1	2.6	TN
let-7d	5.9×10^{-1}	1.6×10^{-1}	0.7	1.5	TN
miR-24	2.8×10^{-1}	2.4×10^{-1}	1.3	0.8	TN
miR-29c	3.4×10^{-1}	1.5×10^{-1}	1.0	2.5	TN
miR-194	1.7×10^{-1}	5.2×10^{-2}	1.6	2.1	TN
miR-221	7.0×10^{-1}	1.1×10^{-2}	-0.5	-2.5	FP
miR-191	5.8×10^{-1}	3.2×10^{-6}	0.6	4.3	FP
miR-328	6.2×10^{-1}	2.9×10^{-5}	-0.6	-2.8	FP
miR-21	2.6×10^{-3}	5.2×10^{-6}	3.9	4.2	TP
miR-184	4.4×10^{-5}	7.7×10^{-7}	5.7	4.3	TP
miR-518b	5.9×10^{-4}	1.9×10^{-10}	4.9	5.1	TP
miR-572	3.7×10^{-3}	1.1×10^{-7}	5.4	4.9	TP
miR-601	2.6×10^{-2}	1.2×10^{-7}	4.1	4.0	TP
miR-622	6.0×10^{-4}	8.2×10^{-9}	6.1	6.6	TP
miR-32	3.9×10^{-3}	1.3×10^{-1}	4.3	1.1	FN
miR-497	2.7×10^{-1}	1.6×10^{-1}	0.6	-0.9	TN

8 TP+6 TN+1 FN+3 FP = 78% accuracy.

Eighteen miRs were selected for single-well qRT-PCR validation of the TLDA data. The *P*-values denote the level of significance in expression between normal and malignant tissues using the single-well PCR, or the TLDA platforms. M-columns indicate the fold change between normal and cancer tissues using the two respective techniques. Classification indicate the TLDA compared with the PCR (gold standard) results: TP, true positive; TN, true negative; FP, false positive; FN, false negative.

tumour-normal expression; and (c) the ability to classify samples on the basis of miR levels. We first evaluated the broad patterns of miR expression using unsupervised pattern recognition³³ to both visualise the data set and discover unexpected trends. Clustering with a divisive hierarchical algorithm (Figure 2) showed a clear separation between the 6 normal (blue bars on the right side of the figure) and the 34 tumour samples (red bars).

Next, standard statistical methodologies were used to assess tumour-normal differential expression in this data set. Among the 208 miR species expressed in at least 10 of the 40 samples, 81 (38.9%) were differentially expressed between tumour and normal tissues at a 5% false-discovery rate. Table 3 lists the 30 most significantly differentially expressed miRs: 26 were overexpressed in tumours, whereas only 4 were underexpressed. The magnitude of this differential expression was considerable, with some miRs reduced to only 5% of their normal tissue levels (eg, miR-125b), whereas others were induced more than 50-fold (eg, miR-196a and miR-622).

Finally, supervised machine learning was used to determine if it were possible, as reported earlier,²¹ to classify tissues on the basis of miR expression profiles. We used the *k*-nearest neighbours machine learning combined with leave-one-out cross-validation to predict the tumour-normal status for each sample. This machine-learning approach achieved 100% accuracy, verifying that miR profiles could distinguish normal from malignant breast tissue samples.

DISCUSSION

Increasing evidence supports the use of miR profiling to characterise human tumours. To date, six distinct predictive signatures have been reported: for CLL,¹⁶ hepatocellular cell,³⁴ esophageal squamous cell,³⁵ colon,³⁶ and two for lung cancer.^{9,22} However, all these evaluations have used frozen tissues. In this study, we provide the first characterisation of the technical robustness of high-throughput global miR profiling of 365 human miRs using a qRT-PCR-based assay. We show high sensitivity and specificity in this approach, by systematically evaluating 40 FFPE breast tissue samples. This method facilitates screening for both high and low expressors in a single experiment. In addition, very small quantities of input total RNA are required (~ 1 – $1.5 \mu\text{g}$), and no labourious miR fractionations are required, thereby minimizing RNA loss.

