Int J Clin Exp Pathol 2016;9(9):9311-9317
www.ijcep.com /ISSN:1936-2625/IJCEP0035148

Original Article

Expression of glutaminase 1 gene in breast cancer and its clinical significance in neoadjuvant chemotherapy

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Received July 6, 2016; Accepted July 20, 2016; Epub September 1, 2016; Published September 15, 2016

Abstract: Objective: To analyze the expression of glutaminase 1 (GLS1) gene in breast cancer and its clinical significance in neoadjuvant chemotherapy. Methods: A total of 134 female patients with breast cancer who were treated in Department of Thyroid and Breast Surgery, Nanjing Drum Tower Hospital were selected. After being diagnosed by biopsy, they were randomly divided into two groups (n=67). Before modified radical mastectomy, the experimental group received a combined TEC (taxotere + epirubicin + cyclophosphamide) regimen. Then surgery was performed after three to four weeks of chemotherapy. Mammary glands of the control group were directly removed by modified radical mastectomy without receiving TEC regimen. After surgery, the cancerous tissues were subjected to immunohistochemical staining for GLS1 protein. Results: According to whether tumor cells penetrated the basement membrane, 5 patients had noninvasive carcinoma, and 129 had invasive carcinoma. According to the pathological morphology, 128 cases had ductal carcinoma, 2 had mucinous carcinoma (2 cases were complicated with ductal carcinoma), and 4 had lobular carcinoma. GLS1 gene was expressed differently in normal and cancerous tissues of the 134 patients, but the expression levels were significantly higher in cancerous ones ($P<0.05$). GLS1 gene expression level of the experimental group was significantly lower than that of the control group ($P<0.05$). Univariate analysis showed that GLS1 gene expression level was positively correlated with lymphatic metastasis and pathological stage ($P<0.05$). Conclusion: GLS1 gene was highly expressed in the tumor tissues of patients with breast cancer. Neoadjuvant chemotherapy before modified radical mastectomy significantly reduced such expression level. GLS1 gene expression level was positively correlated with lymphatic metastasis and pathological stage, which can thus be used as an evaluation index for therapeutic effects. GLS1 can be employed as one of the specific targets for breast cancer therapy and development of targeted therapy drugs.

Keywords: Breast cancer, neoadjuvant chemotherapy, glutaminase 1 gene, targeted therapy

Introduction

Breast cancer, as malignant tumor generated in the epithelial tissues of mammary glands, accounts for over 20% of all female malignant cases and has greatly threatened them [1]. To improve the quality of life of these patients, surgery has been combined with adjuvant therapies [2]. The reason and mechanism of breast cancer are relatively complex. Family heredity, radiation exposure, chemical stimulation, proto-oncogene activation and biological factor stimulation can cause malignant proliferation and cancerous changes of the epithelial tissues [3].

The adjuvant therapies for breast cancer are generally determined based on clinical classification and staging. Currently, breast cancer patients are commonly treated by surgery in combination with postoperative chemotherapy to minimize recurrence and to increase disease-free and 5-year survival rates. Chemotherapy regimens mainly include TAC (taxotere + adriamycin + cyclophosphamide) [4-7], AC-P (adriamycin + cyclophosphamide + paclitaxel), AC-T (adriamycin + cyclophosphamide + taxotere), AC (adriamycin + cyclophosphamide), TC (taxotere + cyclophosphamide) [6] and FAC (fluorouracil + adriamycin + cyclophosphamide). By interfering with various phases of cell cycle, chemotherapeutic agents inhibit DNA replication or cell mitosis, thus destructing normal tissues and cells owing to the lack of specificity [3, 4]. In contrast, breast cancer can be effectively

Table 1. Baseline clinical data (X±s)

Group	Case number (n)	Age (year)	Body weight (kg)	Menstrual cycle (day)	Disease course (month)
Control	67	52.7±6.4	65.7±12.4	29.4±3.5	5.2±2.1
Experimental	67	56.2±8.2	63.2±7.8	30.2±4.1	6.8±4.3
T value	-	0.78	0.42	0.35	-
P value	-	>0.05	>0.05	>0.05	-

treated by using drugs for chemotherapy and targeted therapy simultaneously.

