Int J Clin Exp Med 2019;12(5):5475-5486
www.ijcem.com /ISSN:1940-5901/IJCEM0076915

Original Article

Cyclin G1 mediates the poor prognosis of breast cancer through expanding the cancer stem cells

Yuting Luan[1*], Tao Han[2*], Ran Liu[1*], Xiaodan Yang[2*], Qing Li[1]

[1]Department of Clinical Pharmacy, Shenyang Pharmaceautical University, Shenyang 110840, Liaoning Province, P. R. China; [2]Department of Oncology, Cancer Center of People's Liberation Army, General Hospital of Shenyang Military Region, Shenyang 110840, Liaoning Province, P. R. China. [*]Equal contributors.

Received August 14, 2016; Accepted October 5, 2018; Epub May 15, 2019; Published May 30, 2019

Abstract: Background: Breast cancer stem cells are a subpopulation of tumor cells with the capacity of self-renew and differentiate into distinct cell types that comprise the bulk of breast cancer. Cyclin G1 has been previously reported as a critical oncogene overexpressed in some cancers. However, the role of cyclin G1 in breast cancer stem cells remains unknown. Methods: The expansion of breast cancer stem cells in cyclin G1 over-expressing cells were assessed by spheroids formation and limited dilution assays. Co-expression of cyclin G1 and CD133/CD90 was observed in breast cancer cells by real-time PCR. In addition, a tissue microarray was used to examine 135 pairs of breast cancer samples and corresponding adjacent normal mucosae. The expression of cyclin G1 was determined by immunohistochemistry, and further was confirmed by real-time PCR and Western blot. Kaplan-Meier survival curve was used to determine the correlation of cyclin G1 expression with overall survival of patients. Results: Forced cyclin G1 expression was found remarkably enhanced the expansion of breast cancer stem cells in vitro. Correlated expression of cyclin G1 and tumor stem cell markers was observed in breast cancer. Cyclin G1 expression was detected increased in breast cancer tissue by immunohistochemistry, and further was confirmed at both level of mRNA and protein. Elevated expression of cyclin G1 was highly associated with lower overall survival in breast cancer patients. Compared with non-triple negative breast cancer, high expression of cyclin G1 was found in triple negative breast cancer and played a positive role in chemotherapy resistance. Conclusion: Our findings indicates that cyclin G1 plays an important role in expansion of cancer stem cells and outcome of poor prognosis in breast cancer. Thus, our study reveals a novel biomarker in breast cancer and indicates that cyclin G1 may be a promising therapeutic target for triple negative breast cancer.

Keywords: Breast cancer, cancer stem cells, cyclin G1, prognosis, expansion

Introduction

Breast cancer is one of the most common malignant tumors in women and the second cancer-related cause of death in USA, with an estimated 249,260 new cases and 40,890 deaths in 2016 [1]. Influenced by the characteristics of heterogeneity, the biological performance of breast cancer patients varied with different molecular typing. Progesterone receptor (PR), estrogen receptor (ER), and human epidermal growth factor receptor 2 (HER-2) are widely used in breast cancer as molecular markers for cancer treatment and prognosis evaluation. The expression of these molecular markers can be used to classify breast cancer into different subtypes. Moreover, in the early 20th century, some studies have confirmed that the prognosis of patients with breast cancer is closely related with those different molecular subtypes [2, 3]. In particular, due to the lack of effective treatment strategies, a heterogeneity subtype which we called triple negative breast cancer (TNBC, refers to estrogen ER, PR and HER-2 tumors are all negative), is prone to recurrence and chemoresistance and have the worst prognosis [4]. Compared with other types of breast cancer, TNBC is highly invasive, with high histological grade and early recurrence and metastasis [5]. Recently, some treatment strategies and methods for breast cancer have been improved, but it is still unsatisfactory on early detection and standard treatment. How to explore more effective treatments has become a research hotspot.

Table 1. Clinicopathologic characteristics

Variable		Number and value (range)		*P*
		Cyclin G1-high expression (*N* = 68)	Xyclin G1-low expression (*N* = 67)	
Median Age (range. yrs)		50 (32-82)	51 (29-83)	.85
Gender	Female	68	67	-
Tumor stage	T1/2	60 (52.2%)	55 (47.8%)	.32
	T3/4	8 (40.0%)	12 (60.0%)	
Nodal stage	N0/1	41 (53.2%)	36 (46.8%)	.44
	N2/3	27 (46.6%)	31 (53.4%)	
Triple Negative*	Yes	21 (67.7%)	10 (32.3%)	.03
	No	47 (45.2%)	57 (54.8%)	
AJCC stage	I-II	40 (52.6%)	36 (47.4%)	.55
	III	28 (47.5%)	31 (52.5%)	
Her2	Positive	19 (48.7%)	20 (51.3%)	.81
	Negative	49 (51.0%)	47 (49.0%)	
ER[a]	Positive	40 (46.0%)	47 (54.0%)	.17
	Negative	28 (58.3%)	20 (41.3%)	
PR[a]	Positive	23 (35.9%)	41 (64.1%)	.00
	Negative	45 (63.4%)	26 (36.6%)	
Tumor diameters	> 5	8 (40.0%)	12 (60.0%)	0.32
	< 5	60 (52.2%)	55 (47.8%)	

Note: *Results of the analysis have statistics significance. By comparing the general clinical data of the two groups of cyclin G1 expression, we found that the general clinical data characteristics of the two groups of patients were no significantly different ($p > 0.05$), but the incidence of triple-negative breast cancer was significantly higher in the cyclin G1 high expression group ($p < 0.05$), and the PR negative rate was higher in the cyclin G1 high expression group ($p < 0.001$). [a]ER: Estrogen Receptor; PR: Progesterone Receptor.

