Different miRNA expression profiles between human breast cancer tumors and serum

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A bunch of microRNAs (miRNAs) have been demonstrated to be aberrantly expressed in cancer tumor tissue and serum. The miRNA signatures identified from the serum samples could serve as potential noninvasive diagnostic markers for breast cancer. The role of the miRNAs in cancerigenesis is unclear. In this study, we generated the expression profiles of miRNAs from the paired breast cancer tumors, normal, tissue, and serum samples from eight patients using small RNA-sequencing. Serum samples from eight healthy individuals were used as normal controls. We identified total 174 significantly differentially expressed miRNAs between tumors and the normal tissues, and 109 miRNAs between serum from patients and serum from healthy individuals. There are only 10 common miRNAs. This suggests that only a small portion of tumor miRNAs are released into serum selectively. Interestingly, the expression change pattern of 28 miRNAs is opposite between breast cancer tumors and serum. Functional analysis shows that the differentially expressed miRNAs and their target genes form a complex interaction network affecting many biological processes and involving in many types of cancer such as prostate cancer, basal cell carcinoma, acute myeloid leukemia, and more.

Keywords: breast cancer, miRNA, biomarker, tumor, serum

INTRODUCTION

Breast cancer remains one of the leading causes of cancer death among women worldwide (Siegel et al., 2014). One in every eight women in the United States will develop breast cancer in her lifetime (Desantis et al., 2014). Mammography and ultrasound, as the current standard diagnostic tools, have been successful in the detection of early-stage breast cancer. However, there is a need to develop new, minimally invasive diagnostic approaches to improve diagnosis rates of breast cancer. Recently, microRNAs (miRNA), a class of small non-coding RNAs encoded in the genomes of animals and plants (Carrington and Ambros, 2003; Bartel, 2004; Asli et al., 2008), have been proposed as promising biomarkers of early breast cancer diagnosis and accurate prognosis (Schrauder et al., 2012; Chan et al., 2013; Mar-Aguilar et al., 2013b; Ng et al., 2013). Mature miRNAs are approximately 22 nt long, and derived from larger 60–110 nt hairpin precursor transcripts that serve as substrates for the dsRNA endoribonuclease Dicer (Ke et al., 2003). Active miRNAs regulates gene expression by controlling stability or translation of mRNAs through base pairing to partially complementary sites, predominately in the 3′ untranslated region (UTR) of target mRNAs (Zhao and Srivastava, 2007). miRNAs are involved in highly regulated processes such as proliferation, differentiation, apoptosis and metabolic processes (Pescador et al., 2013), and contribute significantly to the pathophysiology of breast cancer

by facilitating invasion and metastasis, epithelial to mesenchymal transition, and maintenance of breast stem cells (Ryu et al., 2011). Dysregulated expression of miRNAs has been potentially associated with cancers, and these miRNAs can serve as potential biomarkers for the diagnosis of various cancers and other diseases (Chen et al., 2008). miRNAs can be readily detected in tumor biopsies (Jiang et al., 2009) and found stable in serum and plasma, and other body fluids (Blondal et al., 2013). The previous study (Mar-Aguilar et al., 2013b) showed that combination of miR-145, miR-155, and miR-382 could distinguish breast cancer from normal controls. Currently, more than 2500 mature miRNAs have been discovered in mammalian systems and deposited in the publicly available miRNA database miRBase (Release 20; http://microrna.sanger.ac.uk/) (Griffiths-Jones, 2010; Kozomara and Griffiths-Jones, 2014).

To determine the expression pattern of all known miRNAs, many methods were developed for miRNA profiling. Quantitative polymerase chain reaction (qPCR) is a sensitive technique for estimating expression levels of microRNAs. Jun Lu et al. used a bead-based flow cytometric miRNA expression profiling method to classify poorly differentiated tumors and highlighted the potential of miRNA profiling in cancer diagnosis (Lu et al., 2005). Analysis of 54 Luminal A-like breast cancer blood samples and 56 normal blood samples using microarrays (Mcdermott et al., 2014), indicated that the expression profiles of 3 miRNAs

(miR-29a, miR-181a, and miR-652), in combination with mammography, has potential to facilitate accurate subtype-specific breast tumor diagnosis. Recently, next generation sequencing technologies have been developed to revolutionize miRNA profiling by providing a highly quantitative estimate of known individual miRNA species (Huang et al., 2009; Dhahbi et al., 2011; Schotte et al., 2011), as a replacement for microarrays. Additionally, deep sequencing of miRNAs has the potential for discovering novel miRNAs, even those that occur at low frequencies (Lu et al., 2009; Ryu et al., 2011; Wei et al., 2013). However, few studies have compared between serum and tumor miRNA expression. A recent study (Chan et al., 2013) investigated the miRNA signature of breast cancer tumors ($n = 32$) and serum samples ($n = 22$), and concluded that some miRNAs displayed opposite expression pattern in tissue and serum, previously reported in breast cancer (Cuk et al., 2013).

