

miRNA-218 contributes to the regulation of *D-glucuronyl C5-epimerase* expression in normal and tumor breast tissues

Tatiana Y. Prudnikova,¹ Luydmila A. Mostovich,¹ Vladimir I. Kashuba,^{2,3} Ingemar Ernberg,³ Eugene R. Zabarovsky^{3,4} and Elvira V. Grigorieva^{1,3,*}

¹Institute of Molecular Biology and Biophysics; Siberian Department of Russian Academy of Medical Sciences; Novosibirsk, Russia; ²Institute of Molecular Biology and Genetics; Ukrainian National Academy of Sciences; Kiev, Ukraine; ³Department of Microbiology; Tumor and Cell Biology (MTC); Karolinska Institute; Stockholm, Sweden;

⁴Linköping University; Institute of Clinical and Experimental Medicine; Linköping, Sweden

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microRNAs (miRNAs) are key posttranscriptional regulators of gene expression. In the present study, regulation of tumor-suppressor gene *D-glucuronyl C5-epimerase (GLCE)* by miRNA-218 was investigated. Significant downregulation of miRNA-218 expression was shown in primary breast tumors. Exogenous miRNA-218/anti-miRNA-218 did not affect *GLCE* mRNA but regulated *GLCE* protein level in MCF7 breast carcinoma cells in vitro. Comparative analysis showed a positive correlation between miRNA-218 and *GLCE* mRNA, and negative correlation between miRNA-218 and *GLCE* protein levels in breast tissues and primary tumors in vivo, supporting a direct involvement of miRNA-218 in posttranscriptional regulation of *GLCE* in human breast tissue. A common scheme for the regulation of *GLCE* expression in normal and tumor breast tissues is suggested.

Introduction

D-glucuronyl C5-epimerase (GLCE, NM_015554) is one of the key enzymes in biosynthesis of heparan sulfate proteoglycans (HSPGs), which are involved in cell-cell and cell-matrix interactions, intercellular signaling and appropriate cell micro-environment.^{1–4} Recently, it was shown that *GLCE* possesses a tumor-suppressor activity in breast and small-cell lung cancer^{4,5} and *GLCE* expression is significantly downregulated in primary breast tumors and in breast and lung cancer cell lines compared with controls.^{6,7} However, the molecular mechanisms of *GLCE* inactivation in cancer cells remain unclear.

Previous studies show that the b-catenin-TCF4 transactivation complex regulates *GLCE* expression in human colon cancer cells in vitro,⁸ miR-218 directly represses expression of *GLCE* in DLD human colon cancer cells in vitro and rat retina in vivo,⁹ and that overexpression of the EJ-ras oncogene decreases *D-glucuronosyl C-5 epimerase* mRNA levels in rabbit endothelial cells.¹⁰ All the results provide important background for complex study of the molecular mechanisms of *GLCE* inactivation in breast cancer.

We recently showed complex transcriptional regulation for *GLCE* expression in breast cancer, where the combined effects of chromatin structure and TCF4/ β -catenin complex (but not promoter methylation) are necessary to maintain *GLCE* expression.¹¹ However, a possible contribution of some other molecular

mechanisms was indicated as well. Based on previous observations,⁹ miRNA-218, as post-transcriptional regulator of *GLCE*, is a strong candidate to be studied.

So far, no functional interrelation for miRNA-218 and *GLCE* in breast cancer has been demonstrated. On the one hand, tumor-suppressor function was shown both for *GLCE*^{5,12} and miRNA-218.^{13–16} On the other hand, miRNA-218 is supposed to negatively regulate *GLCE* expression in cancer cells.⁹

In the present study, miRNA-218 expression levels were determined in primary breast tumors and control breast tissues, and the contribution of miRNA-218 in posttranscriptional regulation of *GLCE* expression in breast cancer cells in vitro and primary tumors in vivo was investigated.

