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mRNA expression profiles show differential regulatory effects of microRNAs between estrogen receptor-positive and estrogen receptor-negative breast cancer

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Abstract

Background

Recent studies have shown that the regulatory effect of microRNAs can be investigated by examining expression changes of their target genes. Given this, it is useful to define an overall metric of regulatory effect for a specific microRNA and see how this changes across different conditions.

Results

Here, we define a regulatory effect score (RE-score) to measure the inhibitory effect of a microRNA in a sample, essentially the average difference in expression of its targets versus non-targets. Then we compare the RE-scores of various microRNAs between two breast cancer subtypes: estrogen receptor positive (ER^+) and negative (ER^-). We applied this approach to 5 microarray breast cancer datasets and found that the expression of target genes of most microRNAs was more repressed in ER^- than ER^+. That is, microRNAs appear to have stronger RE-score in ER^- breast cancer. These results are robust to the microRNA target prediction method. To interpret these findings, we analyzed the level of microRNA expression in previous studies and found that higher microRNA expression was not always accompanied with higher inhibitory effects. However, several key microRNA processing genes, especially, *Ago2* and *Dicer*, were differentially expressed between ER^- and ER^+ breast cancer, which may explain the different regulatory effects of microRNAs in these two breast cancer subtypes.

Conclusions

Regulatory effect score is a promising indicator to measure microRNAs' inhibitory effect. Most microRNAs exhibit higher RE-scores in ER^- than in ER^+ samples, suggesting that they have stronger inhibitory effect in ER^- breast cancers.

Background

MicroRNAs (miRNAs) are a class of small noncoding (19–24 nt) RNAs that regulate the expression of target mRNAs at the post-transcriptional level [1, 2]. In higher eukaryotic organisms, it is estimated that miRNAs account for about 1% of genes and regulate the expression of more than 30% of mRNAs [3].

It has been shown that miRNAs play critical roles in a variety of biological processes such as cell proliferation[4], apoptosis [5], development [6], and differentiation [7]. In humans, strong links between cancer and miRNA deregulation have been suggested by recent studies [8, 9]. A lot of known miRNAs are found to be located in the fragile sites (regions with high frequencies of copy number alterations in cancers) of human chromosomes, indicating that many miRNAs may be linked to carcinogenesis [10]. Furthermore, it has been shown that aberrant expression of miRNAs contributes to carcinogenesis by promoting the expression of proto-oncogenes or by inhibiting the expression of tumor suppressor genes. For instance, the down-regulation of *let-7*, which represses expression of the proto-oncogene RAS, has been found in a large proportion of lung cancer specimens [11]. Another examples are *miR-15* and *miR-16*, which repress the anti-apoptotic factor BCL2 in Chronic lymphocytic leukemia (CLL) [12]. In addition, some recent studies suggest that expression profiles of miRNAs are

informative for the classification of human cancers. Based on miRNA-expression profiles, Lu *et al*. classify 334 leukemia and solid cancers, which agrees well with the developmental lineage and differentiation state of the tumors [13]. Rosenfield *et al.* demonstrate that by using miRNA as biomarkers, tumors can be classified into sub-classes according to their primary origins[14]. Nowadays, miRNAs are thought of as promising biomarkers for cancer diagnosis and prognosis.

It has been proposed that animal miRNAs regulate gene expression mainly by inhibiting translation of their target mRNAs [15, 16]. More recent studies, however, demonstrate that expression regulation at the mRNA level (via mRNA degradation or deadenylation) also serves as a critical mechanism for miRNA function in animals [17-23]. Over-expression of miRNA in cell lines cause moderate down-regulation of a large number of transcripts, many of which contain the complementary sequences of the over-expressed miRNA in their 3'UTRs [23]. Conversely, gene expression analysis from miRNA knockdown animals reveals that miRNA recognition motifs are strongly enriched in the 3'UTRs of up-regulated genes, but depleted in the 3'UTRs of down-regulated genes[20]. Motivated by these findings, several studies conducted have demonstrated the effectiveness to investigate miRNA regulation by examining their target mRNA expression levels [24-27]. For example, Yu *et al.* show that miRNA targets have lower expression levels in mature mouse and Drosophila tissues than in embryos via global analysis of miRNA target gene expression [27].

In this study, we seek to investigate the differential miRNA regulation between estrogen receptor positive (ER^+) and negative (ER^-) breast cancers via its targets' expressions. Breast cancer is a common disease, which ranks first, in terms of annual

mortality in women worldwide [28]. According to the estrogen receptor (ER) status and responsiveness to estrogen, breast cancer can be divided into two subtypes: ER^+ and ER^-. The links between miRNA expression and breast cancer have been shown using miRNA microarray techniques [13, 29]. Specifically, the expression differentiation of miRNAs between ER^+ and ER^- breast cancers has been investigated in [30-32]. In comparison with the large number of mRNA expression datasets [33-41], the miRNA expression datasets for ER^+ and ER^- breast cancer are still limited. Moreover, results and conclusions from these studies are generally not consistent and sometimes even conflicting [30-32]. In this study, we take advantage of those mRNA expression data sets to investigate the differential miRNA regulation between ER^+ and ER^- breast cancers.

For each miRNA, we calculate a regulatory effect score (RE-score), which measures the expression difference between the targets and non-targets of this miRNA in an expression profile. Then, we compare the RE-scores of miRNAs in ER^+ tumor samples with those in ER^- samples to identify RE-changing microRNAs. We applied our method to five independent microarray datasets that include gene expression profiles for both ER^+ and ER^- samples. In all of them, our results indicate that the majority of RE-changing miRNAs showed higher RE-scores in ER^- than in ER^+, suggesting stronger inhibitory effects of miRNAs on their targets in ER^- breast cancer. To check the robustness, we performed the same analyses by using different miRNA target prediction methods, RE-score calculation methods, RE-changing miRNAs identification threshold and obtained consistent results. Moreover, we examined the expression levels of genes in the miRNA biogenesis pathway and found that *Ago1* and *Ago2* genes (encoding argonautes, the key proteins forming the RISC complex) had

significantly higher expression levels in ER^- than in ER^+ breast cancer. This may suggest higher RISC activities and therefore higher efficiency of miRNAs to down-regulate target gene expression in ER- breast cancer.

Results and discussion

Identification of RE-changing miRNAs between ER^+ and ER^- breast cancers

To measure the inhibitory effect of a miRNA, we calculate a score named regulatory effect score (RE-score), denoted as the difference of average ranks between its non-target and target genes. It should be noted that the RE-scores for different miRNAs may not be directly comparable, because they regulate different sets of target genes. However, we can compare the RE-scores for the same miRNA in different conditions (i.e. using different expression profiles). A higher RE-score indicates lower expression levels of target genes and thereby a stronger inhibitory effect of the corresponding miRNA. Given a breast cancer microarray dataset, we calculate the RE-scores for each miRNA in all samples. Then, we compare the RE-scores in ER^+ and ER^- samples to identify miRNAs that show different regulatory effects between these two breast cancer subtypes. We refer to these miRNAs as RE-changing miRNAs. Using ER^+ as the reference, some RE-changing miRNAs show stronger inhibitory effect, while the others show weaker inhibitory effect in ER^- breast cancer. The false discovery rate (FDR) was estimated using a similar method to the SAM analysis [42]. The flow diagram of our analysis is shown in Figure 1.

Most miRNAs show stronger inhibitory effects in ER^- than in ER^+ breast cancer

We applied our analysis to five carefully selected large scale microarray datasets, each containing at least 30 expression profiles for both ER^+ and ER^- breast cancer samples. Among these datasets, four were measured by one-channel affymetrix GeneChips and one was measured by two-channel cDNA arrays (see Materials and Methods for details about these data sets). For each dataset, we calculated the RE-scores of each miRNA in all samples. To do this, we needed to determine the target and non-target gene sets for miRNAs. Several computational methods have been developed to identify microRNA targets and the predictions could be considerably different (Additional data file 1, the distribution of miRNA targets numbers for different prediction tools). In our analysis, the target genes for miRNAs were predicted by using the PITA algorithm, which has been shown to have high prediction accuracy [43]. Subsequently, we computed the t-scores (ER^- versus ER^+) to measure the difference of RE-scores between ER^- and ER^+ samples. A positive t-score for a miRNA suggests that this miRNA has higher overall RE-scores, and thereby stronger inhibitory effect to its targets, in ER^- samples. Conversely, a negative ER^-/ER^+ t-score indicates stronger inhibitory effect of a miRNA in ER^+ samples. For example, to estimate the RE-score of miR-371 in a sample of the HE (Hess *et al.* [44]) dataset, first, we grouped the total 14327 genes in the HE dataset into two sets, one with 2054 targets, the other with 12273 non-target genes. Second, we sorted the 14327 expressions and computed the average ranks of the 2054 targets and 12273 non-targets, respectively. The RE-score for miR-371 in each sample was calculated as the average rank of non-targets subtracted by the average rank of the targets. We performed the RE-score calculation in 82 ER^+ samples and 51 ER^- samples and found that the RE-scores in ER^- are significantly higher than those in ER^+ samples (T test,

P=3.74E-15). We also compared the RE-scores in ER^+ with those in ER^- in the other 4 datasets. As shown in Figure 2a, in all of the 5 datasets, the RE-scores for miR-371 are significantly higher in ER^-. Namely, miR-371 represses the expression of its target mRNAs more efficiently in ER^- breast cancers. In the next section we discuss the results based on other miRNA target prediction methods.