The objective of this pilot study was to discover a panel of miRNAs as potential novel breast cancer biomarkers and try to find the mechanism of miRNA regulation. Thus, we have used a deep sequencing approach to identify dysregulated miRNAs in human breast cancer tissues vs. adjacent tissues and breast cancer serum vs. serum from healthy female controls. To investigate the biological functions of the candidate dysregulated miRNAs, downstream miRNA target genes were predicted using 11 established miRNA target prediction programs stored in miRecords (http://miRecords.umn.edu/miRecords) (Xiao et al., 2009). In particular, we have focused on the mechanism of profiling miRNA expression associated with breast cancer through examining the expression of their targets, followed by pathway analyses. Finally, we identified a bunch of miRNA and their targets that affect breast cancer tumorigenesis and progression.

MATERIALS AND METHODS

PATIENTS
The patients examined in this study underwent surgery at the Taizhou Central Hospital between 2012 and 2013. All patients had not been previously treated by chemotherapy and radiotherapy when undergoing surgery and provided informed consent to participate in the study. Fresh frozen breast cancer tumors, adjacent normal tissues, and preoperative serum from 8 patients with breast cancer and control serum sample from 8 healthy female volunteers were obtained from the Taizhou Central Hospital.

RNA ISOLATION, LIBRARY CONSTRUCTION, AND SEQUENCING
Total RNA was isolated for each of tissue and serum samples and treated with Trizol reagent (Invitrogen) according to the manufacturer's instructions. The total RNA quantity and purity were analyzed using Bioanalyzer 2100 and RNA 6000 Nano LabChip Kit (Agilent). The RIN value is >7.0. To eliminate the biological variations caused from the different levels of gene expression between samples, the RNA from all tumor samples were pooled together. Similarly, the RNA from all adjacent normal tissue samples, serum samples were pooled, respectively. Thus, approximately 1 ug of total pooled RNA were used to prepare small RNA library according to protocol of TruSeq™ Small RNA Sample Prep

Kits (Illumina). We performed the single-end sequencing (36 bp) on an Illumina Hiseq2500 at the WS-BIO (Hangzhou, China) following the vendor's recommended protocol. Sequencing reads can be accessed through GEO database under accession number GSE56614.

READ MAPPING AND DIFFERENTIAL EXPRESSION ANALYSIS
Adapter dimers, junk, low complexity, common RNA families (rRNA, tRNA, snRNA, snoRNA) and repeats were discarded followed the procedures as described in a previous study (Li et al., 2010). Next, small RNA sequencing reads were aligned against 2578 mature miRNA sequences from miRBase build 20 using Bowtie 1.0.0 (Langmead et al., 2009) allowing at most two mismatches. The other parameters are default.

Expression values are quantified by aggregating reads into counts and differential expression analysis is performed based on normalized deep-sequencing counts in RPM (Reads Per Million mapped reads) (NOISeq) (Tarazona et al., 2012). The miRNAs whose expression levels are two or more fold change with $q = 0.8$ are defined as significantly differentially expressed miRNAs. Correlations between groups were calculated with Pearson.

PREDICTION OF miRNA TARGETS AND ANALYSIS OF THEIR EXPRESSION CHANGE
We predicted the targets of the differentially expressed miRNAs using the database miRecords (http://mirecords.umn.edu/miRecords) (Xiao et al., 2009). The target genes were further filtered by oncomine database that collects cancer microarrays (http://www.oncomine.org, v4.5) and integrates a data-mining platform. We only retained the target genes whose expression changes were at least two fold with p value less than or equal to 0.05 between human breast cancer and human normal breast according to the data in oncomine. Moreover, the satisfied expression change of each gene was supported at least by five datasets in oncomine.