Results

miRNA-218 expression is downregulated in human primary breast tumors in vivo. miRNA-218 expression in primary breast tumors and normal breast tissues (morphologically normal, maximally distant part of the same mammary gland) was determined by the TaqMan MicroRNA Assay (Invitrogen), which uses looped-primer RT-PCR real-time quantification to accurately detect mature miRNAs (Fig. 1).

According to the RT-PCR data, miRNA-218 expression was very heterogeneous in normal breast tissues. However, evident

*Correspondence to: Elvira Grigorieva; Email: elv_grig@yahoo.com

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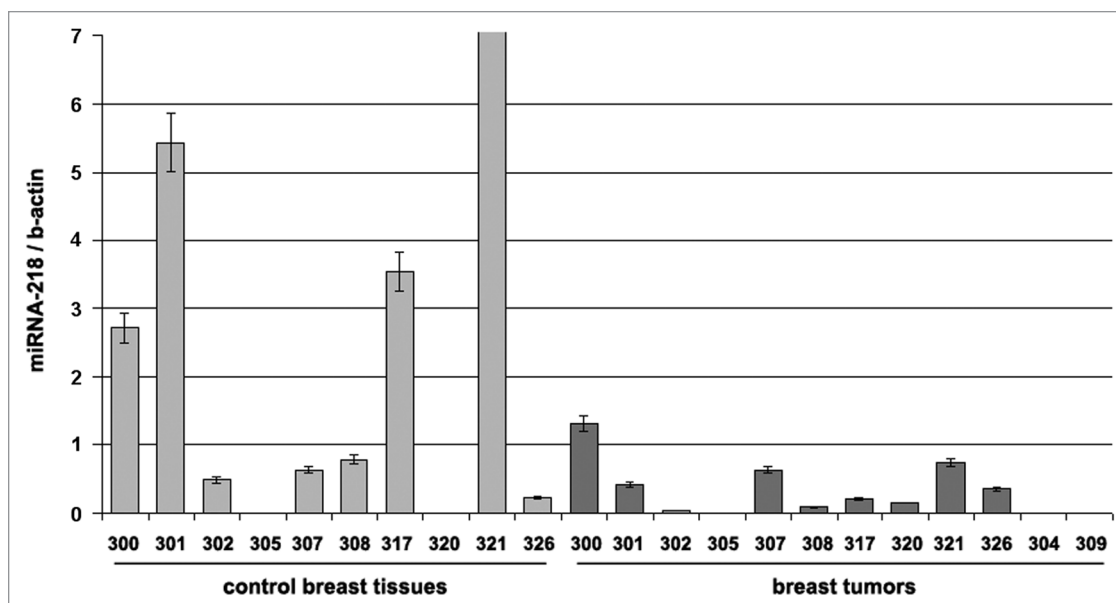


Figure 1. miRNA-218 expression in the human breast tumors and breast tissues in vivo. miRNA-218 expression normalized to that of β -actin, 300–326 patients, control and tumor breast tissues (matched pairs from each patient), bars represent the mean \pm SD from triplicate experiments (OriginPro 8.1).

miRNA-218 downregulation was observed in most of the corresponding breast tumors (patients NN 300, 301, 302, 308, 317 and 321) resulting in a more homogeneous, low-level expression of miRNA-218 in cancer samples. The results showed that miRNA-218 expression is significantly decreased (2- to 15-fold) in human breast tumors compared with normal breast tissues.

Correlation of miRNA-218 and *GLCE* expression in breast tumors in vivo. To investigate a possible interrelation between miRNA-218 and *GLCE* expression, *GLCE* mRNA and protein levels were determined in the same breast tumors and normal tissues by TaqMan-based qReal-time RT-PCR and western blot analysis, respectively (Fig. 2A and B).

