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ORGINAL ARTICLE

Upregulated Tie2 expression in plasma: a potential non-invasive biomarker for the diagnosis of breast cancer

Qingzhu Song[1], Fenglan Zhang[2], Tian Yuan[2], Yulong Wei[2]

[1] Department of Clinical Laboratory, Bayannur Hospital, Bayannur, Inner Mongolia, 015000, China
[2] Department of Pathology, Bayannur Hospital, Bayannur, Inner Mongolia, 015000, China

Corresponding author: Y. Wei
<weiyulong5566@163.com>

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ABSTRACT. Breast cancer is by far the most common malignancy found in women and causes a significant public health problem around the world. Early diagnosis of cancer plays an important role in successful treatment and survival of patients. This study aims to investigate the possibility of plasma Tie2 to be used as a biomarker for diagnosis of breast cancer. In total, 20 healthy volunteers and 33 breast cancer patients were considered for this study. The level of Tie2 in plasma was detected using the ELISA technique and immunohistochemistry was performed to measure the expression of Tie2 in normal and breast cancer tissues. Plasma concentrations of Tie2 were significantly higher among breast cancer patients compared to healthy subjects, and both mRNA and protein expression of Tie2 were higher in breast cancer tissue than in normal tissue. Plasma concentrations of Tie2 were positively correlated with the grade of breast cancer. Finally, in vitro knockdown of Tie2 expression in a breast adenocarcinoma cell line inhibited the proliferation of these cells. It is concluded from the results that Tie2 might be a useful plasma biomarker for the early detection of breast cancer and could be developed to be a target for novel drug discovery.

Key words: Tie2, biomarker, breast cancer, non-invasive diagnosis, plasma

INTRODUCTION

Breast cancer is by far the most common malignancy in women (median age 64 years) and is considered as an increasing public health concern. The incidence of breast cancer is rapidly rising across the world [1]. In the United States, breast cancer has the highest incidence and is the second most common cause of cancer-related death following lung cancer [2]. In China, breast cancer has been the fifth leading cause of tumor-related deaths, with an increasing number of patients in both urban and rural areas, which generates a heavy burden from both social and economic perspectives [3]. Tremendous efforts have been invested in discovering pathogenesis of breast cancer, which is related to primary genetic mutations, reproductive history, and lifestyle factors, such as weight gain, alcohol consumption, and lack of physical exercise [4]. Heterogeneity between tumors and intratumoral heterogeneity were found in breast cancer, which make it difficult to develop an effective prevention strategy against this disease [5]. Thus, current breast cancer therapies are still intimidating and need more efforts to develop more effective prevention approaches [6].

Early diagnosis of cancer plays an important role in treating and increasing survival ratio in patients. It was reported that an early diagnosis, when patients have smaller primary tumors, makes the survival rate higher and remarkably reduces probability of death from cancer [2]. The etiology of most cancers is complicated, which greatly increases the difficulty of cancer diagnosis, especially early diagnosis. Imaging methods based on immunohistochemistry, biopsy pathology, and a few classical biomarkers do not satisfactorily perform widespread population screening for cancers [7]. Cancer diagnosis methods, for example, colonoscopy and stool assays for colon cancer, are restricted due to high cost and low accuracy, respectively [7]. As an easy and non-invasive approach for cancer diagnosis, the diagnosis with blood biomarkers is considered an excellent alternative for an effective widespread screening of cancers compared to conventional methods of diagnosis [7].

As one of the endothelial cell (EC)-specific receptor tyrosine kinases, Tie2 plays a crucial role in remodeling and developing blood and lymphatic vessels and maintaining EC survival [8]. Tie2 is encoded by TEK (or CD202b) gene and is regulated directly by multimeric angiopoietin (Ang) ligands [9]. Tie2 is involved in the regulation of many pathophysiological activities, including endothelial inflammation, apoptosis, endotoxic shock, and sepsis [10]. It was also demonstrated that Tie2 was closely linked to cancers, for example, Tie2 was involved in tumor metastasis of oral squamous cell carcinomas [11]. Interestingly, it was found that Tie2 might be able to induce dormancy in breast cancer cells and be a target for preventing

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breast cancer [12]. Plasma Tie2 was stated to be a tumor vascular response biomarker for VEGF inhibitors in metastatic colorectal cancer [13]. In addition, Tie2 levels were used for the prognosis of lung cancer [14]. Although Tie2 has been investigated for a few decades, knowledge on considering Tie2 as a biomarker for breast cancer remains limited. In this study, the concentration Tie2 in blood was measured in a healthy group and a group of individuals with breast cancer. Subsequently, the possibility of considering Tie2 as a biomarker for diagnosing breast cancer was analyzed and discussed. The findings of the study might provide useful implications for research in pathogenesis of breast cancer and finding an early and non-invasive detection method for breast cancer.

MATERIALS AND METHODS

Ethics

The clinical study was approved by the Ethics Committee of Bayannur Hospital (BH1/01/2019) and was conducted according to the declaration of Helsinki. The study was performed in adherence with the relevant ethical guidelines of Bayannur Hospital, and all participants gave written informed consent prior to enrollment in this study.