FUNCTIONAL ANALYSIS OF TARGET GENES
In order to infer the potential functions of the differentially expressed miRNAs, we performed the functional analysis of their target genes using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) v6.7 (Huang Da et al., 2009a,b). Functional categories were clustered using the Functional Annotation Clustering tool, and representative GO categories and KEGG pathways from each clustered set with a p-value < 0.05 were selected and taken into consideration for further analysis.

RESULTS

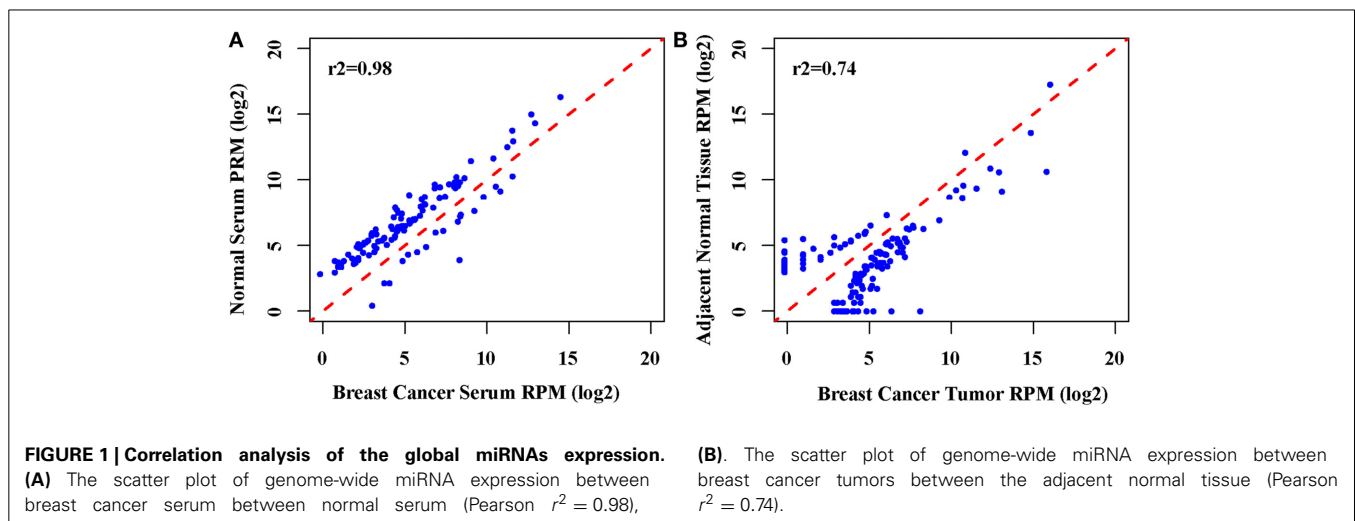### miRNA EXPRESSION PROFILING OF BREAST CANCER TUMOR AND SERUM
In order to identify the potential miRNA signatures for breast cancer detection, we employed RNA-seq to generate the expression profiles of miRNAs in the paired tumors, normal tissues, and serum samples from 8 patients. The serum samples from another 8 healthy individuals were used as normal controls for serum. We obtained more than eight million reads for each sample (Table S1). The comparison showed that the expression profiles

of breast cancer tumors and normal tissues were closely correlated (Pearson $r^2 = 0.74$). In contrast, the expression profiles of serum from breast cancer patients and from healthy individuals are much highly correlated (Pearson $r^2 = 0.98$) (**Figure 1**). Further examination found 174 significantly differentially expressed miRNAs between tumors and normal tissues, 120 miRNAs up-regulated and 54 down-regulated in tumors. There are 109 significantly differentially expressed miRNAs between the serum from patients and the serum from healthy individuals, 18 miRNAs up-regulated and 91 down-regulated in the serum from patients. Only 3 up-regulated miRNAs and 7 down-regulated miRNAs are in common (Table S2). This suggests that miRNAs may be released into the serum selectively. The miRNA expression profiles change differently in tumors and serum as a consequence of breast cancer.