Glutaminase 1 (GLS1) gene, which is located on 2q32.2, was separated from blood platelets and found by Sahai in 1983 [8]. Glutamine is a neurotransmitter, the activity of which is affected by GLS1 gene expression product. The product can degrade glutamine in tissue cells. Recently, GLS1 has been closely associated with some cancers. Since c-Myc and its target gene GLS1 are involved in human peripheral T-cell lymphoma [9], GLS1 may be one of the poor prognostic factors. Moreover, GLS1 is also the downstream target of the miR-192-204-HOPPIP axis in hepatocellular carcinoma, indicating that GLS1 plays an important role in this cancer and can regulate it through miRNA. In addition, glutaminase can regulate the glucose metabolism in tumor cells as the Warburg effect that tends to convert glucose into lactose, without using oxygen. As the downstream target gene of GLS1, c-Myc regulates oncogenic transcription factor through miRNA [10]. Since then, GLS1 has been detected in a wide variety of tumor organs and tissues, such as lung cancer, liver cancer, ovarian cancer, bladder cancer and leukemia.

In this study, we collected the clinical data and pathological samples from 134 patients with breast cancer, aiming to detect GLS1 gene expression level, and to explore its clinical significance in neoadjuvant chemotherapy.

Materials and methods

Subjects

A total of 134 female patients with breast cancer who were treated in Department of Thyroid and Breast Surgery, Nanjing Drum Tower Hospital were selected. This study has been approved by the ethics committee of our hospital, and written consent has been obtained from all patients. Their clinical characteristics, such as age, onset time, childbearing history and menstrual history, were recorded and analyzed. Based on clinical manifestations, imageological examination and pathological test, the selected patients were staged and classified, and a corresponding therapeutic regimen (TEC, taxotere + epirubicin + cyclophosphamide) was established.

Inclusion criteria: Patients diagnosed as breast cancer by pathological examinations.

Exclusion criteria: 1) Patients complicated with malignant tumors in other systems or with metastasis; 2) patients with undefined diagnosis; 3) patients with cognitive or mental disorders; 4) patient samples could not be collected; 5) patients or family members did not comply with treatment; 6) patients quitted this study before it finished; 7) patients could not be examined or treated due to poor physical state.

Neoadjuvant chemotherapy regimen

TEC regimen was performed three to four weeks before surgery, and lasted for three weeks. Afterwards, modified radical mastectomy was conducted. Doses: Taxotere (Rhone-Poulenc Rorer, France), 75 mg/M2 d1; epirubicin (Pharmorubicin, Pfizer, USA), 100 mg/M2 d1; cyclophosphamide (Baxter, USA), 500 mg/M2 d1.

Immunohistochemical assay

Paraffin sections of breast cancer tissues were deparaffinized, hydrated, incubated with 3% H_2O_2 at 20°C for 10 min, rinsed with distilled water and soaked in PBS for 5 min (the procedure was repeated twice). Subsequently, the sections were blocked by 5% PBS-diluted goat serum (Life Technologies, USA) and incubated at 20°C for 10 min. After the goat serum was discarded, primary antibody was dropped (GLS1, ab60709, Abcam, USA), and the sections were incubated at 37°C for 1-2 h or stored in a 4°C refrigerator overnight. Then a working solution of biotinylated secondary antibody

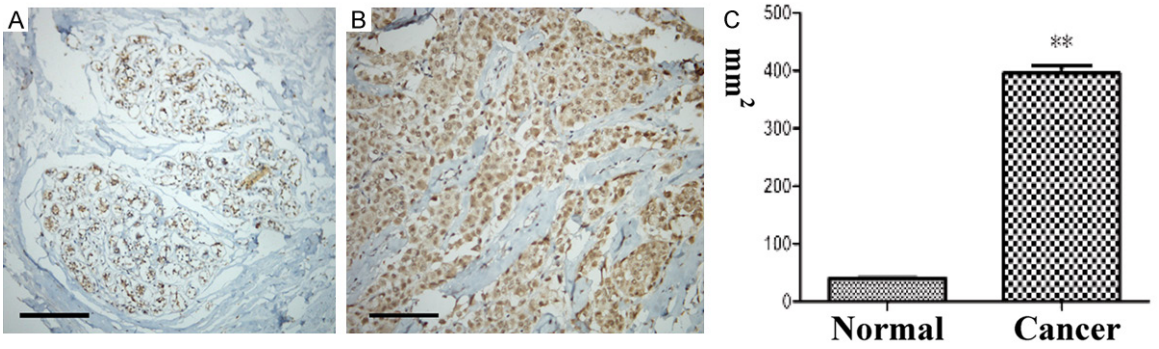

Figure 1. GLS1 expressions in normal (A) and breast cancer (B) tissues (magnification: ×200; bar: 50 μm); (C) Statistical analysis results for immunohistochemical staining.