Healthy control and breast cancer patient's characteristics

A total of 20 healthy volunteers (as control) and 33 cancer patients were registered for the study in Bayannur Hospital. Physical examination was performed on healthy volunteers to rule out serious diseases and any cancers. Breast cancer patients were diagnosed with histologically confirmed first primary invasive breast cancer and classified, based on the Elston and Ellis grading method, according to a previous study's protocol [15]. Breast cancer patients were routinely treated with radiotherapy and systemic adjuvant chemotherapy; no target drugs were used, and patients visited for a follow-up within 3 years, from May 2016 to May 2019. The Women's CARE Study protocol was approved by the institutional review boards at Bayannur Hospital. Volunteers in the healthy group and breast cancer group were carefully selected. The exclusion criteria for the healthy group included cancer patients, patients with a history of infectious diseases other severe diseases (cardiovascular diseases, diabetes, etc.), and prophylactic oophorectomy. For breast cancer patients, exclusion criteria included male breast cancers, patients with secondary cancer, incomplete staging data, incomplete data regarding estrogen receptors (ER), progesterone receptors (PR), and human epidermal growth factor receptor (HER2) and patients who received bilateral prophylactic mastectomy or prophylactic oophorectomy.

Blood sampling for healthy group and breast cancer patients

Blood samples for measurement of Tie2 were drawn from healthy volunteers and breast cancer patients directly in 6 ml ethylenediaminetetraacetic acid vacutainers. For breast cancer patients, blood samples were collected from patients when they were diagnosed with breast cancer, before surgery, and before getting treatments such as chemotherapy and radiotherapy. Thereafter, plasma was separated by centrifugation at $3000 \times g$ for 10 minutes, followed by being immediately aspirated, separated into aliquots, and frozen at–80 °C in the laboratory. Samples were only thawed once for each analysis, which was performed in triplicate.

Circulatory levels of Tie2 detected by ELISA

Circulatory levels of Tie2 were tested by enzyme-linked immunosorbent assays (ELISAs) using Tie2 human ELISA kit (Thermo Fisher, Catalog number: BMS2042) according to the manufacturer's instruction. Microwell strips were washed twice with around 00 µL Wash Buffer, per well, with thorough aspiration of microwell contents between washes. Then, 90 µL of sample diluent was added to sample wells, followed by adding 10 µL of each sample in duplicate to sample wells. Subsequently, 50 µL of Biotin-Conjugate was added into all wells and incubated overnight at 4 °C on a microplate shaker. The next day, all wells were emptied and washed with washing buffer six times. Thereafter, diluted 100 µL of Streptavidin-HRP was added into the wells, followed by being incubated at room temperature (18 °C to 25 °C) for 1 hour on a microplate shaker. Then, all microwell strips were washed six times, followed by adding 100 µL of TMB Substrate Solution, and incubated at room temperature (18 °C to 25 °C) for 10 minutes. Enzyme reactions were stopped by adding 100 µL of Stop Solution into each well. The OD value was read using a spectrophotometer, with 450 nm as the primary wavelength. Standard samples for standard curve and blank (for negative control) were set and performed following the above protocol.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis

The total RNA was isolated from needle tissues from breast cancer patients and healthy volunteers using TRIzol® reagent (Catalog number: 15596026, Invitrogen; Thermo Fisher Scientific, Inc.), followed by quantification using NanoDrop™ 2000/2000c Spectrophotometers (Thermo Fisher Scientific, Inc.). Subsequently, 1 mg of mRNA was reverse transcribed to cDNA using a TAKARA PrimeScript™ RT reagent kit (Catalog number: RR047A, TAKARA, Beijing, China) according to the manufacturer's protocol. The qPCR analysis was performed using the ABI StepOnePlus Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) with q-PCR Master Mix (Toyobo Life Science, Osaka, Japan). Cycling conditions were as follows: initial denaturation at 95 °C for 2 minutes; 40 cycles at 95 °C for 15 seconds, 60 °C for 30 seconds, and 70 °C for 30 seconds. The PCR primer sequences are listed in *table 1* table 1. Target gene expression was normalized to that of GAPDH. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method.

Table 1
Primers for RT-qPCR.

Gene name		Sequence	Tm
Tie2	Sense	CCAGCCCTGCTGATACCAAA	60.03
	Anti-sense	GCACCTTCCACAGTTCCAGA	59.89
GAPDH	Sense	CCATGTTGCAACCGGGAAG	59.41
	Anti-sense	GCCCAATACGACCAAATCAGAG	59.39

IMMUNOHISTOCHEMISTRY ASSAY

Breast cancer tissues were fixed with 4% paraformaldehyde (Sigma, Saint Louis, MO, USA) and embedded in paraffin. Thereafter, tissue slides were prepared (5 μm). Antigen retrieval was carried out with pH9 antigen retrieval buffer (DAKO S2375) at 95 °C for 20 minutes, followed by incubating with primary Tie2 (D9D10) antibody (Cell Signaling Technology, rabbit monoclonal, Catalog number: 7403) or Rabbit mAB IgG Isotype Control (Rabbit (DA1E) mAb IgG XP® Isotype Control, Cell Signaling Technology, #3900) at 1:100 dilution, ER clone SP1 (Neomarkers, USA) at 1:100 dilution, PR clone PgR636 (DAKO, Denmark) at 1:200 dilution, and c-erbB-2 (HER2) (DAKO, Denmark Code A0485) at 1:600 dilution at 4 °C overnight. Subsequently, slides were incubated with biotinylated anti-IgG secondary antibodies (Vector Labs). The signal was detected by incubation with ABC Elite (Vector Labs) for 30 minutes and 3,3′-diaminobenzidine (Dako) for 5 minutes at room temperature. The tumor was considered as ER or PR positive when 10% or more of the invasive tumor nuclei were stained for the respective antibody, regardless of staining intensity. For HER-2, its status was defined according to ASCO CAP guidelines 2007, presenting as 0 (no staining), 1+ (weak, incomplete membrane staining in any proportion or weak, complete membrane staining in <10% of cells), 2+ (weak, complete membrane staining in >10% of cells or complete intense membrane staining of >10% but <30% of invasive tumor cells), or 3+ (complete intense membrane staining of >30% of invasive tumor cells). Patients with expressions of 0 or 1+ were regarded as negative. The expression level of 3+ was regarded as HER2 overexpressed (positive).