Many of the differentially expressed miRNAs have been reported to involve in cancerogenesis (**Table 1**). For example, the previous studies showed that miR-132 (Li et al., 2013), miR-125b (Zhang et al., 2011), miR-34c (Yang et al., 2013), and miR-485 (Anaya-Ruiz et al., 2013), functioning as suppressors, played an important role in breast cancer by suppressing cell proliferation

and migration. Notably, miR-382 (Mar-Aguilar et al., 2013b), miR-224 (Huang et al., 2012), and miR-1246 (Pigati et al., 2010) were also reported as valuable potential biomarkers of breast cancer and diseases. Additionally, miR-598 and miR-184 were also reported to be down-regulated in esophageal cancer (Zhao et al., 2013) and prostate cancer (Walter et al., 2013), respectively.

Since there is only a few differentially expressed miRNAs common to tumors and serum, we further examined the expression change pattern of the other differentially expressed miRNAs in tumors and serum. Our results showed that the expression of 28 dysregulated miRNAs was changed differently between breast cancer tumor tissue and serum (**Table 2**). Among these miRNAs, 27 miRNAs were up-regulated in breast cancer tumors when compared to the normal tissues. In contrast, these 27 miRNAs were down-regulated in the serum from breast cancer patients when compared to the serum from healthy individuals. Additionally, only one miRNA was down-regulated in tumors and up-regulated in the serum from patients. This inconsistent expression change pattern indicates different molecular mechanisms in the tumors and the serum because the expression level of their target genes would be regulated differently.

FIGURE 1 | Correlation analysis of the global miRNAs expression. **(A)** The scatter plot of genome-wide miRNA expression between breast cancer serum between normal serum (Pearson $r^2 = 0.98$), **(B)**. The scatter plot of genome-wide miRNA expression between breast cancer tumors between the adjacent normal tissue (Pearson $r^2 = 0.74$).

Table 1 | Ten miRNAs differentially expressed in both tumor tissue and serum.

miRNA	Expression change[a]	miRbase accession number	Expression change[b]	References
miR-132-5p	Down	MIMAT0004594	Down	Li et al., 2013
miR-125b-1-3p	Down	MIMAT0004592	Down	Li et al., 2013; Mar-Aguilar et al., 2013a
miR-34c-5p	Down	MIMAT0000686	Down	Yang et al., 2013
miR-382-3p	Down	MIMAT0022697	Down	Li et al., 2013; Mar-Aguilar et al., 2013b
miR-485-5p	Down	MIMAT0002175	Down	Anaya-Ruiz et al., 2013
miR-323b-3p	Down	MIMAT0015050	NA	NA
miR-598-3p	Down	MIMAT0003266	NA	NA
miR-224-5p	Up	MIMAT0000281	Up	Huang et al., 2012
miR-1246	Up	MIMAT0005898	Up	Pigati et al., 2010
miR-184	Up	MIMAT0000454	NA	NA

[a] Expression change in this study.

[b] Expression change in the previous studies. NA, Not Available.

COMPLEX NETWORKS UNDERLYING INTERACTION OF 10 COMMON miRNAs AND THEIR TARGET TARGETS

We predicted target genes of the 10 common differentially miR-NAs by at least two algorithms from the miRecords database. We

**Table 2 | Inconsistent expression change of 28 miRNAs between tumor tissue and serum.**

miRNA	Regulation		miRNA	Regulation	
	Serum	Tissue		Serum	Tissue
hsa-miR-370-3p	Down	Up	hsa-miR-455-3p	Down	Up
hsa-miR-381-3p	Down	Up	hsa-miR-212-5p	Down	Up
hsa-miR-483-5p	Down	Up	hsa-miR-9-3p	Down	Up
hsa-miR-125b-5p	Down	Up	hsa-miR-205-5p	Down	Up
hsa-miR-508-3p	Down	Up	hsa-miR-132-3p	Down	Up
hsa-miR-9-5p	Down	Up	hsa-miR-432-5p	Down	Up
hsa-miR-377-3p	Down	Up	hsa-miR-1197	Down	Up
hsa-miR-382-5p	Down	Up	hsa-miR-134-5p	Down	Up
hsa-miR-99a-5p	Down	Up	hsa-miR-539-3p	Down	Up
hsa-miR-107	Down	Up	hsa-miR-100-5p	Down	Up
hsa-miR-485-3p	Down	Up	hsa-miR-500a-3p	Down	Up
hsa-miR-758-3p	Down	Up	hsa-miR-433-3p	Down	Up
hsa-miR-497-5p	Down	Up	hsa-miR-504-5p	Down	Up
hsa-miR-204-5p	Down	Up	hsa-miR-363-3p	Up	Down

only retained the target genes that were further validated by five of the 13 separate publications providing mRNA profiling data of breast cancer tissues in oncomine database.