Recent findings support the concept that cancers are maintained in a hierarchical organization of rare "cancer stem cells", which are integral to the development of human cancer [6]. Therefore, tumors might have a built-in population of drug-resistant cancer stem cells that can survive chemotherapy and repopulate the tumor [7]. Accumulating evidence supports that cancer stem cells exist in different solid tumors, including brain [8], colon [9], breast [10], stomach [11], liver [12], skin [13], pancreas [14], and head and neck cancers [15]. Cancer stem cells have also been identified by several cell surface antigens such as CD133 [16], CD90 [17], epithelial cell adhesion molecule [18], and CD24 [19]. These cancer stem cells are likely to share many of the properties of normal stem cells that provide for a long life span, including relative quiescence, resistance to drugs and toxins. Based on the hypothesis of cancer stem cells, eradication of the stem-cell compartment of a tumor also may be essential to achieve stable, long-lasting remission, and even a cure of cancer [20, 21].

Cyclin G1 was initially discovered as a novel member of cyclin family with homology to c-src [22]. Although cyclin G1 belongs to a subgroup of cyclins, which also includes cyclin G2 and cyclin I, the precise role of cyclin G1 on cellular growth control is still controversial [23-28]. Importantly, cyclin G1 is transcriptionally activated by p53 and p73, and in turn, it negatively regulates p53 family proteins [29]. In reported experimental, loss of cyclin G1 is associated with significantly lower tumor stem cells in liver cancer [30]. And it was also reported that cyclin D1, another important cyclin family member, may serve as a potential target against breast cancer stem cells through Notch related pathway [31]. Although these data suggests a link between cyclin G1 and cancer and tumor stem cells, the precise role of cyclin G1 in breast cancer remains largely unknown.

In this study, we investigated the role of cyclin G1 in self-renewal and prognosis of breast cancer and preliminarily explored the underlying clinical significance, which will provide new insight in breast cancer prevention and therapy.

Table 2. Univariate and multivariate analyses of the primary cohort

Variable	*N*	Time (months)**	*P* value as Univariate	*P* value as Multivariate
Cyclin G1 expression*			0.002	0.006
High	68	88.491 ± 5.170		
Low	67	118.015 ± 3.797		
Tumor stage			0.251	-
T1/2	115	107.428 ± 3.787		
T3/4	20	95.900 ± 10.132		
Nodal stage*			0.001	0.001
N0/1	77	114.312 ± 4.119		
N2/3	58	94.516 ± 5.974		
Tumor diameter (cm)			0.931	-
≤ 5	115	106.149 ± 3.828		
> 5	20	103.150 ± 9.870		
AJCC grade			0.002	-
I-II	76	114.092 ± 4.167		
III	59	95.135 ± 5.904		
Her2			0.022	0.061
Positive	40	116.925 ± 5.465		
Negative	95	101.131 ± 4.451		
ER			0.104	-
Positive	85	109.888 ± 4.322		
Negative	50	98.260 ± 6.094		
PR			0.225	-
Positive	64	111.109 ± 4.670		
Negative	71	100.370 ± 5.233		
Triple Negative*			0.000	0.000
Yes	31	77.903 ± 7.872		
No	104	113.381 ± 3.604		

Note: *Results of the analysis have statistics significant. **Results were presented as mean ± standard error.

Materials and methods

Patients and breast cancer samples

Thirty nine breast cancer samples were randomly retrieved from breast cancer patients who underwent curative resection in General Hospital of Shenyang Military Region (Shenyang, China) from September 2010 to December 2012. The procedure of human sample collection was approved by the Ethic Committee of General Hospital of Shenyang Military Region. Tissue microarray which contains 135 cases breast cancers and 40 cases of peritumor tissues was obtained from Shanghai Outdo Biotech Company.

Cell lines and recombinant virus

MCF-7 and MDA-MB-231 cells were purchased from Cell Bank of Type Culture Collection of Chinese Academy of Sciences, MCF-7 cell line derived from a breast cancer patient whose ER(+), PR(+), Her(-), and MDA-MB-231 cell line has the feature of triple negative breast cancer. All those cell lines were cultured in DMEM (Invitrogen Corporation, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Gibco, Invitrogen). MCF-7 and MDA-MB-231 cells were transferred by pc3.1a-cyclin G1 plasmid and were established as described previously.

Real-time PCR

Quantitative PCR was performed using SYBR Green PCR Kit (Applied Biosystems, Foster City, CA) and ABI 7900HT Fast Real-Time PCR System (Applied Biosystems). The mRNA level of specific genes was normalized against 18S. The sequences of primers used in this study were listed in **Table 3**.