CELL CULTURE

Breast adenocarcinoma cell line MCF7 was purchased from Yuchicell Biological Technology (Shanghai, Catalog number: SC0144). HCC1428 cells were purchased from COBIOER (Nanjing, Catalog number: CBP60412). MCF7 and HCC1428 cells were cultured in T75 flask with Dulbecco's Modified Eagle Medium (DMEM, Thermo Fisher, Catalog number: 11995073) containing 0.01 mg/mL human recombinant insulin and 10% fetal bovine serum (FBS, Thermo Fisher, Catalog number: 16140071). Cells were passaged when the confluency reached more than 90%.

SIRNA SYNTHESIS AND TRANSFECTION

Three siRNAs against Tie were designed using siRNA design tool of GeneScript (https://www.genscript.com/tools/sirna-target-finder) and their sequences were AAGACCAGCACGTTGATGTGA, AAGGCGCGCATCAAGAAGGAT, AAGTACCTGATATTCTAGAAG, which were synthesized by bsyntech (Suzhou, China). For transfection, MCF7 cells were seeded at 1×10^5 cells/well in 24-well plates. After 3 hours of incubation at 37 °C, during which the MCF7 had adhered to wells, cells were transfected with 50 nM siRNA together with transfection reagent (Lipofectamine 3000, Thermo Fisher, Catalog number: L3000008). The knockdown efficiency was checked using qRT-PCR and western blot assays.

WESTERN BLOTTING

Cells were washed with phosphate-buffered saline, collected with a cell scraper, and lysed with medium RIPA buffer (Catalog number: P0013C, Beyotime Biotechnology, Shanghai, China) containing 1% Protease Inhibitor Cocktail for general use (Catalog number: P1005, Beyotime Biotechnology, Shanghai, China). The supernatant was cleaned by centrifugation at 14,000 rpm for 10 minutes at 4 °C to obtain total cell lysates. Cell lysates were separated by 6% to 20% SDS-PAGE. Proteins were transferred to a polyvinylidene difluoride membrane and western blot analysis was performed with indicated antibodies (primary antibody: Tie2 (D9D10) Rabbit mAb from CST (Catalog number: 7403S) and β-actin mouse mAb from Santa Cruz (Catalog number: sc-47778)).

CELL VIABILITY TEST

MCF7 cells were plated at subconfluent density in 96-well plates, and 24 hours later they were transfected with siRNAs against Tie2 and cultured in an incubator for 48 hours at 37 °C. Then, 100 ul Cell Counting Kit-8 solutions were added in wells of 96-well plates, containing siRNA transfected cells, followed by incubating for two hours and testing OD value using EnVision Microplate Readers (PerkinElmer).

STATISTICAL ANALYSIS

Statistical comparisons were performed using two-tailed unequal variance *t*-test and a $P < 0.05$ was considered to be significant.

RESULTS

Characteristics of healthy control subjects and breast cancer patients

As shown in *table 2* table 2, all healthy volunteers were women aged 38 to 63 years (mean age of 48.45 ± 1.81 years). The average height and body weight of volunteers were 161.8 ± 0.60 cm and 53.45 ± 0.60 kg, respectively. Breast cancer and other serious diseases were ruled out for the healthy volunteers. Body mass index (BMI) of the participants was calculated, and it was indicated that the BMI of volunteers was 20.44 ± 1.19. The participants who had breast cancer (*table 3* table 3) were women and aged 38 to 61 years (mean age of 50.03 ± 1.13 years). The height and body weight of volunteers were 157.8 ± 4.55 cm and 50.76 ± 0.49 kg, respectively. The BMI of breast cancer patients was 19.27 ± 1.13. Breast cancers in patients were confirmed using the immunochemistry approach. All patients received 3 years follow-up visit. The TNM staging and ER, PR, and HER2 status of breast cancer patients are listed in *table 4* table 4; it was found that the majority of breast cancer patients had ER and/or PR positive, HER2 negative (66.7%) tumors, while 27.3% had ER and/or PR positive, HER2 positive. One patient (3.0%) had ER^- PR^- $HER2^+$ tumor, and one patient (3.0%) had ER^- PR^- $HER2^-$ tumor.

Table 2
Health set information.