Pearson correlation coefficients were calculated for miRNA-218/*GLCE* mRNA and miRNA-218/*GLCE* protein levels both in the normal breast tissue and breast primary tumors (Fig. 2C and D). A positive correlation between miRNA-218 and *GLCE* mRNA levels was shown for both normal breast tissues ($r = +0.45$, $p < 0.23$) and breast tumors ($r = +0.79$, $p < 0.01$), indicating a possible co-regulation for their expressions in breast tissue. On the contrary, a negative correlation between miRNA-218 expression and *GLCE* protein levels was shown for both groups ($r = -0.45$, $p < 0.26$ for normal breast tissues and $r = -0.40$, $p < 0.30$ for breast tumors). This suggests an involvement of miRNA-218 in post-transcriptional regulation of *GLCE* protein levels in human breast tissues and tumors in vivo, although a moderate correlation between miRNA-218 and *GLCE* protein levels in the samples indicates a possible involvement of some other molecular mechanisms as well.

miRNA-218 regulates *GLCE* protein level but not mRNA in breast cancer cells in vitro. To confirm the ability of miRNA-218 to directly regulate *GLCE* protein content in breast tissue, a functional study was performed in MCF7 breast carcinoma cells in vitro. Mimic miRNA-218 and anti-miRNA-218 oligonucleotides

were transfected into MCF7 cells and, subsequently, miRNA-218, *GLCE* mRNA and *GLCE* protein levels were analyzed (Fig. 3).

The results showed that transfection with either miRNA-218 or anti-miRNA-218 did not affect *GLCE* mRNA level in MCF7 cells. However, *GLCE* protein contents were significantly decreased in the miRNA-218-transfected cells, showing an ability of miRNA-218 to directly regulate *GLCE* protein level in breast cancer cells in vitro.

Interestingly, transfection of MCF7 cells with anti-miRNA-218 resulted in the decreased miRNA-218 content in the cells (by 2–3-fold) but did not increase the *GLCE* protein level. This supports the existence of some other molecular mechanisms or miRNAs, which cooperate with miRNA-218 in the control of *GLCE* protein levels.

Taken together, these results complement and extend the published data on miRNA-218 expression in breast cancer, and show an involvement for miRNA-218 in posttranscriptional regulation of *GLCE* expression in breast cancer cells in vitro and normal breast tissues and primary tumors in vivo.

Discussion

microRNAs (miRNAs) are small non-coding endogenously produced RNAs that play key roles in controlling the expression of many cellular proteins.^{17,18}

One of the key results of the study is the specific downregulation of miRNA-218 in human breast tumors compared with the surrounding normal breast tissues. The results stay in line with the published data on miRNA-218 deregulation in different tumors—a decreased miRNA-218 expression was shown in glioblastoma,¹⁹ glioma cell lines and primary tumors,²⁰ non-small cell lung cancer,²¹ clear cell renal cell carcinoma²² and gastric

