Original Article

Decreased Expression of Bioinformatically Predicted piwil2-targetting microRNAs, miR-1267 and miR-2276 in Breast Cancer

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Abstract

Purpose: Human Piwil2, a member of Piwi subfamily of Argonaute proteins, is primarily expressed in testis, where it regulates self-renewal of germ cells. However, its ectopic expression has been reported with several tumors, including breast cancer. The upregulation of piwil2 in various stages of breast cancer suggested its suitability as a novel tumor biomarker. Considering the vital role of microRNAs (miRNAs) in regulating the expression of most human genes, we hypothesized a concomitant downregulation of the bioinformatically-predicted piwil2-targetting microRNAs in breast cancer.

Method: We employed different bioinformatic tools to predict piwil2-targeting miRNAs. Then, from the list of predicted miRNAs, we chose two less studied miRNAs (miR-1267 and miR-2276) for experimental validation. Using a real-time RT-PCR approach, we quantified the relative expression of the miRNAs in 31 pairs of formalin-fixed paraffin-embedded tumor/non-tumor tissue samples.

Results: Our data revealed a noticeable but not statistically significant (P = 0.133) downregulation of miR-1267 in tumor samples, compared to non-tumor samples obtained from the same patients. Downregulation of miR-1267 was more significant in higher grades of malignancies (fold change = 2.39, P = 0.033) and also in lymph nodes containing high-grade tumor cells (fold change = 6.66, P = 0.02). Interestingly, a significant upregulation of miR-1267 was observed in tumors at high stages (stage 3a, 3b), compared to low stages (stage 2a, 2b) (fold change = 8.05, P = 0.048). Similar patterns of expression alteration were also observed for miR-2276.

Conclusion: Altogether, our findings suggest a probable tumor suppressor role for miR-1267 and miR-2276 in breast tumor initiation and progression, but a probable promoting role for them in invasion and metastasis.

Keywords: Biomarker, breast cancer, gene expression, microRNAs, piwil2

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Introduction

With an estimated incidence rate of 29%, breast cancer is the most common type of cancer in women. It is also the second leading cause of cancer-related death in women, with an estimated death rate of 15%.[1] In Iran, breast cancer has become the most common primary female cancer, with an estimated prevalence rate of 23.65 per 100,000.[2] Most of breast cancer fatality is attributed to its late diagnosis, when the tumor has progressed towards higher grades and stages of malignancies.[3] Therefore, it is vital to find suitable tumor markers for non-invasive early detection and reliable classification of tumors.

The Piwi subfamily of Argonaute proteins are solely expressed in germ lineage cells, and are essential for stem cell self-renewal in various organisms.[4] They also play important parts in RNA silencing [5] translational regulation,[5] and gametogenesis.[6] In humans, all four members of the family (PIWIL1 (HIWI), PIWIL2 (HILI), PIWIL3, and PIWIL4 (HIWI2)) are primarily expressed in testis.[7] Ectopic expression of human PIWIL2 has been reported with several tumor cells and tissues including prostate, gastrointestinal, ovarian and endometrial cancers.[8] Re-expression of PIWIL2 has also been reported in breast cancer, where it acts as an inhibitor of apoptosis.[8–10] Furthermore, the ectopic expression of piwil2 in various stages of breast cancer suggested its suitability as a novel tumor marker.[11]

MicroRNAs (miRNAs) are a new class of single-stranded non-coding RNAs with a size of 19–24 nucleotides.[12] More than 2500 microRNAs have been so far discovered in humans and the list is still growing. They act mostly through imperfect binding to the 3′-UTR of their target mRNAs, and by reducing the half-life of their targets or inhibiting their translation negatively regulate their expression. The miRNAs are involved in regulation of most cellular processes including embryonic development, cell proliferation, cell differentiation, apoptosis, etc.[12,13] Aberrant expression of miRNAs has also been reported for a variety of diseases including cancer.[14] Due to their small sizes, miRNAs are very stable molecules in biological samples such as formalin-fixed paraffin-embedded (FFPE), serum, and urine. For this reason, they are suitable for use as biomarkers, compared to mRNA or protein molecules.[15,16]

Considering the ectopic expression of *piwil2* in breast tumor tissues and its potential application as a tumor marker, we hypothesized here that the expression of miRNAs that target piwil2 might be also altered in breast cancer. Using bioinformatic approaches,

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we predicted two putative piwil2-targetting microRNAs (miR-1267 and miR-2276), and then evaluated their expression alterations in tumor vs. non-tumor tissue samples of breast.