Health group	Age (year)	Tie2 serum level (ng/mL)	Body Mass Index (BMI)
1	38	20	22.0
2	36	24	20.7
3	60	23	18.5
4	63	24	19.5
5	55	25	21.8
6	56	21	20.8
7	51	24	21.6
8	55	25	20.2
9	56	21	20.6
10	57	20	19.9
11	50	21	19.7
12	45	20	19.5
13	51	23	21.2
14	46	22	22.2
15	38	21	20.4
16	39	20	20.7
17	41	19	21.2
18	45	20	18.4
19	46	21	18.3
20	41	21	21.6

Table 3
Breast cancer patients information.

Cancer patients	Age (year)	Tie2 serum level (ng/mL)	Tumor grade*	Body Mass Index (BMI)
1	55	25	1	20.5
2	38	24	1	19.6
3	39	23	1	19.9
4	51	26	2	19.1
5	61	28	2	19.9
6	58	29	3	18.7
7	58	30	3	20.4
8	55	26	2	16.9
9	45	27	2	19.0
10	56	20	1	19.5
11	57	28	2	19.1
12	61	29	2	17.7
13	55	30	3	17.6
14	39	31	3	19.2
15	55	28	2	18.9
16	54	27	2	19.7
17	51	26	2	17.8
18	45	29	2	18.8
19	49	31	3	20.2
20	55	32	3	20.7
21	48	29	2	20.1
22	46	25	1	18.5
23	47	23	1	19.1
24	50	26	2	22.2
25	38	28	2	18.9
26	39	30	3	19.2
27	50	31	3	17.9
28	51	30	3	19.9
29	47	29	2	21.8
30	48	31	3	19.1
31	49	30	3	19.3
32	51	29	2	20.5
33	50	28	2	19.6

*Based on Elston-Ellis grading method, grade 1 denotes as score 3-5, grade 2 denotes as score 6-7, and grade 3 denotes as score 8-9.

Breast cancer patients have elevated Tie2 plasma levels that are positively correlated with grading of cancer

To compare the difference of Tie2 level in the blood between healthy volunteers and breast cancer patients, an ELISA assay against Tie2 was performed. As shown in *table 2*, *table 3*, and *figure 1*A, it was indicated that the Tie2 concentration in the blood of healthy volunteers was 21.75 ± 0.42 ng/mL and it was 27.82 ± 0.48 ng/mL in breast cancer patients. Statistically significant difference was found between mean Tie2 levels as per independent *t*-test in healthy patients versus patients diagnosed with early-stage breast cancer ($P < 0.0001$). ROC curve was generated to

Table 4
TNM staging, ER, PR, and HER2 status of breast cancer patients.

Characteristics	Overall (n=33)	
Tumour Size, n (%)		
< 2 cm	23	69.7
>2-5 cm	7	21.2
>5 cm	3	9.1
Lymph node, n (%)		
Negative	21	63.6
Positive	11	36.4
Stage, n (%)		
I	13	39.4
II	15	45.5
III	5	15.2
Estrogen Receptor, n (%)		
Positive	20	60.6
Negative	13	39.4
Progesterone Receptor, n (%)		
Positive	24	72.7
Negative	9	27.3
Human Epidermal Growth Receptor, n (%)		
Positive	10	30.3
Negative	23	69.7
IHC subtypes, n (%)		
ER and/or PR positive HER2 negative	22	66.7
ER and/or PR positive HER2 positive	9	27.3
ER negative PR negative HER2 positive	1	3.0
ER negative PR negative HER2 negative	1	3.0

assess the predicted risk values for breast cancer diagnosis using Tie2, which showed that the area under the curve was 1.0 (95% CI: 1.0∼1.0, Supplementary figure 1). To demonstrate the relation between Tie2 concentration in blood and grading of breast cancer, a correlation analysis was performed that showed that Tie2 blood level was significantly correlated to the grading of breast cancer ($P < 0.0001$, $r^2 = 0.8871$, *figure 1*B). These data demonstrated that Tie2 blood concentration was closely related to breast cancer. Moreover, it was found that BMI of healthy volunteers was significantly higher than that of breast cancer patients ($P < 0.001$, *figure 1*C). It was found that BMI was negatively related to plasma Tie2 level ($R^2 = 0.1087$, $P = 0.0159$, ***figure 1***D).

Breast cancer tissue expresses higher Tie2 protein levels than normal tissue

To further demonstrate the relation between Tie2 and breast cancer, an IHC assay detecting Tie2 expression was carried out. It was shown that the protein level of Tie2 was higher in breast cancer tissue, whereas it was lowly expressed in normal breast tissue (*figure 2*). Together with results of Tie2 concentration in the blood of both groups, it was found plausible that Tie2 might be treated as a biomarker for detecting breast cancer.

Breast cancer tissue expresses higher Tie2 mRNA levels than normal tissue

To further verify, mRNA expression of Tie2 was investigated in needle breast tissues. As shown in *figure 3*, it was indicated that the mRNA expression of Tie2 was significantly higher (***$P < 0.001$) in breast tissues of breast cancer patients than healthy individuals.

To further dissect survival situation in breast patients, the number of deaths was calculated in the patients with low Tie2 blood concentration and in those with high Tie2 blood concentration, in which less than 30 ng/mL was defined as low Tie2 blood concentration, while more than 30 ng/mL was defined as high Tie2 blood concentration. It was found that 2 of 23 patients with low Tie2 blood concentration died, while 5 of 10 patients with high Tie2 blood concentration died.

