Predictive and prognostic value of selected microRNAs in luminal breast cancer

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The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest

Author contribution statement

MA prepared tissues for molecular analyses, including RNA extraction, cDNA synthesis, performed RT-qPCR assays, analyzed data and drafted the manuscript. JL collected normal breast tissues from reductive mammoplasty and assisted in histopathological evaluation of tissue samples. HP contributed in data analysis and manuscript drafted. MS and SPS collected clinical follow-up data. PL performed immunohistochemistry of all cases. SS contributed in the design of the study. LA assisted in the statistical analyses. RH performed histopathological evaluation of fresh frozen sections stained by H&E. RH and CJ designed and supervised the study and revised the manuscript. All the authors read and approved the final manuscript.

Keywords

breast cancer, luminal subtype, Endocrine therapy, Endocrine resistance, biomarkers, MicroRNAs

Abstract

Word count: 233

Breast cancer (BrC) is the most frequent malignancy and the leading cause of cancer death among women worldwide. Approximately 70% of BrC are classified as luminal-like subtype, expressing the estrogen receptor. One of the most common and effective adjuvant therapies for this BrC subtype is endocrine therapy. However, its effectiveness is limited, with relapse occurring in up to 40% of patients. Because microRNAs have been associated with several mechanisms underlying endocrine resistance and sensitivity, they may serve as predictive and/or prognostic biomarkers in this setting.
Hence, the main goal of this study was to investigate whether miRNAs deregulated in endocrine-resistant BrC may be clinically relevant as prognostic and predictive biomarkers in luminal BrC patients treated with adjuvant endocrine therapy.
A global expression assay allowed for the identification of microRNAs differentially expressed between luminal patients with or without BrC recurrence after endocrine adjuvant therapy. Then, six microRNAs were chosen for validation using quantitative reverse transcription polymerase chain reaction in a larger set of tissue samples. Thus, miR-30c-5p, miR-30b-5p, miR-182-5p and miR-200b-3p were found to be independent predictors of clinical benefit from endocrine therapy. Moreover, miR-182-5p and miR-200b-3p displayed independent prognostic value for disease recurrence in luminal BrC patients after endocrine therapy.
Our results indicate that selected miRNAs may constitute clinically useful ancillary tools for management of luminal BrC patients. Nevertheless, additional validation, ideally in a multicentric setting is required to confirm support our findings.

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Data availability statement

Generated Statement: All datasets generated for this study are included in the manuscript and the supplementary files.

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Keywords: Breast cancer, luminal subtype, endocrine therapy, endocrine resistance, biomarkers, microRNAs

Abstract

Breast cancer (BrC) is the most frequent malignancy and the leading cause of cancer death among women worldwide. Approximately 70% of BrC are classified as luminal-like subtype, expressing the estrogen receptor. One of the most common and effective adjuvant therapies for this BrC subtype is endocrine therapy. However, its effectiveness is limited, with relapse occurring in up to 40% of patients. Because microRNAs have been associated with several mechanisms underlying endocrine resistance and sensitivity, they may serve as predictive and/or prognostic biomarkers in this setting.

Hence, the main goal of this study was to investigate whether miRNAs deregulated in endocrine-resistant BrC may be clinically relevant as prognostic and predictive biomarkers in luminal BrC patients treated with adjuvant endocrine therapy.

A global expression assay allowed for the identification of microRNAs differentially expressed between luminal patients with or without BrC recurrence after endocrine adjuvant therapy. Then, six microRNAs were chosen for validation using quantitative reverse transcription polymerase chain reaction in a larger set of tissue samples. Thus, *miR-30c-5p*, *miR-30b-5p*, *miR-182-5p* and *miR-200b-3p* were found to be independent predictors of clinical benefit from endocrine therapy. Moreover, *miR-182-5p* and *miR-200b-3p* displayed independent prognostic value for disease recurrence in luminal BrC patients after endocrine therapy.

Our results indicate that selected miRNAs may constitute clinically useful ancillary tools for management of luminal BrC patients. Nevertheless, additional validation, ideally in a multicentric setting is required to confirm support our findings.

1 Introduction

Breast cancer (BrC) is the second most common cancer worldwide and the most frequent cancer among women. Despite advances in screening, early diagnosis and the development of treatment strategies, BrC still constitutes the leading cause of cancer-related deaths in women (Bray et al., 2018). BrC is a highly heterogeneous disease with distinct biological features and clinical outcomes. Based on gene expression profiling, BrC is often classified into four well-established intrinsic subtypes (Table 1) (Sørlie, 2004; Parker et al., 2009). However, due to financial constraints, surrogate approaches have been developed for routine clinical practice using more widely available immunohistochemistry (IHC) assays for estrogen receptor (ER), progesterone receptor (PR) and Ki-67 index, together with IHC and/or *in situ* hybridization for human epidermal growth factor 2 receptor (HER2) overexpression/amplification (Senkus et al., 2015).

In addition to surgery, strategies for BrC patients include neoadjuvant, adjuvant and palliative treatments. Adjuvant systemic therapy, aiming to prevent BrC recurrence by eradicating micrometastatic tumors present at diagnosis, can be comprised from one to three modalities: chemotherapy, anti-HER2 therapy (trastuzumab) and endocrine therapy (ET). ER and HER2 *status* are used as predictive factors to select patients for specific adjuvant therapies (Table 1). ET, which blocks ER activation, is recommended for patients with ER-positive disease, to stop or slow the growth of hormone-sensitive BrC (Curigliano et al., 2017). Most luminal A tumors, except those with the highest risk of relapse, do not require adjuvant chemotherapy, whereas most luminal B tumors, especially those with HER2 overexpression, benefit from chemotherapy in addition to trastuzumab (Slamon et al., 2011). Although ET results in substantial improvement of patients' outcome, resistance to treatment has become a major limitation (Zhang et al., 2014a), affecting 30-40% of ER-positive BrC patients, with all those treated in the metastatic setting eventually progressing (Normanno et al., 2005; Murphy

and Dickler, 2016). According to 3rd ESO–ESMO International Consensus Guidelines, endocrine resistance may be defined as primary endocrine resistance when patients relapse within the first 2 years of adjuvant ET, or as secondary (acquired) endocrine resistance, when patients relapse while on adjuvant ET after the first 2 years of treatment or within the 12 months after completing treatment (Cardoso et al., 2017).

MicroRNAs (miRNAs), a class of small (~22 nucleotides) non-coding single-stranded RNAs, have shown promise for assisting in clinical management of BrC, as diagnostic, prognostic or predictive biomarkers (Amorim et al., 2016), namely by their assessment in liquid biopsies (plasma, serum, urine) (Schwarzenbach et al., 2014). Indeed, several studies associated miRNAs deregulation with endocrine resistance and prognosis in luminal BrC (Rodriguez-Gonzalez et al., 2011; Muluhngwi and Klinge, 2015; Barbano et al., 2017; Muluhngwi and Klinge, 2017). Whereas decreased ER expression and endocrine resistance may be due to *miR-221/222* overexpression (Zhao et al., 2008; Rao et al., 2011; Wei et al., 2014; Song et al., 2017), *miR-342-3p* expression positively correlated with ER mRNA transcript levels, being downregulated in tamoxifen refractory BrC (Cittelly et al., 2010). Moreover, miRNAs regulating growth, survival and apoptosis of BrC cells may also be implicated in loss of responsiveness to ET by endowing tumor cells with alternative proliferative and survival stimuli (Thiantanawat et al., 2003). Indeed, *miR-519a* associated with worse prognosis of luminal BrC patients, directly targeting the transcripts of *cyclin dependent kinase inhibitor 1A* (*CDKN1A*) and *phosphatase and tensin homolog* (*PTEN*), allowing for enhanced signaling of the *phosphoinositide3-kinase* (PI3K) growth and survival pathway (Ward et al., 2014) and reducing sensitivity and tumor cell apoptosis in response to apoptotic stimuli (Breunig et al., 2017). Furthermore, miRNA-mediated endocrine resistance might be related with epithelial-to-mesenchymal transition (EMT) and metastatic potential of BrC cells, as members of the *miR-200 family (miR-200f)* were found downregulated in endocrine-resistant BrC *vs.* endocrine-sensitive cell lines, acting as major regulators of EMT (Burk et al., 2008; Manavalan et al., 2013).