Materials and Methods

Clinical samples

Archival Formalin-Fixed Paraffin-Embedded (FFPE) tissue samples were obtained from the pathology department of Shariati Hospital in Tehran, Iran (Table 1). The samples contained tumor, marginal/non-tumor, and lymph node specimens from the same patients. Hematoxilin/Eosin-stained sections from each sample were re-examined by an expert pathologist to confirm the initial diagnosis and classification of samples.

Deparaffinization and RNA extraction

Small punches of FFPE blocks were deparaffinized with three times washing in xylene and absolute ethanol, respectively, and then treated with proteinase K (Fermentas, Lithuania) diluted (20 mg/mL) in PK buffer (1 mM NaCl, 1 mM EDTA, 5 mM Tris-HCl, pH 7.4) for 3 h at 56°C.[17] Total RNA was then extracted by TRIzol reagent (Invitrogen, USA), according to the manufacturer's instruction, in an RNase-free condition. The quantity and quality of the extracted RNAs were determined with measuring the 260/280 nm absorbance with the ND-1000 NanoDrop (Thermo Fisher Scientific, USA) Spectrophotometer (BioTek UK) and agarose gel electrophoresis. RNase-free DNase treatment was also performed to remove any possible traces of genomic DNA within the samples.

cDNA synthesis and real-time PCR

The miRNAs expression levels were quantified using ParsGenome's miRNA amplification Kit (Iran). Initially, a polyA tail was added to the extracted RNAs by incubating 2 µg of RNA with

Table 1. Clincopathological features of patients diagnosed with invasive ductal carcinoma (IDC) type of breast cancer. Non-tumor/marginal samples obtained from the same patients.

Case #	Time span (year)	Age	Grade	Stage	ER	PR	HER2
1	2	38	2	2a	Weak +	+	+++
2	4	56	3	2a	+	+	-
3	4	40	3	2b	-	-	+++
4	4	45	2	2b	-	-	N.A.
5	5	47	3	2b	N.A.	N.A.	N.A.
6	5	30	3	2b	N.A.	N.A.	N.A.
7	5	39	3	2a	N.A.	N.A.	N.A.
8	4	44	2	2b	+	+	-
9	6	51	2	2b	N.A.	N.A.	N.A.
10	2	50	2	3a	+	+	+++
11	2	47	3	3a	-	-	+++
12	2	36	3	3a	-	-	-
13	2	36	3	3a	N.A.	N.A.	N.A.
14	3	60	1	3a	+	+	-
15	2	49	1	3b	+	+	-
16	6	72	3	3a	-	-	-
17	5	41	2	3a	N.A.	N.A.	N.A.
18	4	56	3	3a	+	+	-
19	4	44	2	3a	N.A.	N.A.	N.A.
20	4	48	3	3a	N.A.	N.A.	N.A.
21	5	46	2	3a	+	+	++
22	2	60	3	2a	N.A.	N.A.	N.A.
23	2	35	2	2b	N.A.	N.A.	N.A.
24	2	42	3	3b	-	-	+++
25	2	45	2	2a	+	+	++
26	3	37	1	2b	+	+	N
27	5	55	2	2a	N.A.	N.A.	N.A.
28	5	45	1	2b	N.A.	N.A.	N.A.
29	5	68	3	1	N.A.	N.A.	N.A.
30	6	42	3	3a	N.A.	N.A.	N.A.
31	2	35	3	3a	-	+	-
32	2	80	3	2b	-	-	-
33	3	45	2	1	+	+	-
34	4	55	3	3a	+	+	+++
35	5	51	3	1	N.A.	N.A.	N.A.
36	5	54	3	1	N.A.	N.A.	N.A.