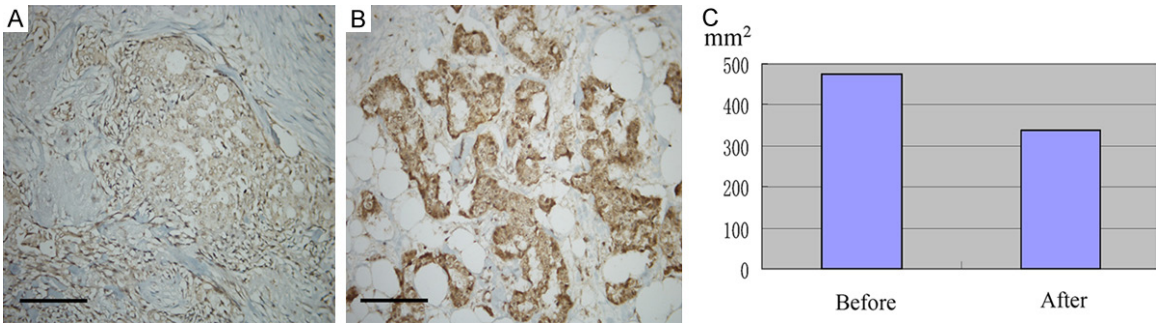

Figure 2. GLS1 expressions in breast cancer tissues before (A) and after (B) neoadjuvant chemotherapy (magnification: ×200; bar: 50 μm); (C) statistical analysis results for immunohistochemical staining.

(Perkin-Elmer) was dropped onto the sections to incubate them at 37°C for 10 min, which were incubated at 37°C for another 10 min after addition of horseradish peroxidase (Sigma, S7571, USA). One to two drops of DAB Plus Chromogen were added into 1 ml of DAB Plus Substrate (TA-125-HDX, Thermo Scientific, USA), and then the mixture was dropped onto the sections to incubate them at 37°C for 10 min. Finally, the sections were washed and sealed.

Statistical analysis

All data were analyzed by using SPSS 20.0. The continuous variable conforming to normal distribution were expressed as mean and standard deviation. Otherwise, they were expressed as median and interquartile range. The categorical variables were expressed as percentages. The continuous variables were compared by Student's t test, while the categorical ones were compared with Pearson χ^2 test. Univariate analysis was performed to study the correlations between GLS1 and various factors.

Results

Baseline clinical data

Most patients were aged 50-60 years old. Their age, body weight, menstrual cycle and disease course were similar (P>0.05) (**Table 1**). The childbearing history, radiation exposure history and family disease history were also compared (Supporting Information, Table S1). Their pathological classifications are shown in Table S2 and Figure S1.

GLS1 expressions in normal and breast cancer tissues

GLS1 gene was expressed differently in normal and cancerous tissues of the 134 patients, but the expression levels were significantly higher in cancerous ones (P<0.05) (**Figure 1**).

GLS1 expression levels before and after neoadjuvant chemotherapy

After 3-4 week of neoadjuvant chemotherapy and surgery, breast cancer tissues of the

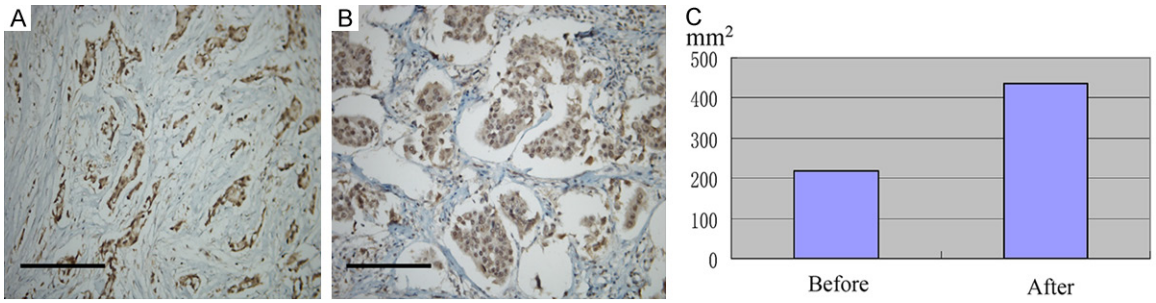

Figure 3. GLS1 expressions in breast cancer tissues of patients with postoperative recurrence (A) and just after surgery (B) (magnification: ×200; bar: 50 μm); (C) Statistical analysis results for immunohistochemical staining.

patients were collected and subjected to immunohistochemical staining (**Figure 2**). Obviously, neoadjuvant chemotherapy significantly reduced GLS1 expression level ($P<0.05$).