Herein, we aimed to identify miRNAs able to predict endocrine resistance among luminal BrC patients undergoing ET, through the comparison of expression levels between BrC samples of patients that develop endocrine-resistance in long term follow-up with those that did not develop endocrine-resistance. This might allow for the stratification of luminal BrC cases into a low-risk subgroup, for whom additional adjuvant systemic treatment can be safely omitted, and patients who are at high-risk for recurrence potentially allowing the detection of resistance to ET at an early stage.

2 Material and methods

2.1 Patients and samples collection

For this study, 136 BrC tissue samples were prospectively collected, after informed consent, from patients with luminal BrC and without metastasis at diagnosis, aged between 40 and 75 years, submitted to adjuvant ET (with or without other adjuvant modalities), after first line surgical treatment, from 1995 to 2002 at the Portuguese Oncology Institute of Porto (IPO-Porto). Furthermore, 26 normal breast tissue samples were collected from reduction mammoplasties of contralateral breast of BrC patients. All specimens were obtained from patients without BrC hereditary syndrome and showed no evidence of preneoplastic/neoplastic lesions. After surgical resection, samples were immediately frozen at -80°C. Relevant clinical and pathological data was retrieved from patients' charts. Five-µm frozen sections were cut and stained with hematoxylin-eosin (H&E) staining for confirmation of BrC by an experienced pathologist, ensuring that samples contained at least 70% of tumor cells, and confirm

that tissues obtained from reduction mammoplasties harbored normal epithelial cells. This study was approved by institutional ethical committee (CES-IPOFG-120/015).

2.2 Breast cancer subtyping

IHC was performed to identify the molecular subtype of each tumor tissue included in this study. Commercially available antibodies were used for ER (Clone 6F11, mouse, Leica), PR (Clone 16, mouse, Leica), HER2 (Clone 4B5, rabbit, Roche) and Ki-67 (Clone MIB-1, mouse, Dako). IHC was carried out in BenchMark ULTRA (Ventana, Roche) using ultraView Universal DAB Detection Kit (Ventana, Roche) according to manufacturer's instructions. Each case was evaluated by an experienced pathologist and was classified according to the College of American Pathologists recommendations (Fitzgibbons et al., 2014). Each case was categorized according to ESMO guidelines (Senkus et al., 2015). Cutoffs for Ki-67 and PR expression were 15% and 25% of positive cells, respectively.

2.3 RNA extraction from fresh frozen tissues

Total RNA was extracted from fresh frozen tissues using the TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's recommendations. RNA concentrations and purity ratios were ascertained using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and RNA samples were stored at -80ºC.

2.4 MicroRNAs cDNA synthesis

cDNA synthesis was performed in a Veriti® Thermal Cycler (Applied Biosystems, Foster City, CA, USA) using miRCURY LNA™ Universal RT microRNA PCR (Exiqon, Vedbaek, Denmark) following manufacturer's instructions. cDNA samples were then stored at -20ºC.

2.5 Global focus microRNA PCR panel

Global miRNAs' expression was evaluated using a Cancer Focus microRNA PCR Panel, 384 well (V4.R) (Exiqon). Each plate, besides containing 80 lyophilized LNA™ miRNA primer sets focusing on cancer relevant human miRNAs, also contained interplate calibrators, candidate reference genes [miRNAs and small nuclear RNAs (snRNAs)] and one water blank. In each well, it was added 0.05 µL of cDNA previously synthesized, 5 µL of SYBR® Green master mix (Exiqon) and 4.95 µL of nuclease-free water (Exiqon). Quantitative reverse transcription polymerase chain reactions (RT-qPCR) were performed in the LightCycler 480 instrument (Roche Diagnostics, Manheim, Germany) according to the following conditions: 95ºC for 10 minutes and 45 cycles at 95ºC for 10 seconds and 60ºC for 1 minute.

The median values of *miR-103a-3p*, *miR-207*, *miR-191-5p* and *SNORD38B* were used for normalization, as these genes were the most stably expressed candidate reference genes (data not shown). Differences in expression values for target miRNAs were calculated using the $2^{-\Delta\Delta CT}$ method. The selection of deregulated miRNAs for further validation was performed considering prominent fold change, good sensitivity for qRT-PCR detection (Ct values, in general, below 30), and novelty.

2.6 Individual assays

Initially, cDNA samples were diluted 80x in sterile distilled water (B. Braun, Melsungen, Germany). Then, on ice, per each well of a 384-well plate it was added: 5 µL of NZYSpeedy qPCR Green Master Mix (2x) (NZYTECH, Portugal), 1 µL of miRNA specific primer mix (microRNA LNA™ PCR primer set, Exiqon), and 4 µL of previously diluted cDNA. Each amplification reaction was performed in

triplicate on a LightCycler 480 instrument (Roche Diagnostics, Manheim, Germany). Each plate also contained 2 negative template controls. RT-qPCR protocol consisted in a denaturation step at 95ºC for 2 minutes, followed by 40 amplification cycles at 95ºC for 5 seconds and 60ºC for 20 seconds. Melting curve analysis was performed according to instrument's manufacturer recommendations.

SNORD38B was used as a reference gene for data normalization, as this gene was the most stably expressed over the whole range of the samples used for the global expression assay. Notwithstanding, the stability *SNORD38B* expression was empirically validated in additional samples. Relative miRNAs expression in each sample was calculated by the $2^{-\Delta\Delta CT}$ method.

2.7 Statistical analysis

To ascertain statistical significance for continuous variables comparisons made between independent samples, non-parametric Mann-Whitney U tests were performed. Fold changes for single miRNAs were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Spearman nonparametric correlation test was performed to assess the association between continuous variables. Chi-square test or Fisher's exact test were used as appropriate to compare proportions between two groups.

Some clinicopathological features were grouped, including pT stage (T1&T2 and T3&T4), pN stage (N0&N1 and N2&N3) and grade [grade (G)1&G2 and G3] (Lakhani, 2012). Age was categorized into four groups (≤44; 45-64; 65-74; ≥75), and miRNA expression levels were categorized according to 25^{th} or 75^{th} percentile. For the survival analysis, Cox-regression univariable and multivariable models were computed to assess standard clinicopathological variables and miRNAs prognostic value. Hazard Ratios (HR) along with respective 95% Confidence Interval (95%CI) were reported. Multivariable Cox models only included the statistically significant variables. Kaplan-Meier with log rank test was used to construct and compare survival curves according to categorized miRNAs expression levels. Endocrine resistance-free survival (ERFS) was defined as the time between surgery and the recurrence dates. Recurrences occurring after 12 months of completing ET were not considered events for this analysis. Disease-free survival (DFS) was defined as the time between surgery date and recurrence date. Distant metastasis-free survival (DMFS) was defined as the time between surgery and the development of distant metastases.

Statistical analysis was performed using SPSS software (SPSS Version 20.0, Chicago, IL) and two-tailed p-values were considered statistically significant when $p<0.05$. Graphs were built using GraphPad 6 Prism (GraphPad Software, USA).

3 Results

3.1 Characteristics of study populations

The discovery cohort, used for global expression assay analysis, consisted of four luminal A and four luminal B tumors from patients which relapsed, and the same number of patients that did not relapse after adjuvant ET. Patients that relapsed during adjuvant ET or within the first 12 months of completing adjuvant ET were considered endocrine-resistant (Table 2).

The validation cohort was composed by 162 patients, from which 136 fresh frozen luminal BrC tissues and 26 normal breast tissues were collected. From the 136 luminal BrC, 40 derived from patients which recurred and 96 from patients that did not. Among 40 patients with BrC recurrence, 22 were considered endocrine-resistant. Clinical and pathological characteristics of patients and controls included in this study are shown in Table 3. Endocrine-sensitive and endocrine-resistant groups did not differ

significantly concerning age distribution (p=0.127). As expected, among endocrine-resistant BrC cases, luminal B tumors were more common (p=0.004), and consequently, the same trend was depicted for HER2-positivity (p=0.024) and high Ki-67 index (p<0.001). Moreover, this group also showed more moderate- and high-grade (G2 and G3) BrC cases (p<0.001). For the remaining clinicopathological features or treatment modalities no significant differences were depicted.