Knockdown of Tie2 inhibits proliferation of MCF2 cells

To question effects of Tie2 on breast cancer, a siRNA-based knockdown assay was performed. It was indicated that three siRNAs against Tie2 could significantly inhibit the mRNA expression of Tie2 (*figure 4*A, n = 5, **$P < 0.01$, ***$P < 0.001$). To further check knockdown efficiency of siRNAs, a western blot assay was performed, which indicated that three siRNAs could suppress the protein level of Tie2 in MCF2 cells (*figure 4*B). Importantly, it was found that the suppression of Tie2 via siRNA could significantly inhibit the growth of breast cancer cells (*figure 4*C, n = 7, ***$P < 0.001$). Thus, it was confirmed that Tie2 played an important role in cancer cell proliferation *in vitro*.

Knockdown of Tie2-inhibited proliferation of HCC1428 cell line

To further investigate effects of Tie2 on breast cancer, another breast cancer cell line named HCC1428 was used. siRNAs against Tie2 were transfected in HCC1428 cells, which indicated that three siRNAs against Tie2 could significantly inhibit mRNA expression of Tie2 (*figure 5*A, n = 5, *$P < 0.5$, **$P < 0.01$, ***$P < 0.001$). To further check the knockdown efficiency of siRNAs, a western blot assay was performed, which indicated a successful knockdown of the protein level of Tie2 in HCC1428 cells (*figure 5*B). Moreover, suppression of Tie2 significantly inhibited the growth of HCC1428 cells (*figure 5*C, n = 7, ***$P < 0.001$). Thus, it was further confirmed that Tie2 played an important role in cancer cell proliferation in vitro.

DISCUSSION

Although breast cancer is thought as a single disease, it shows significant heterogeneity in histology, genomic

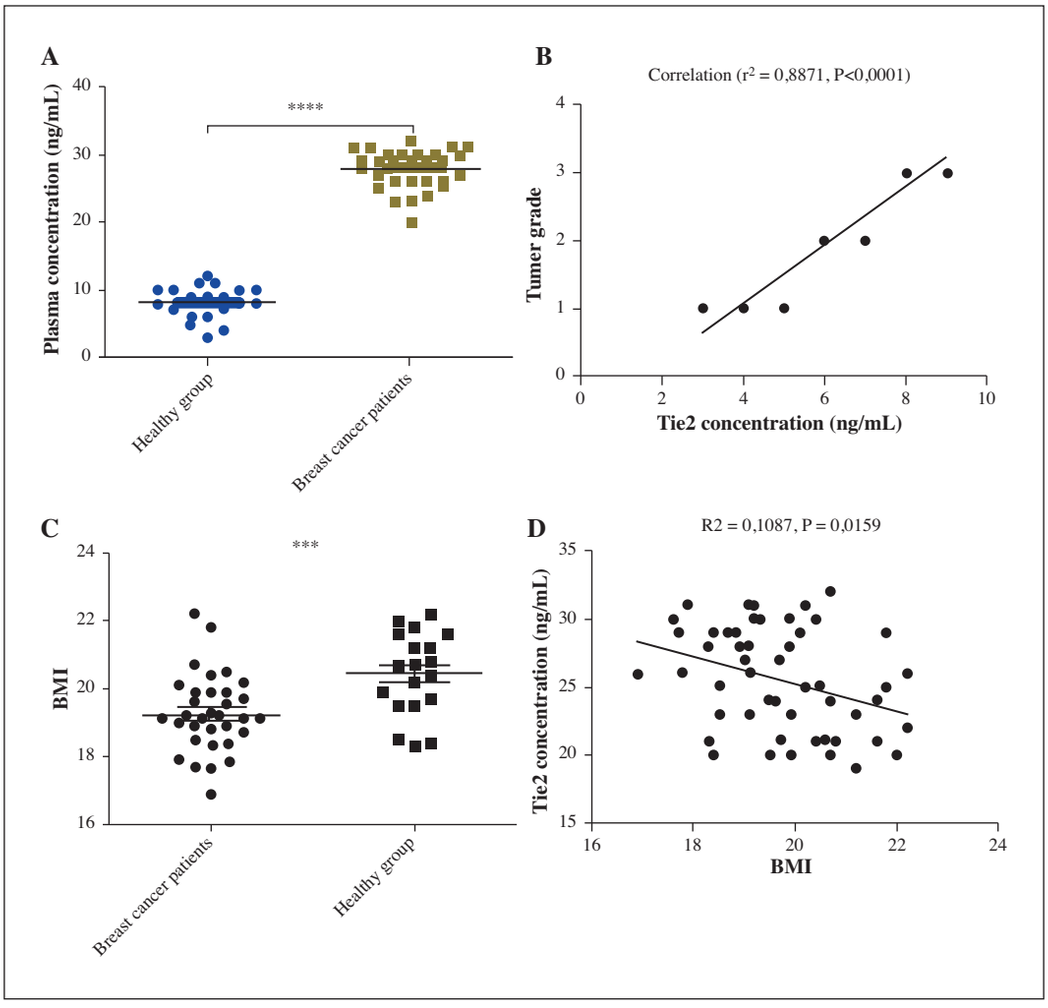

Figure 1

Blood levels of Tie2 in healthy group and breast cancer patients and correlation between Tie2 concentration in blood and grading of breast cancer. (A) The Tie2 concentration in blood of breast cancer patients was significantly higher than in healthy volunteers ($P < 0.0001$); (B) Tie2 blood level was significantly positively correlated with the grading of the breast cancer ($P < 0.0001$, $r^2 = 0.8871$); (C) BMI of healthy volunteers was significantly higher than that of breast cancer patients ($P < 0.001$); (D) BMI was negatively related to plasma Tie2 level ($R^2 = 0.1087$, $P = 0.0159$).

