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Identification and characterization of microRNAs expressed in human breast cancer T-47D cells in response to prolactin treatment by Solexa deep-sequencing technology

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ABSTRACT

MicroRNAs (miRNAs) are key regulators of gene expression and perform critical roles in various biological processes. To investigate the functional roles of miRNAs in the prolactin receptor (PRLR) signaling pathway in breast cancer, we constructed two small RNA libraries from human breast cancer T-47D cells treated with or without prolactin (PRL). The miRNA expression profiles were systematically screened using Solexa deep-sequencing technology. More than 40 miRNAs were significantly differentially expressed, from which 4 miRNAs were chosen for validation by stem-loop real-time PCR. In addition, 3 novel miRNAs were selected for verification by PCR. Furthermore, upstream miRNA target genes were predicted using different algorithms, GO and KEGG analyses revealed that these targets were highly related to the PRLR signaling pathway. This study provides a reference for elucidating the complex miRNA-mediated regulatory networks of PRL/PRLR signaling pathway that affect breast cancer tumorigenesis and progression.

1. Introduction

Breast cancer is the most common type of tumor in women [1]. The potential mechanisms that regulate breast cancer progression are still poorly understood. Many factors and genes are involved in breast cancer pathogenesis. Human prolactin (PRL) is a pleiotropic hormone synthesized and secreted by anterior pituitary lactotrophic cells. It promotes the growth of normal and malignant breast tissues and can prevent apoptosis [2], enhance tumor cell proliferation [3], promote angiogenesis [4], and increase cell motility and metastasis [5]. PRL mediates its effects via the prolactin receptor (PRLR) [6] and stimulates signaling via the Jak2-STAT5 [7], Ras-Raf-MAPK, and PI3K-Akt pathways [8]. The mechanism through which the PRL/PRLR interaction triggers the activation of signaling networks remains enigmatic. However, many retrospective studies have shown that PRLR is expressed in the majority of human breast cancers and is elevated in comparison with normal breast tissue [9]. Inappropriate PRL/PRLR expression is also usually associated with breast cancer tumorigenesis and tumor progression [10].

MicroRNAs (miRNAs) are a class of non-protein-coding small RNAs of 19–23 nt in length that have key roles in regulating gene expression at the post-transcriptional level. These small non-coding RNAs act as negative regulators by binding their target messenger RNAs in the UTR and CDS regions and play important roles in various biological processes including cell growth, differentiation, signal transduction, metabolism and development [11]. Abnormal miRNA expression has been reported in various diseases including human breast cancer. In breast cancer, expression of miRNAs is frequently deregulated and can modulate virtually all relevant stages of breast cancer progression [12,13]. Several studies have assessed the expression of miRNA subsets through microarray or quantitative real-time PCR analysis and demonstrated that many miRNAs are deregulated in breast cancer compared to normal tissues [14,15]. However, these assays were not used to systematically analyze the miRNA expression profiles of breast cancer in the PRLR signaling pathway.

Solexa deep-sequencing technologies have revolutionized miRNA profiling in various model systems. Deep sequencing of miRNAs provides a highly quantitative estimate of known individual miRNA species and has the potential for novel miRNA discovery, including the discovery of those that occur at low frequencies [16]. This large-scale massively parallel sequencing technology utilizes sequencing by synthesis (SBS) with a simply operated automatic platform. Solexa deep-sequencing technologies have also led to a sharp rise in the rate of novel miRNA discovery [17].

To profile the small RNAs expressed in breast cancer and to reveal deeper insights into miRNA expression alterations in response

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to PRL treatment in T-47D cells, we constructed and sequenced two small RNA libraries prepared from T-47D cells treated with or without PRL. Using Solexa deep-sequencing technology, we identified and characterized the miRNAs in the two libraries. Our results provide a foundation for exploring the effect of miRNAs in the PRLR signaling pathway on breast cancer tumorigenesis and development.

2. Materials and methods

2.1. Cell culture and prolactin treatment

T-47D breast cancer epithelial cells (American Type Culture Collection, Manassas, VA), in which PRLR is highly expressed, were cultured in Dubelcco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (Invitrogen, Carlsbad, CA) and incubated at 37 °C, 5% CO_2. The cells were subcultured in six-well plates, and divided into two groups. One group was grown in normal medium as a control (denoted as NYDT47DNON) and another was exposed to 150 μg/μl human recombination prolactin (Peprotech, CA) for 72 h (denoted as NYDT47DPRL). Triplicates of each group were collected.

2.2. RNA extraction, construction and Solexa sequencing of small RNA libraries

Total RNA was isolated from the treated and untreated T-47D cells using Trizol reagent (Invitrogen, Carlsbad, CA). The RNA integrity was evaluated using an Agilent 2100 BioAnalyzer (Agilent Technologies, Palo Alto, CA).