Western blot

Tumor specimens or breast cancer cell extract were prepared in lysis buffer [Tris-HCl (20 mM), pH 7.4, NaCl (150 mM), glycerol (10%), Nonidet P-40 (0.2%), EDTA (1 mM), EGTA (1 mM), PMSF (1 mM), NaF (10 mM), aprotinin (5 mg/ml), leupeptin (20 mM), and sodium orthovanadate (1 mM)] and centrifuged at 12,000 g for 30 min. Protein concentrations were measured using the BCA assay. Immunoblotting was performed using a primary antibody specific for cyclin G1 (Santa Cruz Biotechnology, sc-8016), and immunocomplexes were incubated with goat anti-mouse fluorescein-conjugated secondary antibodies and then detected using an Odyssey fluorescence scanner (Li-Cor, Gene Company). GAPDH was used as a loading control (Santa Cruz Biotechnology, sc-47724).

Table 3. Sequence of primers for Real-time PCR

Primer	Sequence (5' to 3')
Cyclin G1 forward primer	AAGCAGCTCAGTCCAACACA
Cyclin G1 reverse primer	TGGCTTTGACACAGAGACATTT
18s forward primer	CGGCTACCACATCCAAGGAA
18s reverse primer	GCTGGAATTACCGCGGCT
OCT4 forward primer	CTTGCTGCAGAAGTGGGTGGAGGAA
OCT4 reverse primer	CTGCAGTGTGGGTTTCGGGCA
NANOG forward primer	CAGGGCTGTCCTGAATAAGC
NANOG reverse primer	GATTTGTGGGCCTGAAGAAA
CD133 forward primer	TTTGGATTCATATGCCTTCTGT
CD133 reverse primer	CCATTGGCATTCTCTTTGAA
CD90 forward primer	GGGAGACCTGCAAGACTGTT
CD90 reverse primer	CGGAAGACCCCAGTCCA

Spheroid assay

MCF-7 cells over-expressing cyclin G1 or GFP cells were plated in ultra-low attachment Microplates and cultured in DMEM (Gibco, Invitrogen) supplemented with 10% FBS for seven days. The number of spheroids was counted and representative views were shown.

In vitro limiting dilution assay

MCF-7 cyclin G1 or their GFP cells were seeded into 96-well ultra-low attachment culture dishes at cell doses indicated in **Figure 1B** and incubated under spheroid condition for seven days. Colony formation was assessed by visual inspection. Based on the frequency of wells without colony, proportion of T-ICs was determined using Poisson distribution statistics and the L-Calc Version 1.1 software program (Stem Cell Technologies, Inc., Vancouver, Canada).

Statistical analysis

Statistical analysis in this study was calculated with SPSS 18.0 (SPSS Inc., USA). Data were expressed as "Mean ± SEM". The significance of mean values between two groups was analyzed by Student's t test. All differences were two-sided. A *P*-value less than 0.05 were considered statistically significant. Overall survival (OS) was defined as the interval between the dates of surgery and death. Survival curves were calculated using the Kaplan-Meier method and compared with a log-rank test.

Results

Cyclin G1 increases the proportion of tumor stem cells in breast cancer cells

Considering the important role of cancer stem cells in tumorigenesis and chemotherapy, we investigated the effect of cyclin G1 on breast cancer stem cells expansion and studied the correlated clinical significance. Our data showed that enforced cyclin G1 expression remarkably induced the spheroid formation capacity of MCF-7 cells, forced expression of cyclin G1 in breast cancer cells generated more and larger spheroids compared with the control cells infected with GFP (**Figure 1A**, Supplementary Figure 1A). And limiting dilution assay revealed that overexpression of cyclin G1 increased the proportion of cancer stem cells (**Figure 1B**, **1C**). More important evidence come from the clinical sample test, expression of CD133 and CD90, which are considered as potential markers of cancer stem cell, were markedly up-regulated in parallel with the increase of cyclin G1 in breast cancer cells. (**Figure 1D**, Supplementary Figure 1B). These data suggested that expression of cyclin G1 was correlated with breast cancer stem cells. Furthermore, we determined the change in expression levels of chemo-resistence-related genes in cyclin G1 overexpression cell lines, Oct4, Nanog, Bmi1 were significantly upregulated (**Figure 1E**, Supplementary Figure 1B). These results suggested a crosstalk between cyclin G1 signaling and stemness gene expression and hint us a possibility that cyclin G1 could be a biomarker for the stemness-maintain and chemo-resistence of breast cancer.

Enhanced expression of cyclin G1 in breast cancer

To examine the clinical relevance of expression deregulation of cyclin G1 in breast cancer patients, we purchased two breast cancer tissue microarrays from Shanghai Outdo Biotech Company and were used for immunohistochemical staining. It was found that the expression of cyclin G1 was significantly elevated in breast cancer than peri-tumor tissues (**Figure 2A**, **2B**). The results (**Figure 2C**, **2D**) were further confirmed by real-time PCR and