There have been extensive evaluations conducted on the inter- and intra-experimental replicabilities for mRNA expression profiling platforms;^{37–40} none have been undertaken earlier for this relatively new field of miR profiling. To address this gap, we describe a comprehensive assessment of the viability of coupling sample extraction from archival FFPE blocks to miR expression profiling using the TLDA platform. We established three criteria for evaluating this methodology: technical reproducibility, independent validation, and the generation of biologically relevant data.

We first showed that there is a high degree of inter-experimental reproducibility for TLDA analyses of FFPE samples. Three distinct statistical techniques were used: unsupervised machine learning (clustering), distributional assessments (scatter plots), and evaluation of the reproducibility of tumour-normal differences. In each case, the technical reproducibility was high, with Spearman's correlations above 0.9 and technical replicates clustering together (Figure 1b). These technical replicates also show highly similar expression profiles, with few outliers (Figure 1a). The resulting biological information was also extremely robust, with 92.8% reproducibility of tumour-normal differences between technical replicates. Although these criteria have not been assessed earlier for miR profiling, our results are comparable with similar evaluations of mRNA expression profiling, in which >95% of repeated sample accuracies have been determined for fresh leukaemia cells.⁴¹ Therefore, these results indicate that assessing global miR expression using FFPE samples is feasible using relatively simple experimental

Figure 2 Tumour–Normal Heatmap. The miR profiles of normal and malignant breast samples were visualised using agglomerative hierarchical clustering using Spearman's rho as the distance metric. Rows correspond to tissue samples, and are labelled with a box indicating whether the row represents a tumour (red) or a normal (blue) sample. Each column corresponds to an individual miR sequence. This unsupervised and unbiased analysis shows that the 6 normal samples (blue bars) are completely separated from the 34 cancer samples (red bars).

protocols with small quantities of input RNA, and that technical replicates are likely unnecessary.

Having shown technical reproducibility, we then evaluated the second criterion: independent validation. Eighteen miRs were selected for validation using the traditional single-well qRT-PCR assay. Fourteen of these miRs showed concordant results, whereas consistent trends were observed in the remaining four. Importantly, the fold changes predicted for these 18 miRs by the two methods were significantly corre-

lated between the two methods. These results show that miR profiles generated from FFPE blocks using TLDA can be validated using an independent experimental technique. This level of validation is again similar to that observed for mRNA expression profiling.⁴² Discrepancies in validation for mRNA experiments have been attributed to inter-experimental variations, differences in hybridisations, and differences in gene sequences used in arrays vs individual qRT-PCR experiments. Although the latter two issues would not apply to these miR

Table 3 List of 30 most significantly differentially expressed micro-RNAs between malignant vs normal breast epithelial tissues

miRNA	Chromosomal location	Q-value	Fold change (log ₂)
miR-518b	19q13.42	2.11E-16	4.65
miR-572	4p16.1	6.06E-08	4.77
miR-213	1q31.3	1.09E-07	4.06
miR-622	13q31.3	3.32E-07	6.38
miR-601	9q33.3	1.20E-06	3.34
miR-181d	19p13.12	3.16E-06	3.85
miR-184	15q25.1	3.16E-06	3.57
miR-196a	17q21.32 / 12q13.13	3.16E-06	5.89
miR-324-5p	17p13.1	3.16E-06	3.63
miR-221	Xp11.3	1.27E-05	-3.55
miR-328	16q22.1	1.27E-05	-3.38
miR-200c	12p13.31	1.97E-05	3.77
miR-449b	5q11.2	8.44E-05	4.83
miR-21	17q23.2	9.18E-05	3.62
miR-331	12q22	1.16E-04	3.36
miR-646	20q13.33	1.16E-04	3.63
miR-449a	5q11.2	1.71E-04	4.50
miR-487b	14q32.31	1.71E-04	-3.42
miR-191	3p21.31	2.08E-04	3.77
miR-423	17q11.2	4.19E-04	2.84
miR-192	11q13.1	6.15E-04	2.50
miR-374a	Xq13.2	6.15E-04	3.01
miR-125b	11q24.1 / 21q21.1	9.79E-04	-4.07
miR-93	7q22.1	9.79E-04	2.08
miR-155	21q21.3	2.63E-03	2.40
miR-491	9p21.3	2.87E-03	3.70
miR-210	11p15.5	3.04E-03	3.69
miR-187	18q12.2	3.26E-03	2.66
miR-375	2q35	3.26E-03	2.56
miR-151	8q24.3	3.42E-03	2.26

miRs in **bold** are discussed further in the text of the paper.