N.A. = Not available

0.5 µL of polyA polymerase enzyme, 2 µL of 10x buffer, 2 µL of ATP, 0.5 µL of RNase inhibitor (Fermentas, Lithuania), and DEPC-treated water at 37°C for 10 min. Then, for cDNA synthesis, 6 µL of polyadenylated RNA was mixed with 0.5 µL of reverse transcriptase (RT) enzyme, 2 µL of 5x buffer, 0.5 µL of specific linear primers, and 0.5 µL of RNase inhibitor (Ribolock). The mixture was then incubated in a thermal cycler at 42°C for 60 min followed by incubation at 85°C for 1 minute, to heat-inactivate the RT enzyme.

Real-time PCR was performed using 1 µL of cDNA products, 0.5 µL of specific forward primer, 0.5 µL of universal reverse primer (ParsGenome, Iran), 10 µL of SYBR Green PCR master mix (Takara, Japan), and 8 µL of nuclease-free water (CinnaClon, Iran).

The 5s rRNA gene was used as an internal control to normalize the relative expression of miR-1267 and miR-2276 in clinical samples. Real-time PCR reactions were carried out in an ABI-7500 (USA) real-time quantitative PCR instrument with the following conditions: 95°C for 15 minutes, followed by 40 cycles of 95°C for 15 seconds, 63°C for 25 seconds and 72°C for 35 seconds. All PCR reactions were performed at least in duplicates, and a no-RT negative control accompanied each run to monitor the specificity of the reactions. The authenticity of the amplified products was further confirmed by the existence of single melt-curves during real-time amplification, the presence of a single band on Acrylamide gel electrophoresis, and DNA sequencing of the PCR products which had been cloned in a TA cloning vector (Takara, Japan).

Statistical analyses

LinRegPCR (12.x) software (AMC, Amsterdam) was employed to determine the efficiency of each primer pair. All data obtained from the real-time RT-PCR experiments were further analyzed with REST 2009 (TUM, Germany) and GraphPad (CEO, USA) softwares. The expression level of miRNAs in each sample was then normalized to that of 5s rRNA. Moreover, miRNAs expressions in tumor samples were normalized to their matched non-tumor counterparts ($2^{\wedge\text{-}\Delta\Delta CT}$). The data were then analyzed by performing the student t-test, and a $P < 0.05$ was considered as statistically significant.

Results

miR-1267 and miR-2276 have target sites on 3' UTR of piwil2 transcript

Considering the major involvement of piwil2 in breast cancer tumorigenesis, we hypothesized that any factor affecting the expression level of piwil2 may have a role in breast cancer initiation and progression. For this reason, we screened the sequence of piwil2 transcript with RNAhybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid) and miRSearch V3.0 (Exiqon, Denmark) softwares to find putative miRNAs capable of targeting the transcript. Next, we narrowed down the list of predicted miRs to two miRNAs, miR-1267 and miR-2276, based on two criteria: 1) their novelty and lack of previous information on their contribution in breast cancer; and 2) their shared genomic locations with breast cancer hot spots.

Down-regulation of miR-1267 in breast tumor samples

To evaluate the expression of miR-1267 in tumor and non-tumor samples, we employed a real-time PCR approach and the $2^{-\Delta\Delta ct}$ formula to measure gene expression alterations. After setting up the reactions and confirming the authenticity of the amplified products (Figure 1), miR-1267 expression was quantified in tumor and non-tumor breast samples. The expression level of an internal control, 5S, was used as an internal control to normalize the expression level of miR-1267. LinReg analysis showed a reliable efficiency of 1.8 for miR-1276 primers to amplify the endogenous microRNA in real-time PCR.

The relative expression of miR-1267 in 31 tumor samples was compared with their matched non-tumor samples obtained from the same patients. The obtained data, analyzed with the Graphpad software, demonstrated a noticeable, but not statistically significant ($P = 0.133$), down-regulation of miR-1267 (Figure 2A) in tumor samples. When we compared the expression alterations in different grades of malignancy, we observed a significant down-regulation of miR-1267 in high-grade tumors, in comparison to the low-grades (fold-change = 2.39, $P = 0.033$, Figure 2B). The same pattern of expression was observed in samples obtained