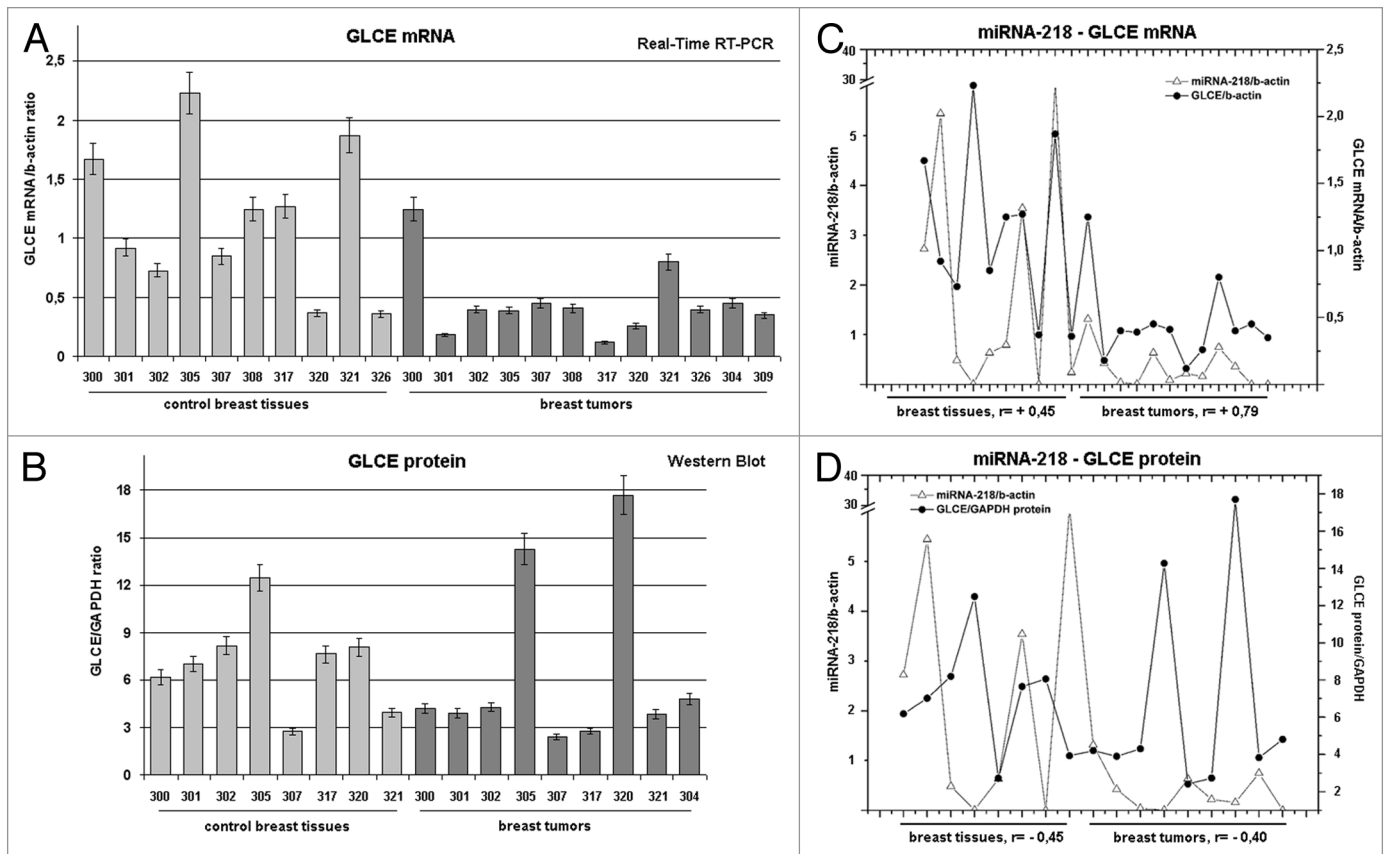


Figure 2. GLCE expression in human breast tumors and control tissues in vivo. **(A and B)** GLCE mRNA and protein levels in the same clinical samples; GLCE expression normalized to that of β -actin and GAPDH, respectively. 300–326 patients, control and tumor breast tissues (matched pairs from each patient), bars represent the mean \pm SD from triplicate experiments (OriginPro 8.1). **(C and D)** Correlation analysis between miRNA-218 and GLCE mRNA **(C)** or GLCE protein **(D)** expression in breast tissues and tumors in vivo. Pearson's correlation coefficients are shown (OriginPro 8.1).

cancer.²³ In addition, significant loss of miRNA-218 was shown during prostate cancer progression,²⁴ although unchanged miRNA-218 expression was shown in mouse colon tumors.²⁵ In a number of functional studies, a tumor-suppressor role for miRNA-218 was shown in non-small cell lung cancers,¹³ bladder cancer,¹⁴ nasopharyngeal carcinoma¹⁵ and renal cell carcinoma.¹⁶

However, it is not clear whether miRNA-218 has tumor-suppressor activity in human breast tissue/tumors as well.