3.2 Global focus microRNA PCR Panel analysis

In the global expression assay, one luminal A case with recurrence was excluded from the analysis, due to low RT-qPCR success rate (25% of the miRNAs did not amplify, and the remaining showed Ct values higher than 30). Likewise, three (*miR-202-3p*, *-206* and *-20b-5p*) out of the 80 miRNAs were excluded due to low real-time PCR success rates. MiRNAs with fold variation values higher than 1 were selected, resulting in a panel comprising 56 miRNAs (Table 4).

3.3 Gene-specific assays

From the global expression assay analysis, *miR-30b-5p*, *miR-181a-5p*, *miR-182-5p*, *miR-200b-3p* and *miR-205-5p* were selected for further validation. All these miRNAs disclosed prominent fold change and good sensitivity for qRT-PCR detection. *MiR-30b-5p* was chosen because several studies focused on other members of the *miR-30 family (miR-30f)* and, to the best of our knowledge, its predictive potential for endocrine therapy had not been assessed previously (Cheng et al., 2012; Bockhorn et al., 2013; Zhang et al., 2014b; D'aiuto et al., 2015; Yang et al., 2017). *MiR-181a-5p* and *miR-200b-3p* were selected to confirm the reported association with endocrine-resistance in *in vitro* studies (Hiscox et al., 2006; Maillot et al., 2009; Manavalan et al., 2011; Vesuna et al., 2012; Manavalan et al., 2013). Furthermore, *miR-182-5p* was also selected to better ascertain its role in endocrine resistance due to controversial results in global focus microRNA PCR panel, since it was overexpressed in luminal B tumors from recurrent patients and downregulated in luminal A tumors from recurrent patients. Finally, *miR-30c-5p* was chosen as a positive control since higher expression levels of this miRNA had been positively associated with benefit of ET, in multivariable analysis, in advanced ER-positive BrC (Rodriguez-Gonzalez et al., 2011).

To determine "baseline" miRNA expression, 26 normal breast tissues were also analyzed, and we found that *miR-181a-5p* (p=0.0007), *miR-182-5p* (p<0.0001) and *miR-200b-3p* (p<0.0001) expression levels were significantly higher whereas *miR-205-5p* expression levels were significantly lower (p=0.0056) in luminal BrC tissues (Figure 1). No differences were depicted for the remainder miRNAs.

Furthermore, *miR-30c-5p* (p=0.0041), *miR-30b-5p* (p=0.0396) and *miR-200b-3p* (p=0.0293) were significantly downregulated in tumor tissues from endocrine-resistant BrC compared to endocrine-sensitive tumors (Figure 2), while no differences were depicted for the remainder miRNAs.

3.4 Association between miRNAs expression and clinicopathological features

MiR-30c-5p expression levels were significantly associated with PR-positive and HER2-negative tumors (p=0.0314 and p=0.0462, respectively). Moreover, *miR-30b-5p* expression levels were also higher in HER2-negative tumors (p=0.0447). Additionally, high grade (G3) BrC displayed significantly lower *miR-205-5p* levels (p=0.0268) compared to G1/G2 BrC (Figure 3).

3.5 Survival analyses

All survival analyses were restricted to 15 years of follow-up. The median follow-up time was 121 months (17.6-180 months). At 15 years of follow-up, 79 (58.1%) patients were alive. Of these, 76

patients (55.9%) were alive with no evidence of cancer and 3 patients (2.2%) with cancer. Additionally, 57 patients (41.9%) had deceased, 31 of which due to BrC (22.8%).

Firstly, ERFS was calculated and, in univariable analysis, most standard clinicopathological parameters were significantly associated with ERFS. Specifically, HER2-positivity (HR = 3.46, p=0.010), high Ki-67 index (HR=5.82, p<0.001), high grade (G3) (HR=2.69, p=0.028) and luminal B subtype (HR=5.11, p=0.009) disclosed worse ERFS. Furthermore, lower *miR-30c-5p*, *miR-30b-5p*, *miR-182-5p* and *miR-200b-3p* levels predicted decreased ERFS (Table 5, Figure 4). In multivariable analysis, all miRNAs identified in the univariable model remained independent predictors of improved ERFS adjusted to molecular subtype (Table 5). To disclose the potential of miRNAs expression level as predictors of ERFS for each molecular subtype, a stratified analysis by luminal subtype was performed (Table 6). However, miRNAs only retained statistical significance in luminal B tumors.

Furthermore, to assess the miRNAs prognostic value, DFS analysis was also performed. In an univariable analysis, HER2-positivity (HR = 3.33, p=0.0002), high Ki-67 index (HR=2.48, p=0.010) and high grade (G3) (HR=2.21, p=0.016) associated with worse DFS, as expected. Interestingly, lower *miR-30c-5p*, *miR-30b-5p*, *miR-182-5p* and *miR-200b-3p* expression levels associated with decreased DFS (Table 5, Figure 5). Nonetheless, only *miR-200b-3p* and *miR-182-5p* were independent prognostic predictors adjusted for HER2 *status* in the multivariable model (Table 5). After stratifying the analysis according to HER2 *status*, both miRNAs retained statistical significance in both HER2-positive and HER2-negative BrC (Table 6).

Finally, DMFS was also performed, disregarding locoregional recurrences. In the same line as for DFS, HER2-positivity (HR = 3.39, p=0.001), high Ki-67 index (HR=2.27, p=0.029) and high grade (G3) (HR=2.25, p=0.020) associated with worse DMFS, in a univariable analysis. Besides, lower *miR-30c-5p*, *miR-30b-5p*, *miR-182-5p* and *miR-200b-3p* expression levels also associated with decreased DMFS (Table 5). In multivariable analysis, *miR-182-5p* retained statistical significance adjusted for HER2 *status* and tumor grade, whereas *miR-200b-3p* only retained statistical significance for HER2 *status* (Table 5). After stratifying analysis according to HER2 *status* and grade, *miR-182-5p* retained statistical significance in both low/intermediate and high-grade cancers, as well as in HER2-negative tumors, whereas *miR-200b-3p* retained statistical significance in HER2-positive BrC (Table 6).

4 Discussion

BrC remains the most common malignancy in women and a major cause of morbidity and mortality (Bray et al., 2018). De-escalation of both systemic and local adjuvant treatment, paralleling trends in surgery, is critical to provide patient-tailored treatment and avoid harmful side effects (Hwang, 2014; Senkus et al., 2015). Indeed, identification of luminal BrC patients with low recurrence risk after or while on ET, for which additional adjuvant systemic treatment can be safely omitted, is very important. Importantly, the identification of high-risk luminal BrC patients requiring more aggressive treatment regimens might avoid recurrence and subsequent metastatic disease, currently affecting approximately 40% of luminal BrC patients after adjuvant ET (Guarneri and Conte, 2004; Normanno et al., 2005; Murphy and Dickler, 2016). Thus, identification of biomarkers providing predictive and prognostic information in this group of patients is clinically relevant. Assessment of specific miRNAs expression deregulation, which has been associated with several mechanisms underlying endocrine resistance and sensitivity (Muluhngwi and Klinge, 2015; Muluhngwi and Klinge, 2017) might provide such kind of information. Nonetheless, most of those studies have been performed in cancer cell lines and display several limitations, including absence of epithelial-stromal and tumor-host interactions, that could modulate sensitivity *in vivo* (Shekhar et al., 2003). Conversely, tissue analysis from patients treated

with ET may allow for broader insight into biologically and clinically relevant miRNAs that may serve as markers of response or resistance to ET. Thus, we focused on the identification of aberrantly expressed miRNAs in endocrine-resistant BrC, exploring its predictive and prognostic value in luminal BrC patients treated with adjuvant ET.