The Q-value represents the P-value, adjusted for a false-discovery rate for multiple comparisons.

experiments, we would attribute the miR discrepancy to inter-experimental variations of these very sensitive qRT-PCR-based assays.

Indeed, we have assessed the reliability of shifting from high-throughput miR profiling to qRT-PCR. The expressions of four genes (RNU6B, miR-328, miR-221, and miR-213) along with two normalisation controls (RNU44 and RNU48) were further evaluated in 4 normal and 12 tumour samples in triplicate (Supplementary Figure 2). A variance analysis was

performed to quantify the relative contributions of technical and biological variability. For all the 4 genes across the 16 samples, the technical variability was smaller than the biological variability (Supplementary Figure 3a). We further considered the differential expression between tumour and normal samples identified by TLDA and qRT-PCR. Good concordance was observed for three genes (RNU6B, miR328, and miR213), both in terms of statistical significance (Supplementary Figure 3b) and magnitude of differential expression (Supplementary Figure 3c), further corroborating that the TLDA platform leads to reproducible measurements of miR expression, as confirmed by qRT-PCR.

The capabilities and robustness of global miR profiling using FFPE samples are far superior to that of mRNA profiling, which is not technically reliable because of RNA degradation.²⁵ This issue was specifically addressed in a direct comparison of mRNA profiling of FFPE vs frozen tissues, showing a correlation coefficient of only 0.28.²⁹ The only mRNA assay that could be conducted on FFPE samples is the measurement of already-identified transcripts, such as the 21-gene signature⁴³ for breast cancer (<http://www.genomichhealth.com/oncotype>), which would not allow for the discovery of novel biologically relevant sequences.

Interesting information has emerged in the evaluations of global miR profilings; in a direct comparison between FFPE and snap frozen samples of a normal human thyroid cell line, this group showed higher miR expression levels observed from FFPE when compared with that from frozen samples. This group hypothesised that this unexpected finding might relate to miRs being resistant to the methylol cross-linking between RNA and proteins, which occurs during tissue fixation.⁴⁴ Similarly, a systematic comparison of miR profiling between frozen and FFPE normal murine tissues showed an excellent correlation of 0.89²⁹ with remarkable stability even in FFPE blocks that have been in storage for 10 years,²⁹ which is corroborated in yet another recent study reporting similarly high correlations (0.85 to 0.95) using miR microarray analyses.⁴⁵ Our own data using two corresponding pairs of frozen and FFPE invasive breast cancer samples yielded the same level of outstanding consistency, with Spearman's correlations of 0.94 or greater (Figure 1c).

Even more useful is the ability to conduct *in situ* hybridisation of miRs in FFPE breast cancer samples, which provides additional valuable information on the cellular localisation of relevant miRs in disease processes.³⁰ Hence, our study contributes to the emerging literature on the utility and robustness of miR profiling of FFPE samples, underscoring opportunities to expeditiously acquire clinically and biologically relevant insight into the role of miRs in human malignancies.

Statistical analysis identified a distinct subset of breast cancer-associated miRs, similar to earlier-reported miR evaluations, including upregulation of miR-21, miR-155, miR-191, miR-196a, miR-200c, miR-210, and miR-213, as well as downregulation of miR-125b and miR-221.^{10,12,30,46} Micro-