GLS1 expression levels before and after breast cancer recurrence or metastasis

The patients with postoperative recurrence had significantly higher GLS1 expression levels that those just after surgery ($P<0.05$). In addition, such level significantly rose upon lymphatic metastasis ($P<0.05$) (**Figure 3**).

Regression analysis for prognostic factors of breast cancer

Univariate analysis was performed considering pathological classification (invasive or noninvasive), distant metastasis, cancer tissue volume, recurrence, age, body weight and menopause. GLS1 gene expression level was positively correlated with lymphatic metastasis and pathological stage ($P<0.05$). However, it was not correlated with tumor size, recurrence or metastasis ($P>0.05$) (**Table 2**).

Discussion

Breast cancer, which is experiencing increasing cases, is commonly treated by combining surgery with postoperative radiotherapy and chemotherapy to maximally inhibit the growth and proliferation of tumor cells [1]. Nevertheless, researchers are devoted to finding preoperative chemotherapy strategies to shrink tumors as well as to minimize surgical areas and physical and psychological damages to women. Moreover, currently available targeted therapy drugs based on gene specificity mainly exert their effect through the HER2 receptor family [11] on tumor cell surfaces. In the beginning, they can markedly improve the prognosis and quality of life, but the therapeutic effects are weakened as cancer proceeds due to increased drug resistance. Thereby motivated, the patients with breast cancer were herein given 3-4 weeks of TEC regimen [12] before surgical resection, and GLS1 expression levels before and after surgery were compared to provide valuable evidence for developing novel targeted therapy drugs.

In recent years, tumor metabolism has been highlighted. Most tumor cells are bound to undergo the Warburg effect [13] that means they still manage to proliferate under hypoxic or anaerobic conditions by elevating glycolytic and lactic acid fermentation rates. In extreme cases, the rates can still be raised under normoxic conditions. Dang [14] reported that tumor cells adapted themselves to proliferative requirements by changing various respects of metabolism. Notably, the Warburg effect occurs evidently depending on intracellular glutamine levels, manifested as overconsumption in human body [15]. Therefore, it is also referred to as glutamine addiction [16]. During this process, entrance into the citric acid cycle (a part of the tricarboxylic acid cycle) requires a large quantity of extracellular glutamates, consuming considerable bioenergy [17]. This process in predominantly controlled by glutaminase, a GLS-encoded protein product [18].

In this study, GLS1 gene was expressed differently in normal and cancerous tissues of the 134 patients, but the expression levels were significantly higher in cancerous ones ($P<0.05$). High GLS1 expression suggested that cells vigorously proliferated in these patients. GLS1 expression has been reported to increase not

Table 2. Univariate analysis for prognostic factors of breast cancer

Variable (n=134)	Case number	GLS1 (>++)	P
Age (year)			0.149
≥45	102	71 (70.0)	
<45	32	30 (93.8)	
Body weight (kg)			0.135
≥60	78	51 (65.4)	
<60	56	50 (89.3)	
Tumor diameter (cm)			0.372
≥10	45	25 (55.6)	
5~10	39	29 (74.4)	
≤5	50	47 (94.0)	
Differentiation degree			0.451
Low	31	14 (45.2)	
Medium	44	38 (86.3)	
High	59	49 (83.1)	
Tumor type			0.563
Lobular carcinoma	4	4 (100.0)	
Ductal carcinoma	128	95 (74.2)	
Mucinous carcinoma	2	2 (100.0)	
TNM stage			<0.001
I	35	24 (68.6)	
II	37	24 (64.9)	
III	24	15 (62.5)	
IV	38	38 (100.0)	
Regional lymph node staging			<0.001
Nx	12	2 (16.7)	
N0	15	10 (66.7)	
N1	27	19 (70.4)	
N2	38	30 (78.9)	
N3	42	40 (95.2)	
Distant metastasis			0.783
Mx	38	20 (52.6)	
M0	53	38 (71.7)	
M1	43	43 (100.0)	
Surgical type			0.463
Modified radical mastectomy	102	81 (79.4)	
Others	32	30 (93.8)	
Menopause			0.141
Yes	44	36 (81.2)	
No	90	65 (72.2)	
Childbearing			0.283
Yes	84	56 (66.6)	
No	50	45 (90.0)	