The first step of this study consisted on the profiling of miRNAs expression patterns, looking for differences between endocrine-sensitive and endocrine-resistant luminal BrC. Hence, *miR-30c-5p*, *miR-30b-5p*, *miR-181a-5p*, *miR-182-5p*, *miR-200b-3p* and *miR-205-5p* were selected for validation in a larger set of luminal BrC and normal breast tissues. Upregulation of *miR-181a-5p* and *miR-182-5p* and downregulation of *miR-205-5p* in this BrC tissue cohort was consistent with previous publications (Hui et al., 2009; Li et al., 2014a; Zhang and Fan, 2015), providing indirect validation of our methodological approach. Contrarily, downregulation of *miR-200b-3p* in tumor compared to normal tissues has been previously reported (Ye et al., 2014; Yao et al., 2015). However, these studies have used non-cancerous tissues from breasts harboring carcinoma as controls, which may not represent truly normal breast tissues. Our results have also successfully confirmed the biomarker potential of *miR-30c-5p*, which was downregulated in endocrine-resistant BrC patients and independently predicted better ERFS in luminal B BrC patients. Moreover, *miR-30c-5p* expression correlated with PR-positivity and HER2-negativity, two of the most important predictive factors for ET sensitivity (Konecny et al., 2003). In fact, higher PR expression has suggest better sensitivity to ET and activation of HER2 signaling has been known as one of the factors most prominently contributing to endocrine resistance (Moon et al., 2011; AlFakeeh and Brezden-Masley, 2018). Likewise, *miR-30b-5p* and *miR-200b-3p* displayed the same trend and together with *miR-182-5p*, also independently predicted for improved ERFS in luminal B BrC patients. The lack of significance in luminal A subtype might be due to the small number of cases and events in our series. Importantly, we were able to validate in primary BrC the association between *miR-200b-3p* and endocrine-resistance, previously reported in *in vitro* models (Manavalan et al., 2013). Interestingly, several members of *miR-30f* have been reported as markers of favorable prognosis in BrC (Cheng et al., 2012; Bockhorn et al., 2013; Zhang et al., 2014b; D'aiuto et al., 2015; Croset et al., 2018) and our study also revealed that *miR-30b-5p* might be predictive of response to ET. Finally, concerning *miR-182-5p*, our results extended previous observations on the correlation with clinical benefit from therapy with tamoxifen in advanced-stage BrC, only showed in univariable analysis (Rodriguez-Gonzalez et al., 2011).

In addition to their predictive value, *miR-30b-5p* and *miR-30c-5p* also displayed prognostic potential in univariable analysis. Lower levels of these miRNAs were associated with decreased DFS and DMFS. *MiR-30f* members and their role as tumor suppressor during BrC have been previously reported (Bockhorn et al., 2013; Zhang et al., 2014b). Indeed, decreased levels of *miR-30f* members in BrC patients has been associated with poor relapse-free survival (Croset et al., 2018). Remarkably, we have also showed that *miR-182-5p* and *miR-200b-3p* are not only predictive, but also independent prognostic markers in multivariable analysis. Downregulation of these miRNAs was associated with decreased DFS in both HER2-positive and HER2–negative BrC and both miRNAs independently predict DMFS in HER2-negative and HER2-positive cancers, respectively. The role of *miR-200b-3p* as a prognostic marker in BrC is not a novelty (Ye et al., 2014; Yao et al., 2015). Indeed, members of *miR-200f* are known to act as enforcers of epithelial phenotype through either Zinc finger E-box-binding homeobox (ZEB)-dependent or –independent pathways (Li et al., 2014b). Intriguingly, most *in vitro* studies consistently attributed an oncogenic role to *miR-182-5p* (Chiang et al., 2013; Zhan et al., 2017). Though, higher *miR-182-5p* expression levels were associated with poor clinical outcome in BrC patients (Song et al., 2016), contrarily to our findings. It should be recalled, however, that *miR-182-5p* is a member of a miRNA family comprising three homologous, coordinately expressed, miRNAs (*miR-183*, *miR-182* and *miR-196*) that are clustered in chromosome 7q32.2 and that members of this cluster

have been linked to both pro- and anti-metastatic behavior in BrC, suggesting that *miR-183/96/182* cluster members may have divergent functions which are regulated in a context- and tissue-dependent manner (Lowery et al., 2010; Li et al., 2014a; Hong et al., 2016). Furthermore, the 7q32.2 locus has been considered a metastasis suppressor locus, enduring genetic copy number losses in BrC progression (Png et al., 2011). Thus, the association between *miR-182-5p* downregulation and worse prognosis probably results from a complex molecular scenario and additional studies are required to discriminate which members of the *miR-183/96/182* cluster may contribute and to which extent to BrC prognosis.

BrC tissues displayed higher *miR-182-5p* and *miR-200b-3p* levels compared to normal breast, whereas *miR-30b-5p*, *miR-30c-5p*, *miR-182-5p* and *miR-200b-3p* downregulation associated with decreased DMFS. Once development of solid neoplasms results from multiple sequential steps in which malignant cells undergo widespread modifications to successfully migrate and colonize other organs, we are tempted to speculate a context-dependent role of these miRNAs that may contribute to the emergence of malignant phenotype. Indeed, decreased *miR-200f* members expression might be associated with EMT initiation enabling cells with invasive features, whereas subsequent upregulation might be associated with MET, facilitating colonization (Gravgaard et al., 2012; Hilmarsdottir et al., 2014).

Globally, our results suggest a panel of miRNAs that might be tested in primary tumor tissues to assess the likelihood of recurrence and resistance to ET in newly diagnosed luminal BrC. Nevertheless, these miRNAs need to be carefully validated, ideally in multicenter studies, to generate more conclusive results. Furthermore, *in vitro* studies, including gain and loss of function assays following *in vitro* treatment with ET, are also critical to functionally characterize the role of these miRNAs. As future perspective, we intend to evaluate the potential role of these miRNAs in tumor dissemination. Additionally, we also intend to assess the expression of these miRNAs in liquid biopsies, evaluating their potential as non-invasive biomarkers. Indeed, miRNAs in circulation would enable the repeated noninvasive monitoring of miRNA expression profile changes during treatment's course, which could allow for early detection of ET resistance and/or recurrence, potentially improving the management and care of luminal BrC patients.

5 Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

6 Author Contributions

MA prepared tissues for molecular analyses, including RNA extraction, cDNA synthesis, performed RT-qPCR assays, analyzed data and drafted the manuscript. JL collected normal breast tissues from reductive mammoplasty and assisted in histopathological evaluation of tissue samples. HP contributed in data analysis and manuscript drafted. MS and SPS collected clinical follow-up data. PL performed immunohistochemistry of all cases. SS contributed in the design of the study. LA assisted in the statistical analyses. RH performed histopathological evaluation of fresh frozen sections stained by H&E. RH and CJ designed and supervised the study and revised the manuscript. All the authors read and approved the final manuscript.

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564

10 Figure legends

Figure 1. Scatter-plots of *miR-181a-5p* (A), *miR-182-5p* (B), *miR-200b-3p* (C) and *miR-205-5p* (D) relative expression levels in luminal tumor tissues and normal breast tissues. A ** denotes *p*-value <0.01, a *** denotes *p*-value <0.001 and a **** denotes *p*-value <0.0001 by non-parametric Mann-Whitney U test. Y-axis denotes $2^{-\Delta\Delta CT}$ values multiplied by 1000.

Figure 2. Scatter-plots of *miR-30c-5p* (A), *miR-30b-5p* (B) and *miR-200b-3p* (C) relative expression levels in tumor tissues from endocrine-sensitive and –resistant patients. A * denotes *p*-value <0.05 and a ** denotes *p*-value <0.01 by non-parametric Mann-Whitney U test. Y-axis denotes $2^{-\Delta\Delta CT}$ values multiplied by 1000.