We calculated the ER^-/ER^+ t-scores (measuring the difference of RE-scores in ER^- versus ER^+ samples) for 470 human miRNAs in all of the 5 datasets. Interestingly, we found that most miRNAs exhibit higher RE-scores in ER^- than in ER^+ samples, as suggested by the distributions of their t-scores in Figure 2b. We calculated the significance of the t-scores based on the permutation test using a similar method to SAM [42] (see Materials and method for detail). At the 0.05 significance level (FDR $\leqslant$0.05), we identified 109, 188, 15 and 306 RE-changing miRNAs from a total of 475 miRNAs in HE (Hess *et al.* [44]), MI (Miller *et al.* [38]), MN (Minn *et al.* [39]) and VA (van't Veer *et al.* [34]) datasets, respectively, and all of them show higher inhibitory effect in ER^- breast cancer. In the WA (Wang et al. [40]) dataset, we identified 377 RE-changing miRNAs, among which 373 were with higher inhibitory effect and only 4 were with lower inhibitory effect in ER^- breast cancer. This suggests that most miRNAs exhibit stronger inhibitory effects to the expression of their targets in ER^- with respect to ER^+ breast cancer. This conclusion can still be drawn when we relaxed the FDR threshold to 10% and 20%, as illustrated in Figure 2c. The t-score, p-value and FDR of each miRNA from all datasets were provided in the Additional data file 2.

Use of other miRNA target prediction algorithms

Next, we investigated whether similar results can be obtained using other miRNA target prediction methods. It has been shown previously that distinct miRNA prediction methods may result in considerably different target gene sets (Additional data file 1, the distribution of miRNA targets numbers for different prediction tools). To rule out the possible bias introduced by PITA, we repeated our analysis using three other miRNA target prediction methods: TargetScan[3], PicTar[45] and miRanda[46]. We chose these three out of a handful of miRNA target prediction methods not only because they have been prevalently used but also because they are, in some sense, complementary to the PITA method. Almost all miRNA target prediction methods first scan the 3'UTR of transcripts for potential miRNA binding sites that are complementary to the seed region of miRNAs. TargetScan and PicTar meet stringent seed pairing criteria, whereas the criteria are moderately stringent in PITA and miRanda. To further increase the prediction accuracy, PITA takes into account the local accessibility of the potential binding sites, whereas miRanda and PicTar apply a different strategy: they filter out those miRNA binding sites in non-conserved regions. TargetScan, the most widely used prediction method, considers both site conservation and context accessibility.

The results based on PicTar and miRanda are illustrated in Figure3a and 3b. As shown, the t-scores for RE-score comparison in ER^- versus ER^+ are more likely to be positive values in all 5 datasets, suggesting stronger inhibitory effects of miRNAs to their targets in ER^- breast cancer. Since both PITA and miRanda can require moderately stringent miRNA seed:target complementarity, in order to obtain more reliable target and non-target gene sets for miRNAs, we also tried another strategy:

combining the prediction results of PITA and miRanda methods. For each miRNA, we define its target genes as those predicted by both methods and its non-target genes as those predicted by neither. This will presumably decrease both false positive and false negative prediction rates. Based on this target and non-target gene sets definition, we again obtain similar results as shown in Figure3c.

TargetScan is currently the most widely used microRNA target prediction tool, which relies on strict miRNA seed region complementarity [3, 47]. In addition, the conservation of binding site, the context of the miRNA-binding site, the proximal AU composition, and proximity to sites for co-clustered miRNAs can enhance the targeting efficacy of a binding site[48]. Choosing different parameters in target prediction results in quite different performances[49]. Among the parameters, site conservation and site accessibility (measured as context score) are the two most important ones[50, 51]. To evaluate the performance of different TargetScan cutoffs in the RE-score comparison, we chose three target sets: the binding site is conserved, the context score is more than -0.20, and all potential targets, which we referred to as ConservedTS, ContextTS, and AllTS, respectively. These three TargetScan predictions are quite different. On average, 210, 765, and 2026 targets per miRNA are predicted in ConservedTS, ContextTS and AllTS, respectively. After integrating mRNA expression data with all three target sets for comparing the RE-scores in ER^- and ER^+, we found again, that t-scores for ER^- versus ER^+ are more likely to be positive in all 5 datasets, as illustrated in Figure3d-f. This demonstrates that the observation of higher RE-scores in ER^- breast cancer, for most miRNAs, is not likely caused by a bias from the miRNA prediction method. Complete results based on three

TargetScan predictions, miRanda, PicTar, and the intersection of PITA and miRanda can be found in Additional data file 2.

Use of alternative methods to compare the miRNA inhibitory effects

To further substantiate our findings, we also used two alternative methods to investigate the inhibitory effects of miRNAs in ER^+ and ER^- breast cancers. The first method is similar to the one described above, but we use a different way to calculate the RE-scores for miRNAs in an expression profile. Instead of computing the average rank difference between the target and non-target gene sets for a miRNA, we calculate the RE-score as follows: (1) calculate the relative expression levels of each gene across all of the samples by subtracting the mean and then dividing by the standard deviation; (2) calculate the RE-score of a miRNA by comparing the relative expression levels of its target and non-target genes. For clarity, we will refer to these two RE-score calculation methods as rank comparison and expression comparison. Similar to what we have found by using rank comparison method, RE-scores obtained from expression comparison tend to be higher in ER^- samples as indicated by the t-score (ER^- versus ER^+) distribution (Figure4a). These results are not dependent on the miRNA target prediction method because similar results are obtained using PITA and miRanda (complete results are shown in Additional data file 3). As a matter of fact, the t-scores, obtained by using expression comparison and rank comparison, are highly correlated. For example, for the VA dataset, these two methods yield two sets of t-scores with a correlation coefficient of 0.928 (Figure4b). As shown, 432 out of 466 miRNAs have positive t-scores from both methods, confirming stronger inhibitory effects of miRNAs in ER^- breast cancer.

The other method, referred to as ARR(Adapted Ranked Ratio), is similar to the ranked ratio (RR) method proposed by Yu *et al.*[27]. First, the expression levels of each gene in ER^+ and ER^- samples were compared and a t-score (ER^+/ER^-) was calculated to measure the expression differentiation of this gene in the two breast cancer subtypes. The t-scores for all genes were then ranked and genes were divided into two groups, ones with high t-scores and ones with low t-scores. For each miRNA, the ARR value was calculated by dividing the number of target genes in the "low" ranked group by the "high" ranked group. The ARR value is an indicator of the distribution of a miRNA's targets within all genes. A low ARR value (ARR<1) indicates that this miRNA has more targets in genes with higher t-scores, i.e. genes that are lowly expressed in ER^- samples. That is, the target genes of this miRNA tend to have lower expression levels in ER^- breast cancer. We calculated the ARR values for all miRNAs in each of these 5 datasets. The numbers of miRNAs with ARR<1 and ARR>1 are listed in Table 1. As shown, more miRNAs have ARR<1 in all datasets, indicating their stronger inhibitory effect in ER^- breast cancer.

Although the ARR method is similar to the RR(Ranked Ratio) method described by Yu *et al.*[27], they differ in some ways. The RR value for a miRNA in a tissue is calculated by dividing the number of targeted genes with 'low' expression by the number of target genes with 'high' expression after the expression levels of each gene across a series of tissues are ranked and split into 'high' and 'low' halves. In our ARR method, we first performed t-test to compare the expressions of each gene between ER^+ and ER^- samples. The t-scores were ranked and genes were divided into two halves, one with high rank and the other with low rank. The ARR value of each gene was then calculated by dividing the number of targets with high rank by the number

of targets with low rank. Comparing with the RR method described by Yu *et al.*[27], our method is different in three perspectives: First, for each gene, the expressions were compared between ER^+ and ER^- samples. To reveal the expression difference between two groups, t-score is more effective than the ranks across all samples. Second, the ARR value from our method is actually an indicator of difference between the expression distribution of a microRNA's target genes and that of all genes. Therefore, it reflects directly the regulatory effect on target genes of a microRNA. Third, for a microRNA, only one ARR value is obtained based on the whole dataset with our method, and the ARR value facilitates a global inspection of the inhibitory acitivities differences of a microRNA between two sample groups.