Two small RNA libraries of NYDT47DNON and NYDT47DPRL were constructed using previously described methods [18]. Small RNAs (18–30 nt) were obtained from the total RNA, 5′ and 3′ adaptors were ligated to the small RNAs, the adaptor-ligated RNAs were subsequently transcribed into cDNA by RT-PCR, and the samples were amplified by PCR. The PCR products were purified and subjected to Solexa sequencing (Illumina, CA) at the Beijing Genomics Institute (BGI, Shenzhen, China).

2.3. Sequencing data analysis

The raw sequence reads were generated by the Illumina Genome Analyzer. To identify conserved miRNAs, the filtered sequences were initially used to search miRBase18.0 (http://www.mirbase.org/) with BLASTN, with a maximum allowance of two mismatches in which gaps were counted as mismatches. The criterion was then implemented according to a reported miRNA protocol [19]. All unannotated RNAs were subjected to novel miRNA prediction using Mireap software (https://sourceforge.net/projects/mireap/). The expression of known miRNAs was compared between the two groups to screen for differentially expressed miRNAs. The miRNA expression in the two groups was visualized by plotting the log2-ratio and scatter plot of the data.

2.4. miRNA validation by stem-loop quantitative real-time PCR and novel miRNA identification by PCR

A stem-loop primer based PCR assay was used to confirm the known and novel miRNAs. We selected four differentially expressed known miRNAs and 3 novel miRNAs obtained from the Solexa deep sequencing results as candidates for further confirmation. miRNA-specific stem-loop reverse transcription primers were synthesized by Life Technologies Corporation (Life Technologies, Shanghai, China). Primer sequences are listed in Table S5.

Total RNA was isolated from T-47D cells treated with or without PRL for 72 h using Trizol. First-strand cDNA was synthesized using the PrimeScript RT Reagent kit (TaKaRa, Dalian, China). To quantify the four known miRNAs, real-time PCR reactions were performed using the SYBR Green I kit and an ABI StepOne Plus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Each reaction was performed in triplicate, and U6 snRNA was used as a normalization control. The relative expression levels were determined using the comparative threshold cycle $2^{-\Delta\Delta^{Ct}}$ analysis method. The alteration in miRNA expression was calculated as the fold change relative to control. Student's *t*-tests were used for statistical analyses with two-tailed distributions and two-sample unequal variance parameters.

Three novel miRNAs were verified by PCR using Fermentas PCR Mix (Fermentas International Inc., USA). U6 snRNA was also used as a control. PCR products were subjected to 8% neutral polyacrylamide gel electrophoresis.

2.5. miRNA target prediction and functional annotations

The potential targets of miRNAs were predicted using TargetScan, PicTar, miRDB and microRNA.org programs with the default parameters. In our study, we subjected four verified known miRNAs and three novel miRNAs to target prediction. Intersecting elements identified in each miRNA-target prediction program were manually selected as miRNA targets. To investigate the possible biological processes regulated by the miRNAs, the MAS3 program (http://bioinfo.capitalbio.com/mas3/) was used to determine the putative functions of potential miRNA target genes by annotation using Gene Ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses.

3. Results

3.1. Solexa sequencing data of two small RNA libraries

Detailed sequencing data for the two libraries are displayed in Table S1 (Supplementary). More than 10 million pieces of raw data were harvested from the two libraries. Small RNA annotation results are summarized in Tables S1A and B, including details of categories and their corresponding unique reads and total read numbers. The common and specific tags in the two libraries are listed in Table S1. The total number of common sRNA tags (97.94%) was much higher than the number of specific sRNA tags in the two libraries (1.03%, 1.03%). Moreover, the general trend of the small RNA size distribution of the two libraries was similar, both centering approximately 19–25 nt. The length distribution of the small RNAs annotated as miRNAs are summarized in Fig. 1, and details of the tag lengths are shown in Tables S1 C and D. All tags peaked at 21–23 nt. Over 60% of the tags in the NYDT47DNON (61.74%) and NYDT47DPRL (60.23%) libraries belonged to canonical 22 nt miRNAs, which is in accordance with the lengths of mature miRNAs.