One miRNA often regulates more than one target genes. A gene is also often regulated by more than one miRNAs. Therefore, there is a many-to-many interaction relationship between miR-NAs and their target genes. Table S3 lists all the potential miRNA-target interactions which may play an important role in breast cancer. We further employed Cytoscape, a tool to construct the miRNA-mRNA interaction network. Our results show a complex network consisting of the differentially expressed miRNAs and their target genes (**Figure 2**).

FUNCTIONAL ANALYSIS OF THE DIFFERENTIALLY EXPRESSED miRNAs

What functions and pathways could be affected by the complex interactions between the miRNAs and their target genes? To address this issue, we inferred the functions of miRNAs from their target genes because miRNAs exert their functions through regulating their target genes. The functional analysis show that target genes are involved in a variety of positive regulation processes like nitrogen compound metabolic process, biosynthetic, cell motion, cell proliferation, and other biological processes like hormone metabolic process, mammary gland development, wound healing and gland development (**Figure 3**). Further analysis found that their target genes were enriched in the pathways
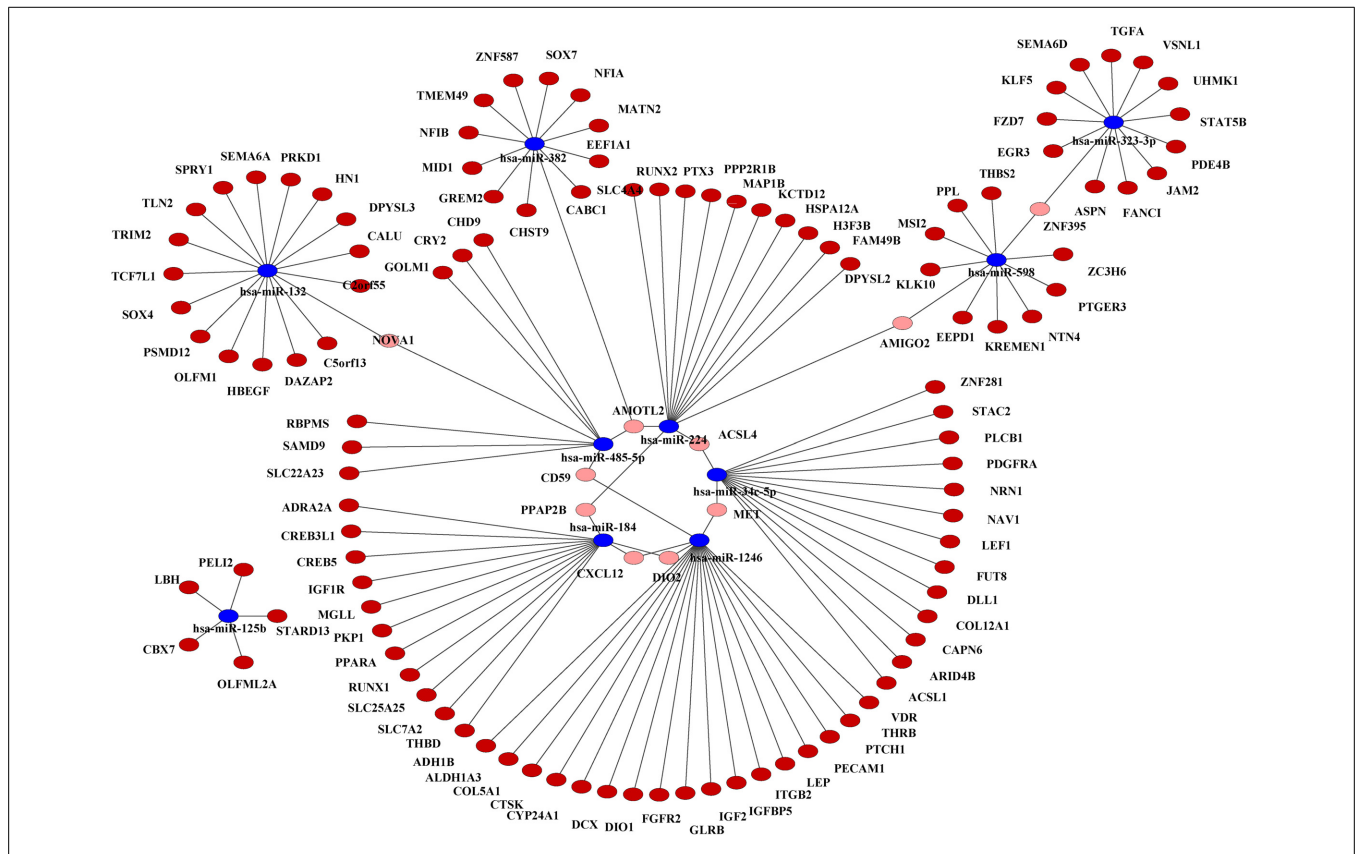
FIGURE 2 | Interaction network of the 10 common differentially expressed miRNAs and their target genes in breast cancer. Target genes associated with only one miRNA are indicated in dark red. Target genes associated with more than one miRNAs are indicated in pink. The 10 common miRNAs are indicated in blue.