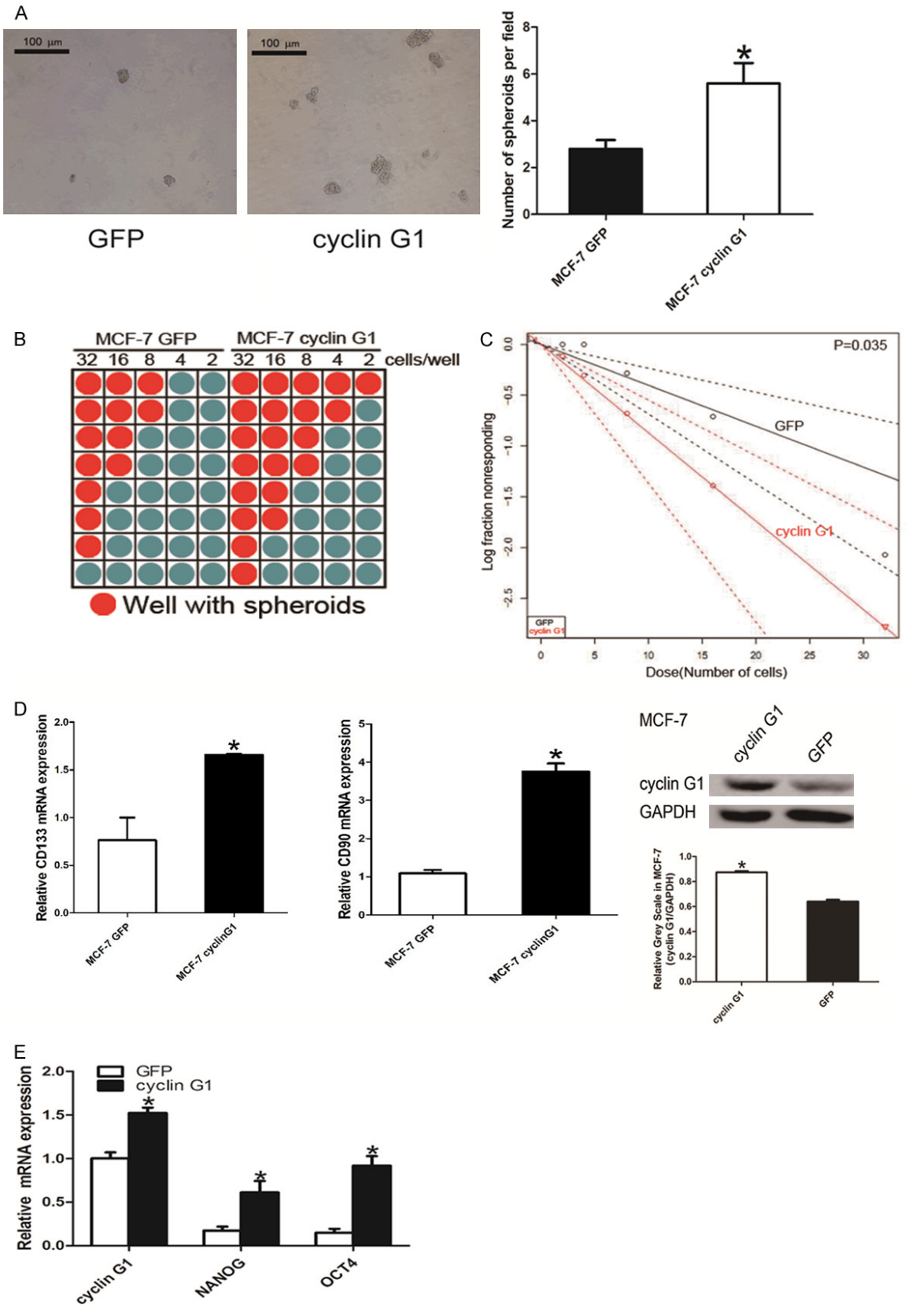

Figure 1. Cyclin G1 increases the proportion of cancer stem cells in breast cancer cells. A. 1 × 10^6 cells/mL MCF-7 cells expressing cyclin G1 or GFP were plated in ultra-low attachment Microplates at 5 × 10^3 cell per well and cultured in DMEM (Gibco, Invitrogen) supplemented with 10% FBS for seven days. Representative pictures were shown and

the number of spheroids was counted. *P < 0.05. B, C. MCF-7 cells expressing cyclin G1 or GFP were seeded into 96-well ultra-low attachment culture dishes at indicated cell doses (8 wells per dose) and incubated under spheroid condition for seven days. Based on the frequency of wells without colony, proportion of stem cells was determined using Poisson distribution statistics and the L-Calc Version 1.1 software program (Stem Cell Technologies, Inc., Vancouver, Canada). Limiting dilution assay of MCF-7 GFP and MCF-7 cyclin G1 cells was performed and the proportion of stem cells was shown. *P < 0.05. D, E. Expression of Cancer Stem Cell markers (CD133 and CD90) and stemness related genes (NANOG and OCT4) in MCF-7 GFP and MCF-7 cyclin G1 cells by real-time PCR analysis. *P < 0.05, the identification of MCF-7 GFP and MCF-7 cyclin G1 celllines by western blot and the relative grey-scale map (cyclin G1/GAPDH) of the celllines was shown.

Western blot analyses. This finding prompted us to speculate whether cyclin G1 played an important role in breast cancer.