According to our results, miRNA-218 expression significantly decreased in breast tumors, which correlated with GLCE mRNA level both in normal breast tissues ($r = +0.45$) and breast tumors ($r = +0.79$). This implicates coordinate expression, which looks logical for GLCE protein regulation—the more GLCE mRNA in the cell, the more miRNA-218 needed to keep GLCE protein at a stable level. Possibly, a feedback loop may exist between miRNA-218 and GLCE expression, as it was recently shown for some other genes.^{26,27}

miRNA-218 negatively regulates expression of GLCE protein in MCF7 breast cancer cells in vitro and primary breast tumors in vivo.

However, simultaneous general downregulation of miRNA-218 and GLCE in breast tumors raises the question of the functional interrelation between a potential tumor-suppressor,

miRNA-218, and its downstream target, tumor-suppressor gene GLCE in breast cancer.

It is known that the tumor-suppressor activity of miRNA-218 is achieved through the inhibition of cancer-promoting genes related to mTOR-Akt signaling pathway,²⁸ MMP-9 and IKK- β /NF- κ B pathway,²⁰ SLIT-ROBO pathway,^{15,23,29} transcription factors HNF-6/OC-1, OC-2³⁰ and Runx2,³¹ some other downstream targets such as paxillin,²¹ actin filament bundling proteins LIM and SH3 protein 1 (LASP1)³² and epithelial cell-specific marker LAMB3.³³ To avoid the tumor-suppressor effect, miRNA-218 seems to be inactivated during the carcinogenesis.

However, miRNA-218 inactivation also supposes simultaneous increase of potential miRNA-218 targeted tumor-suppressor proteins (GLCE, RASSF1A and claudin-6³⁴). The upregulation of tumor-suppressor genes could counteract malignant transformation and may be suppressed by some other molecular mechanisms, not directly related to miRNA-218. Possibly, a clonal selection of the cells with simultaneous inactivation of both miRNA and its target tumor-suppressor genes is a necessary step for tumor development. The hypothesis is supported by the recent publication on a previously unappreciated miRNA-regulation mechanism by which a single miRNA may target both oncogenes and tumor suppressors, simultaneously or

sequentially, in tumorigenesis and normal development.³⁵

From the presented results and our previous study,¹¹ a possible molecular mechanism of GLCE inactivation in breast tumors, and its relation to miRNA-218, could be suggested (Fig. 4). In normal breast tissue, *GLCE* expression is transcriptionally regulated by the direct interaction of TFC4/b-catenin transcription complex with the *GLCE* promoter; GLCE protein level is then adjusted by miRNA-218 at the posttranscriptional level. In breast tumors, TCG4/b-catenin-*GLCE* promoter interaction is blocked due to overall chromatin inactivation at the *GLCE* promoter site, resulting in the decreased *GLCE* mRNA and protein levels, in spite of the impairment of the regulating effect of miRNA-218.¹¹ The scheme suggests a multilevel mechanism for GLCE regulation in normal breast tissue and a model for its deregulation in breast tumors.

Materials and Methods

Patients and tissue samples. All tissue samples were obtained from primary breast tumors during the radical surgery at Central Municipal Hospital N1, Novosibirsk, Russia, “snap-frozen” in liquid nitrogen and stored at -70°C. Regions were manually dissected from the frozen blocks to provide a consistent tumor cell content of more than 70% in tissues used for analysis. The prevalent histological type of tumors was duct infiltrating adenocarcinoma of different degree of malignancy. Most patients were at the second stage of malignancy progression according to the TNM formula. All patients provided written informed consent and the study protocol was approved by the Local Ethics Committee in accordance with the Helsinki Declaration of 1975.

Cell lines, cell culture and miRNA-218 or anti-miRNA-218 transfection. The human breast cancer cell line MCF7 was obtained from the Karolinska Institute. Cells were maintained in IMDM medium supplemented with 2mM L-glutamine, 100 units penicillin, 100 µg/ml streptomycin and 10% (v/v) fetal bovine serum at 37°C in a humidified 5% CO₂. For analysis, cells were harvested using trypsin/EDTA (Sigma).