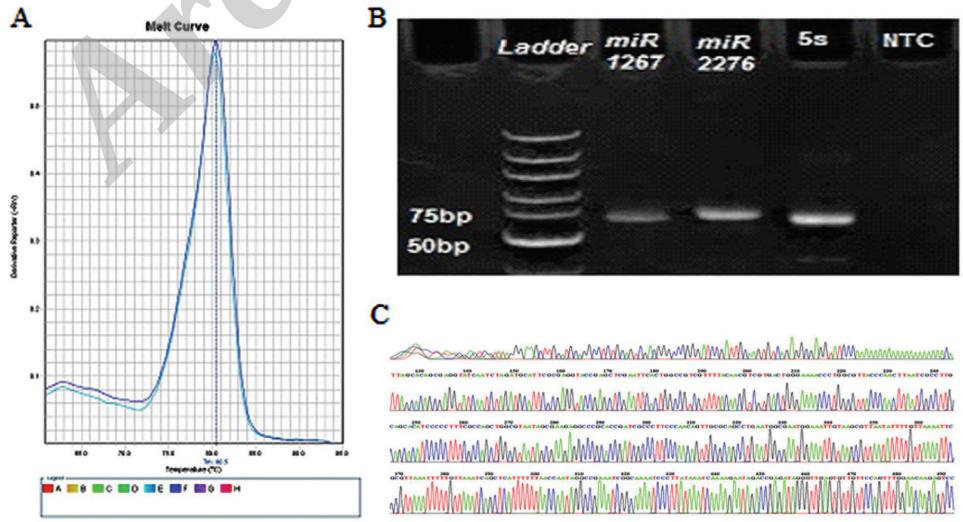

Figure 1. Confirming the authenticity of the amplified products by **A)** inspecting the single melt curve peak of the products, **B)** single-band products with correct sizes on agarose gel electrophoresis, and **C)** DNA sequencing of the amplified products.

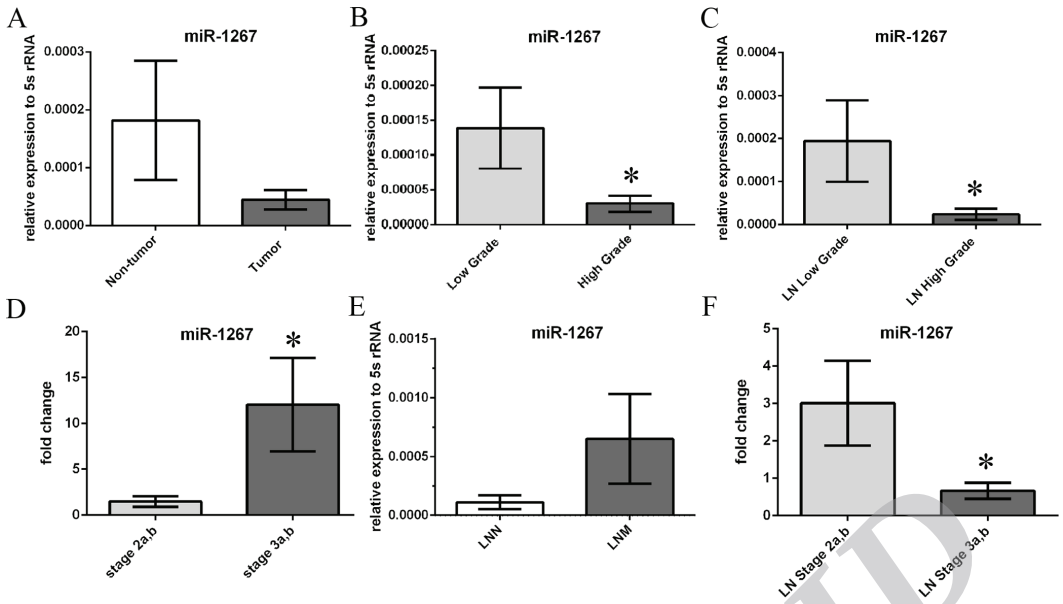

Figure 2. A) Evaluating the relative expression level of miR-1267 in breast tumor samples in comparison to non-tumor samples obtained from the margin of same tumor, normalized to that of house-keeping gene, 5s. **B)** A comparison between the expression levels of miR-1267 in breast tumors with different stages. **C)** A similar comparison in lymph node metastases (LNM) vs. lymph node negative (LNN) samples. **D)** Note that when the expression of miR-1267 was compared between tumor vs. non-tumor samples in each stage subgroup the difference was statistically significant (*). A *P* value less than 0.05 considered as statistically significant.