Clinical significance of cyclin G1 expression in breast cancer

Then we preliminarily analyzed the clinical implication of cyclin G1 expression in breast cancer. High levels of cyclin G1 turned out to be associated with a poor survival of breast cancer (**Figure 3A**). We then examined the relationship between cyclin G1 expression in tumor tissues and the clinicopathological characteristics of the 135 patients (**Table 1**). Correlation regression analysis indicated that prognosis was correlated with several individual parameters, including cyclin G1 expression, nodal stage, AJCC grade and Her2 expression (**Table 2**). These individual parameters were further analyzed with a multivariate Cox proportional hazard model. Results indicated that cyclin G1 expression, nodal stage, and triple negative (Her2-, ER- and PR-) were significant and independent factors that affected the survival of breast cancer patients (**Table 2**; **Figure 3B**). Among these factors, cyclin G1 expression level had the significant hazard ratio (HR) value for cumulative survival (HR, 2.592; 95% confidence interval, 1.306-5.147; P = 0.006). We also analyzed the nodal stage and triple negative (Her2-, ER- and PR-), they all have the significant hazard ratio (HR) value for cumulative survival. For nodal stage N2/3, the HR is 2.979, 95% confidence interval is 1.553-5.713, P = 0.001. For triple negative breast cancer, it has the greatest hazard ratio, the HR is 3.207, 95% confidence interval is 1.704-6.038, P < 0.001. Since triple negative breast cancer has the greatest hazard ratio value for cumulative survival, we further analyzed the expression level of cyclin G1 expression between triple negative breast cancer and non triple negative breast cancer, we found that patients with triple negative breast cancer tended to have high cyclin G1 expression (**Figure 3C**). As known, triple negative breast cancer patients more inclined to chemotherapy-resistance. To clarify the role of cyclin G1 played in chemotherapy-resistance, we treated the triple negative breast cancer cell line MDA-MB-231-GFP and MDA-MB-231-cyclin G1 with 5-Fu for 24 h and measured the cell apoptosis by Flow Cytometry, results showed that cyclin G1 overexpression may reduce the rate of cell apoptosis to 5-Fu (**Figure 3D**). All above results suggested that through expanding the cancer stem cells cyclin G1 may play an important role in chemotherapy-resistance and mediate the poor prognostic of triple negative breast cancer.

Discussion

In recent years, the theory of cancer stem cells has attracted more and more attention from scientists. The proposal of cancer stem cells provides a reasonable explanation for the mechanism of anti-tumor drug resistance and tumor recurrence. Nevertheless, more further studies still need to solve scientific problems such as the mechanism of drug resistance in cancer stem cells, the accurate biomarkers to identify the stemness from tumor cell clolny, the effective targeted spot in cancer stem cells, and the regulation of cancer stem cells from dormancy to activation. Some researches have been close to the answers, but far from being enough. For example, in breast cancer tissues, there is also proved a small number of cells with stem cell-like properties that have self-renewal, indefinite proliferation, and multi-differentiation capabilities, we definate breast cancer stem cells [32]. More and more evidence favors the breast cancer stem cell as the fundamental cause of tumor recurrence, drug resistance, and responsible for the poor prognosis and chemotherapy resistance for breast cancer patients [33]. Futhermore, clinical research reveal that conventional chemo-radiotherapy can't eliminate the stem cells of