MCF7 cells were transfected with miRNA-218 and anti-miRNA-218 oligonucleotides as described previously.⁹ Briefly, miRNA-218 LNA-enhanced oligonucleotides possessing 2-O-Me modifications on the outermost 6 nucleotides (Exiqon, final concentration of 10 nmol/L) or anti-miRNA-218 LNA-modified oligonucleotides (final concentration of 10 nmol/L) were transfected using Lipofectamine 2000 (Invitrogen). Forty-eight hours later, total RNA or protein was isolated. Sequences were as follows: miR-218, 5-AAC CAC ATG GTT AGA TCA AGC ACA A-3'; anti-miR-218, 5-GTT AGA TCA AGC ACA-3.

Analyses of miRNA-218 expression using the TaqMan MicroRNA assay. Total RNA was extracted from the cells using

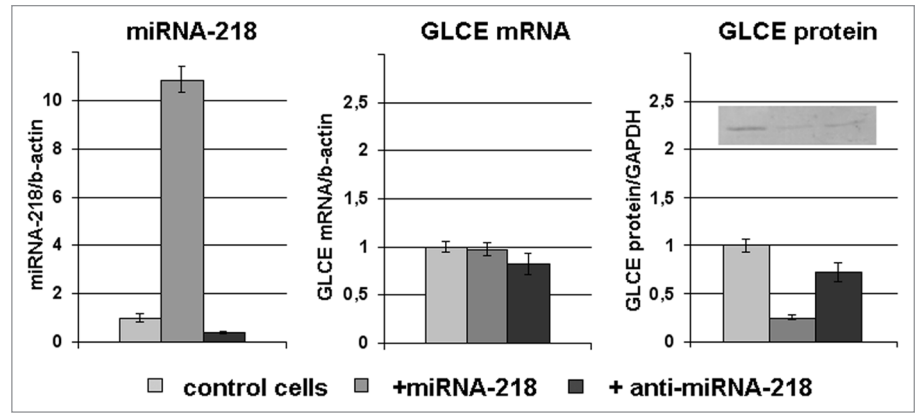


Figure 3. Transfection of MCF7 breast carcinoma cells with mimic miRNA-218 and anti-miRNA-218 oligonucleotides. miRNA-218 expression, TaqMan MicroRNA Assay; GLCE mRNA expression, Taqman-based qReal-Time RT-PCR; GLCE protein level, western blot. Inset: a representative blot. Bars represent the mean ± SD from triplicate experiments (OriginPro 8.1).

the TRIZOL reagent (Invitrogen) according to the manufacturer's instructions. miRNA-218 quantification was performed using the BioRad IQ5 Multicolor Real-Time PCR Detection System (BioRad) and the two-step RT-PCR TaqMan MicroRNA assay (Invitrogen). At the reverse transcription (RT) step, cDNA was synthesized from total RNA samples using specific miRNA primers and reagents from the TaqMan MicroRNA Reverse Transcription Kit. At the PCR step, PCR products were amplified from cDNA using the TaqMan MicroRNA Assay/TaqMan Universal PCR Master Mix. The following conditions were used for PCR: 95°C, 10 min; 95°C, 15 sec; 60°C, 60 sec; 40 cycles. β-actin (*ACTB*) was used as the housekeeping gene.