RNA-21 (Table 3), which is overexpressed ~10-fold in our cohort, is among the most-studied miRs in human cancers. Another highly dysregulated miR is miR-196a, which is overexpressed ~64-fold in this study; this miR has been suggested to target HOXB8 and is significantly differentially expressed between normal and malignant pancreatic tissues.^{47,48} Similar to the Sempere *et al*'s³⁰ study, miR-200c was overexpressed (~16-fold), and miR-221 was downregulated (also by ~16-fold) in our cohort (Table 3). Micro-RNA-200c can reduce the expression of transcription factor 8 and increase the expression of E-cadherin,^{30,46} which might account for an increased propensity for invasion. Micro-RNA-221 targets p27⁴⁹ and kit,⁵⁰ and is included in the predictive signature sets for both CLL¹⁶ and lung cancer,²² suggesting an important role for this miR in cancer progression. Downregulation of miR-125b has been consistently detected in breast cancers^{10,12} and has been shown to suppress both expression and function of ERBB2 and ERBB3.⁵¹

This study includes a more up-to-date and comprehensive evaluation of human miRs, enabling the identification of several less well-characterised miRs that are significantly altered in invasive breast cancers. As shown in Table 3, we have identified new breast-related miRs, which might provide additional insight into the complexities of human breast cancer dysregulation. These novel miRs include the less-characterised miRs, such as upregulated miR-423, miR-449, miR-449b, miR-518b, miR-572, miR-601, miR-622, miR-646, and downregulated miR-328 (Table 3).

The overexpression of HER-2/neu and ER expression are two important prognostic factors for human breast cancer. The association of miR with HER-2/neu or ER expression in breast cancer has been investigated earlier,^{10,12} noting distinct miR subsets correlating with HER-2/neu, PR, and ER status.^{10,12} On the other hand, Sempere *et al*'s³⁰ observed no clear association of miR expression with either ER or HER-2/neu. Similarly, in our study, no association of miR with HER-2/neu or ER expression was identified, which might be attributable to the small number (four cases) of HER-2/neu positive tumours in our cohort.

In conclusion, we have successfully conducted global miR profiling for human breast FFPE samples, showing that this platform achieves excellent inter-experimental reproducibility and biological accuracy, rendering technical replicates likely unnecessary. Such high-throughput profiling offers the rich resource of archival clinical FFPE blocks, from which biological insight could now be acquired in an expeditious manner, for the further elucidation of the role of miRs in human malignancies.

Supplementary Information accompanies the paper on the Laboratory Investigation website (<http://www.laboratoryinvestigation.org>)