from lymph nodes of the patients. miR-1267 was significantly down-regulated in lymph nodes biopsies containing high-grade breast cancer cells, compared to the ones with low-grades cancer cell (fold change = 6.66, *P* value = 0.02, Figure 2C). On the other hand, we also examined the expression alteration of miR-1267, based on the stage of tumor samples. Surprisingly, we found a significant upregulation of miR-1267 in high stages (stage 3a, b) of breast cancers, compared to the low stages (stage 2a, b) (fold change = 8.05, *P* value = 0.048, Figure 2D). Similarly, there existed a noticeable, but not statistically significant, upregulation of miR-1267 in lymph nodes containing metastatic tumor cells, compared to normal lymph nodes (p value = 0.14, Figure 2E). In contrast, we had a significant down-regulation of miR-1267 in lymph nodes containing high stages of breast cancer metastasis (fold change = 4.51, *P* value = 0.03, Figure 2F), in comparison to those containing low stages metastatic tumor cells.

miR-2276 expression alteration in tumor and non-tumor tissue samples of breast

As described above, we also measured the expression alteration of miR-2276 in different clinical specimens of breast and lymph nodes. LinReg analysis indicated a PCR reaction efficiency of 1.8 for the primers amplifying miR-2276.

Real-time PCR data revealed a significant downregulation of miR-2276 expression in invasive breast carcinoma samples (*P* value < 0.05), compared to the non-tumor samples obtained from the same patients (fold change = 3.96, *P* value = 0.045, Figure 3A). The observed downregulation was more prominent in high-grade samples (fold change = 1.63, *P* value = 0.038, Figure 3B), compared to the low grades. Similarly, the same observation was made in lymph nodes containing high grade metastatic cells, in comparison to the lymph nodes having low grade metastatic cells (fold change = 5.97, *P* value = 0.03 , Figure 3C).

Interestingly, similar to what we reported for miR-1267, the same expression alteration pattern was observed for miR-2276 with different stages of tumor progression. Our results showed a significant upregulation of miR-2276 in breast tumors with high stages (stage 3a, b), in comparison to low stage (stage 2a, 2b) tumors (fold change = 5.021, *P* value = 0.012, Figure 3D). Furthermore, while we had a noticeable upregulation of miR-2276 in lymph nodes with metastatic breast tumor cells, compared to the normal lymph nodes, the difference was not statistically significant (*P* value = 0.12, Figure 3E). However, within metastatic lymph nodes, we observed a significant downregulation of miR-2276 in lymph nodes containing metastatic cells from high stages tumors, in comparison to low-stage metastatic lymph nodes (fold change = 6.57, *P* value = 0.028 , Figure 3F).

Our data failed to find any significant correlation between the expression alteration of the miRNAs with the personal characteristics of the patients including age and the (right/left) location of tumors (data not shown).

Discussion

Piwil2 is a member of piwi gene family with a crucial role in the self-renewal maintenance of stem and germ cells. It is also a key player in the process of tumor initiation and progression, and has the potential for use as a common cancer biomarker, as well as a molecular target for cancer therapy.[10] *Piwil2* is specifically expressed in premeiotic germ cells; however, its ectopic expression has been also reported in other tumors, including breast cancer.[8,18–20]

We hypothesized here that overexpression of *piwil2* in tumor cells could be partly due to downregulation of piwil2-targetting microRNAs. Accordingly, we used different bioinformatic approaches to scan the sequence of *piwil2* to find miRNAs with potential target sites on the transcript. Among the list of predicted piwil2-targetting microRNAs, we chose two, miR-1267 and miR-