Although the calculations of RE-score and ARR value are completely different, the results of RE-score and ARR value are highly consistent. We compared the results of the RE-scores from expression comparison methods and ARR methods. First, we computed the spearman correlation of the RE-scores and the ARR values for each microarray dataset. As illustrated in Table 2, the inhibitory activities calculated by these two different methods are highly correlated, with the correlation coefficients ranging from 0.578 to 0.861, which provides further confirmation that more microRNAs show higher inhibitory effects in ER^- breast cancers. Second, we overlapped the microRNAs with higher or lower inhibitory activity in ER^- cancers predicted by the RE-score and ARR values (Table 3). If a microRNA reveals t-score(ER^-/ER^+)>0 in the RE-score comparison and ARR<1 in the adapted ranked ratio calculation, it is predicted with higher activity in ER^- cancers consistently by both methods, whereas a microRNA with both t-score<0 and RR>1 shows consistently higher activity in ER^+. The overlapped consistent miRNAs are more than

80%, indicating that these two methods are in strong agreement. Furthermore, the miRNAs with consistently higher activity in ER^- are much more than those with consistently lower activity in ER^+, again indicating that most miRNAs exhibit higher regulatory effect in ER^- than in ER^+ samples. Some significant miRNAs can be identified by both methods. For example, it has been reported that miR-206, which regulates the estrogen receptor, showed higher activity in ER^- cancers than ER^+ cancers[52]. In our calculations, for all 5 microarray datasets, the ARR values of this microRNA are all below 1, and the t-scores for expression comparison of RE-scores between ER^- and ER^+ cancers are all above 0 (Table 3). These results are consistent with the activity difference between ER^+ and ER^- reported by Adams *et al.*[52].

Differential regulatory effects of miRNAs can not be explained by miRNA expression difference between ER^+ and ER^- cancer

To understand why miRNAs tend to have stronger inhibitory effects on their targets in ER^- breast cancers, we asked whether they are more highly expressed in ER^-. Using miRNA microarray technologies, expression levels of miRNAs have been previously measured and compared in ER^- and ER^+ samples in 3 different studies [30-32]. Iorio *et al.* identified 11 miRNAs that were differentially expressed between ER^+ and ER^-, among which 8 were down-regulated in ER^- [31]. In contrast, many more miRNAs were reported to be differentially expressed by Blenkiron *et al.* [30] and Mattie *et al.* [32]. Specifically, Blenkiron *et al.* identified 35 differentially expressed miRNAs, among which 11 were up-regulated and 24 were down-regulated in ER^-. Mattie *et al.*, however, reported that the majority of differentially expressed miRNAs were down-regulated in ER^- (40 out of 43). These 3 miRNA expression studies do not support the idea that miRNAs tend to be more highly expressed in ER^- than ER^+ breast cancer. It

should be noted that the three studies obtained substantially different results due to the technological issues of miRNA microarray experiments.

In addition, to measure the correlation between miRNAs' inhibitory effects and their expression levels, we computed the spearman correlations of the t-scores for miRNA expression comparison and those for miRNA RE-score comparison. As illustrated in Table 4, there is only a very weak positive correlation between them; particularly, the miRNA expression data published by Mattie *et al.*[32], shows almost no correlation with miRNA regulatory effects predicted from all the five mRNA expression datasets. This further indicates that the stronger inhibitory effect of miRNAs in ER^- cannot be explained by their expression levels.

Some microRNAs reveal big inconsistency between their expressions and RE-scores. For example, many studies have suggested that the expression levels of *Dicer*, the key gene to the generation of microRNAs, vary in different cancer subtypes [53-55]. In our study, *Dicer* is significantly down-regulated in ER^- with respect to ER^+ cancers (see next section for details). A possible mechanism is that it is regulated epigenetically[56]. Six microRNAs, *miR-103*, *miR-122a*, *miR-130a*, *miR-148a*, *miR-19a*, and *miR-29a* are commonly predicted to target *Dicer* by the four prediction methods, PITA, miRanda, PicTar and Targetscan. We investigated the expressions of these microRNAs in two distinct datasets respectively published by Blenkiron *et al.*[30] and *Mattie et al.*[32]. The expressions of these microRNAs are mostly lower in ER^- (Figure 5), which is opposite to our inference that they may be up-regulated to transcriptionally repress *Dicer* in ER^-. We then compared the RE-scores of these microRNAs in ER^+ and ER^- cancers. To our surprise, almost all microRNAs show

stronger inhibitory effects in ER^- cancers (Figure 5), which may explain why *Dicer* is expressed less in ER^-. Especially, *miR-122a*, which was reported to target Dicer and function in various cellular stresses[57, 58], expresses significantly lower in ER^- but shows significantly higher inhibition in ER^-, strongly indicating that the differential regulatory effects of miRNAs can not be explained by miRNA expression difference between ER^+ and ER^- cancer.

Several studies have reported that good classification for cancer subtypes can be achieved from the expressions of miRNAs[13, 14]. Because striking differences of the RE-scores for a set of miRNAs between ER^+ and ER^- are observed, the RE-score of miRNA can be a promising predictor for breast cancer subtype classification. We used the RE-scores of the top 8 significantly RE-changing miRNAs in the MN[39] datatset to classify the ER^+ and ER^- subtypes. As expected, the accuracy was up to 89.29%. The RE-score profiles of these miRNAs are plotted in Figure 6. The classification accuracies were comparable to even better than that estimated from the classification using the expressions of top 35 differentially expressed miRNAs in the dataset published by Blenkiron *et al.*[30], 85.76%, suggesting that it is an alternative way to predict ER status of breast cancer based on miRNA regulatory effect or miRNA targeted mRNA expression rather than based on miRNA expression.

Differential expression of miRNA processing genes between ER^+ and ER^- breast cancers

In addition to miRNA abundance, post-transcriptional regulation of miRNA expression may also be important for the inhibitory effect of miRNAs to their targets. Deregulation of genes required for miRNA biogenesis may be expected to lead to

global changes in miRNA expression as well as the inhibitory effect of miRNAs. Therefore, we examined whether miRNA processing genes are differentially expressed in ER^+ and ER^- breast cancers.

We found that among the miRNA processing genes, *Ago1* and *Ago2* were significantly up-regulated in ER^- with respect to ER^+ samples in all datasets with combined P-values of 4.0E-8 and 2.0E-10, respectively; whereas *Dicer* and *TRBP* were significantly down-regulated with combined P-values of 8.8E-6 and 2.9E-10, respectively (Figure 7b and Additional data file 4). Differential expression of *Ago1*, *Ago2* and *Dicer* between ER^+ and ER^- breast cancer has been previously investigated and consistent results were reported by Blenkiron *et al.* [30]. As shown in Figure 7a, several proteins play a critical role in the miRNA processing pathway. DROSHA, a dsRNA-specific ribonuclease, digests the pri-miRNA in the nuclease to release hairpin, precursor miRNA (pre-miRNA) [7]; then DICER, a member of the RNase III nuclease, cleaves the pre-miRNA into a single-stranded mature miRNA with the assistance of TRBP [59]; finally, the mature miRNA is incorporated into the RNA-induced silencing complex (RISC) consisting of DICER, TRBP, AGO and several other proteins [60-62]. Among the 8 human AGO proteins, AGO1 and AGO2 are known to play the most important roles in transcriptional silencing mediated by miRNAs or siRNAs. Assembly of human RISC minimally requires AGO2, DICER, and TRBP, among which AGO2 is the catalytic engine owing to its endonuclease activity and the DICER-TRBP complex acts simply as a platform [60, 63, 64]. The relatively lower abundance of AGO1 and AGO2 proteins in ER^+ breast cancer may limit the activity of functional RISC complex, which would in turn lower the inhibitory effect of miRNAs on their targets. Moreover, since the expression levels of

Dicer and *Ago* genes are anti-correlated in ER^+ and ER^-, there is no necessary link between the mature miRNA expression levels and the RISC activity. This may also explain the global up-regulation of miRNA expression levels in ER^+ observed by Blenkiron *et al.* since *Dicer* is significantly up-regulated [30].

It seems that the key genes in microRNAs biogenesis pathway are subjected to delicate regulation and their differential expression is likely to be associated with distinct tumor subtypes. More interestingly, genes in this pathway are not consistently regulated: *Dicer* and *TRBP* which are involved in miRNA maturation and RISC complex assembly are down-regulated whereas the catalytic engine of the RISC complex is up-regulated in ER^- relative to ER^+ breast cancer. As a result, the capability of miRNAs (or more precisely the RISC complex) to repress their targets may not be reflected by their expression levels. Using microRNA microarray experiments, Blenkiron *et al.* found that the most differentially expressed miRNAs between ER^+ and ER^- are down-regulated in the latter [30]. They also examined the correlation between miRNA expression and changes in mRNA levels of direct target but failed to detect the enrichment for down- or up-regulation of predicted target miRNAs consistent with miRNA expression differentiation in most cases. This can be explained by the hypothesis that many miRNAs act at the level of translation rather than mRNA stability; nevertheless, this can also be explained by the discordance of expression change between the key miRNA processing genes. Our results demonstrate that miRNAs tend to have stronger inhibitory effect to their mRNA targets in ER^- breast cancer, suggesting that the AGO proteins (up-regulated in ER^- at the mRNA level), the catalytic engine of the RISC complex, may eventually determine the efficiency of miRNAs to down-regulate their targets. In addition,

deregulation of the key genes in the miRNA biogenesis pathway may be related to tumorigenesis of certain cancer types, as has been suggested by the fact that down-regulating DICER expression promoted tumorigenesis in vitro and in a mouse lung cancer model[65].

