Original Articles

miR-22 as a prognostic factor targets glucose transporter protein type 1 in breast cancer

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A B S T R A C T
It has been reported that miR-22 plays an important role and may be a promising therapeutic target in cancer. In this study, we found that GLUT1 is a direct target of miR-22. The ectopic expression of miR-22 inhibited breast cancer cell proliferation and invasion by targeting GLUT1. A reverse correlation between the expression of miR-22 and GLUT1 was observed in breast cancer tissue samples. Furthermore, miR-22 was significantly correlated with the TNM stage, local relapse, distant metastasis, and survival of breast cancer patients. Our data suggest that miR-22 functions as a tumor suppressor and is a promising prognostic biomarker in breast cancer.

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Introduction

Breast cancer is one of the most common malignant diseases in women worldwide [1]. Although improvements in detection and treatment have decreased breast cancer mortality in recent years, the prevention and therapy of breast cancer remain a major public health concern [2]. MicroRNAs (miRNAs) are a class of small non-coding RNAs (18–22 nt) that silence their cognate target genes by either degrading mRNA molecules or inhibiting their translation [3]. They can act as oncogenes or tumor suppressors and are often dysregulated in tumors [4,5]. These dysregulations are believed to be involved in cancer progression and can be prognostically indicative of human cancers. Increasing studies show that miRNAs are involved in the tumorigenesis and progression of breast cancer. In this study, we focus on miR-22, which has been found to be aberrant in a variety of cancers, overexpressed in prostate cancer, and down-regulated in cholangiocarcinoma, multiple myeloma, colorectal cancer, and hepatocellular carcinoma [6,7]. In a previous study, we found that the over-expression of miR-22 can inhibit gastric cancer cell growth in vitro and in vivo and induces apoptosis through targeting the Wnt-1 signaling pathway [8]. Moreover, the up-regulation of miR-22 expression has been reported to suppress gastric cancer cell migration and invasion by targeting the Sp1 gene [9].

In addition, miR-22 has been shown to inhibit estrogen signaling by directly targeting the estrogen receptor alpha mRNA [10], and its overexpression induces growth suppression and senescence-like phenotypes in human breast epithelial and cancer cells [11].

Cancer cells utilize glucose as the main energy substrate using only these facilitative transport systems (GLUTs) based on their increased metabolic activity and rapid proliferation [12]. Glucose transporter 1, which is also known as solute carrier family 2 (SLC2A1), facilitates glucose transporter member 1 (GLUT1), a unipporter protein that is encoded by the SLC2A1/GLUT1 gene in humans [13]. GLUT1 facilitates the transport of glucose across the plasma membranes of mammalian cells. Previous literature has reported that GLUT1 expression is associated with increased malignant potential, invasiveness, and poor prognosis in lung, colorectal, gastric, and ovarian cancers. In addition, GLUT1 expression is increased in breast cancer with higher grade and proliferative activity [14]. In this study, we proved that miR-22 can directly target the GLUT1 gene, and its expression is negatively correlated with GLUT1 expression in breast cancer. Moreover, the correlation of miR-22 and GLUT1 expression is a valuable marker of breast cancer prognosis.

Materials and methods

Cell culture

The human breast cancer cell lines MDA-MB-231 and MCF-7, which were obtained from American Type Culture Collection and freshly recovered from liquid nitrogen (3 months), were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen, CA, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Cappinist, Brazil) in a humidified incubator at 37 °C containing 5% CO₂.
Clinical samples