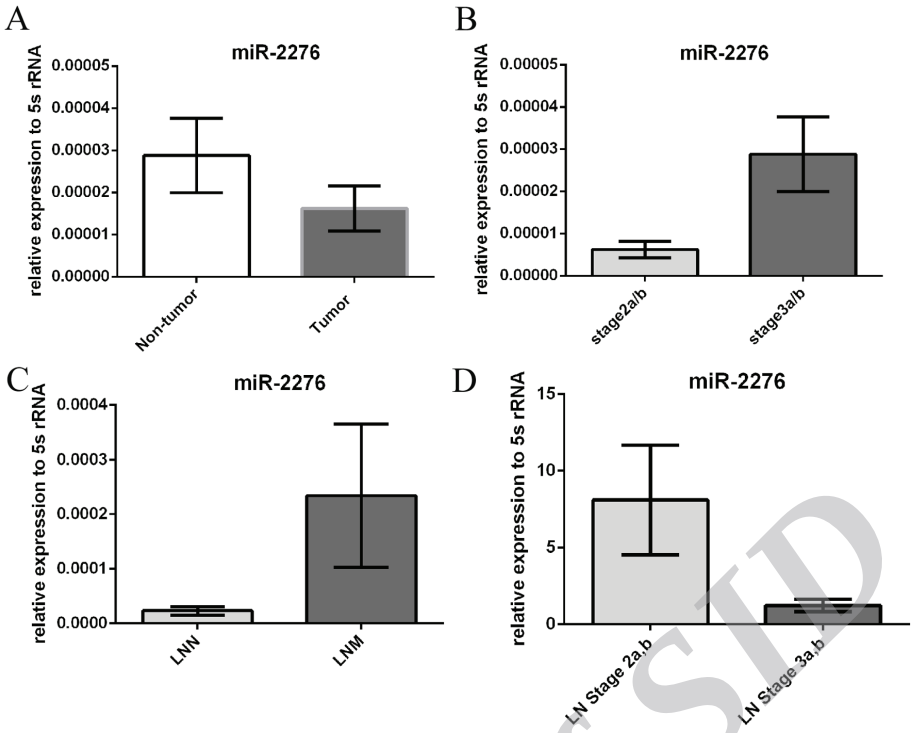

Figure 3. **A)** Evaluating the relative expression level of miR-2276 in breast tumor samples in comparison to non-tumor samples obtained from the margin of same tumor, normalized to that of house-keeping gene, 5s. **B)** A comparison between the expression levels of miR-2276 in breast tumors with different stages. **C)** A similar comparison in lymph node metastases (LNM) vs. lymph node negative (LNN) samples. **D)** Note that when the expression of miR-2276 was compared between tumor vs. non-tumor samples in each stage subgroup the difference was statistically significant (*). A *P* value less than 0.05 considered as statistically significant.

2276, with no previous knowledge on their roles in tumorigenesis. As expected, a noticeable downregulation of both genes was found in breast tumor tissues, in comparison to non-tumor tissues obtained from the same patients. The downregulation of the genes was more significant in high-grade tumors. Surprisingly, the expression of both miRNAs was significantly upregulated in higher stages of tumor samples, suggesting a dual function of the genes in initiation and invasion of breast tumorigenesis.

To date, there is no direct report on expression alteration or function of miR-1267 and miR-2276 in tumorigenesis. However, a 2.5 fold upregulation of miR-1267 is reported by Yu *et al.*[21] in a microRNA microarray of 10 patients with gastric cancer. A similar 2.9 fold overexpression of miR-1267 is reported in a microarray analysis of serum samples from gastric cancer patients.[22] The inconsistency between these reports and ours could be due to the fact that they used different methodology, different type of tumors, small number of samples, and also not discriminating between different grades and stages of tumors. However, in agreement with our findings, more than 40% loss of heterozygosity (LOH) at the chromosomal location of miR-1267 suggests a tumor suppressor role for it in ovarian cancer.[23]

A 4.5-fold increase in the expression of miR-2276 is reported in a deep sequencing analysis of colorectal cells transfected with STAT3 siRNA, confirming that the oncogenic STAT3 is a negative regulator of miR-2276.[24] The data is in agreement with our findings on a tumor-suppressor role of miR-2276 in breast tissue.

In conclusion, our data suggest a potential link between down-regulation of the aforementioned bioinformatically predicted piwil2-targetting microRNAs and piwil2 overexpression in breast cancer. However, one limitation of our study is the small sample size. While the results emphasize the potential application of these miRNAs in diagnosis and therapy of breast tumors, functional analysis is needed to experimentally confirm a link between miR-1267 and miR-2276 with piwil2, and their contribution to initiation and progression of breast cancer.

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