involved in many cancers including prostate, colorectal, basal cell carcinoma, acute myeloid leukemia (**Figure 4**). These results indicate that these 10 common differentially expressed miRNAs play a key role in cancerogenesis by impacting various biological processes.

DISCUSSION

There are only 10 common differentially expressed miRNAs between breast cancer tumors and the serum (Table S2). Moreover, the expression change pattern of the differentially expressed miRNAs is different between the tumors and the serum (**Table 2**). These results indicate that only a small portion of miRNAs are released into the serum selectively. This is consistent with the previous study (Chan et al., 2013). To date, at least

four possible different explanations have been proposed for the origin of serum miRNAs. The first and controversial hypothesis proposed that miRNAs may originate in large part from cells circulating in the blood (Duttagupta et al., 2011; Pritchard et al., 2012). However, this proposal has been challenged by other studies, such as filtering and differential centrifugation experiments suggesting that miRNAs are not derived from blood cells (Brase et al., 2010). The second hypothesis is that genetic exchange of mRNA and miRNA between cells can be accomplished through microvesicles, or exosome-mediated transfer (Valadi et al., 2007). Microvesicles are shed from the plasma membrane into the extracellular environment and released into the blood stream to facilitate communication between cells. Vesicles released from human and murine mast cell lines contain over 1200 mRNA and
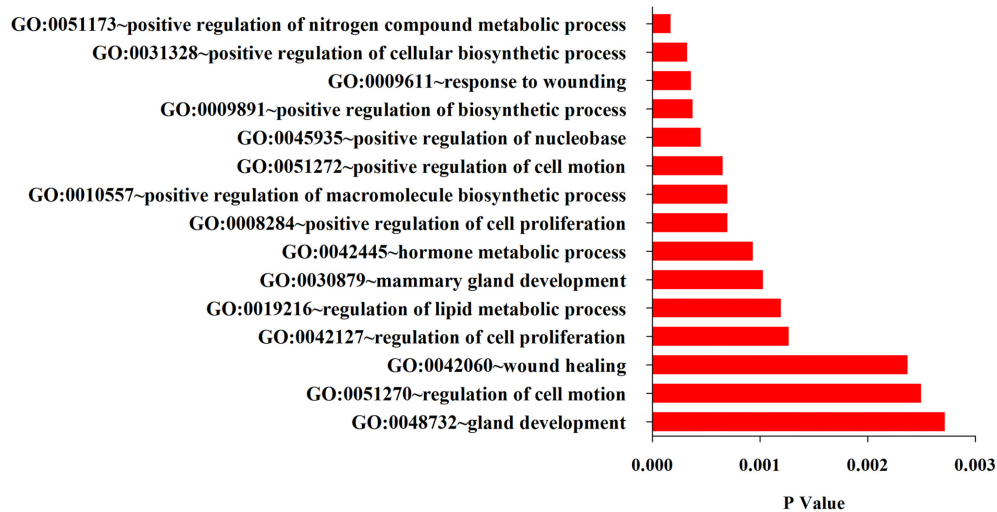