Paraffin-embedded tumor tissues were obtained from 122 female breast cancer patients who were diagnosed by two pathologists in Sun Yat-Sen University Cancer Center, China, during the period from October 2000 to December 2006. The IHC of ER, PR, and HER-2 status were performed in the Pathology Department of Sun Yat-Sen University Cancer Center. All of the patients recruited in this study did not receive any chemotherapy and radiotherapy preoperation, and their complete clinicopathological data, including age, histological type, lymph nodes status, tumor size, stage, ER status, PR status, HER-2 status, local relapse, and distant metastatic relapse, were available and reviewed. The histological type, which was reclassified according to the WHO classification and tumor stage, was based on the TNM staging system (Amer-ican Joint Committee on Cancer Classification, 7th edition, http://www.cancerstaging.org). Follow-up was updated by a review of the records and telephone calls. The date of death and the date of relapse were used to calculate the overall survival (OS) and disease-free survival (DFS). This study was approved by the Ethics Committee of Sun Yat-Sen University Cancer Center Health Authority. The collection and use of tissues followed procedures that are in accordance with the ethical standards as formulated in the Helsinki Declaration.

Quantitative RT-PCR analysis (qRT-PCR)

The total RNA from the cells with that was extracted the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription and qRT-PCR reactions were performed using a qSYBR-Green-containing PCR kit (Qigene, Germantown, MD, USA). The fold change was determined as 2^-ΔΔCt, where Ct is the fractional cycle number at which the fluorescence of each sample passes the fixed threshold. The ΔCt was calculated by subtracting the Ct of miRNA 0.6 from the Ct of the miRNA of interest. The ΔΔCt was calculated by subtracting the ΔCt of the reference sample (paired non-tumorous tissue for surgical samples, and MCF-10A cells for nine breast cancer cell lines) from the ΔCt of each sample. The primers for the qRT-PCR detection of GLUT1 mRNA were synthesized by Invitrogen. All real-time PCR assays were performed using the Bio-Rad IQTM 5 Multicolor Real-Time PCR Detection System (USA).

In situ hybridization (ISH) and immunohistochemistry (IHC) analysis

The miR-22 miRCURY LNA™ Detection probe (Exiqon, Vedbaek, Denmark) was used for IHC according to a previously described standard method [15]. The 5′-3′ sequence was ACAGTCTTCAACTGGCAGCTT with a DIG label at both the 5′ and 3′ ends. The IHC studies were performed using the standard streptavidin-peroxidase staining method as described previously [8]. The intensities of miR-22 and GLUT1 staining were scored in the range of 0–4 as follows: 0–1 (no staining), 1–2 (weak staining), 2–3 (medium staining), and 3–4 (strong staining). The percentages of miR-22 and GLUT1 cells in three representative high-power fields of individual samples were analyzed. The expression scores equaled the scores of the intensities corres-ponding to the percentages of positive cells, with a maximum of 4 and a minimum of 0. The individual samples were evaluated by at least two pathologists in a blind manner, and the expression scores greater or equal to 2 were defined as high ex-pression, whereas those that were less than 2 were associated with low expression.

RNA interference

The sense sequences of siRNA oligonucleotides targeting the GLUT1 transcript were as follows: si-GLUT1, 5′-AUCAUCAGCAUUGAAUUCCTT-3′. Scrambled siRNA was used as a negative control. The cells were plated in culture dishes or in 96 or 24-well plates for 24h and transfected with siRNA using Lipofectamine 2000. After 48 h, the cells were harvested for use in other assays or for RNA and protein extraction. 40 nM siRNA or scramble siRNA was transfected into cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA).

miR-22 mimics and LNA-modified anti-miR-22 oligonucleotide transfection

The miR-22 mimics (5′-AACUGCCACGAAGAGAUCGGU-3′), scrambled mimics (5′-IUCUGCCAACGUCAGCACUGTT-3′), anti-miR-22 LNA oligonucleotide (miR-22 in-hibitor), (5′-NLA-ACAGUUUCUCAACGUCACACGUU-3′), and control LNA oligonucleotide (miR-ctrl), (5′-NLA-CAGUUCUUGGUUUCAGUACAA-3′) were synthesized by GenePharma and were transfected into cells using Lipofectamine 2000, 40 nM mimic or anti-miR LNA oligonucleotides were transfected into cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA).

Colony formation assay

Six-well plates were covered with a layer of 0.6% agar in medium supplemented with 20% fetal bovine serum. The cells were prepared in 0.3% agar and seeded in triplicate. After the plates were incubated at 37 °C for two weeks, the colonies were counted.