3.2. Differential expression of known miRNAs between the two libraries

All small RNAs annotated as miRNAs were blasted using miRBase18.0, and the matched sequences were annotated according to their similarities to known mature miRNA sequences deposited in miRBase18.0. The results showed that 4844 and 4800 unique reads (representing 798 and 821 mature miRNAs) were identified in the NYDT47DNON and NYDT47DPRL libraries, respectively. Details for known miRNAs are shown in Table S2, including the sequence reads and hairpin structure information. Compared with the parent miRNAs, all known miRNAs identified showed a poly-U attached to the 3′ terminus and one or more nucleotides deleted from the 5′ terminus, both of which have been previously reported [20]. In addition, 428 known mature miRNAs were coexpressed in

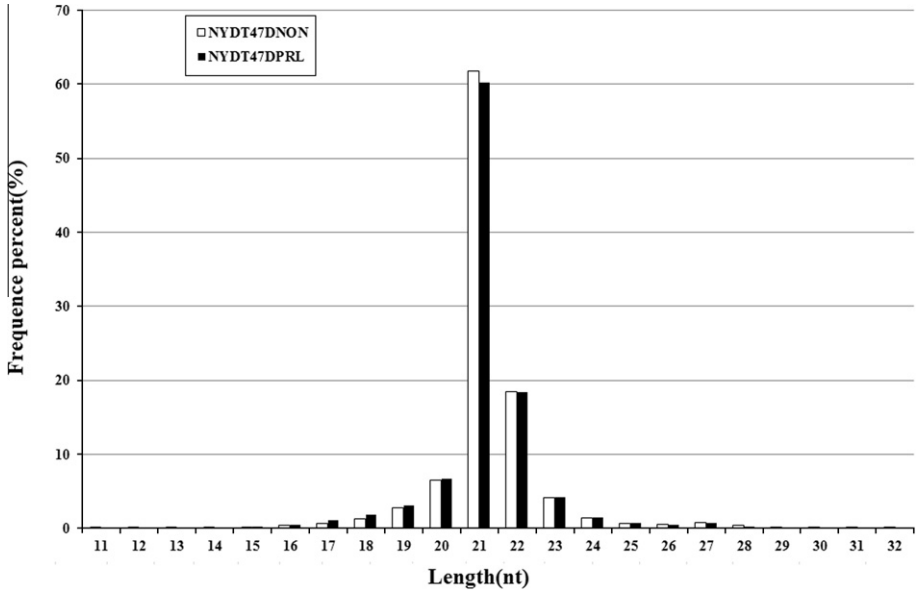

Fig. 1. The length distributions of miRNA reads produced by small RNA sequencing in NYDT47DNON and NYDT47DPRL cells. A total of 12,367,076 reads in T-47D and 10,056,034 reads in T-47DPRL ranged in length from 11 to 32 base pairs. The length distributions peaked at 22 bp, which is consistent with the expected miRNA length.

both of the libraries. Detailed information for the 13 miRNAs that were differentially expressed between the two libraries is presented in Table 1. These coexpressed miRNAs were also demonstrated by plotting the log2-ratios and scatter plot (Fig. 2). It showed that 42 miRNAs were significantly differentially expressed between the two libraries. In comparison to the NYDT47DNON group, 6 miRNAs were down-regulated and 36 miRNAs were up-regulated in the NYDT47DPRL group.

3.3. Identification of known miRNAs and their functional annotations

To validate the Solexa sequencing miRNA expression results for the two libraries, we selected miR-449c-5p, miR-16-5p, miR-15b-5p, and miR-193a-3p for stem-loop real-time PCR quantification. The relative quantification results are depicted in Fig. 3A. The results showed that miR-449c-5p was down-regulated, while miR-16-5p, miR-15b-5p and miR-193a-3p were up-regulated. These results paralleled the Solexa sequencing data.

The above four miRNAs were subjected to further target gene prediction and functional analyses. We found 249 potential targets of the four miRNAs. Next, GO and KEGG analyses were performed. Fig. 3B summarizes the categorization of miRNA target genes according to the biological process, cellular component or molecular function. The genes were classified into 20 categories based on biological processes, as shown in Fig. 3B. The four most over-represented biological process GO terms were regulation of transcription, development, transcription and signal transduction. The cellular component GO terms revealed that the miRNA-target genes were related to 10 cellular parts. The three most frequent terms were nucleus, cytoplasm and plasma membrane. Furthermore, the biological interpretation of these targets was annotated using a KEGG pathway analysis. A total of 93 pathways were identified, and 20 categories are shown in Fig 3C. The most represented pathway was the Wnt signaling pathway. Focal adhesion, the MAPK signaling pathway, cell cycle, and the Jak-STAT signaling pathway were also included.

3.4. Identification of novel miRNAs in the two libraries and the functional annotation of the predicted target genes

Within the Solexa sequencing results, all unannotated RNAs were used to predict novel miRNAs. Unannotated unique reads in the two libraries were analyzed using Mireap software (http://sourceforge.net/projects/mireap/) to predict novel miRNAs. As a result, 46 novel miRNAs were coexpressed in both libraries, 69 specific novel miRNAs were identified in the NYDT47DNON library, and 40 specific novel miRNAs were found in the NYDT47DPRL library. Details for the novel miRNAs are shown in Table S4. Similar to known miRNAs, most novel miRNAs also carried 3′ or 5′ end variants, differing in one or more nucleotides.