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1. Calin GA, Croce CM. MicroRNA signatures in human cancers. *Nat Rev Cancer* 2006;6:857–866.
2. Esquela-Kerscher A, Slack FJ. Oncomirs—microRNAs with a role in cancer. *Nat Rev Cancer* 2006;6:259–269.
3. Lee RC, Feinbaum RL, Ambros V. The *C. elegans* heterochronic gene *lin-4* encodes small RNAs with antisense complementarity to *lin-14*. *Cell* 1993;75:843–854.
4. Griffiths-Jones S, Grocock RJ, van Dongen S, *et al*. miRBase: microRNA sequences, targets and gene nomenclature. *Nucleic Acids Res* 2006;34(Database issue):D140–D144.
5. Bentwich I, Avniel A, Karov Y, *et al*. Identification of hundreds of conserved and nonconserved human microRNAs. *Nat Genet* 2005;37:766–770.
6. Lewis BP, Shih IH, Jones-Rhoades MW, *et al*. Prediction of mammalian microRNA targets. *Cell* 2003;115:787–798.
7. Calin GA, Sevignani C, Dumitru CD, *et al*. Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. *Proc Natl Acad Sci USA* 2004;101:2999–3004.
8. Volinia S, Calin GA, Liu CG, *et al*. A microRNA expression signature of human solid tumors defines cancer gene targets. *Proc Natl Acad Sci USA* 2006;103:2257–2261.
9. Yanaihara N, Caplen N, Bowman E, *et al*. Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. *Cancer Cell* 2006;9:189–198.
10. Iorio MV, Ferracin M, Liu CG, *et al*. MicroRNA gene expression deregulation in human breast cancer. *Cancer Res* 2005;65:7065–7070.
11. Zhang L, Huang J, Yang N, *et al*. MicroRNAs exhibit high frequency genomic alterations in human cancer. *Proc Natl Acad Sci USA* 2006;103:9136–9141.
12. Mattie MD, Benz CC, Bowers J, *et al*. Optimized high-throughput microRNA expression profiling provides novel biomarker assessment of clinical prostate and breast cancer biopsies. *Mol Cancer* 2006;5:24.
13. Roldo C, Missiaglia E, Hagan JP, *et al*. MicroRNA expression abnormalities in pancreatic endocrine and acinar tumors are associated with distinctive pathologic features and clinical behavior. *J Clin Oncol* 2006;24:4677–4684.
14. Chan JA, Krichevsky AM, Kosik KS. MicroRNA-21 is an antiapoptotic factor in human glioblastoma cells. *Cancer Res* 2005;65:6029–6033.
15. Calin GA, Dumitru CD, Shimizu M, *et al*. Frequent deletions and down-regulation of micro-RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. *Proc Natl Acad Sci USA* 2002;99:15524–15529.
16. Calin GA, Ferracin M, Cimmino A, *et al*. A microRNA signature associated with prognosis and progression in chronic lymphocytic leukemia. *N Engl J Med* 2005;353:1793–1801.
17. Si ML, Zhu S, Wu H, *et al*. miR-21-mediated tumor growth. *Oncogene* 2007;26:2799–2803.
18. Meng F, Henson R, Lang M, *et al*. Involvement of human micro-RNA in growth and response to chemotherapy in human cholangiocarcinoma cell lines. *Gastroenterology* 2006;130:2113–2129.
19. Tricoli JV, Jacobson JW. MicroRNA: potential for cancer detection, diagnosis, and prognosis. *Cancer Res* 2007;67:4553–4555.
20. Blenkiron C, Goldstein LD, Thorne NP, *et al*. MicroRNA expression profiling of human breast cancer identifies new markers of tumour subtype. *Genome Biol* 2007;8:R214.
21. Lu J, Getz G, Miska EA, *et al*. MicroRNA expression profiles classify human cancers. *Nature* 2005;435:834–838.
22. Yu SL, Chen HY, Chang GC, *et al*. MicroRNA signature predicts survival and relapse in lung cancer. *Cancer Cell* 2008;13:48–57.
23. Macabeo-Ong M, Ginzinger DG, Dekker N, *et al*. Effect of duration of fixation on quantitative reverse transcription polymerase chain reaction analyses. *Mod Pathol* 2002;15:979–987.
24. Bresters D, Schipper ME, Reesink HW, *et al*. The duration of fixation influences the yield of HCV cDNA-PCR products from formalin-fixed, paraffin-embedded liver tissue. *J Virol Methods* 1994;48:267–272.
25. Cronin M, Pho M, Dutta D, *et al*. Measurement of gene expression in archival paraffin-embedded tissues: development and performance of a 92-gene reverse transcriptase-polymerase chain reaction assay. *Am J Pathol* 2004;164:35–42.