MTT assay and apoptosis

The cell viability was examined by the 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. The cells were transfected with scramble, miR-22 mimics, control, or siRNA, seeded at a density of 5000 cells per well in 96-well plates and incubated at 37 °C for 24 h. The cells were then incubated with MTT for additional 48 h, and the MTT assay was performed according to the manufacturer’s instructions (Molecular Probes, Eugene, OR, USA). The absorbance values were determined at 570 nm using a Spectra Max 250 spectrophotometer (Mo-lecular Devices, Sunnyvale, CA, USA). Annexin V/propidium iodide staining and flow cytometry were performed using the Annexin V-fluorescein isothiocyanate Apop-to-sis Detection Kit (KeyGen, Nanjing, China) according to the manufacturer’s guidelines.

 Luciferase assays

The full length of the 3′ untranslated regions (UTRs) of the GLUT1 genes was amplified by PCR from MDA-MB-231 genomic DNA and inserted into the pGL3 control vector (Promega, Madison, WI, USA) using the XbaI site immediately downstream from the stop codon of luciferase. We also generated several inserts with deletions of 5 bp from the site of perfect complementarity of the GLUT1 gene using the QIAGEN XL-site directed Mutagenesis Kit (QIAGEN, Valencia, CA, USA). MDA-MB-231 cells were cotransfected using nucleoporation (Amaza Biosystems) according to the manu-facturer’s protocol (solution V, program T-016) using 5 μg of the firefly luciferase report vector and 0.5 μg of the control vector containing Renilla luciferase (pRL-TK, Promega). Each nucleoporation used 50 nM miR-22 or a scrambled oligonucleotide and LNA-modified anti-miR-22 oligonucleotide or a control oligonucleotide. The Firefly and Renilla luciferase activities were measured consecutively using the dual luciferase assay (Promega) 48 h after transfection.

Western blot

The proteins from breast cancer cell lines were extracted using RIPA lysis buffer with a protease inhibitor. The protein concentration in the lysates was measured with the Protein BCA Assay Kit (Bio-Rad, USA), and 20 μg of the total protein mixed with 2 × SDS loading buffer was loaded per lane. The proteins in the lysates were separated by 12% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore, USA). To block nonspecific binding, the membranes were incubated at room temperature for 1 h with 5% skim milk powder. The PVDF membranes were then incubated for 12 h at 4 °C with an anti-serum containing antibodies against GLUT1 and anti-β-actin, which were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). A peroxidase-conjugated sec-ondary antibody (1:5000 dilution) and ECL Western blot detection reagents were used to visualize the target proteins (ECL, New England Biolabs, USA), which were quantified with a Bio Image Intelligent Quantifier 1-D (Version 2.2.1, Nilson-Biolmage, Ltd., Japan).

Cell migration and invasion assays [15]

Cell migration was examined using wound-healing assays. An artificial “wound” was created on a confluent cell monolayer through scratching, the cells were then treated with 10 μg/ml mitomycin C for 2 h, and photographs were obtained using an inverted microscope (Olympus, Tokyo, Japan) at 24 h. For the cell invasion assay, the cells were seeded onto the basement membrane matrix present in the insert of a 24-well culture plate (EC matrix, Chemicon, Temecula, CA, USA), and fetal bovine serum was added to the lower chamber as a chemoattractant. After 48 h, the non-invading cells and EC matrix were gently removed with a cotton swab. The invasive cells located on the lower side of the chamber were stained with Crystal Violet, counted, and imaged.