From the novel miRNAs, we chose to validate three novel miRNAs that were coexpressed in the two libraries (denoted as novel-miR-01, novel-miR-02, novel-miR-03). The three novel miRNAs are listed in Table 2 with the corresponding genome location, mature sequence length, precursor length and minimum free energy. Their predicted secondary structures were determined using Mireap according to a principle of minimum folding energy, and the results are represented in Fig. 4A. Furthermore, we designed miRNA-specific stem-loop PCR primers and successfully detected the expression of the three novel miRNAs in T-47D cells treated with or without PRL. In addition, we predicted the potential targets of the three novel miRNAs. A total of 485 candidates were predicted as putative targets. GO and KEGG analyses were also used to explore the functional annotations of these targets genes. These genes were classified into 20 categories based on biological processes as shown in Fig. 4C, and the four most over-represented biological process GO terms were regulation of transcription, transcription, signal transduction, and development. The cellular component GO analysis showed that these genes were related to 10 main cellular components, such as the nucleus and cytoplasm, functioning on protein binding, zinc ion binding and metal ion binding. The KEGG analysis revealed that target genes were enriched in focal adhesion, the regulation of actin cytoskeleton and the MAPK signaling pathway.

4. Discussion

Breast cancers are sensitive to hormones such as PRL, which was originally identified as a neuroendocrine hormone of pituitary origin. Although the pleiotropic actions of PRL are recognized, its role in regulating growth and differentiation of mammary tissues is not well understood [21]. Retrospective studies indicate that women on estrogen and prolactin hormone replacement therapy had

Table 1
Significantly differentially expressed miRNAs in NYDT47DNON and NYDT47DPRL libraries.

miR-name	NYDT47DNON-std	NYDT47DPRL-std	Fold-change(log2 NYDT47DPRL/NYDT47DNON)	*p*-value	Sig-lable
miR-1226-3p	0.1628	1.6066	3.30283818	0.000133497	**
miR-345-3p	0.2443	1.5062	2.62418758	0.000986277	**
miR-331-3p	4.2341	24.0991	2.50885202	1.45E-39	**
miR-2277-5p	0.2443	1.3054	2.41776618	0.003519966	**
miR-3127-3p	0.2443	1.3054	2.41776618	0.003519966	**
miR-1307-5p	7.8168	34.04	2.12258101	3.15E-45	**
miR-4470	0.4885	2.1087	2.10992339	0.000563693	**
miR-3158-3p	0.3257	1.3054	2.00287632	0.00928829	**
miR-4774-5p	0.3257	1.3054	2.00287632	0.00928829	**
miR-500a-5p	0.3257	1.3054	2.00287632	0.00928829	**
miR-500b	0.3257	1.3054	2.00287632	0.00928829	**
miR-188-5p	0.4885	1.8074	1.88748536	0.003058607	**
miR-766-3p	0.7328	2.7112	1.88744013	0.000265883	**
let-7e-3p	0.57	1.9078	1.74287611	0.003921638	**
miR-3676-3p	4.8041	14.9615	1.63891674	5.16E-15	**
miR-629-3p	0.7328	2.2091	1.59196732	0.003547195	**
miR-503	0.6514	1.9078	1.55029431	0.007955302	**
miR-378c	5.7812	15.9657	1.46553492	1.00E-13	**
miR-18a-5p	4.5598	11.8487	1.37768633	9.82E-10	**
miR-4741	1.9542	4.619	1.24100242	0.000448173	**
miR-20b-3p	1.4656	3.3136	1.17690805	0.004416153	**
miR-324-5p	20.2748	44.2821	1.12703596	5.90E-24	**
miR-940	12.6209	26.1074	1.048644	2.77E-13	**
miR-296-5p	16.9364	34.8433	1.04075402	4.95E-17	**
miR-184	40.7939	83.7444	1.03763928	1.71E-38	**
miR-339-5p	33.7099	68.9837	1.03308316	8.69E-32	**
miR-15b-5p	522.5038	1054.2361	1.01268457	0	**
miR-100-5p	0.2443	1.1045	2.17666765	0.012050669	*
miR-4485	0.4071	1.3054	1.68103682	0.020755495	*
miR-363-5p	0.3257	1.0041	1.62428733	0.04889635	*
miR-4454	0.3257	1.0041	1.62428733	0.04889635	*
miR-1284	0.4071	1.205	1.56557802	0.034896822	*
miR-624-5p	0.4885	1.3054	1.41806148	0.040807852	*
miR-3124-5p	0.8142	2.1087	1.37289873	0.010714088	*
miR-16-5p	1110.1475	2074.8338	0.9022444	0	*
miR-193a-3p	295.9796	527.0678	0.83249081	6.78E-161	*
miR-449b-3p	4.7226	2.1087	−1.16322748	0.000986622	**
miR-449c-5p	2090.1779	874.1956	−1.25759772	0	**
miR-105-5p	2.3613	1.1045	−1.09618793	0.02831823	*
miR-4742-3p	2.4427	1.1045	−1.14508327	0.020790189	*
miR-3193	2.117	0.9037	−1.22810544	0.023461651	*
miR-3936	1.2214	0.4017	−1.60434538	0.039555731	*