only in breast cancer patients but also in babies and children who have traumas and bone fractures awaiting repair and recovery. Such increase is a physiological process to main normal cellular functions [19]. Evaluating the onset, progression and outcomes of breast cancer depending only on GLS1 content is inaccurate due to the lack of cell specificity [20]. Besides, whether GLS1 expression level is associated with the differentiation degrees of tissues and cells remains unclear. Re gardless, this study provided reasonable evidence for the diagnosis, treatment, prognostic evaluation, recurrence and metastasis of breast cancer, potentially allowing the development of novel targeted therapy drugs.

After 3-4 weeks of preoperative TEC regimen, GLS1 expression level was correlated with clinical stage and lymphatic metastasis ($P<0.05$). Since increase in GLS1 expression commonly predicts enhanced anabolism and weakened catabolism, tumor cells may remarkably increase in the patients with lymphatic metastasis and high clinical stage. However, it does not necessarily mean that tumors are larger at higher GLS1 levels. Moreover, such level was not correlated with the pathological classification of breast cancer. Given that neoadjuvant chemotherapy significantly reduced GLS1 expression level ($P<0.05$), the chemotherapeutic agents effectively suppressed the proliferation of tumor cells. The results are of great clinical significance. However, only clinical phenotype studies were conducted herein, so animal researches are in need to clarify whether this regimen was able to improve the survival rate and to prolong the disease-free survival.

We also found that tumor size was not correlated with GLS1 expression, but Zu et al. [19] reported that tumor size was correlated with the expressions of gastric cancer markers and prognosis. Similarly, Yang et al. [20] reported that tumor size was not apparently correlated with the proliferation or metabolic degree of tumor cells. Thus, for breast cancer, tumor size was not significantly correlated with its metabolism. In the patients with lowly differentiated or

undifferentiated tumors, although tumors remained small, most of them had undergo distant metastasis or lymphatic metastasis [10]. Accordingly, the correlation between tumor size and metabolic degree still needs in-depth studies.

Our study still has limitations. For instance, further *in vitro* and *in vivo* studies are needed to detect the dynamic changes of GLS1 gene expression during breast cancer treatment. Meanwhile, the mechanism by which GLS1 gene participates in the onset and progression of breast cancer remains elusive. Li et al. [9] found that c-Myc and its target gene GLS1 played crucial roles in peripheral T-cell lymphoma. GLS1 may be one of the factors responsible for poor prognosis. Furthermore, GLS1 is the downstream target of the miR-192-204-HOPPIP axis in hepatocellular carcinoma [21]. Based on these, we will be inspired to further analyze the relationship between GLS1 gene and breast cancer.

In summary, GLS1 gene is of noticeable clinical significance in evaluating the recurrence, metastasis and deaths of breast cancer patients. To observe the therapeutic effects of neoadjuvant chemotherapy, GLS1 is a potentially eligible index and one of the specific targets for breast cancer therapy and development of targeted therapy drugs.

Acknowledgements

This study was financially supported by National Natural Science Foundation of China (No. 81372294, 81372455).

Disclosure of conflict of interest

None.

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Table S1. Other baseline clinical data (%)

Disease history	Group	Case number	Yes	No
Childbearing history	Experimental	67	40	27
	Control	67	31	36
	χ^2 value	-	2.42	
	P value	-	>0.05	

Table S2. Clinical pathological classification

Case number (n)	Lobular carcinoma	Ductal carcinoma	Mucinous carcinoma
134	4 (4/134)	128 (128/134)	2 (2/134)

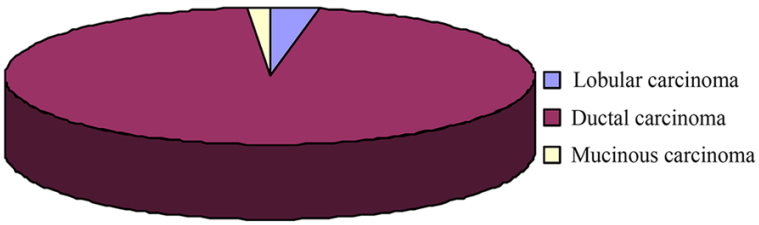

Figure S1. Clinical pathological classification.