Up-Regulation of *Mir-105-5p* Promotes The Development of Breast Cancer By Targeting *AKT1 / GRB2* Genes In Patients With Breast Cancer

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Research Article

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Abstract

Objective: miR-105-5p either acts as an oncomiR or tumor suppressor that has been shown to have various expression levels in a wide range of diseases and targets RAC-alpha serine/threonine-protein kinase (AKT1) and Growth Factor Receptor-bound protein 2 (GRB2) genes. GRB2 is a signaling protein that takes part in various signaling pathways. Due to the fact that miRNAs by acting on target genes, and regulates the expression of them, so the aim of this study is to evaluate the expressions of miR-105-5p, AKT1, and GRB2 genes and the correlation between them in breast cancer (BC) tissue compared to normal-adjacent tissues (NATs) in women with breast cancer.

Materials and methods: We utilized bioinformatics analysis to searching for target genes of miR-105-5p. Then, 35 BC and NATs tissues were taken. In this experimental study, quantitative Real-Time PCR was performed to evaluate miR-105-5p, AKT1 and GRB2 genes expression, and the correlation between them was evaluated. The relation between expression and features of clinicopathological was explored.

Results: The miR-105-5p expression was increased significantly in BC tissues in comparison to NATs (P<0.05). The expression levels of AKT1 and GRB2 genes were decreased significantly in BC tissue in comparison to NATs (P<0.05). An inverse correlation was observed between miR-105-5p, AKT1 and GRB2 expression which was not significant (P>0.05). Also, a direct correlation was observed between GRB2 and AKT1 gene expression (P<0.05).

Conclusion: Our findings revealed that increased expression levels of miR-105-5p in patients with breast cancer caused a decrease in the expression level of its target genes, AKT1 and GRB2, and subsequently lead to tumor progression and invasion of the tumor. miR-105-5p may serve as a promising target for BC therapy.

Introduction

Breast cancer (BC) is widely assumed to be the main cause of cancer death in women around the world. Every year, 2,088,849 new cases are diagnosed with BC with over 626,679 deaths estimated worldwide [1]. It is a multifactorial disorder associated with the effects of multiple genes in combination with environmental factors [2,3,4]. BC is classified into two forms of sporadic BC, which approximately attributes to 95 percent of BCs, and familiar BC which accounts for the rest of the BCs and it is caused by mutations in genes like Breast cancer susceptibility gene 1 (BRCA1) and Breast cancer susceptibility gene 2 (BRCA2)[5]. There are five major molecular subtypes of BC. They are grouped based on the genes expressed: Luminal A BC is hormone-receptor positive (progesterone-receptor positive and/or estrogen-receptor), HER2 negative, and the protein Ki-67 is in low levels. Luminal B BC is hormone-receptor positive (progesterone-receptor positive and/or estrogen-receptor), and either HER2 positive or HER2 negative with high levels of Ki-67. Triple-negative/basal-like BC is HER2 negative and hormone-receptor negative (progesterone-receptor and estrogen-receptor negative). HER2-enriched BC is HER2 positive and hormone-receptor negative (progesterone-receptor and estrogen-receptor negative). Normal-like BC is similar to
luminal A disease: HER2 negative and hormone-receptor positive (progesterone-receptor and/or estrogen-receptor positive), and the protein Ki-67 is in low levels [6].

In the past decade, with the usage of genome-wide studies on DNA sequence, gene expression, and so forth, many aberrant genes and miRNAs have been identified which might contribute to breast tumor [7, 8]. **miR-105-5P** targets many genes in various cancers and can act either as tumor suppressor or oncomiR [9]. Some studies showed that miR-1 to be associated with several diseases such as cancer (BC, human gliomas, colorectal cancer, gastric cancer, prostate cancer, and hepatocellular carcinoma), and heart diseases [9]. miR-105 functions as an oncogene in several of these cancers such as colorectal cancer and BC, and high expression of this miR causes metastasis and invasion. But in cancers such as prostate cancer, hepatocellular carcinoma, human gliomas, and lung cancer, miR-105 functions as a tumor suppressor. miR-105 can alter the expression levels of its target gene, so it has an impact on cancer progression [9]. **miR-105-5P** is secreted by cancer cells and in BC, it works as oncomiR via targeting ZO1 protein, which acts as a tight junction adaptor protein, and therefore, it contributes to metastasis. **miR-105-5P** targets when this micro RNA is suppressed in BCs it can lead to a decrease in the metastasis rate [10, 11].