It has been reported that *Ago2* expresses more in ER^- than in ER^+ breast cancer cell lines, and the event is correlated with active ER signaling [66]. AGO2 enhances cell proliferation, reduces cell-cell adhesion, and increases cell migratory ability, which contributes to the tumor phenotype transformation from ER^+ to ER^- through overexpression of *Ago2*. Either gene amplification or activation of cell signaling cascades make *Ago2*'s expression elevated in ER^- cancer cells. Up to now, no clear evidence or experimental data shows that the gene *Ago2* amplifies in ER^-. Epidermal growth factor receptor (EGFR) and mitogen activated protein kinase (MAPK) signaling cascade are the major signal transduction pathways in ER^- breast cancers[67, 68]. One of the frequent and remarkable features in ER^- is the up-regulated *EGFR*[69]. Adams *et al.* proposed and confirmed that EGF stimulated *Ago2* expression in ER^- cancers and that the event was primarily regulated by the MAPK pathways[66]. In addition, with the overexpression of *Ago2*, the inhibition activity of *miR-206* was elevated, whereas without *Ago2* the activity of *miR-206* remained unchanged even with the overexpression of *miR-206*, suggesting that formation of Ago2-miRNA complexes are the factors to influence the activity for microRNA inhibition function[70]. This is consistent with our finding that the activity of a microRNA cannot be explained merely by its expression level. Upon this suggestion, a hypothesis can be provided that on the elevated expression of *Ago2*, miRNA's inhibition activity is accordingly increasing, which leads to an inappropriately low expression level of

the genes involved in ER^+ cell type and the predominance of oncogenic pathways for cancer cells progressing to ER^-.

Despite growing evidence that *Dicer* mRNA levels vary between different tumor subtypes and that these variations are correlated with cancer progression[53-55], the regulation of Dicer remains unclear. Weisen *et al.* reported that the Type I interferon represses *Dicer*[56]. As known, MAPK signaling pathways are a major cascade in ER^- cancers[68]. Type I interferon signals can be transduced by the MAPK pathway[71]. The activated MAPK pathway in ER^- cancers may enhance the signal of Type I interferon, which results in the inhibition of *Dicer* expression. Another possible explanation of the low expression of *Dicer* in ER^- cancers may be the regulation effect of the miRNAs. DICER's epigenetic regulation could also occur via specific mechanisms involving the DICER 3'UTR and the binding of microRNAs[56]. In this study, we have shown that the activity of miRNAs is stronger in ER^- than in ER^+, and thereby *Dicer* is targeted and suppressed to a lower level in ER^- with respect to ER^+ cancers.

Conclusions

In this study, we created a score referred to as the regulatory effect score (RE-score), to measure the inhibitory effect of a miRNA to its targets. Based on RE-score calculations, we compared the targets-inhibitory effects of miRNAs between two breast cancer subtypes, ER^+ and ER^-. MiRNAs that showed significantly different inhibitory effect were identified for 5 independent datasets. We found that for most miRNAs the target genes were more repressed in ER^- than ER^+, suggesting stronger

inhibitory abilities of miRNAs in ER^-. The exact identity of miRNA targets seems not important since these findings are robust to several distinct methods of miRNA target prediction and further consolidated by another two methods for miRNA regulation comparison. To seek the potential mechanisms that contribute to the inhibitory effect of miRNAs, we explored the miRNA abundance measured by miRNA microarrays and the expression levels of genes involved in miRNA biogenesis and function. Our analysis indicates that high target-inhibitory ability is not necessarily associated with high miRNA expression levels, because previous miRNA expression data do not suggest prevalent over-expression in ER^- breast cancer. However, it is interesting to find that several key miRNA processing genes are significantly differentially expressed between ER^+ and ER^-: *Ago1* and *Ago2* are significantly up-regulated in ER^-, while *Dicer* and *TRBP* are significantly down-regulated. These results imply that the miRNA processing pathway is subject to the subtle regulation and that deregulation of key genes in this is involved in the cancer pathology. This method is easily applied and can be used to investigate the miRNA regulation underlying other microarray datasets.

Materials and methods

Breast cancer microarray Data sets

All the microarray data used in this study was downloaded from public databases or from the websites provided by the original publications. Over 10 breast cancer datasets have been generated in previous studies [33-41, 44]. From these datasets, we chose 5 according to the following criteria: (1) contain at least 30 samples for both ER^+ and ER^- breast cancer; (2) expression of ER^+ and ER^- samples is measured using

the same platform. The first criterion is to ensure a high power of statistical analysis, while the second criterion is to avoid bias introduced by platform effect. Among these 5 datasets, one used cDNA arrays and the other four used oligonucleotide arrays produced by Affymextrix. Sample numbers of ER^+ and ER^- in each data set are listed in table 5. The expression values are represented by normalized log ratios for cDNA microarrays or by log-transformed intensities after RMA normalization for Affymetrix oligonucleotide microarrays [72]. The probe or probeset IDs are mapped to NCBI Refseq IDs. When multiple probe sets are mapped to the same Refseq ID, their values are averaged to represent the expression level of this Refseq gene.

miRNA target predictions

A number of miRNA target prediction approaches have been suggested in the past few years [43, 45-47]. In this paper, we utilize four sets of miRNA target prediction data by the PITA [43] , miRanda [46], PicTar [45]and TargetScan[3, 47], respectively. All miRNA target prediction datasets were downloaded from the most recently updated websites. To facilitate the analysis, the target gene IDs were also converted into NCBI refseq IDs. For each miRNA, the target genes are defined as those presented in the microarray data and predicted to contain at least one binding site at their 3'UTR; the non-target genes are defined as those presented in the microarray data but are not predicted to be regulated by this miRNA.

Measuring a miRNA's inhibitory effect with the average rank difference between its targets and non-targets

To measure the inhibitory effect for a miRNA, we defined a score, regulatory effect score (RE-score), which measures the difference in expression levels between its

target and non-target genes. The RE-score can be calculated in two ways: one is based on the rank comparison and the other is based on expression comparison.

The RE-score based on rank comparison is calculated as the follows. We denote the number of a miRNA's targets and non-targets as N_t and N_n, respectively. After sorting the expressions of all genes, the rank of a target genes and a non-target genes are denoted as R_t and R_n, respectively. The RE-score of a miRNA is defined as the difference of the average rank between its targets and non-targets:

$$S_{RE} = \overline{R}_n - \overline{R}_t = \frac{\sum_{N_n} r_n}{N_n} - \frac{\sum_{N_t} r_t}{N_t}$$

where $\overline{R}_t$ and $\overline{R}_n$ represent the mean of target's ranks and non-target's ranks, respectively. The RE-score is essentially a transformation of the sum rank statistic (the sum of ranks for target genes) used in the Wilcoxon rank sum test. Since genes with high absolute expression values have high rank values, a positive RE-score indicates that the non-target genes of a miRNA tend to be expressed in higher levels than its target genes, presumably due to the inhibitory effect of miRNA to its target genes. The higher the RE-score, the more inhibitory effect of a miRNA to its targets.

Identifying microRNAs which RE-scores are significantly changed

To investigate the difference of a miRNA regulatory effect between ER^+ and ER^- breast cancer, two sample t-test was performed to compare RE-scores and determine whether the RE-scores of a miRNA are significantly different between ER^+ and ER^- cancers.

Since usually hundreds of miRNAs are examined simultaneously, multiple testing corrections needed to be considered. We calculated the false discovery rate (FDR) based on the permutations similar to the method in the SAM analysis [42]. If there were N_1 ER^+, and N_2 ER^- samples, the t-scores obtained from comparing RE-scores for each miRNA were calculated in the original data, denoted as $T_{RES}(r)$ for the rth miRNA. We then permutated the samples; at each permutation, N_1 samples were randomly selected to form one permuted ER^+ group, and the rest of the samples were used as the permutated ER^- group. The permutated t-score, denoted as $T_{RES}(r, k)$, for the r^{th} miRNA in the k^{th} permutation, is recalculated. We then considered the histogram of all $T_{RES}(r, k)$ over all r and k, and used this null distribution to compute an FDR value for a given t-score $T_{RES}(r) = T_{RES}^*$. If $T_{RES}^* \geqslant 0$, the FDR is the ratio of the percentage of all (r, k) with $T_{RES}(r, k) \geqslant 0$, whose $T_{RES}(r, k) \geqslant T_{RES}^*$, divided by the percentage of miRNAs with $T_{RES}(r) \geqslant 0$, where $T_{RES}(r) \geqslant T_{RES}^*$, and similarly if $T_{RES}^* < 0$.

If the FDR for a miRNA is below a predefined threshold, we call this miRNA as a significantly RE-changing miRNA.