Figure 3. Scatter-plots of *miR-30c-5p* relative expression levels according to PR *status* (A) and HER2 *status* (B), *miR-30b-5p* relative expression according to HER2 *status* (C) and *miR-205-5p* relative expression according to grade (D). A * denotes *p*-value <0.05 by non-parametric Mann-Whitney U test. Y-axis denotes $2^{-\Delta\Delta CT}$ values multiplied by 1000.

Figure 4. Endocrine Resistance-free Survival curves *of miR-30c-5p* (A), *miR-30b-5p* (B), *miR-182-5p* (C) and *miR-200b-3p* (D). Abbreviations: P25 – Percentile 25.

Figure 5. Disease-free Survival curves (Kaplan–Meier with log rank test) of *miR-30c-5p* (A), *miR-30b-5p* (B), *miR-182-5p* (C) and *miR-200b-3p* (D). Abbreviations: P25 – Percentile 25.

11 Tables

Table 1. Breast Cancer molecular subtypes characterization (Perou et al., 2000; Sørlie et al., 2001; Oh et al., 2006; Eroles et al., 2012; Haque et al., 2012; Network, 2012; Howell, 2013; Zhang et al., 2014a; Senkus et al., 2015).

Breast cancer subtypes		**Clinicopathological surrogate markers**	**Signature genes**	**Adjuvant systemic therapeutic options**
Luminal A		ER^+ PR high [1] $HER2^-$ Ki-67 low [2]	*ESR1* and/or *PGR*, *KRT8*/18, *GATA3*, *XBP1*, *FOXA1* and *ADH1B*	ET alone in most of cases + Cht if high tumor burden (≥N3, ≥T3)
Luminal B	**$HER2^-$**	ER^+ $HER2^-$ Ki-67 high or PR low	*ESR1* and/or *PGR*, *KRT*8/18, *FGFR1*, *ERBB1*, *MKI67 and*/or *CCNE1*, *CCNB1* and *MYBL2*	ET + Cht for the most of cases
	$HER2^+$	ER^+ $HER2^+$ Any Ki-67 Any PR		ChT + anti-HER2 + ET for all patients
Basal-like		ER^- PR^- $HER2^-$	*KRT*5/6, *KRT*17, *ERBB1* and/or *KIT*, FOXC1, TP63, *CDH3*, VIM and *LAM*	ChT
HER2-enriched		$HER2^+$ ER^- PR^-	*ERBB2* and *GRB7*	ChT + anti-HER2

[1] Suggested cut-off value is 20% [2] Ki-67 scores should be interpreted in the light of local laboratory median values. **Abbreviations**: ER – Estrogen Receptor; PR – Progesterone Receptor; HER2 - Human Epidermal Growth Factor Receptor 2; *ESR1* - Estrogen Receptor 1; *PGR* - Progesterone Receptor; *KRT* – Keratin; *GATA3* - GATA Binding Protein 3; *XBP1* - X-Box Binding Protein 1; *FOX* - Forkhead Box; *ADH1B* - Alcohol Dehydrogenase 1B (Class I), Beta Polypeptide; *FGFR1* - Fibroblast Growth Factor Receptor 1; *ERBB* - Erb-B2 Receptor Tyrosine Kinase; *MKI67* - Marker Of Proliferation Ki-67; *CCN* – Cyclin; MYBL2 - MYB Proto-Oncogene Like 2; *MYBL2* - MYB Proto-Oncogene Like 2; *KIT* - KIT Proto-Oncogene Receptor Tyrosine Kinase; *TP63* - Tumor Protein P63; *CDH* – Cadherin; *VIM* – Vimentin; *LAM* – Laminin; *GRB7* - Growth Factor Receptor Bound Protein 7; Cht – Chemotherapy; ET – Endocrine Therapy; N – Nodal Stage; T – Tumor Size.

Table 2. Clinical and pathological data of luminal tumors included in the discovery cohort.

	Molecular Subtype	Age at diagnosis	Grade	Stage	ChT	RT	Recurrency Site	Endocrine-resistant
Patients who relapsed	**Luminal A**	82	G2	IIIA	NO	NO	Liver	YES
		41	G3	IIA	YES	YES	Bone	YES
		60	UNKN	IA	NO	YES	Contralateral breast	NO
		43	G2	IIB	YES	YES	Lymph nodes	NO
	Luminal B	65	G3	IIIC	YES	YES	Lung	YES
		63	G2	IIIA	NO	YES	Bone	YES
		67	G2	IIB	NO	NO	Bone	NO
		66	G3	IIIA	NO	NO	Locoregional	NO
Patients who did not relapse	**Luminal A**	70	G3	IIB	NO	YES	n.a.	n.a.
		68	G2	IIB	NO	YES		
		69	G2	IIIA	NO	NO		
		69	G2	IA	NO	YES		
	Luminal B	65	G3	IIIC	YES	YES		
		72	G3	IIIC	NO	YES		
		70	G1	IIB	NO	YES		
		73	G1	IIIC	NO	YES		

Abbreviations: ChT – Chemotherapy; RT – Radiotherapy; UNKN – Unknown; n.a. – Not Applicable.

Table 3. Clinical and pathological data of luminal tumors and normal breast samples included in the validation cohort.

Clinipathological features	Endocrine-Sensitive	Endocrine-Resistant	NBr
Patients (n)	114	22	26
Age median (range)	61.5 (43-73)	60 (41-75)	54 (40-70)
	61.0 (41-75)		
Molecular subtype (%)			
Luminal A	53 (46.5)	3 (13.6)	n.a.
Luminal B	61 (53.5)	19 (86.4)	
Histological type (%)			
Invasive carcinoma of NST (IDC)	99 (86.8)	19 (86.4)	
Invasive lobular carcinoma	6 (5.3)	2 (9.1)	n.a.
Other special subtype carcinoma	1 (0.9)	1 (4.5)	
Mixed type carcinoma	8 (7.0)	0 (0.0)	
Progesterone receptor *status* (%)			
Positive	96 (84.2)	15 (68.2)	n.a.
Negative	18 (15.8)	7 (31.8)	
HER2 receptor *status* (%)			
Positive	10 (8.8)	6 (27.3)	n.a.
Negative	104 (91.2)	16 (27.3)	
Ki-67 index (%)			
<15%	89 (78.1)	7 (31.8)	n.a.
>15%	20 (17.5)	11 (50.0)	
UNKN	5 (4.4)	4 (18.2)	
Grade (%)			
G1	19 (16.7)	0 (0.0)	
G2	57 (50.0)	9 (40.9)	n.a.
G3	31 (27.2)	11 (50.0)	
Not determined	7 (6.1)	2 (9.1)	
Pathological T Stage (%)			
pT1	34 (29.8)	5 (22.7)	
pT2	56 (49.1)	14 (63.6)	n.a.
pT3	3 (2.6)	0 (0.0)	
pT4	5 (4.4)	1 (4.5)	
Not determined	16 (14.0)	2 (9.1)	
Pathological N Stage (%)			
pN0	42 (36.8)	8 (36.4)	
p N1	43 (37.7)	8 (36.4)	n.a.
p N2	9 (7.9)	3 (13.6)	
p N3	5 (4.4)	1 (4.5)	
Not determined	15 (13.2)	2 (9.1)	
Adjuvant RT			
Yes	85 (74.6)	19 (86.4)	n.a.
No	19 (16.7)	3 (13.6)	
Not determined	10 (8.8)	0 (0.0)	
Adjuvant ChT			
Yes	39 (34.2)	12 (54.5)	n.a.
No	59 (51.8)	8 (36.4)	
Not determined	16 (14.0)	2 (9.1)	

Abbreviations: NBr – Normal Breast Tissues; NST – No Special Type; IDC – Invasive Ductal Carcinoma; HER2 - Human Epidermal Growth Factor Receptor 2; G – Grade; RT – Radiotherapy; ChT – Chemotherapy; n.a.- Not Applicable.

612 **Table 4.** MiRNAs with fold variation values higher than 1 in the global expression assay.