Other genes such as **AKT1** which are involved in different cell signaling pathways and any dysregulation of their expression may lead to creation of cancer cells [12].

AKT1 is serine-threonine protein kinase which regulates numerous processes such as proliferation, cell survival, growth, and so on [13, 14]. **AKT1** is a mediator of various cellular pathways including **PI3K / PTEN / AKT / mTORC1**, therefore, it has distinct roles in the development of BC [15, 16]. It has been assumed that overexpression and activation of **AKT1** might lead to BC progression by contributing to being resistant to anti-proliferative signals [17, 18].

Growth Factor Receptor-bound protein 2 (**GRB2**) gene is an adapter protein that plays an important link between cell surface growth factor receptors and the Ras signaling pathway and it may have a vital role in cell survival, proliferation, differentiation, and angiogenesis [19]. It has been reported that GRB2 is involved in development and progression of various malignancies such as BC, lung cancer, gastric cancer, colorectal cancer, and so forth [20, 21, 22]. The aim of the current study is to evaluate the expression of miR-105-5p, **AKT1**, and **GRB2** genes and the correlation between them in BC tissues from Iranian patients with BC and adjacent normal tissue (NATs) from same patients.

**Material And Methods**

**Subjects**

In this experimental study, fresh breast cancer (BC) tissues and paired normal adjacent tissues (NATs) (>5 cm from tumor) from 35 women with BC were collected from Imam Khomeini Hospital, Tehran, Iran. Tissues were carried to the laboratory via liquid nitrogen tank. The BC patients had the average age of 50 years when diagnosed (range 30-55 years). Patients did not undergo any radiation therapy or
chemotherapy. The diagnosis, histological grade and the clinical stage of each case were independently confirmed by a pathologist. Patients had no other diseases and the ones that did, were excluded from the study. The study was approved by the Ethics Committee and all of the contributors provided written informed consent.

**RNA isolation and cDNA synthesis**

Total RNA was extracted from the fresh BC tissues using XS spin kit (Macherey-Nagel, Germany, LOT No: 1301/005), based on the manufacturer's instruction. After applying DNase-I for removing DNA contamination, complementary DNA strand (cDNA) was synthesized using the PrimeScript™ RT Reagent Kit (Takara, Japan, Cat No: RR037A) according to the manufacturer's protocol. For evaluation of *miR-105-5p* expression, stem loop cDNA was synthesized. The RNA samples were evaluated on Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) for both quantity and also quality.

**Target genes prediction of miR-105-5p**

miRNA targets were predicted and determined using the algorithms of TargetScan 7.1 [23], miRBase [24], miRanda [25], miRDIP 4.1 [26], miRWALK 3.0 [27]and miRDB 5.0 [28].