ER^+ and ER^- subtypes classification

The miRNA RE-score is a promising feature to classify tumor subtypes, as well as the microRNA expression. In this study, we constructed a multi-miRNA signature and used the algorithm of linear support vector machine. We ranked the p-values that were derived from the comparison of RE-scores or expressions. The top N significant RE-score changing microRNAs or differentially expressed microRNAs were chosen to

perform the classification analysis. To estimate the effect of the classifier, we adapted a leave one out cross validation strategy. Generally, the sample number of ER^+ is different from that of ER^-. If there were N_1 ER^+ and N_2 ER^- samples, assuming that $N_1 > N_2$, in order to balance the sample effect, we randomly selected N_2 ER^+ samples. The total 2* N_2 (N_2 ER^+ and N_2 ER^-) samples were used in the leave one out validation. The classification accuracy was determined by averaging the accuracies of the leave one out validations repeated 100 times.

Abbreviations

ARR: Adapted Ranked Ratio; EGF: Epidermal growth factor; ER^+: Estrogen Receptor Positive; ER^-: Estrogen Receptor Negative; FDR: False Discovery Rate; MAPK: mitogen activated protein kinase; RE: Regulatory Effect; RISC: RNA-induced silencing complex; RR: Ranked Ratio; UTR: Untranslated Region;

Authors' contributions

CC and MG conceived and designed the study. CC extracted the gene expression data. CC and XF preformed the full analysis. CC, XF, PA and MG wrote the manuscript.

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Figure legends

Figure 1 - Schematic diagram for identifying RE-changing miRNAs between ER^- and ER^+ breast cancer samples.

For each miRNA in each sample, a RE-score is calculated by comparing average ranks of its target and non-target genes. RE-changing miR($ER^->ER^+$) and RE-changing miR($ER^-<ER^+$) represent miRNAs that have significantly higher and lower RE-scores in ER^- with respect to ER^+, respectively. RE-invariant miR represents miRNAs that show no significant difference in RE-scores between ER^+ and ER^-. Note that many miRNAs share a same target mRNA, while many mRNAs can also be targeted by a same miRNA, which constitutes a complex miRNA-mRNA network.

Figure 2 - The comparison of RE-scores between ER^+ and ER^- samples in 5 breast cancer expression datasets.

(a) Box plots of RE-scores for miR-371. MiR-371 shows significantly higher RE-scores in ER^- than in ER^+ for all the 5 datasets. The statistical significance level of difference (FDR) for each dataset is also shown. (b) Distributions of the t-scores for RE-score comparison between ER^- and ER^+. The t-scores for 470 miRNAs are calculated by comparing their RE-scores in ER^- with those in ER^+ samples. The t-score distributions for the 5 datasets are shown in different colors. Most t-scores are positive, indicating that most miRNAs exhibit higher RE-scores in ER^- than in ER^+ samples. (c) Proportion of RE-changing miRNAs with higher inhibitory effect in ER^- (red) and RE-changing miRNAs with lower inhibitory effect in ER^- (green) at three different significance levels (FDR$\leq$0.05, FDR$\leq$0.10, and FDR$\leq$0.20). The number on the top of a bar represents how many RE-changing miRNAs identified from the corresponding mRNA microarray dataset. HE, MI, MN, VA and WA represent the

microarray data published by Hess *et al.* [44], Miller *et al.* [38], Minn *et al.* [39], van't Veer et *al.* [34], and Wang *et al.* [40], respectively.

Figure 3 - Distributions of the t-scores for comparison of RE-score based on distinct miRNA target prediction algorithms.

(a) PicTar algorithm, (b) miRanda algorithm, (c) Intersection of miRanda and PITA, (d) TargetScan algorithm, the site is conserved, (e) TargetScan algorithm, site context score is above -0.20, and (f) TargetScan algorithm, all potential targets. The t-score distributions for the 5 datasets are shown in different colours. The t-scores are more likely to be positive values in all 5 datasets, suggesting stronger inhibitory effects of miRNAs to their targets in ER^- breast cancer.

Figure 4 - The results obtained from an alternative RE-score calculation method based on expression comparison.

(a) Distributions of the t-scores calculated by comparing the RE-scores from the expression comparison method. The employed target prediction algorithm is PITA. The t-score distributions for the 5 datasets are shown in different colors. (b) Correlation between the t-scores obtained from the two different RE-score calculation methods. The microarray dataset used was VA, published by van't Veer et *al.* [34]. The correlation coefficient (R) is 0.928, indicating that the t-scores obtained by using expression comparison and rank comparison are highly correlated.

Figure 5 - The expressions and RE-scores of the microRNAs predicted to target *Dicer*.

BL and MA represent the microRNA microarray data published by Blenkiron *et al.*[30] and Mattie *et al.*[32]. HE, MI, MN, VA and WA represent the mRNA microarray data published by Hess *et al.* [44], Miller *et al.* [38], Minn *et al.* [39], van't

Veer et *al.* [34], and Wang *et al.* [40], which were used to infer the microRNA RE-scores. If the difference between ER^+ and ER^- is significant, the plot is flagged with '***'. The expression levels of these six microRNAs are mostly lower in ER^-, however, almost all the RE-scores in ER^- are higher, suggesting that the differential regulatory effects of miRNAs can not be explained by miRNA expression difference between ER^+ and ER^- cancers.

Figure 6 - The RE-score profiles of microRNAs for classification in ER^+ and ER^- breast tumors.

The figure demonstrates unsupervised hierarchical clustering of 57 ER^+ and 42 ER^- in the MN dataset[39] using the top 8 RE-changing miRNAs. A dendrogram of the tumors is shown at the top, with ER^+ marked red and ER^- marked yellow. For hierarchical clustering, RE-scores of each miRNA were mean centered and normalized, and tumors were clustered using Pearson correlation (uncentered) and average linkage (CLUSTER and TREEVIEW software)[73].

Figure 7 - Differential expression of miRNA processing genes between ER^+ and ER^- breast cancer samples.

(a) miRNA biogenesis and function pathway. Genes significantly up-regulated and down-regulated in ER^- with respect to ER^+ are shown in yellow and cyan, respectively. A gene, for example, *Drosha*, is marked grey to denote that it shows no significant differential expression between ER^- and ER^+. (b) Expression levels of *Ago1*, *Ago2*, *Dicer*, and *TRBP* in ER^+ (red) and ER^- (green). The mean and the standard deviation of the expression levels for each gene are shown as bar and vertical line, respectively. Data for a gene is not shown if it is missing in a dataset. The combined P-value for each gene is also shown.

Tables

Table 1 - The number of miRNAs with ARR<1 and ARR>1 in each dataset.

Data	PITA			miRanda		
	ARR<1	ARR>1	Percentage (ARR<1)	ARR<1	ARR>1	Percentage (ARR<1)
HE	279	187	60%	224	188	54%
MI	446	24	95%	380	34	92%
MN	388	79	83%	293	122	70%
VA	407	60	87%	345	71	82%
WA	332	137	71%	299	113	72%

Table 2 - The correlation between the results of ARR method and those of RE-score calculation method.

Data	PITA			PicTar		
	Percentage (ER^->ER^+)	Percentage (ER^-<ER^+)	Spearman Correlation	Percentage (ER^->ER^+)	Percentage (ER^-<ER^+)	Spearman Correlation
HE	58%	23%	0.861	77%	12%	0.752
MI	100%	0%	0.646	60%	16%	0.778
MN	73%	10%	0.668	62%	17%	0.763
VA	86%	2%	0.659	65%	5%	0.578
WA	68%	18%	0.855	59%	22%	0.837

Percentage(ER^->ER^+): The fraction of microRNAs with ARR<1 and T-score < 0, indicating that the microRNAs show higher regulatory activity in ER^- than in ER^+ as consistently supported by both the ARR method and RE-score expression comparison method. Percentage(ER^-<ER^+): The fraction of microRNAs with RR>1 and T-score > 0. These microRNAs show higher regulatory activity in ER^+ as supported by methods. Spearman Correlation: The correlation between ARR value and t-score(ER^-/ER^+).

Table 3 - The regulatory activity of miR-206 predicted by RE-score method and ARR method.

Data	PITA		PicTar	
	ARR	T-score(ER^-/ER^+)	RR	T-score(ER^-/ER^+)
HE	0.986	0.91	0.852	1.98
MI	0.973	1.91	0.977	1
MN	0.97	1.02	0.783	2.56
VA	0.979	2.47	0.823	3.82

WA	0.977	1.57	0.84	3.09

T-score(ER^-/ER^+): The t-score is calculated by performing t-test to measure differentiation of the RE-scores for a miRNA in the two breast cancer subtypes. Note that here RE-scores were calculated with expression comparison method.

Table 4 - Correlation between microRNA RE-scores and their expressions.

		PITA		miRanda	
		Expression		Expression	
		BL	MA	BL	MA
RE-score	HE	0.218	0.023	0.150	-0.069
	MI	0.211	0.056	0.254	-0.072
	MN	0.235	0.089	0.201	-0.085
	VA	0.102	0.015	0.071	-0.044
	WA	0.285	0.121	0.251	-0.055

BL and MA represent the microRNA microarray data published by Blenkiron *et al.*[30] and Mattie *et al.*[32]. HE, MI, MN, VA and WA represent the mRNA microarray data published by Hess *et al.* [44], Miller *et al.* [38], Minn *et al.* [39], van't Veer *et al.* [34], and Wang *et al.* [40], which were used to infer the microRNA RE-scores.

Table 5 - Breast cancer gene expression datasets used in this study.