Statistical analysis

All of the statistical analyses were performed using the SPSS 16.0 software package (SPSS, Chicago, IL, USA). The data are presented as the means ± SD from at least three separate experiments. The chi-squared test was used to investigate the signifi-cance of the correlation of miR-22 expression with clinicopathological features in breast cancer. The Kruskal–Wallis test was used to confirm miR-22 low expression correlated with advanced stages. The disease-free and overall survival curves were plotted using the Kaplan–Meier method and were evaluated for statistical signifi-cance using a log-rank test. Survival was measured from the day of the surgery. Variables with a value of P < 0.05 by univariate analysis were used in subsequent multivariate analysis based on the Cox proportional hazards model. All differences were statistically significant at the level of P < 0.05.
Results

SLC2A1/GLUT1 is a direct target of miR-22

We used two algorithms (TargetScan and Miranda) to identify the miR-22 targets in human breast cancer. Among these candidate target genes, both algorithms predicted SLC2A1 (Fig. 1A), which provides instructions for producing glucose transporter protein type 1 (GLUT1). We confirmed this finding in breast cancer cell lines by performing luciferase reporter assays. The full-length GLUT1 3’-UTR was cloned downstream of the firefly luciferase gene and cotransfected with miR-22 mimics or scrambled oligonucleotides and LNA-modified anti-miR-22 oligonucleotide or a control oligonucleotide. The luciferase activity was measured 48 h after transfection. MDA-MB-231 cells cotransfected with GLUT1 reporter constructs and miR-22 mimics exhibited an approximately 40% reduction in luciferase activity with respect to those cotransfected with the scrambled oligonucleotide, and the relative luciferase activity of the wild-type construct of the 3’-UTR of GLUT1 in MDA-MB-231 cells was significantly increased in the presence of anti-miR-22 (Fig. 1B). Additionally, mutation of the putative miR-22 sites in the 3’-UTR of GLUT1 abrogated the luciferase response to miR-22 (Fig. 1B). Furthermore, to confirm that GLUT1 is a target gene of miR-22, we transfected miR-22 mimics or scrambled oligonucleotides into MDA-MB-231 cell lines, and qRT-PCR and Western blot analyses were performed to detect the expression of GLUT1 regulated by miR-22 in MDA-MB-231 cells. The results showed notable reductions in the mRNA and protein levels of GUL1 and S1P1 (a known target of miR-22) [9] in miR-22-overexpressing MDA-MB-231 cells compared with the negative control (Fig. 1C and D). Taken together, these results indicated that miR-22 down-regulates GLUT1 expression by directly targeting its 3’UTR.

Overexpression of miR-22 inhibits breast cancer cell proliferation, colony formation, invasion and induced apoptosis by targeting GLUT1

To assess the biological effects of miR-22 and GLUT1 in breast cancer, miR-22 mimics, miR-22 inhibitors or GLUT1 siRNA was transfected into breast cancer cells. QRT-PCR and Western blot analysis demonstrated that the transfection of miR-22 mimics, miR-22 inhibitors, or SLC2A1/GLUT1 siRNA was successful in MCF-7 and
MDA-MB-231 cells (data not shown). The proliferation assay showed that the ectopic expression of miR-22 or the suppression of GLUT1 in MCF-7 and MDA-MB-231 cells markedly attenuated cell proliferation compared with the control group (Fig. 2A) colony formation and apoptosis assay showed that the ectopic expression of miR-22 or the suppression of GLUT1 in MDA-MB-231 cells markedly attenuated cell colony formation ability in soft agar and apoptosis compared with the control group (Fig. 2B and C), and that miR-22 inhibition with a specific inhibitor markedly promoted cell proliferation compared with the control group. In addition, GLUT1 siRNA could antagonize the effect of miR-22 inhibition (Fig. 2A–C). A transwell assay was employed to assess the impact of miR-22 on cellular invasion in MDA-MB-231 cells. Our results showed that the ectopic expression of miR-22 or the inhibition of GLUT1 significantly inhibited MDA-MB-231 cell invasion capacity compared with the control group, that miR-22 inhibition promotes MDA-MB-231 cell invasion capacity and that SL2A1/GLUT1 siRNA can antagonize the role of miR-22 inhibition (Fig. 2D).