alterations, gene expression, metastatic behavior, and treatment responses [5]. Early diagnosis of breast cancer is crucial for increasing survival ratio [15]. A non-invasive detection approach is preferable for cancer diagnosis due to less risk [16]. As one of the non-invasive diagnosis methods, plasma biomarkers have been used in cancer detection [17]. In the present study, it was found that Tie2 blood level was higher in breast cancer patients than in healthy individuals, which was also positively correlated with grade of cancer. Patients who had higher Tie2 blood level might have lower survival percentage than patients who had lower Tie2 blood level. It is plausible that circulating Tie2 level might be utilized as a plasma biomarker for breast cancer diagnosis.

As one of the EC-specific receptor tyrosine kinases, Tie2 is needed to remodel blood and lymphatic vessels [8] Accumulating evidence shows that Tie2 closely regulates pathogenesis of cancers. Forget *et al*. reported that a subset of CD14+ monocytes expressed Tie2, and these Tie2-expressing monocytes/macrophages played a maor role in tumor angiogenesis, progression, and metastasis [18]. Angiogenesis is important for tumor development and maintaining tumor microenvironment. It was found that the sensitivity of human tumor cells to immune-mediated lysis through immunogenic modulation was largely enhanced by suppression of the angiopoietin/Tie2 signaling by Trebananib (mL4-3 and L1-7(N)) [19]. In gliomas, it was found that perturbation of Ang-Tie2 pathway using a Tie2 decoy receptor could inhibit invasive tumor outgrowth after anti-angiogenesis therapy [20]. In the present study, we found that compared to healthy subjects, Tie2 blood level was much higher in breast cancer patients irrespective of tumor stages (*table 2* and figure 1). Increased Tie2 blood concentration might be a response to breast cancer in the body, which could be a target for developing anti-cancer therapy against breast cancer. Consistently, Chen *et al*. claimed that stimulation of Tie2 on breast tumor cells could help in breast cancer development [21]. In fact, as a selective Tie2 inhibitor, rebastinib was reported to be a promising therapy for breast cancer patients, although it is still under clinical trial [19].

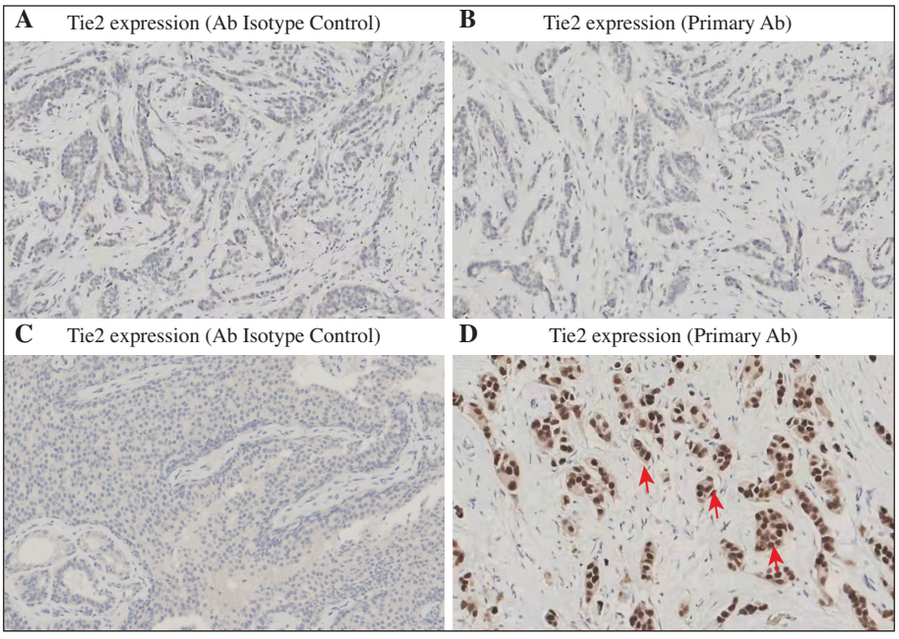

Figure 2
The protein level of Tie2 was highly expressed in breast cancer tissue, whereas it was lowly expressed in normal breast tissue. (A) Tie2 staining using primary antibody isotype control in normal breast tissue, (B) Tie2 staining using primary antibody against Tie2 in normal breast tissue, (C) Tie2 staining using primary antibody isotype control in breast cancer tissue, and (D) Tie2 staining using primary antibody against Tie2 in breast cancer tissue (red arrows indicate tumor cells).