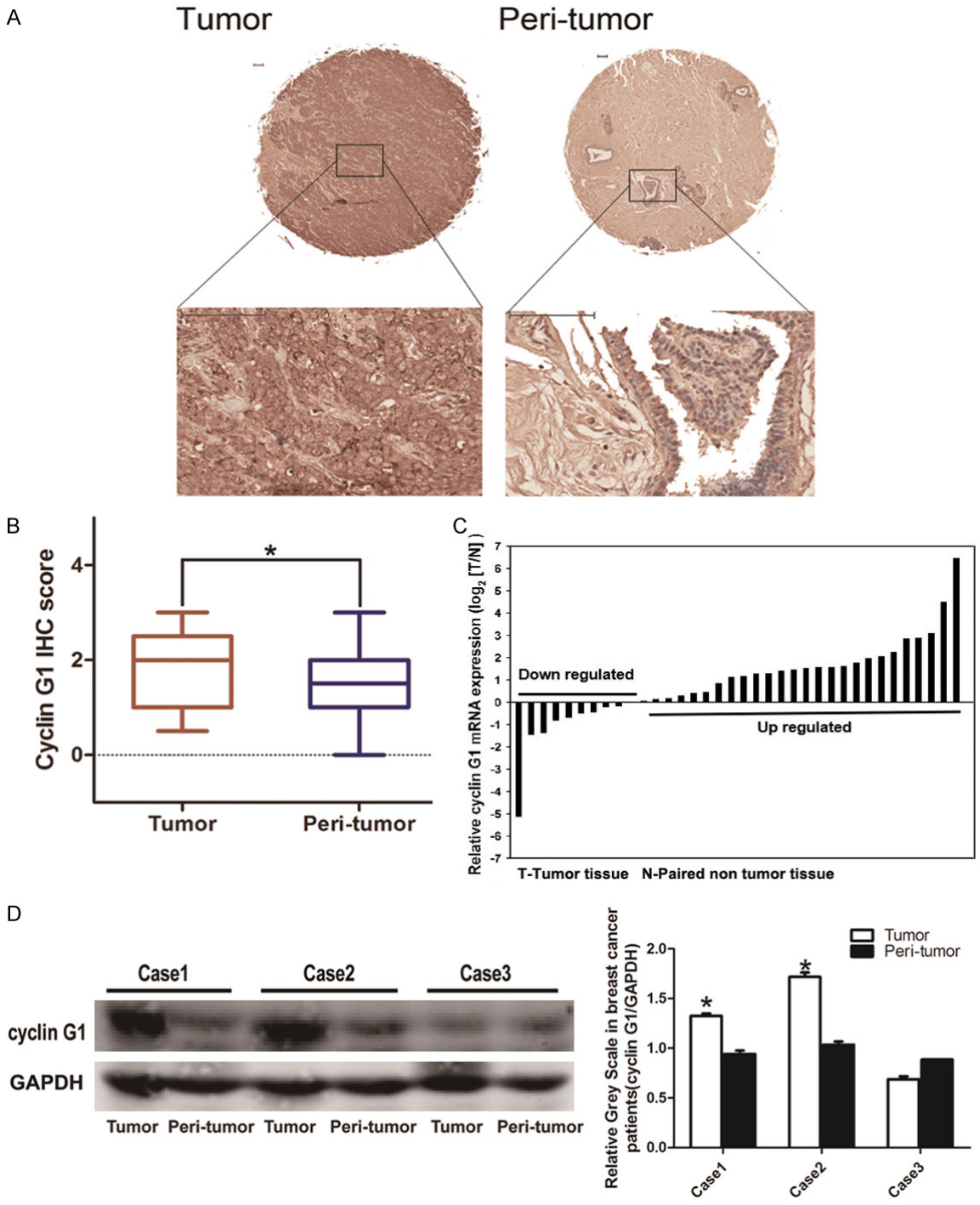

Figure 2. Enhanced expression of cyclin G1 in breast cancer. A, B. Immunohistochemical staining of cyclin G1 expression in tissue microarray (n = 135), then expression of cyclin G1 was analyzed in tumor and peri-tumor tissues of breast cancer patients. The representative view was shown. C. Comparison of cyclin G1 expression in tumor and peri-tumor tissues of breast cancer patients by real-time PCR. D. Representative western blot showing the expression of cyclin G1 protein in tumor and peritumor tissues from 3 patients, the relative grey-scale map (cyclin G1/GAPDH) in breast cancer patients was shown.

those exists in cell cycle G0 period, and those cells may become the origin of the metastasis and recurrence of breast cancer proliferation once again. Animal experiments in vitro also