**Real-time quantitative PCR (qPCR)**

Quantitative Real-time PCR was carried out using ABI 7900HT instrument (ABI Inc.), WizPure™ qPCR Master (SYBR) (Korea, Cat No: w1711). U6 snRNA was used for *miR-105-5p* normalization. Specific primers were brought as follow: U6 forward: 5´- GCTTCGGCAGCACATATAC -3´, and U6 reverse: 5´- ATTCCGTTTCTGGGAGGG-3´, GRB2 forward: 5´-ATTCTCTGGGACATAGAACA -3´, GRB2 reverse: 5´- AGTTCCTCAACACCCATGGAGAGGG-3´, AKT1 forward: 5´-TCTATGCCCTGAGATTGTG -3´, AKT1 reverse: 5´- GATTAATGCCCCTCCTGT -3´, miR-105-5p forward: 5´-TCAAATGCTCAGAGAGGTA-3´ and miR-105-5p reverse: 5´- CCAGTGCAGGGTCCGAGGTA-3. The qPCR reaction for AKT1 and GRB2 genes and miR-105-5p were performed with the following reaction: 20 µL volume reaction containing 1 µl of template cDNA (12.5 ng/µL), 0.5 µl of each 10 pmol/ul primer (Sinaclone, Iran), 10 µl of SYBR Green PCR master mix and 8 µl dH₂O and followed by initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 30 secs, annealing at 60°C for 30 secs. GAPDH gene was used for AKT1 and GRB2 genes for normalization. GAPDH forward (F): 5´- ATTTGGTCGTATTGGGCG -3´ and GAPDH reverse (R): 5´- GTACTCAGCGCCACGATC -3´. The relative gene expression level was assessed by using 2⁻ΔΔCt quantitative method [29], each sample was analyzed in duplicate.

**Statistical analysis**

All experimental information was expressed as mean ± SEM. Statistical comparisons between the BC tissues and NATs were carried out using paired t-test and one-way analysis of variance was carried out to compare the differences among more than two groups followed by Tukey's test. Differences were considered statistically significant at P ≤ 0.05. The correlation between *AKT1*, and *GRB2* genes and *miR-105-5p*
miR-105-5p is upregulated in Breast cancer

Quantitative real-time PCR was used to evaluate the expression levels of miR-105-5p in breast cancer (BC) and paired normal adjacent tissues (NATs). We observed that there was a significant up-regulated in BC samples relative to that in NATs as the control group (Fig. 1) (p=0.0194). This finding indicates that miR-105-5p may be a regulator of molecular mechanisms in BC progression. The relationship between
miR-105-5p expression and clinicopathological characteristics of BC patients including histological grade, clinical stages and patients’ age were examined. An increase in the expression level of miR-105-5p was observed in stage I and grade I compared with other grade (II – III) and stage (II-III). BC patients were divided according to age into the younger and older groups (women aged ≤ 50 and >50 years, respectively). In older women (aged >50 years) miR-105-5p expression was increased compared with younger women (aged ≤ 50 years) but no difference was found in the rate of miR-105-5p mRNA expression in according to age. In our study, statistical analysis showed that the miR-105-5p expression was not significantly related to clinicopathological features including histological grade, clinical stages and patients’ age (p >0.05) (Table 2).

Table 2: Correlation between miR-105-5p expression and Clinicopathological Characteristics in Breast Cancer patients

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>SE of difference</th>
<th>P value</th>
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<td>&lt;50 vs ≥ 50</td>
<td>0.2952</td>
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<td>Grade I vs. Grade III</td>
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<td>Grade II vs. Grade III</td>
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<tr>
<td>Stage I vs. Stage III</td>
<td>0.7593</td>
<td>&lt;0.0001</td>
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<tr>
<td>Stage II vs. Stage III</td>
<td>0.3796</td>
<td>0.2733</td>
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**Prediction of target genes of miR-105-5p**

Various target prediction programs (TargetScan, miRBase, miRanda, mirDIP, miRWALK, and miRDB) were used to explore the potential mechanism and analyze the AKT1 and GRB2 target genes of miR-105-5p (ACESSION: MIMAT0000102). Based on the predicted genes, score class of AKT1 gene is top up 5% as high-throughput miR-105-5p targeting and Score class of GRB2 gene is top up 1% as very high-throughput miR-105-5p targeting.
AKT1 is downregulated in Breast cancer

We first investigated the expression of AKT1 in BC tissues and NATs by quantitative RT-PCR. We observed the expression levels of AKT1 was decreased in BC tissues compared to that in NATs significantly (Fig. 2) \((p= 0.0141)\). Next, we compared the expression levels of AKT1 with the clinicopathological features including histological grade, clinical stages and patients’ age. The lowest expression of AKT1 was observed in Stage III and grade I. But, the AKT1 gene expression was not significantly related to clinicopathological features including histological grade, and clinical stages.