Note: (1) NYDT47DNON-std/ NYDT47DPRL-std: the result of Solexa sequencing exhibited, presented the normalized expression level of miRNA in two libraries. (2) Fold-change(log2 NYDT47DPRL/NYDT47DNON): fold change of miRNAs in both samples. (3) *p*-value: *p* value manifests the significance of miRNA differential between two samples. Less *p* value indicates more significance of difference of miRNA. (4) Sig-lable: significance label. (5) Down-regulated miRNAs were highlighted with gray, others were up-regulated miRNAs.
*: fold change(log2) >1 or fold change(log2) <−1, and $0.01 \leqslant p$-value <0.05.
**: fold change(log2) >1 or fold change(log2) <−1, and *p*-value <0.01.

an increased risk of breast cancer and were more likely to have larger tumors of higher grade and to succumb to their cancer compared to women on estrogen alone [22]. Recently, the ability of hormones, such as estradiol (E2) and progestin (PR), to regulate miRNAs in breast cancer cells in culture has been studied [14,15]. Little evidence has demonstrated that miRNAs are involved in modulating multiple signaling pathways during breast cancer development, but they may serve as potential targets for breast cancer [23,24]. However, no studies have been conducted to identify PRL-mediated regulation of miRNA expression in breast cancer through the PRLR signaling pathway. In the present study, we thoroughly analyzed the miRNA expression profiles of T-47D cells treated with PRL. Solexa deep-sequencing technology and bioinformatics methods were used to determine the miRNA expression in T-47D cells treated with or without PRL. To our knowledge, this is the first Solexa sequencing-based expression profiling study of miRNA alterations associated with the PRL/PRLR signaling pathway in human breast cancer cells.

In this study, high-throughput Solexa sequencing identified 798/821 known and 115/86 novel miRNAs in the two libraries. Forty-two miRNAs were significantly differentially expressed, 36 miRNAs were up-regulated and 6 miRNAs were down-regulated in T-47D cells after PRL treatment. We could ascribe the miRNA expression differences between the two libraries to PRL exposure, which stimulates a series of chain reactions including changes in miRNA expression due to a PRLR-mediated response to PRL. In addition, we observed a diverse population of mature miRNA variants known as isomiRNAs in both libraries. These isomiRNAs might contribute to altered RNA editing, which could impact miRNA processing and miRNA diversity [25]. Subsequently, we quantified four known miRNAs by stem-loop real-time PCR, and the results were consistent with Solexa sequencing. These results demonstrate the reliability of the Solexa sequencing results obtained with the two libraries, which provides a powerful foundation for subsequent miRNA research. In the present study, 46 novel miRNAs were coexpressed and showed a similar number of reads number in both