The expression of GLUT1 is inversely correlated with the expression of miR-22 in breast cancer

Because miR-22 is down-regulated in breast cancer and directly targets GLUT1, we then determined whether GLUT1 protein expression is negatively associated with the miR-22 level in the breast cancer tissue samples. ISH analysis showed that 65.6% (80/122) of the tumor samples presented a low expression of miR-22, whereas the IHC analysis showed that 69.7% (85/122) of the tumor samples presented a low expression of miR-22, the correlation analysis showed that the level of miR-22 was inversely related to the expression of GLUT1 in primary breast cancer tissue (Fig. 3). Furthermore, we found that the overexpression of miR-22 was more likely to be observed in breast cancer with a low level of miR-22, providing more evidence for miR-22 mediated GLUT1 regulation.

Correlation of miR-22 expression with the clinicopathological characteristics of breast cancer

To further evaluate whether the low expression of miR-22 is linked to the clinical progression of breast cancer, we analyzed the association of miR-22 expression with the clinicopathological status of breast cancer patients. As shown in Table 1, miR-22 expression was observed to be closely negatively correlated with the TNM stage, local relapse and distant metastasis ($P = 0.008, 0.004$ and $0.007$, respectively) of breast cancer. We performed Kruskal–Wallis test to confirm miR-22 low expression correlated with advanced stages ($H = 6.462, P = 0.011$). However, there were no significant correlations between miR-22 expression and other clinicopathologic factors, including age, menopause, lymph node (LN) infiltration, tumor size, and ER and PR status ($P = 0.565, 0.055, 0.146, 0.983, 0.552, 0.830, 0.776$ and $0.841$, respectively).

Table 1 Clinico-pathological variables and the expression of miR-22 in total breast cancer patients.

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miR-22 and GLUT1 expression is associated with poor survival of breast cancer patients

To determine whether our observations could be verified in human breast cancer patients, we analyzed the significance of miR-22 and GLUT1 further in terms of clinical prognosis, and a Kaplan–Meier survival analysis was conducted using the patient overall survival (OS) and disease-free survival (DFS). The results showed that patients with low miR-22 expression presented shorter mean months of OS and DFS than patients with high miR-22 expression ($P = 0.006$ for OS, $P = 0.003$ for DFS; Fig. 4A). We also observed that GLUT1 expression was associated with a lower overall survival rate and a lower disease-free survival rate; however, the lower OS was not significant ($P = 0.066$ for OS, $P = 0.014$ for DFS; Fig. 4B). In addition, a low expression of miR-22 and a high expression of GLUT1 were significantly associated with shorter OS and DFS (Fig. 4C). These results indicated that the expression levels of miR-22 and GLUT1 were significantly associated with the patient OS and DFS.

We further performed a Cox multivariate analysis to identify independent prognostic markers for OS. Univariate Cox proportional hazard regression analysis showed that lymph node metastasis, ER status and miR-22 level were predictive factors for prognosis in breast cancer patients. Multivariate regression analysis confirmed that only lymph node metastasis and miR-22 level were independent unfavorable prognostic factors for overall survival (Table 2).