To better understand the relationship between AKT1 expression and patients’ age, we considered the expression of this gene in younger and older groups \((\leq 50 \text{ year and } > 50 \text{ year})\). No significant difference was observed between aged \(\leq 50 \text{ year and } > 50\)-year groups \((P=0.680)\). However, there was a trend toward decreasing AKT1 expression in aged > 50-year groups (Table 3).

Table 3: Correlation between AKT1 expression and Clinicopathological Characteristics in Breast Cancer Patients

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>SE of difference</th>
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<td>&lt;50 vs (\geq 50)</td>
<td>0.6882</td>
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<td>Grade I vs. Grade III</td>
<td>0.8569</td>
<td>0.9801</td>
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<tr>
<td>Grade II vs. Grade III</td>
<td>0.6459</td>
<td>0.6311</td>
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Breast cancer staging

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<table>
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<tr>
<td>Stage I vs. Stage II</td>
<td>1.621</td>
<td>0.3079</td>
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<tr>
<td>Stage I vs. Stage III</td>
<td>1.722</td>
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</tr>
<tr>
<td>Stage II vs. Stage III</td>
<td>0.8608</td>
<td>0.6434</td>
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AKT1 is a target of miR-105-5p

Bioinformatics prediction revealed potential binding sites of miR-105-5p on the 3’-untranslated region (3’-UTR) of AKT1 (Fig. 3a). Spearman’s rank order correlation was used to determine the relation between
miR-105-5p expression and its target gene. We observed a negative correlation between *miR-105-5p* and *AKT1* in BC tissues (slope=-0.008862, P=0.9602) but not significantly (Fig. 3b). In the other hand, the expression of *miR-105-5p* was up-regulated, whereas *AKT1* was downregulated in the tumor tissues. These findings collectively indicate that *AKT1* is a target of *miR-105-5p* in BC.

**GRB2 is downregulated in Breast cancer**

The expression level *GRB2* was quantified by quantitative real-time PCR. We observed that there was a significant decrease in *GRB2* expression in BC tissue samples in comparison to NATs (p= 0.0473) (Fig. 4). We next explored the relationship between *GRB2* and clinicopathological characteristics of BC patients. The lowest expression of *GRB2* was observed in Stage III and grade I. But the significant difference was not found when the gene expression was compared between different histological grades (I to III) and different clinical stages (I to III). Also, the expression of *GRB2 in patients* aged > 50-year groups was lower than aged< 50 year, but no correlation was observed between *GRB2* expression and age (< 50 year and > 50-year groups) (P= 0.585) (Table 4).

Table 4: Correlation between GRB2 expression and Clinicopathological Characteristics in Breast Cancer Patients

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>SE of difference</th>
<th>P value</th>
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<tbody>
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<td>4.104</td>
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<td>Histological grade</td>
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<tr>
<td>Grade I vs. Grade II</td>
<td>7.184</td>
<td>0.9198</td>
</tr>
<tr>
<td>Grade I vs. Grade III</td>
<td>8.849</td>
<td>0.3992</td>
</tr>
<tr>
<td>Grade II vs. Grade III</td>
<td>6.670</td>
<td>0.3946</td>
</tr>
<tr>
<td>Breast cancer staging</td>
<td></td>
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<tr>
<td>Stage I vs. Stage II</td>
<td>10.11</td>
<td>0.5194</td>
</tr>
<tr>
<td>Stage I vs. Stage III</td>
<td>10.74</td>
<td>0.2622</td>
</tr>
<tr>
<td>Stage II vs. Stage III</td>
<td>5.368</td>
<td>0.5123</td>
</tr>
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</table>

**GRB2 is a target of miR-105-5p**
Bioinformatics prediction indicated that GRB2 is the potential target gene of miR-105-5p. The 3′-UTR of GRB2 mRNA has a specific sequence which is complementary to the seed region of miR-105-5p (Fig. 5a).