Plasma biomarkers are great indicators for early detection of many diseases. Changes in circulating concentration of Tie2 have been observed by several groups. It was found that blood levels of tumor angiogenesis- and lymphangiogenesis-related factors including vascular endothelial growth factor (VEGF)-A, -C, and -D, Flt-1, and KDR, were increased in breast cancer [22]. Compared with healthy control, the blood level of Angiopoietin-2, Tie2, and VEGF was higher in patients with acute congestive heart failure (CHF) [23]. Blood levels of VEGF, Ang-1, Ang-2, Tie-2, and platelet VEGF were found to be higher in hypertensive patients than in normotensive controls [24]. Tie2 plasma levels were confirmed to be increased from baseline and remained elevated in the chronic phase of coronary syndromes [25]. In cervical cancer patients, it was demonstrated that plasma concentrations of Ang-1, Ang-2, Tie-2, and Ang-1/Ang-2 ratios were significantly higher than those in healthy individuals [26]. In the present study, it was found that the blood level of Tie2 was significantly higher in breast cancer patients than in the healthy group, and the expression of Tie2 was higher in breast cancer tissue than in normal tissue (*figures 2* and *3*). Significantly, breast cancer patients who had a higher concentration of Tie2 in blood might have lower survival percentage compared to patients who had a lower blood concentration of Tie2. Interestingly, it was found that Tie2 played an essential role in the maintenance of cancer cell growth (*figures 4* and *5*). Thus, Tie2 may serve as an important target for developing anticancer drugs against breast cancer.

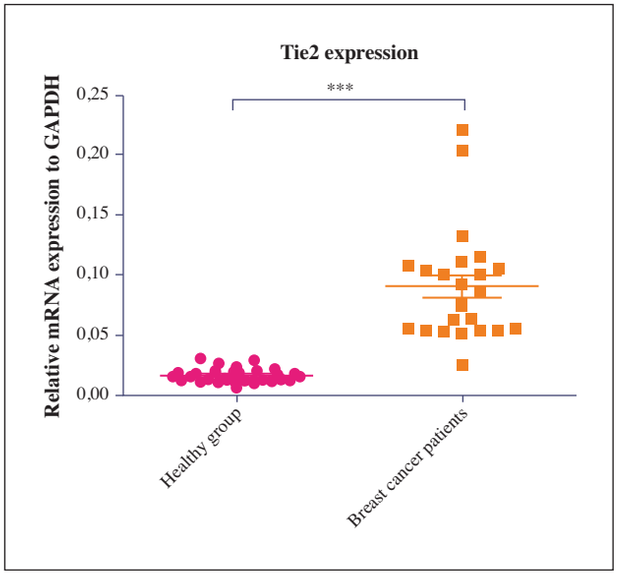

Figure 3
The mRNA expression of Tie2 was higher in breast cancer tissue than in normal breast tissue detected by qRT-PCR assay.

Although our results support that Tie2 might be a potential biomarker for diagnosing breast cancer, there are some shortcomings in the present study. The first shortcoming is the limited number of patients in our cohort, which may overestimate the predictive ability of Tie2 as the biomarker of breast cancer. For example, the AUC of ROC curve of Tie2 is 1, but it may decrease with increase in the number of patients. The second shortcoming of the study is that we did not measure the blood level of Tie 2 during the period of follow-up. Last but not least, the healthy controls in the study were screened by history taking, which may cause undiagnosed cardiovascular disease, diabetes, CKD, and subclinical infections. To this end, for further studies, a larger number of patients should be studied to dissect relationship between Tie2 blood level and survival ratio. Multiple detections of the blood