Dataset ID	Reference	Array Type	Number of Samples	
			ER^+	ER^-
HE	Hess *et al.* [44]	One channel oligo	82	51
MI	Miller *et al.* [38]	One channel oligo	213	34
MN	Minn *et al.* [39]	One channel oligo	57	42
VA	van 't Veer *et al.* [34]	Two channels cDNA	53	44
WA	Wang *et al.* [40]	One channel oligo	209	77

Additional data files

Additional data file 1 –The distribution of miRNA target numbers for different prediction tools.

File format: PDF

Additional data file 1 is a figure plotting the distribution of miRNA target numbers for 4 prediction tools, PITA, miRanda, PicTar, TargetScan. In addition, three different parameters in TargetScan prediction were chosen and denoted as 'Conserved', 'ContextScore ≥ -0.20' and 'All', respectively. On average, 6949, 2026, 1563, 765, 426, and 210 targets per miRNA are predicted in PITA, TargetScan(All), miRanda, TargetScan(ContextScore≥ -0.20), PicTar, and TargetScan(Conserved) respectively.

Additional data file 2 – The comparison results of RE-scores in 5 breast cancer expression datasets.

File format: XLS

Additional data file 2 includes 7 sheets containing the complete results based on RE-scores from rank comparison and 7 miRNA target predictions from PITA, miRanda, PicTar, the intersection of PITA and miRanda, TargetScan(Conserved), TargetScan(ContextScore>=-0.20), and TargetScan(All), respectively. The t-score, p-value, and adjusted p-value (FDR) of all miRNAs in the 5 breast cancer datasets are provided.

Additional data file 3 – The comparison results of RE-scores calculated from expression comparison method.

File format: XLS

Additional data file 3 includes two sheets containing the complete results based on RE-scores calculated from expression comparison. In the two sheets, miRNA target predictions provided by the miRanda and PITA methods are used, respectively.

Additional data file 4 – The expressions of several miRNA processing genes

File format: XLS

Additional data file 4 is a table showing the expression differentiation of several miRNA processing genes between ER^+ and ER^- samples.

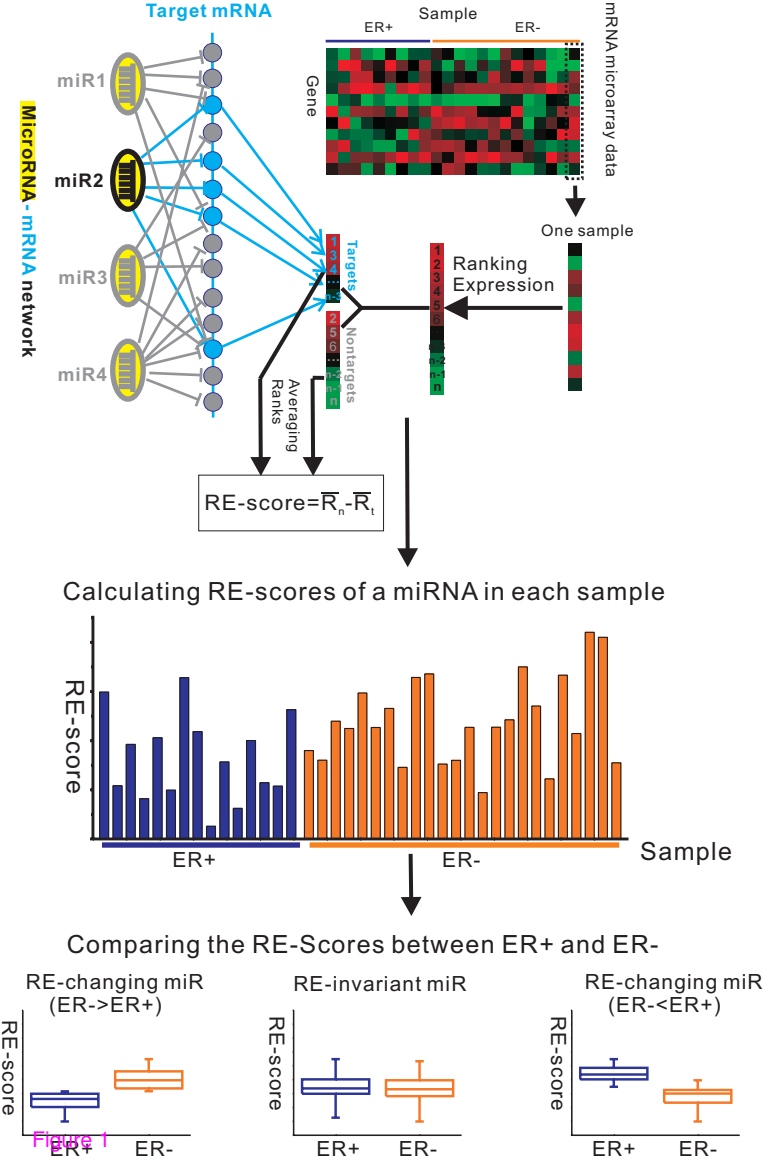
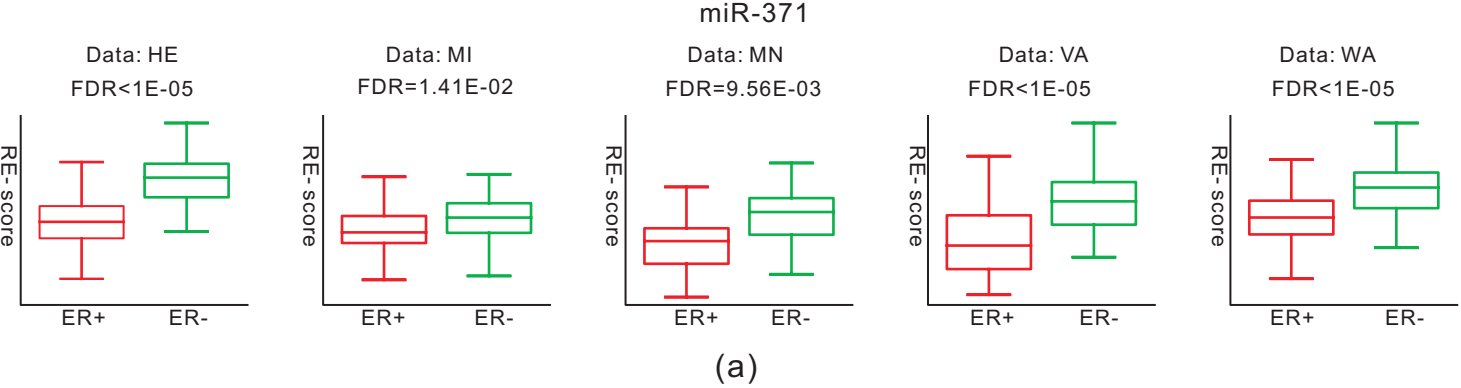
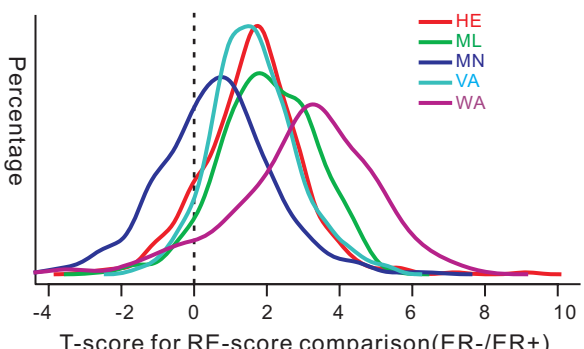

MicroRNA-mRNA network
Target mRNA
miR1
miR2
miR3
miR4
Sample
ER+
ER-
Gene
mRNA microarray data
One sample
Ranking Expression
Targets
Nontargets
Averaging Ranks
RE-score=$\overline{R}_n$-$\overline{R}_t$
Calculating RE-scores of a miRNA in each sample
RE-score
ER+
ER-
Sample
Comparing the RE-Scores between ER+ and ER-
RE-changing miR (ER->ER+)
RE-invariant miR
RE-changing miR (ER-<ER+)
RE-score
ER+
ER-

Figure 1


miR-371
Data: HE
FDR<1E-05
Data: MI
FDR=1.41E-02
Data: MN
FDR=9.56E-03
Data: VA
FDR<1E-05
Data: WA
FDR<1E-05
RE- score
ER+
ER-
(a)


HE
ML
MN
VA
WA
Percentage
-4
-2
0
2
4
6
8
10
T-score for RE-score comparison(ER-/ER+)
(b)

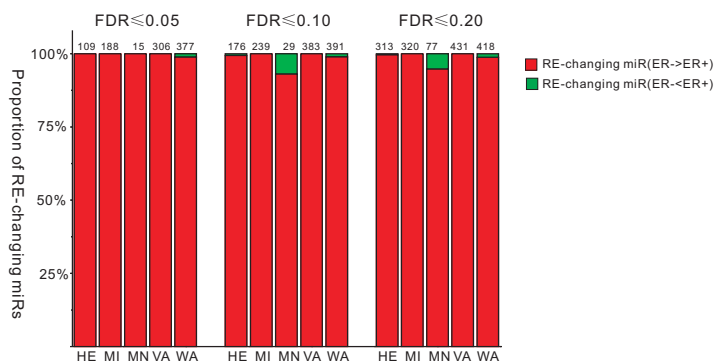

FDR≤0.05
FDR≤0.10
FDR≤0.20
109 188 15 306 377
176 239 29 383 391
313 320 77 431 418
100%
75%
50%
25%
Proportion of RE-changing miRs
HE MI MN VA WA
RE-changing miR(ER->ER+)
RE-changing miR(ER-<ER+)
(c)