Analyses of *GLCE* expression by quantitative TaqMan-based real-time RT-PCR. Quantitative real-time RT-PCR (qRT-PCR) analysis of *GLCE* expression was performed as described earlier.^{11,12} Briefly, total RNA was extracted from the cells using the TRIZOL reagent (Invitrogen), cDNA was synthesized from 1–2 µg of total RNA using a First Strand cDNA Synthesis kit (Fermentas), and 1/10th of the product was subjected to PCR analysis. qRT-PCR was performed using the BioRad IQ5 Multicolor Real-Time PCR Detection System (BioRad) and the *GLCE* TaqMan Custom Assay (Applied Biosystems) under the following conditions: 95°C for 3 min, followed by 40 cycles at 95°C for 10 sec and 60°C for 30 sec. The total reaction volume was 25 µl. β-actin (*ACTB*) was used as the control housekeeping gene. The PCR primers and TaqMan probes used were: *GLCE*-F, 5'-TTC CAA AGT CTA TGC ACA GAG AGC-3'; *GLCE*-R, 5'-TCC ACA TTG TAG CCT TCA AAA GAC A-3'; *GLCE*-probe, 5'-FAM-CCC CTA TCA CCC CGA TGG T-TAMRA-3'; β-actin-F, 5'-GGC ACC CAG CAC AAT GAA G-3', β-actin-R, 5'-GCC GAT CCA CAC GGA GTA CT-3', β-actin-probe, 5'-FAM-TCA AGA TCA TTG CTC CTC CTG AGC GC-TAMRA-3'.

Western blotting. Western blotting was performed as described earlier.¹¹ Briefly, cells were lysed with RIPA-buffer (1% Nonidet P-40, 150 mM NaCl, 0.1% SDS, 50 mM Tris, pH 7.4) containing “complete” Protease Inhibitor Cocktail (Roche),

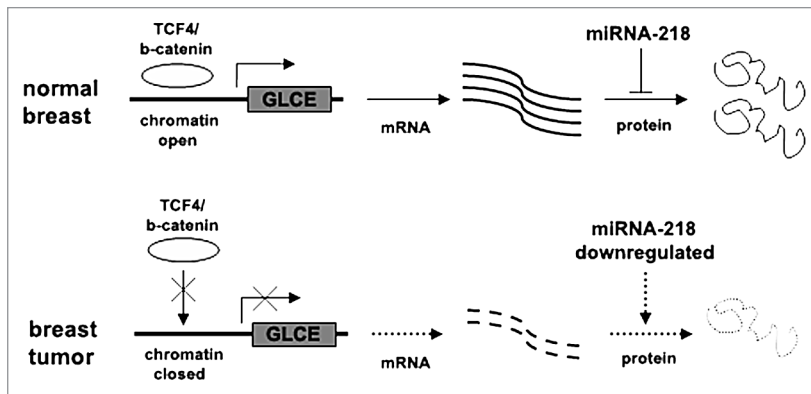


Figure 4. Schematic representation for the regulation of GLCE expression in normal breast tissues and breast tumors.

sonicated and centrifuged for 10 min at 12,000 g. The protein concentration was quantified using Quant-iT Protein Assay Kit (Invitrogen). Total proteins (30 μ g) were treated in NuPAGE LDS Sample Buffer (Invitrogen) with 10% β -mercaptoethanol for 5 min at 100°C, resolved in 10% SDS-PAGE and transferred to PVDF membranes. The membranes were blocked with 5% milk for 1 h and incubated with primary antibodies (rabbit anti-GLCE polyclonal serum (GenScript Corporation), 1:2,000) overnight at 4°C and secondary peroxidase-conjugated antibody [polyclonal goat anti-rabbit (Pierce)] for 1 h at RT. Proteins were

detected with an Opti-4CN Substrate Kit (Bio-Rad) according to the manufacturer's instructions. The gels were scanned using the "DNA Analyzer" system (Vilber Lourmat) and GLCE protein levels were estimated from the intensity of GLCE fragment normalized against the intensity of GAPDH using the TotalLab program (Nonlinear Dynamics).

Statistical analysis. Pearson's correlation was used to determine the association between miRNA-218 and GLCE expression. Correlation coefficients (r) were calculated using a computer program ORIGIN Pro 8.0, $p < 0.05$ was considered statistically significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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