LumA Rec *vs.* LumA NRec		LumB Rec *vs.* LumB NRec		Lum Rec *vs.* Lum NRec	
microRNA	**Fold Change**	**microRNA**	**Fold Change**	**microRNA**	**Fold Change**
miR-196a-5p	2.1281	**miR-9-5p** [1]	2.5978	**miR-9-5p** [1]	1.4448
miR-181b-5p	-1.0119	**miR-210-3p** [1]	1.7178	**miR-149-3p** [1]	1.23995
miR-130a-3p [1]	-1.0519	**miR-182-5p** [2]	1.6028	**miR-126-3p**	-1.0909
miR-29b-3p	-1.1169	**miR-7-5p** [1]	1.3978	**miR-1**	-1.1352
let-7b-5p	-1.1269	**miR-200c-3p**	1.2778	**miR-148a-3p**	-1.1419
let-7i-5p	-1.1369	**miR-31-5p** [1]	1.0928	**miR-30d-5p**	-1.2139
miR-106b-5p	-1.1419	**miR-221-3p**	1.0128	**miR-181a-5p** [2]	-1.4322
miR-132-3p [1]	-1.1519	**miR-125b-5p**	-1.0172	**miR-200a-3p**	-1.5732
miR-26b-5p	-1.1619	**miR-146a-5p**	-1.0372	**miR-205-5p** [2]	-2.3252
miR-19b-3p	-1.1769	**miR-181a-5p** [2]	-1.0622		
miR-192-5p [1]	-1.1969	**miR-205-5p** [2]	-1.1172		
let-7g-5p	-1.2019	**miR-1** [1]	-1.1472		
miR-16-5p	-1.2319	**miR-10b-5p**	-1.4022		
miR-15a-5p	-1.2619				
miR-106a-5p	-1.2669				
miR-20a-5p	-1.2769				
let-7a-5p	-1.3019				
miR-21-5p	-1.3169				
miR-214-3p	-1.3569				
miR-93-5p	-1.4119				
let-7f-5p	-1.4369				
miR-222-3p	-1.4419				
miR-200c-3p	-1.4719				
miR-155-5p	-1.5119				
let-7e-5p	-1.5119				
let-7d-5p	-1.5619				
miR-148a-3p	-1.6369				
miR-181a-5p [2]	-1.6519				
miR-23b-3p	-1.7569				
miR-23a-3p	-1.8069				
miR-19a-3p	-1.8519				
miR-1 [1]	-1.8869				
miR-221-3p	-1.9319				
miR-195-5p	-1.9369				
miR-18a-5p [1]	-1.9919				
miR-30c-5p [2]	-2.0419				
miR-182-5p [2]	-2.1119				
miR-186-5p [1]	-2.1319				
miR-141-3p	-2.1619				
miR-17-5p [1]	-2.1919				
miR-30d-5p	-2.2769				
miR-30b-5p [2]	-2.4819				
miR-101-3p	-2.5319				
miR-200b-3p [2]	-3.0019				
miR-92b-3p [1]	-3.1069				
miR-200a-3p	-3.2169				
miR-205-5p [2]	-4.1269				

613 [1] Cps higher than 30 [2] miRNAs chosen for further validation

614 **Abbreviations**: Lum – Luminal; Rec – Recurrent.

615

Table 5. Univariable and multivariable cox regression models assessing the association between microRNAs expression levels and clinical outcome.

Model	Outcome	Variable	HR (95% CI)	*p*-value
Univariable Analysis	ERFS	**miR-30c-5p expression categorized** ≤P25 >P25	1 0.311 (0.135-0.717)	0.006
		miR-30b-5p expression categorized ≤P25 >P25	1 0.362 (0.156-0.838)	0.018
		miR-182-5p expression categorized ≤P25 >P25	1 0.194 (0.081-0.464)	< 0.001
		miR-200b-3p expression categorized ≤P25 >P25	1 0.217 (0.091-0.518)	0.001
	DFS	**miR-30c-5p expression categorized** ≤P25 >P25	1 0.426 (0.223-0.815)	0.010
		miR-30b-5p expression categorized ≤P25 >P25	1 0.412 (0.208-0.817)	0.011
		miR-182-5p expression categorized ≤P25 >P25	1 0.213 (0.101-0.452)	< 0.001
		miR-200b-3p expression categorized ≤P25 >P25	1 0.226 (0.110-0.465)	< 0.001
	DMFS	**miR-30c-5p expression categorized** ≤P25 >P25	1 0.467 (0.234-0.932)	0.031
		miR-30b-5p expression categorized ≤P25 >P25	1 0.465 (0.224-0.964)	0.040
		miR-182-5p expression categorized ≤P25 >P25	1 0.284 (0.126-0.644)	0.003
		miR-200b-3p expression categorized ≤P25 >P25	1 0.287 (0.131-0.628)	0.002
Multivariable Analysis	ERFS	**miR-30c-5p expression categorized** [1] ≤P25 >P25	1 0.353 (0.152-0.818)	0.015
		miR-30b-5p expression categorized [1] ≤P25 >P25	1 0.367 (1.497-17.112)	0.019
		miR-182-5p expression categorized [1] ≤P25 >P25	1 0.181 (0.075-0.434)	< 0.001
		miR-200b-3p expression categorized [1] ≤P25 >P25	1 0.218 (0.091-0.522)	0.001
	DFS	**miR-182-5p expression categorized** [2] ≤P25 >P25	1 0.194 (0.091-0.415)	< 0.001
		miR-200b-3p expression categorized [2] ≤P25 >P25	1 0.246 (0.119-0.511)	< 0.001
	DMFS	**miR-182-5p expression categorized** [3] ≤P25 >P25	1 0.191 (0.081-0.454)	< 0.001
		miR-200b-3p expression categorized [2] ≤P25 >P25	1 0.314 (0.143-0.691)	0.004

[1] Cox regression model adjusted for molecular subtype. [2] Cox regression models adjusted for HER2 *status*; [3] Cox regression model adjusted for grade and HER2 *status*. **Abbreviations**: ERFS - Endocrine Resistance-free Survival; DFS - Disease-free Survival; DMFS - Distant Metastasis-free Survival.

Table 6. Cox regression models stratified according to the clinicopathological features with statistical significance in the multivariable analysis.

Outcome	Layering Variable	Variable	HR	*p*-value
ERFS	Luminal A	**miR-30c-5p expression categorized** ≤P25 >P25	-	0.555
	Luminal B	**miR-30c-5p expression categorized** ≤P25 >P25	1 0.344 (0.140-0.847)	0.020
	Luminal A	**miR-30b-5p expression categorized** ≤P25 >P25	-	0.661
	Luminal B	**miR-30b-5p expression categorized** ≤P25 >P25	1 0.344 (0.140-0.848)	0.020
	Luminal A	**miR-182-5p expression categorized** ≤P25 >P25	-	0.689
	Luminal B	**miR-182-5p expression categorized** ≤P25 >P25	1 0.145 (0.058-0.364)	< 0.001
	Luminal A	**miR-200b-3p expression categorized** ≤P25 >P25	-	0.699
	Luminal B	**miR-200b-3p expression categorized** ≤P25 >P25	1 0.178 (0.071-0.445)	< 0.001
DFS	HER2-negative	**miR-182-5p expression categorized** ≤P25 >P25	1 0.179 (0.058-0.364)	0.002
	HER2-positive	**miR-182-5p expression categorized** ≤P25 >P25	1 0.197 (0.058-0.364)	0.004
	HER2-negative	**miR-200b-3p expression categorized** ≤P25 >P25	1 0.235 (0.073-0.750)	0.014
	HER2-positive	**miR-200b-3p expression categorized** ≤P25 >P25	1 0.311 (0.113-0.858)	0.024
DMFS	Grade 1&2	**miR-182-5p expression categorized** [1] ≤P25 >P25	1 0.249 (0.076-0.819)	0.022
	Grade 3	**miR-182-5p expression categorized** [1] ≤P25 >P25	1 0.168 (0.044-0.642)	0.009
	HER2-negative	**miR-182-5p expression categorized** [2] ≤P25 >P25	1 0.235 (0.089-0.625)	0.004
	HER2-positive	**miR-182-5p expression categorized**[2] ≤P25 >P25	-	0.053
	HER2-negative	**miR-200b-3p expression categorized** ≤P25 >P25	-	0.066
	HER2-positive	**miR-200b-3p expression categorized** ≤P25 >P25	1 0.219 (0.054-0.884)	0.033

[1] Cox regression model adjusted for HER2 *status*. [2] Cox regression models adjusted for grade. **Abbreviations**: ERFS - Endocrine Resistance-free Survival; DFS - Disease-free Survival; DMFS - Distant Metastasis-free Survival; HER2 – Human Epidermal Growth Factor 2 Receptor.