Figure 2

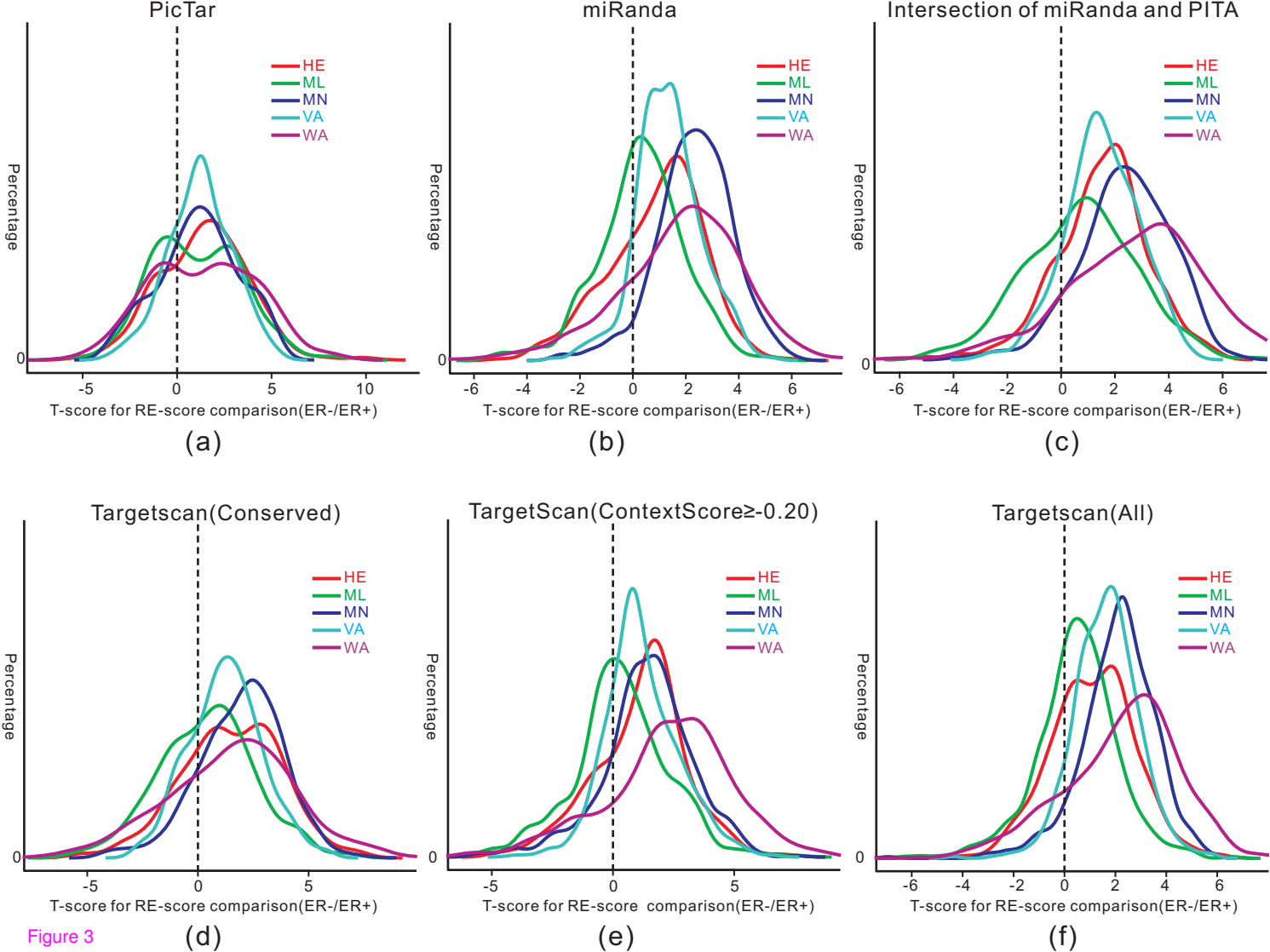
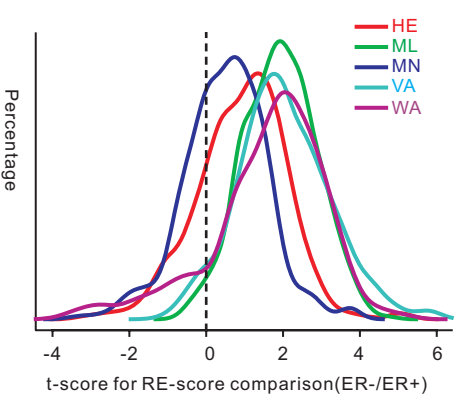
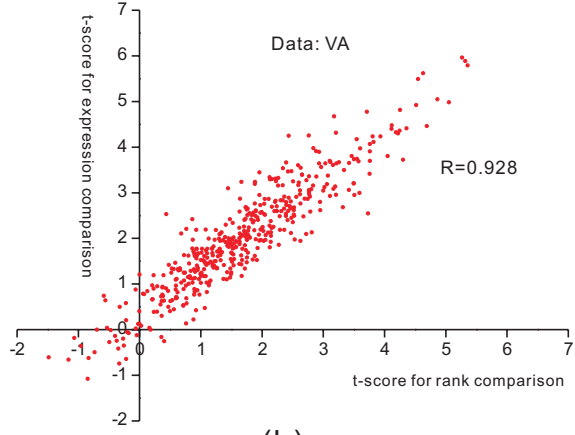

PicTar
miRanda
Intersection of miRanda and PITA
Targetscan(Conserved)
TargetScan(ContextScore≥-0.20)
Targetscan(All)
HE
ML
MN
VA
WA
Percentage
0
T-score for RE-score comparison(ER-/ER+)
(a)
(b)
(c)
(d)
(e)
(f)

Figure 3


HE
ML
MN
VA
WA
Percentage
-4
-2
0
2
4
6
t-score for RE-score comparison(ER-/ER+)

(a)


Data: VA
R=0.928
t-score for expression comparison
t-score for rank comparison
7
6
5
4
3
2
1
0
-1
-2
-2
-1
0
1
2
3
4
5
6
7

(b)