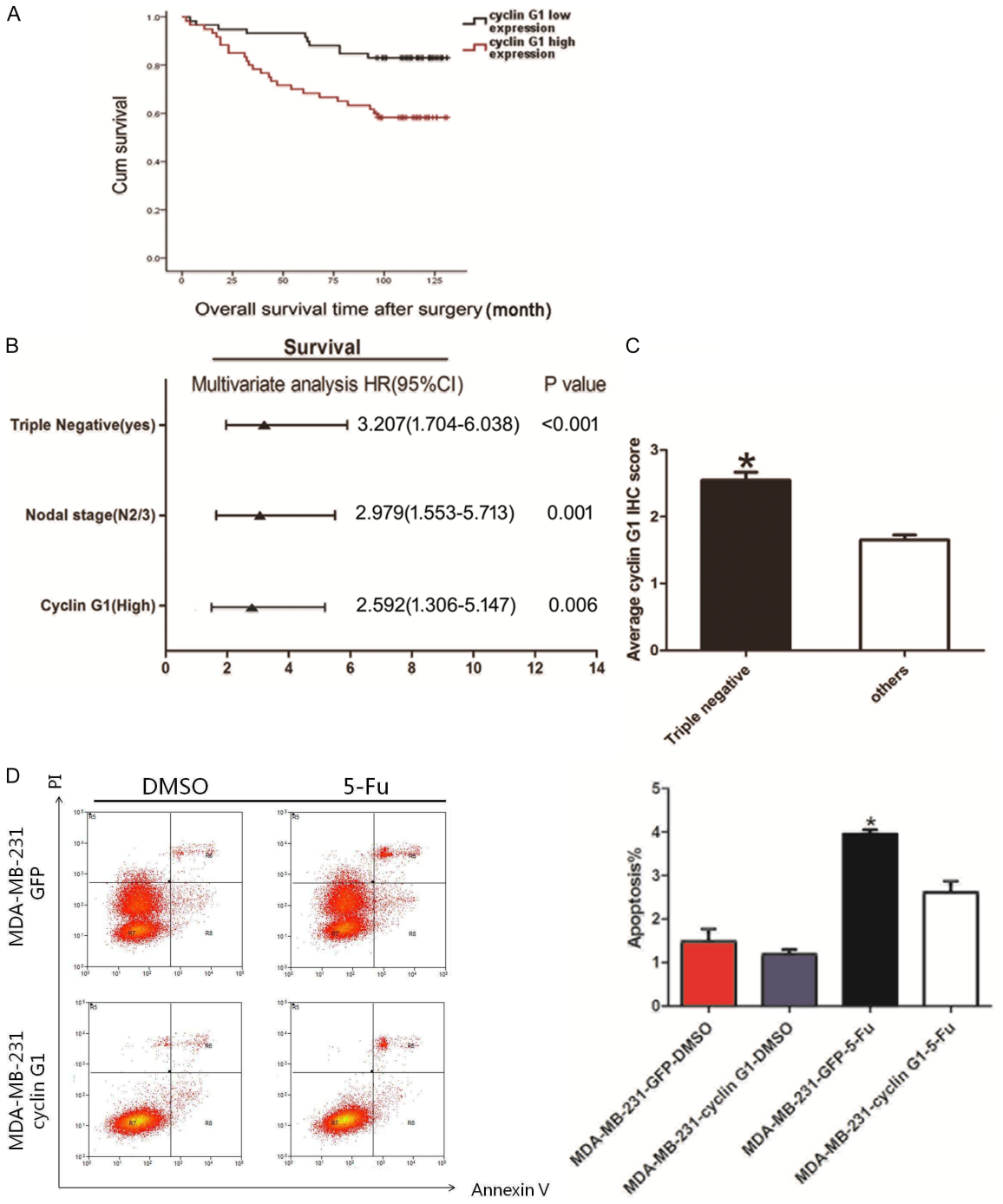

Figure 3. Clinical Significance of cyclin G1 expression in breast cancer. A. Breast cancer patients were divided into a cyclin G1 "high" group (whose fold change of relative expression was higher than the median) and "low" group (whose fold change of relative expression was lower than the median), the overall survival rates of 135 breast cancer patients were compared between the cyclin G1 "high" group and "low" group. B. Multivariate analysis of HRs for overall survival of breast cancer patients in tissue microarray. C. Comparison of cyclin G1 expression between triple negative patients and non triple negative patients of breast cancer in tissue microarray by immunohistochemical staining. D. Apoptosis rates in triple negative cell lines MDA-MB-231-GFP/cyclin G1 treated with 5-Fu (50 ug/ml) for 24 h. The celllines were stained by PI/Annexin v for 15 mins at room temperature and then measured by flow cytometry.

found that breast cancer stem cells have strong tolerance to chemotherapy. Shafee et al. found that breast cancer cells are very sensitive to drugs at the early stage of chemotherapy in

vitro, but immune in the late stage, for drugs have a certain inselective effect on the stem cells [34]. Cancer stem cells also were found induced poor prognosis of breast cancer patients. A clinical research invloved those patients with breast cancer who suffered 6 cycles chemotherapy including paclitaxel and epirubicin, the result revealed that overexpression of HER-2 breast cancer cells have strong resistance to chemotherapy and has a poor prognosis. Chemotherapy can eliminate differentiated breast stem cell, but increased the proliferation of stem cell in the proportion of breast cancer [35]. Other numerous studies have evidenced the existence of breast cancer stem cells and have indicated their significance in prognosis and treatment of the patients [36]. Erol et al. found a flavonoid inhibit the proliferation of cancer stem cells by inducing cell cycle arrest and regulating transcription, and verified its antitumor activity in CD44+/CD24- breast cancer stem cells [37]. Nozaky reported that the high expression of c-Met protein is closely related to two markers of breast cancer stem cells as ALDH1A3 and CD133. Meanwhile, he found that c-Met plays an inhibitory role in breast cancer stem cells with ALDH1 positive, suggesting that c-Met may be a potential therapeutic target for ALDH1 positive breast cancer patient [38].

Some breast cancer patients would experience an inefficient treatment due to the character of chemo-resistance [39]. It is thereby important to design combinatorial therapeutic strategies to overcome the drug resistance of breast cancer. However, specific molecular targets against breast stem cells remain largely vacant. In current study, we elucidated the pivotal role of cyclin G1 in breast cancer stem cells expansion with clinical relevance. To our knowledge, this is the first report on the role of cyclin G1 in breast cancer, breast cancer stem cells and breast cancer prognosis.

Firstly, considering the important role of tumor stem cell in chemo-resistance, we investigated the effect of cyclin G1 on breast cancer stem cells expansion, and the result indicated that enforced cyclin G1 expression remarkably induced the self-renewal ability of breast cancer cells and increased proportion. Previously, CD133 and CD90 have been identified as putative tumor stem cell markers [40, 41]. Intriguingly, over-expression of cyclin G1 significantly increased the expression of CD133 in breast cancer cells. Furthermore, we found that stemness related genes such as Oct4 and Nanog were up-regulated when cyclin G1 was overexpressed in breast cancer cells, suggesting a crosstalk between cyclin G1 signaling and stemness related genes expression. These data indicated that cyclin G1 plays an important role in expansion of breast cancer stem cells and chemo-resistance against breast cancer therapy.

Then we evaluated the role of cyclin G1 in clinical breast cancer samples, as shown in **Figure 2A**, **2B**, cyclin G1 was up-regulated in breast cancer patients through immunohistochemical analysis. Also, cyclin G1 transcripts were significantly increased in breast cancers relative to the noncancerous tissues [28, 42], which was further confirmed by western blot assay (**Figure 2C**, **2D**). These findings further implicated that the high expression of cyclin G1 maybe closely link to the initiation and progression of breast cancer.

What's more, the patients with higher cyclin G1 levels possess low overall survival than those with lower cyclin G1 expression, indicating the significance of cyclin G1 in the prognosis of patients underwent surgical resection. We also analyzed the expression level of cyclin G1 expression between triple negative breast cancer and non-triple negative breast cancer, and found that patients with triple-negative breast cancer tended to have high cyclin G1 expression (**Figure 3C**) and cyclin G1 high expression maybe mediating the progress of stemness-related chemo-resistance in triple negative breast cancer. Thus, cyclin G1 overexpression could serve as a valuable predicting factor for poor survival and chemo-resistance of breast cancer patients.