FIGURE 3 | Top 15 GO terms enriched in the up-and down-regulated target genes of the differentially miRNAs.
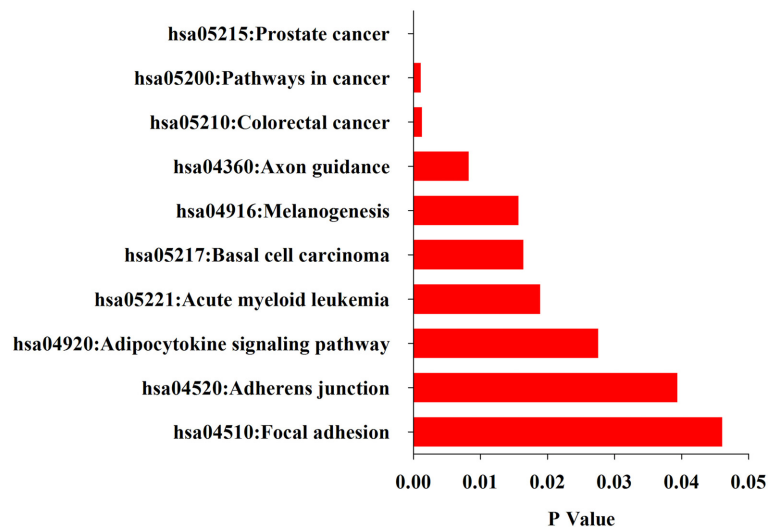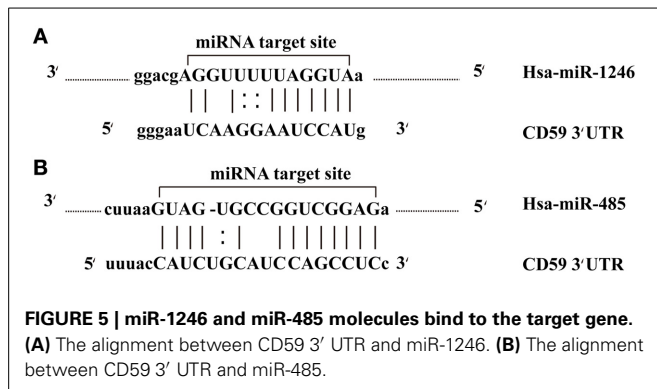

FIGURE 4 | KEGG pathways enriched in the up-and down-regulated target genes of the differentially miRNAs by DAVID.

FIGURE 5 | miR-1246 and miR-485 molecules bind to the target gene.
(A) The alignment between CD59 3′ UTR and miR-1246. **(B)** The alignment between CD59 3′ UTR and miR-485.

approximately 121 miRNA molecules, firstly making the connection between microvesicles and miRNA (Valenti et al., 2007). The third explanation is that passive release occurs during tissue injury (Brase et al., 2010).The high rate of proliferation and cell lysis in cancer might contribute to the abundance of miRNAs in the blood stream. The results presented here establish the foundation to motivate future global investigations of the difference of miRNA expression between breast cancer tumor tissue and serum.

The potential for miRNAs as biomarkers in cancer is being explored, based on the theoretical fact that miRNAs are natural antisense interactors that regulate many gene associated with cell survival and proliferation. In this study, we found that *CD59*, the cluster of differentiation 59, was a common target of miR-1246 and miR-485-5p belonging to the 10 differentially expressed miRNAs (**Figure 5**). MiRNA target sites in the 3′ UTRs of target genes were predicted by miRanda algorithm. *CD59*, also called protectin, is an 18–20 kD phosphatidylinositol-anchored glycoprotein that inhibits the cytolytic activity of complement by binding to C8 and C9, thereby protects the host cell against lysis by the membrane attack complex (MAC) of homologous complement (Meri et al., 1990; Li et al., 2011). The loss of *CD59* may offer a selective advantage for breast cancers, resulting in more aggressive tumors (Madjd et al., 2003). In addition to this, a previous study showed that miR-1246 induced p53-dependent apoptosis triggered by DNA damage (Palma et al., 2012). Perhaps the overexpression of miR-1246 alters the *CD59* expression profiles in breast cancer, resulting in cell apoptosis. Unfortunately, the interaction between *CD59* and miR-1246 has not been verified. On the other hand, miR-485 was also found to act as a tumor suppressor by affecting the proliferation rates and cell migration of breast carcinoma T47D cells (Anaya-Ruiz et al., 2013). Collectively, these results indicate that down-expression of miR-485 facilitates the expression of *CD59*, thereby improving the proliferation rates and cell migration of breast cancer cells. Because miRNAs can regulate cell-fate decisions, we concluded that miR-1246 and miR-485 may regulate the apoptotic vs. proliferative phenotype of breast cancer cells.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fgene.2014.00149/abstract

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