We analyzed 35 BC tissues and NATs to explore the relationship between miR-105-5p and the target GRB2 gene. The Spearman’s correlation demonstrated that miR-105-5p expression had negative correlation with the GRB2 expression (slope = -0.02614, p=0.4563) but not significantly shown in (Fig. 5b). Showed miR-105-5p was up-regulated in BC tissues while GRB2 was down-regulated in BC tissues.

Moreover, we explored the relationship between two target genes, AKT1 gene and GRB2 gene. We observed a significant positive correlation between AKT1 gene and GRB2 gene (slope = 3.582, p<0.0001) in BC tissues. This finding indicates that AKT1 gene and GRB2 gene can be a regulator of signaling pathway in BC progression.

Discussion

Our study indicated that miR-105-5p expression was up-regulated in BC tissues in comparison to NATs. It was statistically significant. In some studies, it was observed that miR-105-5p acts as tumor suppressor. Honeywell et al. and Liu et al. found out that miR-105-5p inhibits prostate tumor growth and human glioma cell progression respectively and its reduced expression can lead to these types of cancers [10, 30]. The same result was found with non-small cell lung cancer [31]. It was also revealed that in some studies, miR-105-5p acts as onco-miR. miR-105-5p expression level was increased in gastric cancer cells and colorectal cancer cells [9, 32]. Our results indicated miR-105-5p acts as onco-miR in BC tissue because the expression level of miR-105-5p is increased in BC tissue compared with NATs, and it is revealed that miR-105-5p may be relevant with cell proliferation. miRNAs have been known to have a role in regulating most biological processes and therefore, they can be used in order to gain an insight into the complex processes, including cell proliferation in cancer. Our bioinformatics studies showed that miR-105-5p targets AKT1 pathway and GRB2 gene. AKT signaling pathway has been a major signaling node within the cells [33]. AKT1 pathway dysregulation could possibly lead to disruption of the balance between survival and death and it can also have a major impact on cancer development and therapy. Our results demonstrated that AKT1 expression level significantly decreased in BC tissue compared with NATs even the expression of this gene is more in the advanced stages of BC tissues. Our results agreement with previous studies. Heng et al. found out that AKT1 expression level was significantly lowered in breast tumor in comparison to matched adjacent normal tissues [34]. We also observed miR-105-5p had negative correlation with expression of AKT1 in BC tissues. This result can be justified by Shen et al.’s finding in which they demonstrated that miR-105-5p can inhibit PI3K/AKT pathway in hepatocellular cancer [12].

GRB2 is an essential signaling mediator in various oncogenic signaling pathways and it has role-playing in several human malignancies [35]. The GRB2 has a wide association with a number of irregularities. So, it is an important target for the design of remedial anticancer strategies [36]. Lin-Yan et al. found that
overexpression of GRB2 had correlation lymph node metastasis [37]. Yu et al. demonstrated that GRB2 is over expressed in gastric cancer [22]. Watanabe et al. found that GRB2 expression was significantly increased in human bladder cancer cell lines [38]. Lim et al. found out that GRB2 downregulation led to AKT inactivation and consequently it caused BC [38]. Our results revealed that GRB2 expression level significantly decreased in BC tissue compared with NATs. Its expression level lowered more in the advanced stages of BC. We demonstrated that miR-105-5p negatively modulated GRB2 by combining with the 3′-UTR of GRB2 gene.

Furthermore, another important aspect of this study showed that strong positively correlated between the expression levels of AKT1 and GRB2 genes. So, that downregulation of GRB2 genes suppressed the activation of AKT1 pathway. Lim et al. found that downregulation of GRB2 can lead to AKT1 inactivation and subsequently can cause lowered expression of AKT1 [39]. These findings support the pivotal role of AKT pathway in modulating the BC development. Based on our results, we speculate that miR-105-5p may contribute to the development of BC via downregulation of AKT1 and GRB2 genes.

miR-105 affects the genes which are involved in intracellular signaling pathways and this leads to the downregulation of GRB2 and GRB2 downregulates AKT1 through p13k signaling pathway. Consequently, these alterations lead to more invasion and metastasis in BC.