In conclusion, elucidating the essential role of cyclin G1 in breast cancer stem cells provides us a new insight into individualized therapy of breast cancer and maybe provide us a new target of triple negative breast cancer which is prone to recurrence, chemo-resistance and have the worst prognosis [42]. Further prospective studies and extensive preclinical modeling will be needed to confirm whether cyclin G1 is a biomarker of breast cancer targeting cancer stem cells, and its role as a target for triple negative breast cancer. Taken together, our study provides new insight into

prognosis and individualized therapy of breast cancer patients.

Acknowledgements

This work was supported by grants from the Postdoctoral Science Foundation of China (No. 2015M582822), National Natural Science Foundation of China (No. 81372329) and the Doctor Scientific Research Foundation of Liaoning Province (No. 201501022). No additional external funding received for this study.

Disclosure of conflict of interest

None.

Abbreviations

TNBC, Triple negative breast cancer; HER2, Human epidermal growth factor receptor type2; ER, Estrogen receptor; PR, Progesterone receptor; PI, Propidium iodide.

Address correspondence to: Qing Li, Department of Clinical Pharmacy, Shenyang Pharmaceautical University, Shenyang 110840, Liaoning Province, P. R. China. Tel: +86-24-43520589; Fax: +86-24-43520589; E-mail: liqinglq@yeah.net; Tao Han, Department of Oncology, PLA Cancer Center, General Hospital of Shenyang Military Region, 83 Wenhua Road, Shenhe District, Shenyang 110840, Liaoning Province, P. R. China. Tel: +86-24-2885-1906; E-mail: than1984@sina.com

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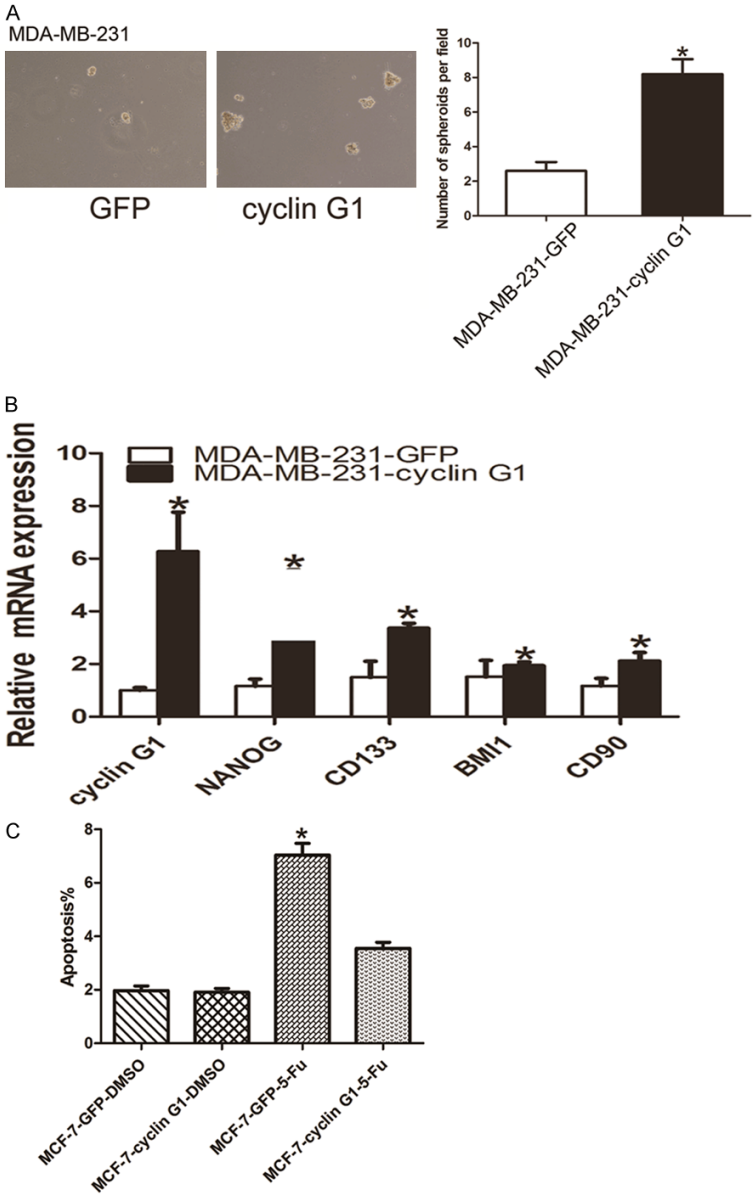

Suplementary Figure 1. Cyclin G1 increases the proportion of cancer stem cells in breast cancer cells. A. 1 × 10^6 cells/mL MDA-MB-231 cells expressing cyclin G1 or GFP were plated in ultra-low attachment Microplates at 5 × 10^3 cell per well and cultured in DMEM (Gibco, Invitrogen) supplemented with 10% FBS for seven days. Representative pictures were shown and the number of spheroids was counted. *P < 0.05. B. Expression of Cancer Stem Cell markers (CD133 and CD90) and stemness related genes (NANOG and BMI-1) in MDA-MB-231 GFP and MDA-MB-231 cyclin G1 cells by real-time PCR analysis. *P < 0.05. C. Apoptosis rates in MCF-7 GFP/cyclin G1 treated with 5-Fu (50 ug/ml) for 24 h. The celllines were stained by PI/Annexin v for 15 mins at room temperature and then measured by flow cytometry, the results was shown.