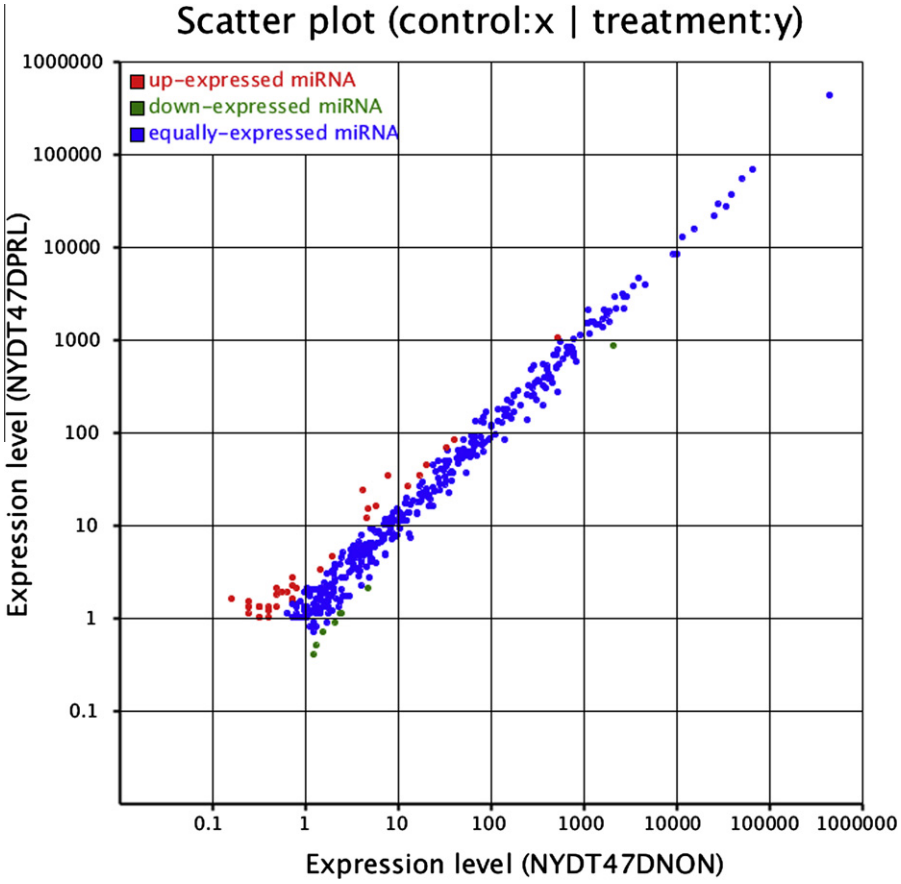

Fig. 2. Differential expression of the conserved miRNAs identified in the NYDT47DNON and NYDT47DPRL libraries. Each point in the figure represents a miRNA. Red points represent up-regulated miRNAs (fold change>2), green points represent down-regulated miRNAs (fold change<0.5), and blue points represent miRNAs that were not differentially expressed (0.5<fold change $\leqslant$2).

libraries. We speculate that these miRNAs might not play an important role in the PRL/PRLR signaling network because their expression did not change significantly after PRL treatment. Compared to the 69 newly-predicted miRNAs identified in untreated T-47D cells, only 40 specific novel miRNAs were expressed in PRL-treated cells. This difference might be due to PRL functioning through PRLR or other molecular elements to elicit changes in the miRNA profile. Three novel miRNAs were verified by stem-loop primer-based PCR, further confirming the reliability of Solexa sequencing.

After validating selected known and novel miRNAs, we predicted the putative miRNA targets using several different algorithms. As Fig. 3 shows, GO terms showed that the target genes of the four known miRNAs were mainly enriched in the nucleus, cytoplasm and membrane cellular components and the biological processes of transcription regulation, development, and transcription and signal transduction. Their molecular functions included protein binding and nucleotide binding. The KEGG results revealed that these genes were tightly related to the Wnt signaling pathway, focal adhesion, the MAPK signaling pathway, and the Jak-STAT signaling pathway. We found that the GO and KEGG results of the three novel miRNAs targets were similar to the four known miRNA targets based on the functional analysis comparison. We speculate that PRL activates PRLR and triggers a cascade of distinct events because the targets of the differentially expressed miRNAs and novel miRNAs were highly related to pathways involved in PRLR signaling, such as the Jak-STAT and MAPK signaling pathways. Upon activation of PRLR, numerous genes and modulating factors distributed in the nucleus or cytoplasm might be involved, as shown by the GO and KEGG results. The predicted miRNA targets and their functions need to be further studied.

The sequence 2–8 nt from the 5′–3′ end in a mature miRNA, called the seed sequence, is highly conserved and binds to the 3′UTR of target mRNAs to exert its function by perfect or non-perfect complementation. The seed sequence of a miRNA often binds numerous targets, and targets can be altered when one or more nucleotide is substituted or edited in this region. Moreover, nucleotide alteration or modification in the seed sequence may result in miRNA dysfunction, leading to altered phenotypic characteristics [26]. As an extension of our research, we systematically analyzed the seed sequences of mature miRNAs for nucleotide editing. We observed 85 mature miRNAs with single nucleotide substitutions in the seed sequence in two samples, and detailed substitution information is shown in Tables S3A and B. We showed that a single mature miRNA might have different types of substitutions. In Table S3C and D, the dominant substitutions appear to be A-to-G, G-to-A, G-to-T, T-to-C, T-to-G and T-to-A. However, these results are not completely consistent with former research regarding RNA modification in rice and Arabidopsis, in which A-to-C and C-to-T were dominant substitutions while T-to-G and T-to-A were not included [27]. We reason that miRNAs may hold different substitution types in different species or tissues. Additionally, highly expressed miRNAs, such as let-7f-5p and let-7e-5p, were found to have a higher editing probability in our analysis (Tables S3E and F), which suggested that they might have a higher probability to target more genes.