**Conclusion**

Our findings demonstrated miR-105-5p was up-regulated in human BC. miR-105-5p promotes cell proliferation in BC by targeting AKT1 and GRB2 genes. Up-regulation of miR-105-5p could promote BC growth by decreasing the activity of AKT1 signaling pathways and GRB2.

Our Insilco analyses showed that AKT1 and GRB2 are the direct targets of miR-105-5p and basically micro RNAs effect on the cell through regulating their target genes. Increased expression of miR-105-5p downregulate the expression of GRB2 and AKT1 and therefore these changes cause tumor progression and tumor invasion. These two genes are important genes in intracellular signaling pathways and reduced expression of them can dysregulate the cell cycle and cause tumor formation. Therefore, identifying a specific biomarker, and gaining an understanding into the underlying mechanism might provide feasible therapeutic approaches to overcome cancer. By targeting miR-105-5p, it might be considered as a possible therapeutic strategy for BC treatment. However, the detailed mechanism of miR-105-5p in BC progression needs further investigations.

**Declarations**

**Conflict interest**

The authors declare no conflict of interests in this study.

**Authors’ contributions**
F.F. Participated in study design. F.R. and F.A. collected the samples. F.F, F.R. and F.A. participated in data collection and evaluation, drafting. F.F and F.R. participated in RT-qPCR analysis and statistical analysis. F.R. prepared lab working, and doing molecular experiments. F.F. and F.R. Contributed extensively in the interpretation of the data and the conclusion. All authors performed editing and approving the final version of this paper for submission, also participated in the finalization of the manuscript, and approved the final draft.

Acknowledgments

This paper is based on MSc thesis of Fatemeh Rezaei, Faculty of Advanced Science and Technology, Tehran Medical Sciences, Islamic Azad University, Tehran, Iran.

Ethical issues

Approval for the study was obtained from the Ethics Committee of Islamic Azad Tehran Medical Sciences University-Pharmacy and Pharmaceutical Branches Faculty. Ethical code is IR.IAU.PS.REC.1398.069.

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Figures

![Bar graph showing the relative expression of miR-105-5p in BC tissues and NATs](image)

**Figure 1**

miR-105-5p is up-regulated in BC tissues and NATs. Relative miR-105-5p expression levels in BC tissues and NATs were determined by qRT-PCR. The expression of miR-105-5p is up-regulated in BC tissues compared with NATs (* p 0.05). U6snRNA was used as an internal control. Abbreviation: BC: breast cancer, NATs: paired normal adjacent tissues.
Figure 2

*AKT1* mRNA expression is downregulated in BC tissues and NATs. Relative *AKT1* expression levels in BC tissues and NATs were determined by qRT-PCR. The expression of *AKT1* is downregulated in BC tissues compared with NATs (*p* < 0.05). *GAPDH* was used as an internal control. Abbreviation: BC: breast cancer, NATs: paired normal adjacent tissues.

Figure 3

(a) Position 93-100 of *AKT1* 3' UTR

(b) Scatter plot showing the correlation between relative *AKT1* mRNA expression and relative *mir-105-5p* expression.
AKT1 is a direct target of miR-105-5p in breast cancer. a) miR-105-5p and its putative binding sequence sites in the AKT1 3'-UTR. b) miR-105-5p expression has negative correlation with AKT1 expression in BC tissues, (slope=-0.02466, P=0.7569).

Figure 4

GRB2 mRNA expression is downregulated in BC tissues and NATs. Relative GRB2 expression levels in BC tissues and NATs were determined by qRT-PCR. The expression of miR-105-5p is significantly downregulated in BC tissues compared with NATs (* p 0.05). GAPDH was used as an internal control.
Abbreviation: BC: breast cancer, NATs: paired normal adjacent tissues.
Figure 5

*GRB2* is a direct target of miR-105-5p in breast cancer. a) miR-105-5p and its putative binding sequence sites in the *GRB2* 3′-UTR. *miR-105-5p* expression had negative correlation with the expression of *GRB2* in BC tissues, (slope = -0.02098, p=0.1664)