Figure 1.TIF

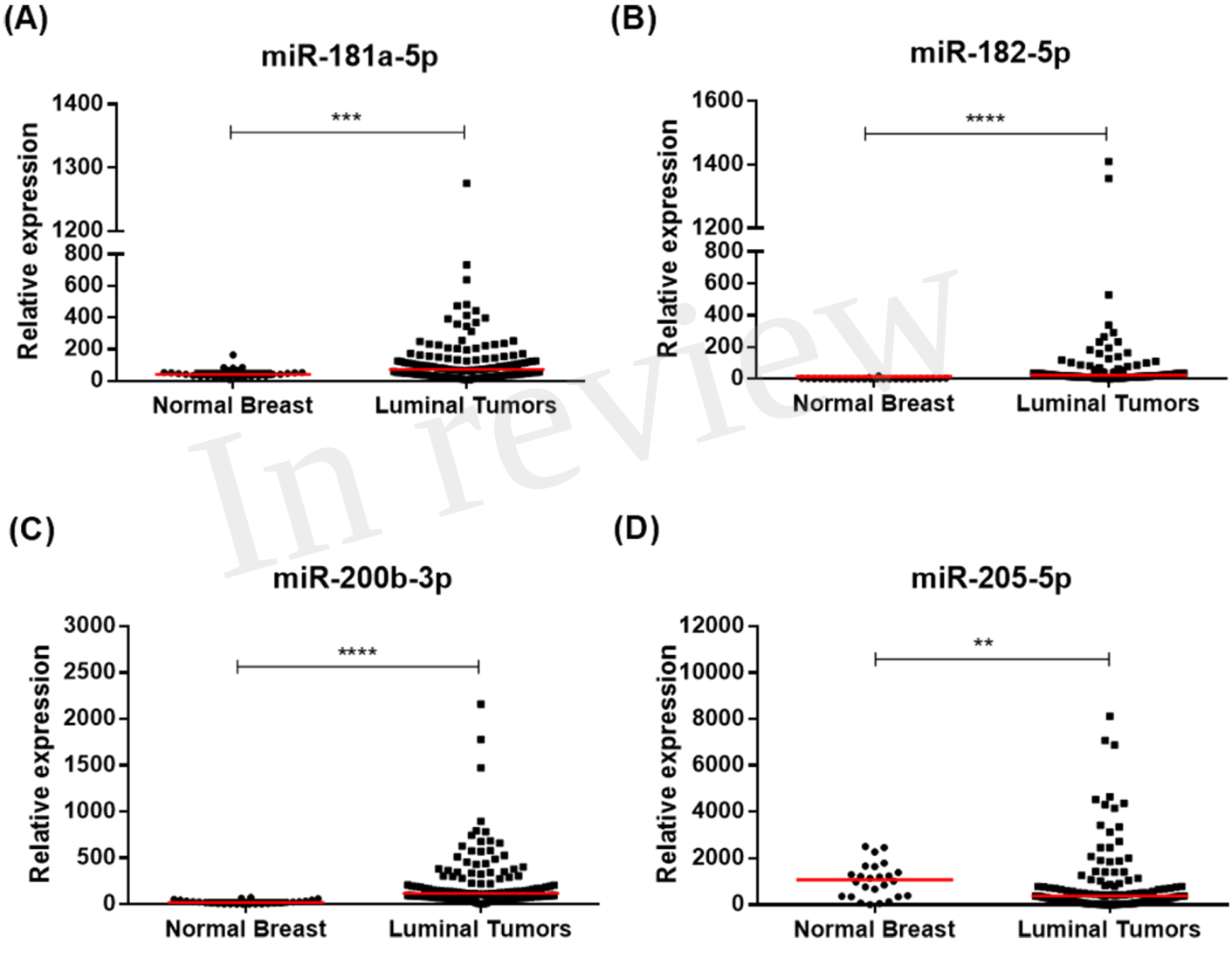
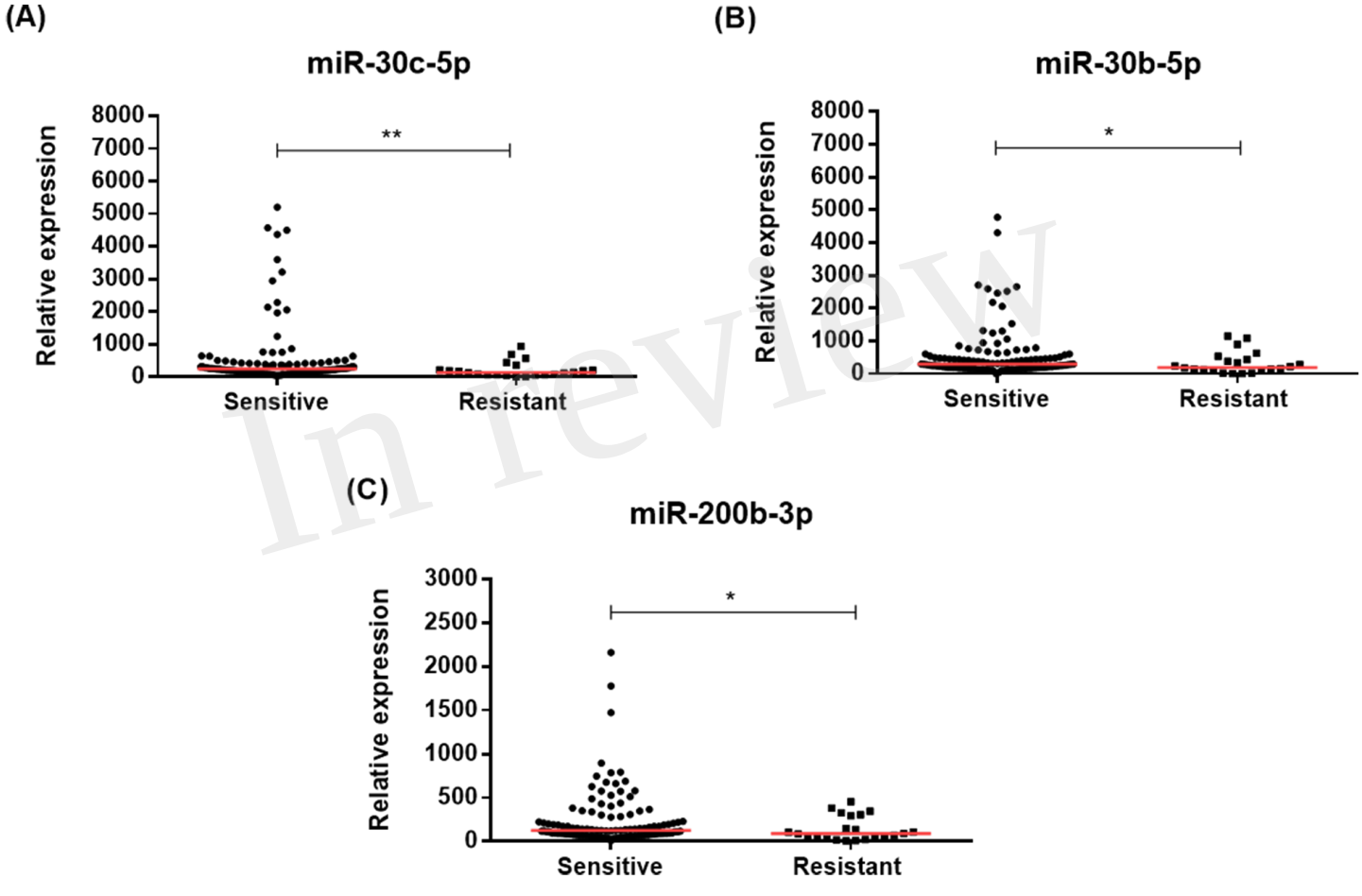