Figure 4

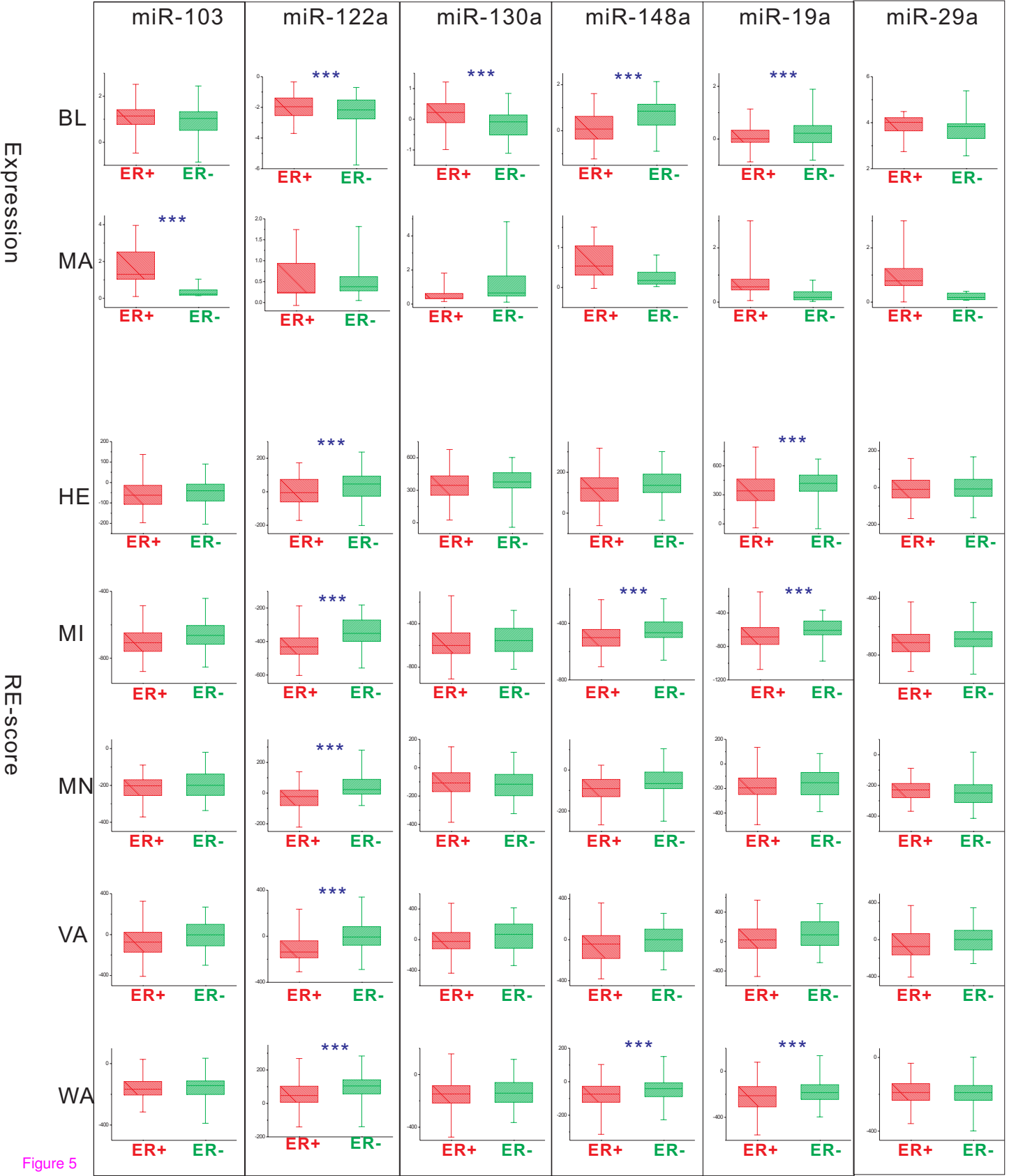

miR-103
miR-122a
miR-130a
miR-148a
miR-19a
miR-29a
BL
MA
HE
MI
MN
VA
WA
Expression
RE-score
ER+
ER-
***

Figure 5

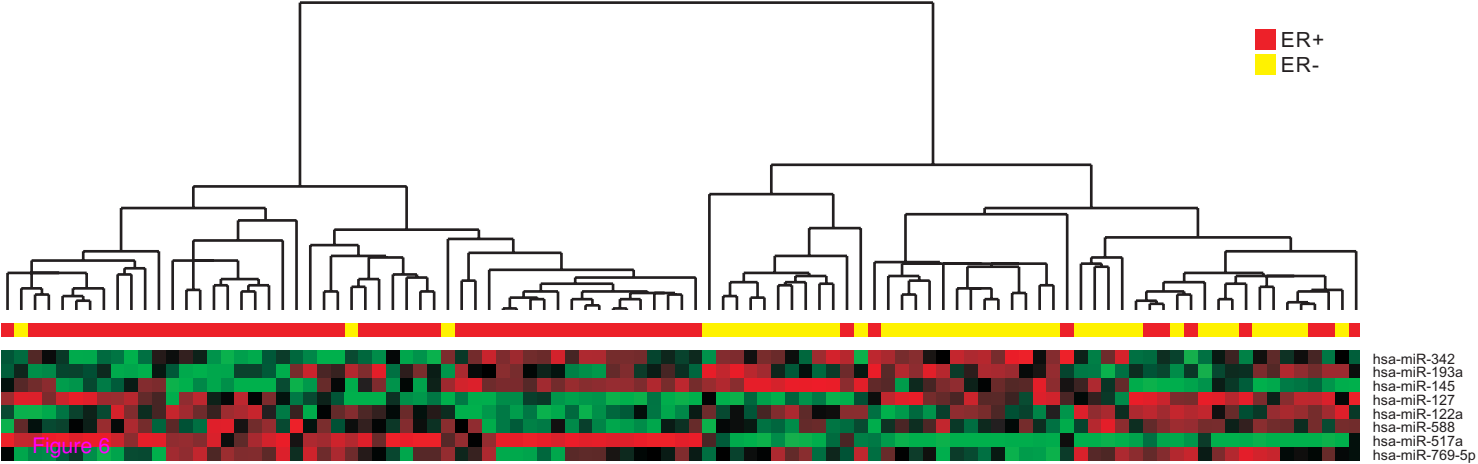
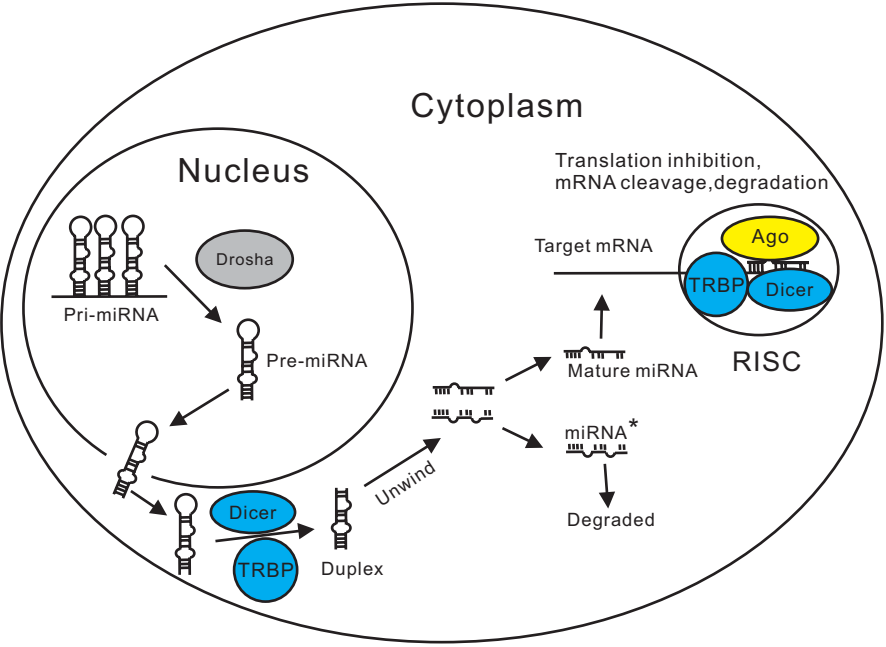
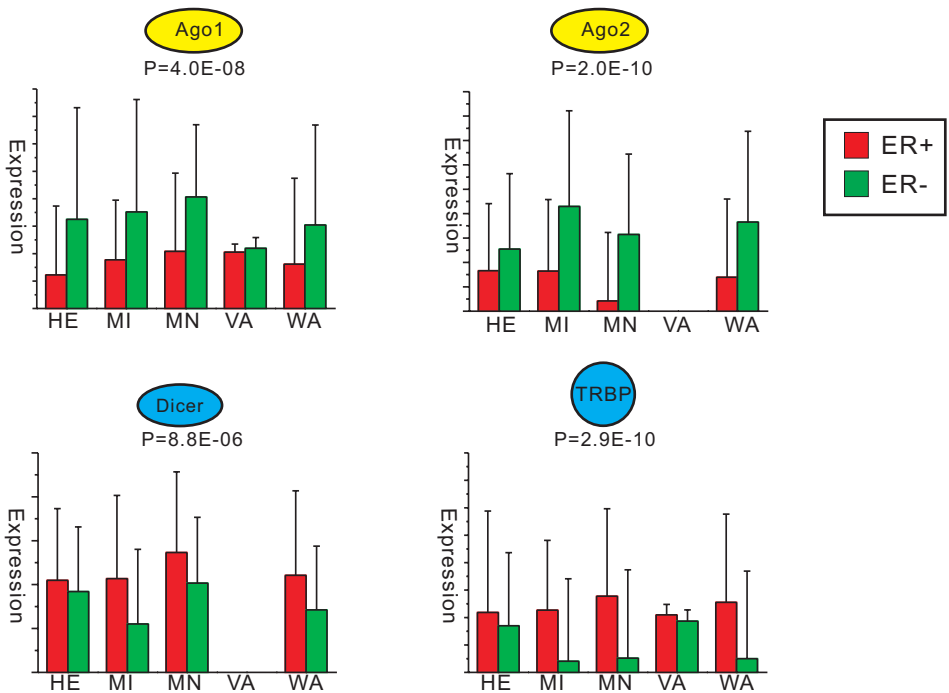

ER+
ER-
hsa-miR-342
hsa-miR-193a
hsa-miR-145
hsa-miR-127
hsa-miR-122a
hsa-miR-588
hsa-miR-517a
hsa-miR-769-5p

Figure 6


Cytoplasm
Nucleus
Translation inhibition,
mRNA cleavage,degradation
Target mRNA
Ago
TRBP
Dicer
RISC
Drosha
Pri-miRNA
Pre-miRNA
Mature miRNA
miRNA*
Degraded
Unwind
Dicer
TRBP
Duplex

(a)


Ago1
P=4.0E-08
Ago2
P=2.0E-10
ER+
ER-
Expression
HE
MI
MN
VA
WA
Dicer
P=8.8E-06
TRBP
P=2.9E-10

(b)

Figure 7

Additional files provided with this submission:

Additional file 1: additional_file_1.pdf, 14K
http://genomebiology.com/imedia/5345595212923035/supp1.pdf
Additional file 2: additional_file_2.xls, 960K
http://genomebiology.com/imedia/3766302742923035/supp2.xls
Additional file 3: additional_file_3.xls, 298K
http://genomebiology.com/imedia/2487469532923035/supp3.xls
Additional file 4: additional_file_4.xls, 21K
http://genomebiology.com/imedia/5048050122923035/supp4.xls