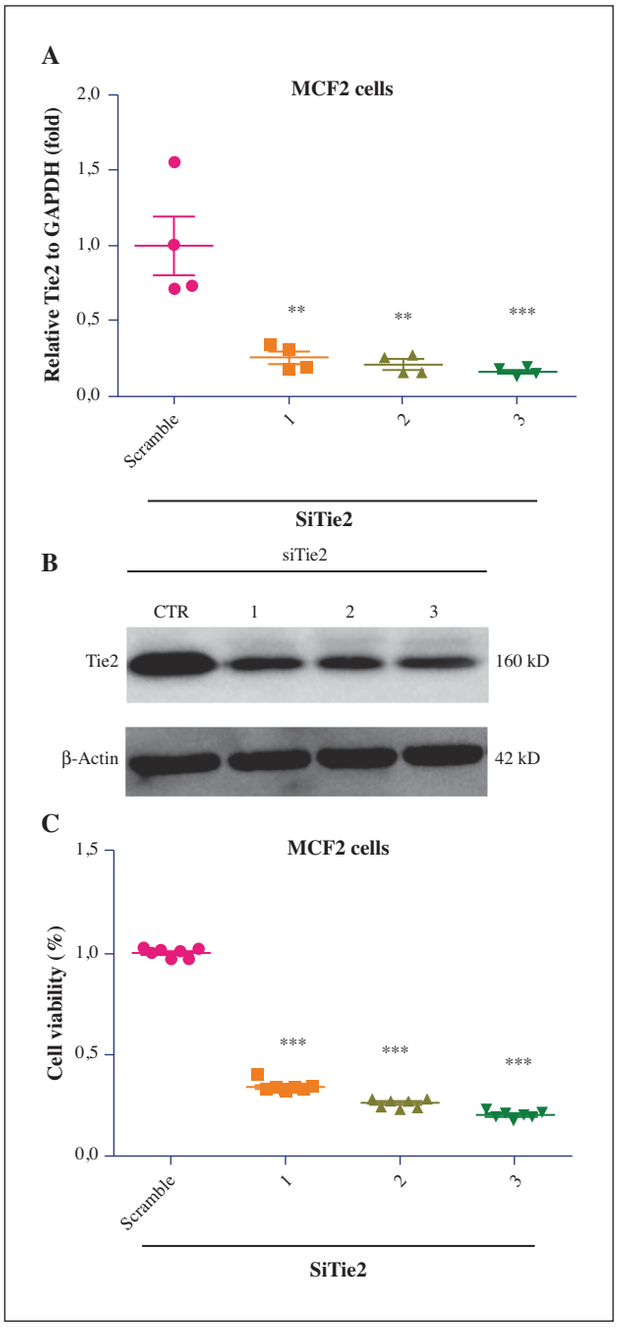

Figure 4
Knockdown of Tie2 suppressed breast cancer growth in MCF7 cell line. (A) qRT-PCR indicated successful knockdown of Tie2 using siRNAs against Tie2 in MCF7 cells (n = 5, $^{**}P < 0.01$, $^{***}P < 0.001$). (B) Western blot indicated gene knockdown of Tie2 using siRNAs against Tie2 in MCF7 cells. (C) Knockdown of Tie2 suppressed breast cancer growth in MCF7 cell line detected by CCK8 assay (n = 7, $^{***}P < 0.001$).

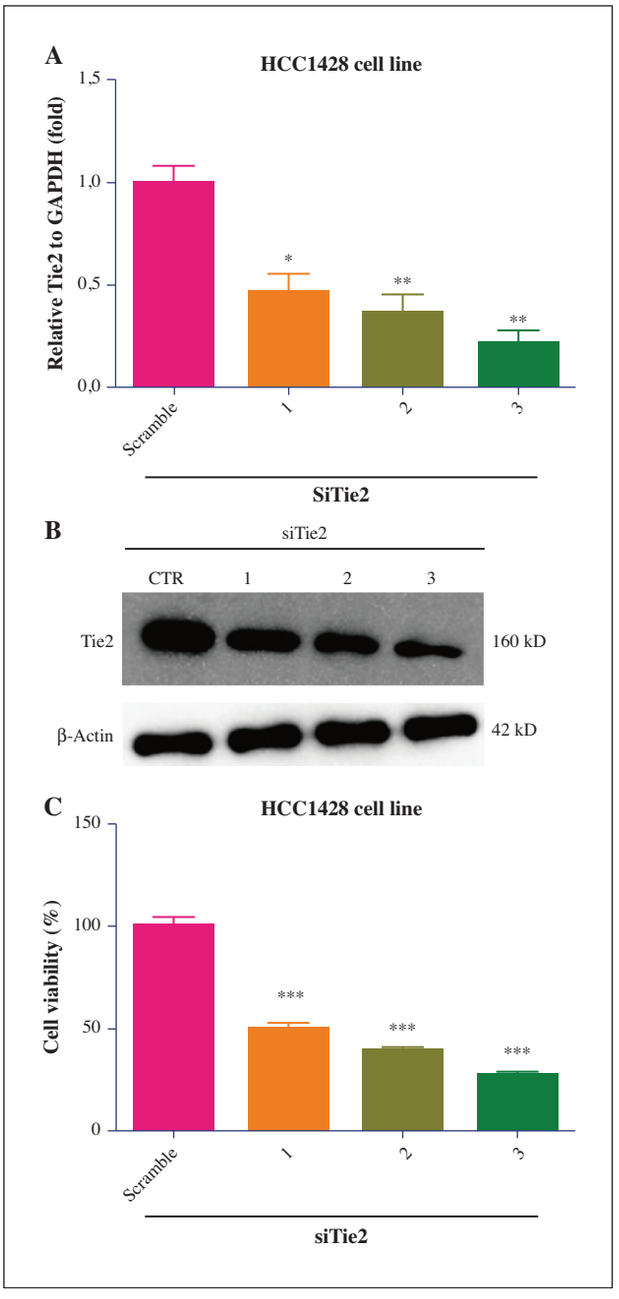

Figure 5
Knockdown of Tie2 suppressed breast cancer growth in HCC1428 cell line. (A) qRT-PCR indicated successful knockdown of Tie2 using siRNAs against Tie2 in HCC1428 cells (n = 5, $^{**}P < 0.01$, $^{***}P < 0.001$); (B) western blot indicated gene knockdown of Tie2 using siRNAs against Tie2 in HCC1428 cells; (C) knockdown of Tie2 suppressed breast cancer growth in HCC1428 cell line detected by CCK8 assay (n = 7, $^{***}P < 0.001$).

level of Tie2 should be performed during the period of follow-up to further ensure the predictive effect of Tie2. Tie2 should also be assessed over the long term to understand whether it is a prognostic and predictive biomarker for breast cancer. The physical examination or lab testing of healthy controls should be performed to rule out possible comorbidities.

In summary, breast cancer patients have higher blood concentration of Tie2 than healthy individuals. Tie2 blood level is positively correlated with the grading of the breast cancer. Breast cancer patients who have a higher level of Tie2 in blood might have a lower survival percentage compared to patients who have a lower blood level of Tie2. It is found that Tie2 aids cancer cell growth in vitro. Thus, Tie2 could be developed to be a target for novel drug discovery against breast cancer and early detection as a plasma biomarker.

AUTHORS' CONTRIBUTION

The study concept and design were executed and approved by all authors; SQ drafted the manuscript; ZF, YT, and SQ were responsible for literature study;

SQ and WY were responsible for data analysis and approval. All authors approved the final manuscript.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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