Figure 2.TIF



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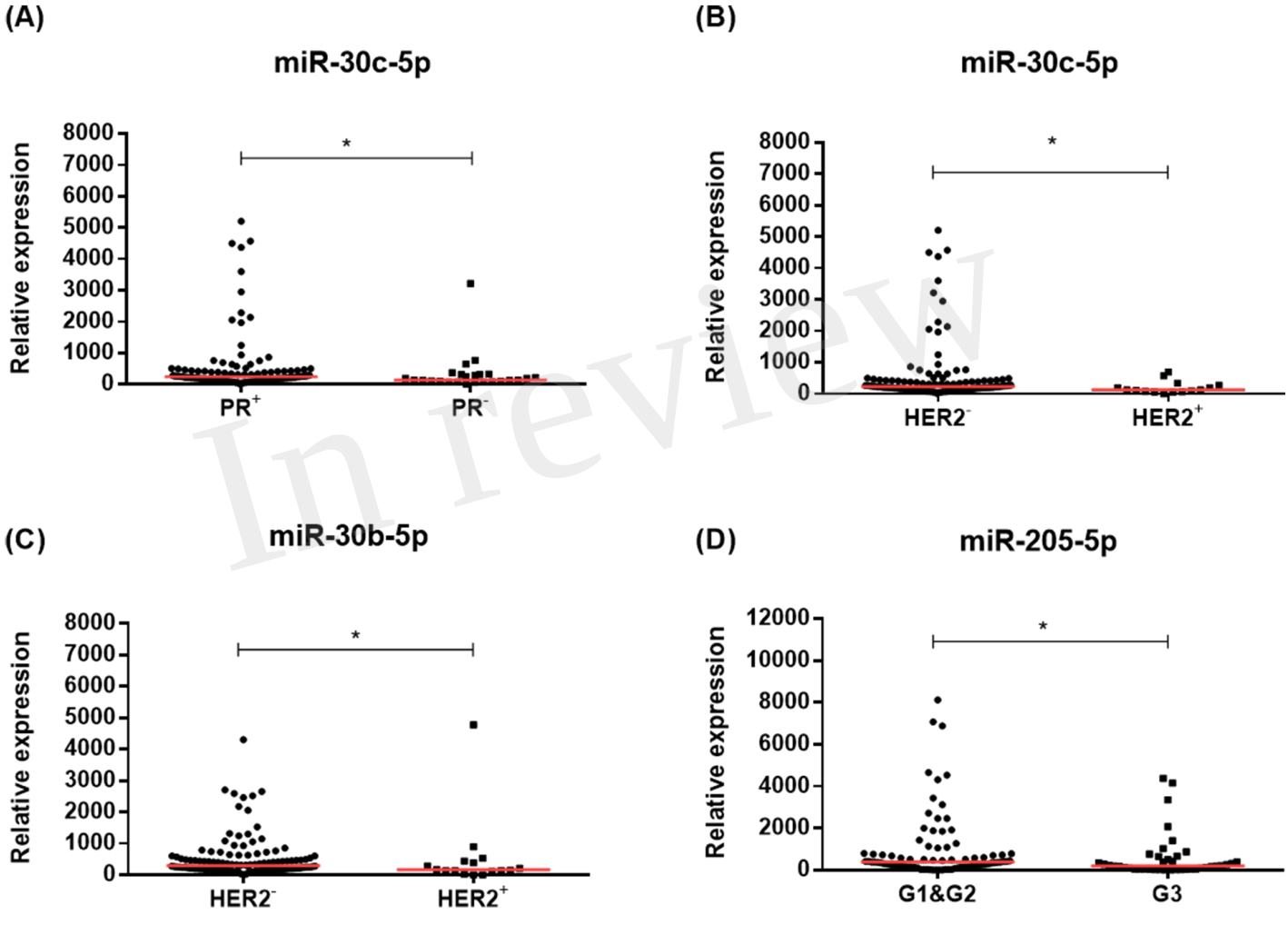

Figure 4.TIF

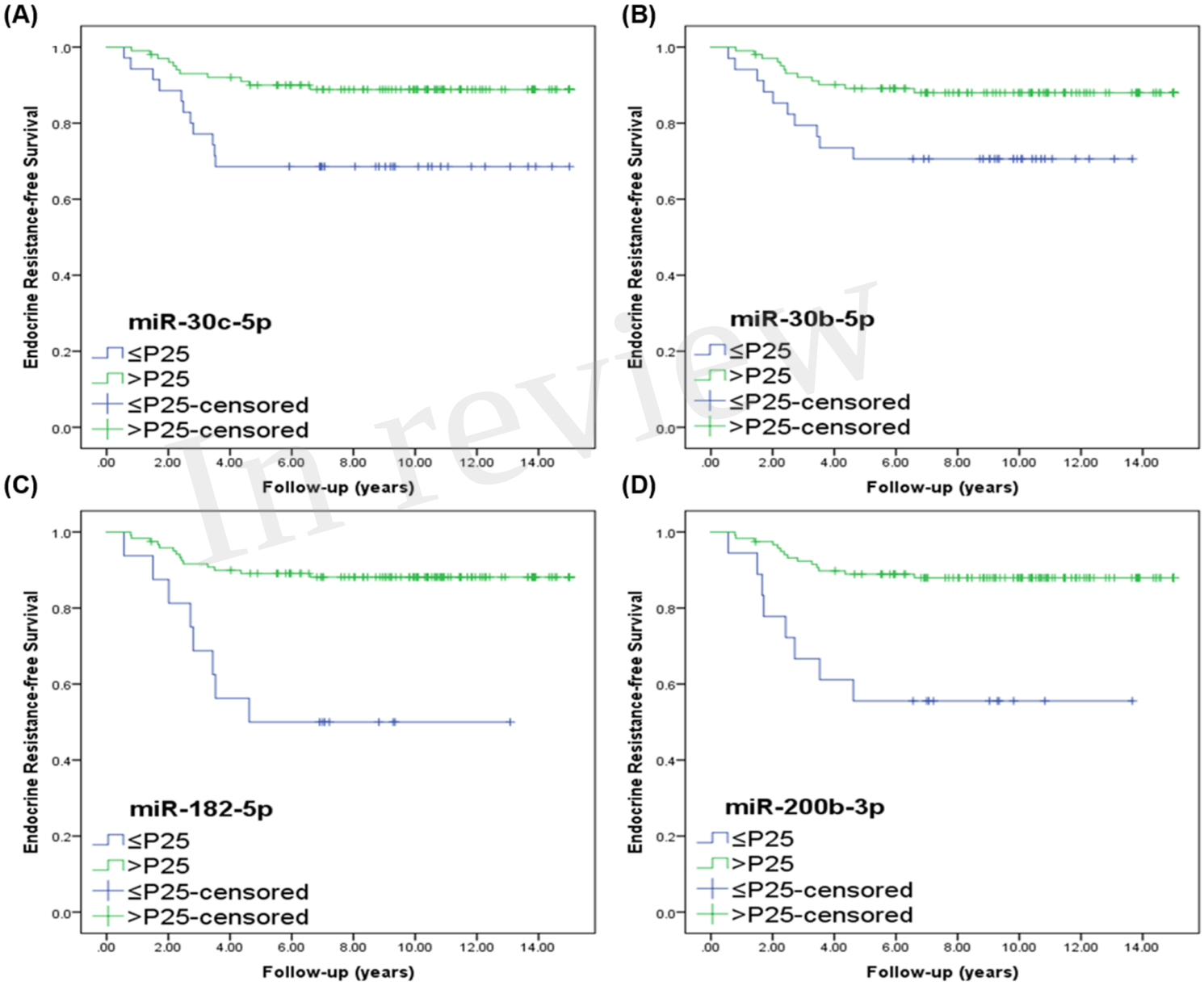

Figure 5.TIF