In summary, we report for the first time the construction of two small RNA libraries from T-47D cells treated with or without PRL,

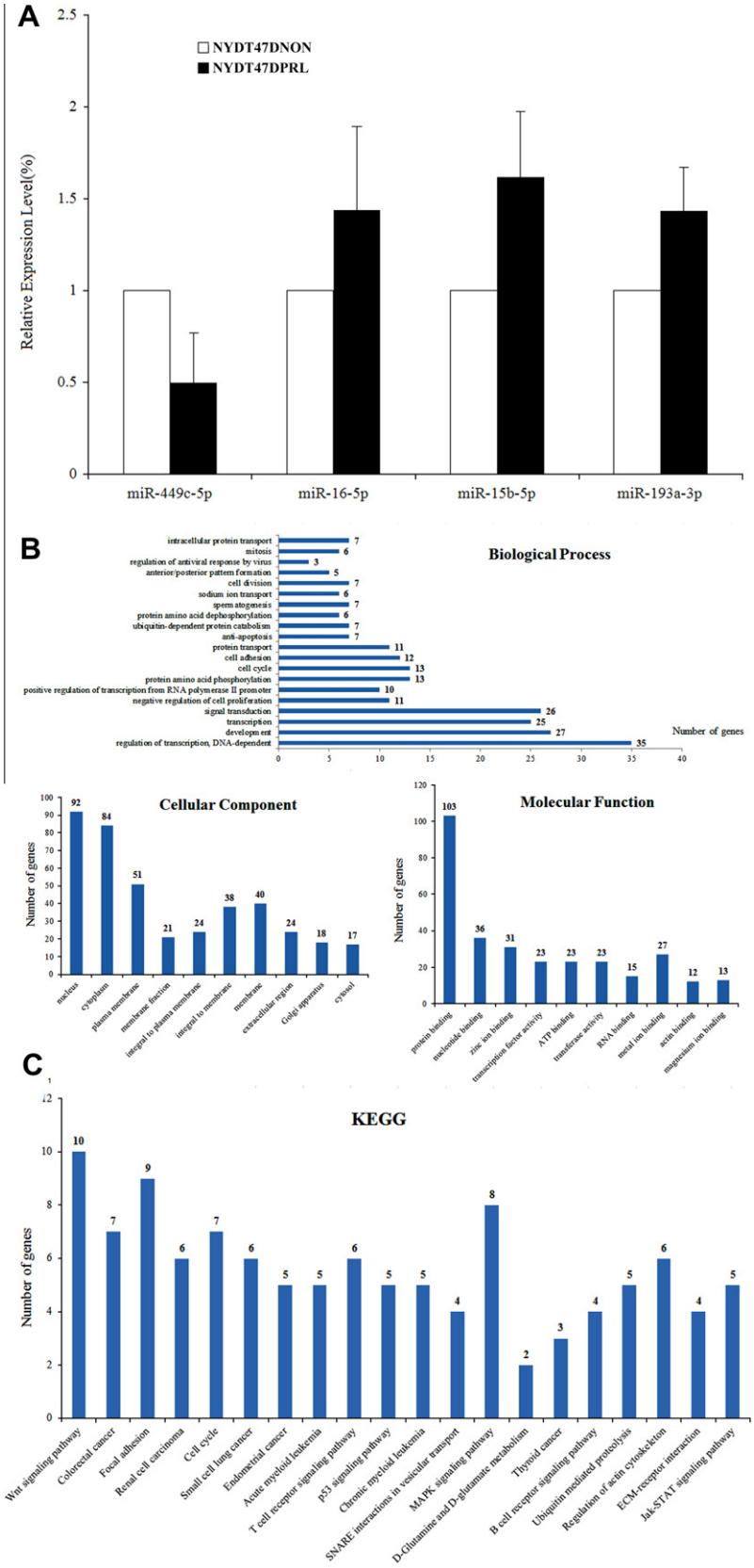

Fig. 3. Verification of four known miRNAs and their target gene functional annotations. (A) Real-time PCR verification of miR-449c-5p, miR-16-5p, miR-15b-5p and miR-193a-3p. The relative quantification results were the same as the sequencing results. (B) GO analysis of the predicted targets of four known miRNAs. Twenty biological process categories and 10 cellular component and molecular function categories were included in the analysis. (C) The KEGG analysis of the predicted targets of four known miRNAs. Twenty categories are represented in the figure.

Table 2
Detailed information of the 3 novel miRNAs in two libraries.