26. Nelson PT, Baldwin DA, Scearce LM, *et al*. Microarray-based, high-throughput gene expression profiling of microRNAs. *Nat Methods* 2004;1:155–161.
27. Chen C, Ridzon DA, Broomer AJ, *et al*. Real-time quantification of microRNAs by stem-loop RT-PCR. *Nucleic Acids Res* 2005;33:e179.
28. Jiang J, Lee EJ, Gusev Y, *et al*. Real-time expression profiling of microRNA precursors in human cancer cell lines. *Nucleic Acids Res* 2005;33:5394–5403.
29. Xi Y, Nakajima G, Gavin E, *et al*. Systematic analysis of microRNA expression of RNA extracted from fresh frozen and formalin-fixed paraffin-embedded samples. *RNA* 2007;13:1668–1674.
30. Sempere LF, Christensen M, Silahdaroglu A, *et al*. Altered microRNA expression confined to specific epithelial cell subpopulations in breast cancer. *Cancer Res* 2007;67:11612–11620.
31. Barsyte-Lovejoy D, Lau SK, Boutros PC, *et al*. The c-Myc oncogene directly induces the H19 noncoding RNA by allele-specific binding to potentiate tumorigenesis. *Cancer Res* 2006;66:5330–5337.
32. Duda RO, Hart PE, Stork DG. *Pattern Classification*, 2nd edn Wiley: New York, 2001.
33. Boutros PC, Okey AB. Unsupervised pattern recognition: an introduction to the whys and wherefores of clustering microarray data. *Brief Bioinform* 2005;6:331–343.
34. Budhu A, Jia HL, Forgues M, *et al*. Identification of metastasis-related microRNAs in hepatocellular carcinoma. *Hepatology* 2008;47:897–907.
35. Guo Y, Chen Z, Zhang L, *et al*. Distinctive microRNA profiles relating to patient survival in esophageal squamous cell carcinoma. *Cancer Res* 2008;68:26–33.
36. Schetter AJ, Leung SY, Sohn JJ, *et al*. MicroRNA expression profiles associated with prognosis and therapeutic outcome in colon adenocarcinoma. *JAMA* 2008;299:425–436.
37. Shi L, Reid LH, Jones WD, *et al*. The MicroArray Quality Control (MAQC) project shows inter- and intraplatform reproducibility of gene expression measurements. *Nat Biotechnol* 2006;24:1151–1161.
38. Frueh FW. Impact of microarray data quality on genomic data submissions to the FDA. *Nat Biotechnol* 2006;24:1105–1107.
39. Guo L, Lobenhofer EK, Wang C, *et al*. Rat toxicogenomic study reveals analytical consistency across microarray platforms. *Nat Biotechnol* 2006;24:1162–1169.
40. Larkin JE, Frank BC, Gavras H, *et al*. Independence and reproducibility across microarray platforms. *Nat Methods* 2005;2:337–344.
41. Kohlmann A, Schoch C, Dugas M, *et al*. Pattern robustness of diagnostic gene expression signatures in leukemia. *Genes Chromosomes Cancer* 2005;42:299–307.
42. Canales RD, Luo Y, Willey JC, *et al*. Evaluation of DNA microarray results with quantitative gene expression platforms. *Nat Biotechnol* 2006;24:1115–1122.
43. Paik S, Shak S, Tang G, *et al*. A multigene assay to predict recurrence of tamoxifen-treated, node-negative breast cancer. *N Engl J Med* 2004;351:2817–2826.
44. Li J, Smyth P, Flavin R, *et al*. Comparison of miRNA expression patterns using total RNA extracted from matched samples of formalin-fixed paraffin-embedded (FFPE) cells and snap frozen cells. *BMC Biotechnol* 2007;7:36.
45. Zhang X, Chen J, Radcliffe T, *et al*. An array-based analysis of microRNA expression comparing matched frozen and formalin-fixed paraffin-embedded human tissue samples. *J Mol Diagn* 2008;10:513–519.
46. Hurteau GJ, Carlson JA, Spivack SD, *et al*. Overexpression of the microRNA hsa-miR-200c leads to reduced expression of transcription factor 8 and increased expression of E-cadherin. *Cancer Res* 2007;67:7972–7976.
47. Yekta S, Shih IH, Bartel DP. MicroRNA-directed cleavage of HOXB8 mRNA. *Science* 2004;304:594–596.
48. Szafranska AE, Davison TS, John J, *et al*. MicroRNA expression alterations are linked to tumorigenesis and non-neoplastic processes in pancreatic ductal adenocarcinoma. *Oncogene* 2007;26:4442–4452.
49. le Sage C, Nagel R, Egan DA, *et al*. Regulation of the p27(Kip1) tumor suppressor by miR-221 and miR-222 promotes cancer cell proliferation. *EMBO J* 2007;26:3699–3708.
50. Lee EJ, Gusev Y, Jiang J, *et al*. Expression profiling identifies microRNA signature in pancreatic cancer. *Int J Cancer* 2007;120:1046–1054.
51. Scott GK, Goga A, Bhaumik D, *et al*. Coordinate suppression of ERBB2 and ERBB3 by enforced expression of micro-RNA miR-125a or miR-125b. *J Biol Chem* 2007;282:1479–1486.