Discussion

Breast cancer is a group of heterogeneous diseases that show various biological and clinical characteristics [16]. miRNAs have been demonstrated to have a close relationship with breast cancer. For instance, in breast cancer, miR-200c restores sensitivity to microtubule-targeting chemotherapeutic agents [17], and miR-31 inhibits local invasion, extravasation, or initial survival at a distant site and metastatic colonization [18]. miR-22 was originally identified in HeLa cells and has been shown to exert growth-suppressive functions in vitro in some cancer cell lines [6,10]. Moreover, the injection of miR-22 has been reported to be an efficient therapy for the suppression of tumor growth and metastasis [11]. In addition, several studies show that many proteins are the targets of miR-22, such as estrogen receptor α (ERα), c-Myc-binding protein (MYCBP), Myc-associated factor X (MAX), and PTEN [10,19–21]. Among the predicted miR-22 targets in TargetScan and Miranda,
miR-22 inhibits breast cancer cell proliferation, colony formation, invasion and induced apoptosis by targeting SLC2A1/GLUT1. (A) MCF-7 and MDA-MB-231 cells were transfected with miR-22 mimics, scramble (miR-scr), miR-22 inhibitors, control (miR-ctr), si-SLC2A1/GLUT1, or control (si-ctr). The cell viability was determined with the MTT assay at 48 h post-transfection. (B) MDA-MB-231 cells were transfected with miR-22 mimics, scramble (miR-scr), miR-22 inhibitors, control (miR-ctr), si-SLC2A1/GLUT1, or control (si-ctr). Colony formation ability in soft agar was determined by flow cytometry at 48 h post-transfection. (C) MDA-MB-231 cells were transfected with miR-22 mimics, scramble (miR-scr), miR-22 inhibitors, control (miR-ctr), si-SLC2A1/GLUT1, or control (si-ctr). Annexin V-FITC/propidium iodide staining and FACS quantification of the number of apoptotic cells were determined by flow cytometry at 48 h post-transfection. (D) MDA-MB-231 cells were transfected with miR-22 mimics, scramble (miR-scr), miR-22 inhibitors, control (miR-ctr), si-SLC2A1/GLUT1, or control (si-ctr). The invasion activity was determined by a transwell assay at 48 h post-transfection.
SLC2A1/GLUT1 attracted our attention. GLUT1, which is a member of the family of glucose transporters, facilitates glucose movement across the plasma membranes in a tumor-specific manner. Accelerated glycolysis is one of the biochemical characteristics of cancer cells that allow them to compensate the inefficient extraction of energy from glucose in order to continue their uncontrolled growth and proliferation [22]. GLUT1 is overexpressed in many tumors, including hepatic, pancreatic, breast, esophageal, brain, renal, lung, cutaneous, colorectal, endometrial, ovarian, and cervical cancers [23]. It has been shown that estrogen plays critical roles in GLUT regulation [24]. However, little is known about the relationship between the expressions of miR-22 and GLUT1 in human breast cancer with the prognosis of breast cancer patients.

To the best of our knowledge, this study provided the first line of evidence that miR-22 restoration reduces proliferation and induced apoptosis through the repression of GLUT1. In this study, we proved
miR-22 and GLUT1 expression is associated with poor survival in breast cancer patients. Low levels of miR-22 and high levels of GLUT1 are correlated with shorter survival. The OS and DFS curves for all studied patients with high or low miR-22 expression (A), high or low GLUT1 expression (B), and the four possible combinations (C) are shown.

Fig. 4.
that miR-22 targets SLC2A1/GLUT1 directly through luciferase reporter assays and that miR-22 expression is negatively correlated with GLUT1 expression in breast cancer tissues. Moreover, we demonstrated that the miR-22 levels in breast cancer tissues are significantly lower than those in noncancerous tissues, as determined by qRT-PCR and IHC. In addition, we used qRT-PCR and IHC to show that the GLUT1 levels in breast cancer tissues are significantly higher than those in noncancerous tissues. Furthermore, decreased miR-22 levels in breast cancer are correlated with the TNM stage, local relapse and distant metastasis, and low expression of miR-22 and high expression of GLUT1 were significantly associated with a shorter OS and a shorter DFS. Based on these data, the miR-22 and GLUT1 expression levels may be useful as prognostic markers for the prediction of survival and relapse in breast cancer patients, although we have not confirmed if miR-22 executes its function mainly through repressing GLUT1. Due to the heterogeneity and complexity of the mechanisms of tumor progression, it is necessary to develop a new method for modeling the interactions and action of these complex relationships and their impact on cancer.

In summary, miR-22 directly targets SLC2A1/GLUT1. Low miR-22 and high GLUT1 expression levels are associated with shorter disease-free survival times and poor overall survival in patients with breast cancer, which suggests that miR-22 and GLUT1 expression may be a valuable marker of breast cancer progression and prognosis.

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Conflict of interest

The authors declare no conflicts of interest.

References

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