Name	Mature sequence (5′–3′)	Genome location	Strand	precursor length (nt)	Minimum free energy (kcal/mol)
novel-miR-01	UCGGGCGGGAGUGGTGGCUUUU	chr6:28918819:28918903	+	85	−22.2
novel-miR-02	UGGGAGGAACAAGUAUGCAUU	chr11:16984501:16984581	–	81	−27.1
novel-miR-03	UCAGGGAGAAAGAAGGGUUAUU	chr11:122928619:122928702	–	84	−21.3

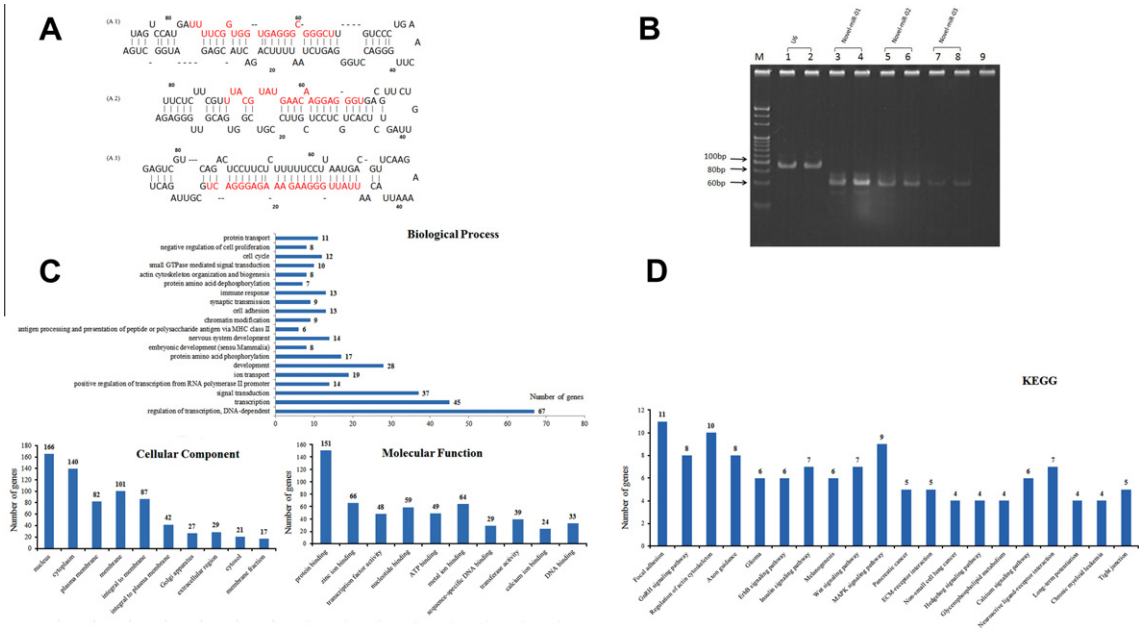

Fig. 4. Secondary structure prediction and verification of three novel miRNAs and their target gene functional analyses. (A) The secondary structures of three novel miRNAs were predicted using Mireap. The mature sequence of each novel miRNA is highlighted in red, and the Arabic number represents the detailed position in the structure. (B) The PCR products of three novel miRNAs are shown in an electrophoretogram. Lanes 1 and 2 are the internal control snRNA U6. Lanes 3 and 4 are novel-miR-01, lanes 5 and 6 are novel-miR-02, lanes 7 and 8 are novel-miR-03, and lane 9 is a blank control. (M: 20 bp marker. 1, 3, 5 and 7 are PCR products of NYDT47DNON. 2, 4, 6 and 8 are PCR products of NYDT47DPRL). (C) The GO analyses of the predicted targets of three novel miRNAs. Twenty categories were involved in biological processes, and 10 categories were involved in both the cellular components and molecular functions. (D) The KEGG analysis of the predicted targets of three novel miRNAs. Twenty categories are represented in the figure.

and we identified a number of miRNAs that were significantly differentially expressed between the two libraries. We also detected several novel putative miRNAs associated with PRL/PRLR signaling and analyzed their potential roles in human breast cancer cells. Moreover, we predicted the targets of several known miRNAs and novel miRNAs and then identified the functional annotations of these targets. Additionally, we extensively analyzed isomiRNA expression and seed sequence editing of known miRNAs. The results of the present study collectively confirm that the Solexa sequencing platform was an effective tool to analyze small RNAs and trace new miRNAs. More importantly, the identification of the entire miRNAome in human breast cancer T-47D cells in this research shall pave the way to establish the complexity of miRNA-mediated regulatory crosstalk associated with PRL/PRLR signaling.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

XC and QJW conceived and designed the study. WH, JY and YJL performed the experiments and analysis. QJW and WH wrote the original manuscript. XC and LG contributed to revisions of the manuscript. All authors read and approved the final version.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.02.016.

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