Value of the level of methylation of RASSF1A and WIF-1 in tissue and serum in neoadjuvant chemotherapeutic assessment for advanced breast cancer

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Abstract. This study assessed the clinical efficacy of the neoadjuvant chemotherapy TAC scheme in treatment of patients with locally advanced breast cancer, and the value of the level of Ras association domain family 1A (RASSF1A) gene methylation and the Wnt inhibitory factor (WIF)-1 gene in tissue and serum of patients in clinical outcome prediction. In total, 126 patients were consecutively selected to receive TAC scheme (docetaxel, pirarubicin/epirubicin and cyclophosphamide) for at least four cycles with the total effective rate. The incidence of complications, progression-free survival and survival rate were recorded. Tumor tissues and peripheral blood samples collected in this study was used to detect methylation positive rate of RASSF1A and WIF-1 by methylation-specific PCR method and the relative level of expression of RASSF1A and WIF-1 mRNA by reverse transcription PCR method. Of the 126 patients, there were 18 cases with complete response (CR), 32 cases with partial response (PR), 50 cases with stable disease (SD), and 26 cases with disease progression (PD) with a total effective rate of 79.37%. Comparison on baseline data of effective group and ineffective group showed no difference (P>0.05), and comparison on adverse reactions occurrence showed no difference (P>0.05). Progression-free survival of the effective group was prolonged with a significant increase in survival rate (P<0.05). Positive rates of RASSF1A methylation and WIF-1 in tissue and serum of the patients in the effective group were significantly lower than those in the ineffective group, but the mRNA of RASSF1A and WIF-mRNA was significantly higher than the ineffective group (P<0.05). The sensitivity of clinical outcome prediction using tissue RASSF1A methylation was 67.0%, the specificity 15.4%, positive predictive value 69.0% and negative predictive value 31.0%. The above-mentioned indexes of tissue WIF-1 were 76.0, 31.4, 72.2 and 27.8, respectively. The indexes of serum RASSF1A were 85.0, 50.0, 76.2 and 23.8%, respectively, and the indexes of serum WIF-1 were 94.0, 75.0, 81.0 and 19.0%, respectively. The receiver operating characteristic curve analysis suggested that the accuracy of clinical outcome prediction using tissue RASSF1A mRNA level was 0.812. The sensitivity 85.2%, the specificity 76.3% and the critical value 0.4256. These indexes of tissue WIF-1 were 0.833, 86.7%, 75.4% and 0.3562 for CR, PR, SD and PD, respectively. These indexes of serum RASSF1A were 0.864, 88.3%, 77.4% and 0.2564, respectively, and for serum WIF-1 were 0.882, 89.4%, 73.5% and 0.1562, respectively. In conclusion, the detection of RASSF1A and WIF-1 gene methylation and level of mRNA expression in tissue and serum of patients with locally advanced breast cancer has an important application value in predicting clinical efficacy of neoadjuvant chemotherapy of the TAC scheme.

Introduction

Like every major malignant tumor threatening the health of women, breast cancer, accounting for 7-10% of the systemic tumors, has a high mortality rate world-wide (1). Locally advanced breast cancer, the major cause of postoperative recurrence and death, can rarely be completely removed by surgical resection. Neoadjuvant chemotherapy can narrow lesions, decrease tumor clinical stage, reduce micro-metastases, increase breast conservation rate and improve the quality of life (2). There remains no unified neoadjuvant chemotherapy regimen, but TAC scheme (docetaxel, pirarubicin and cyclophosphamide) is proved to have better safety and efficacy (3). Appropriate indexes with better sensitivity and accuracy for predicting the clinical outcome are of great value in improving the efficacy and decreasing the side effects of chemotherapy. Several studies (4,5) have confirmed that downregulation or deficiency in expression of tissue and serum Ras association domain family 1A (RASSF1A) gene and the Wnt inhibitory factor (WIF)-1 gene is closely related with the occurrence and metastasis of breast cancer, in which the abnormal methylation of promoter is one of the main mechanisms (6) for the decrease of gene activity. Our study aimed at analyzing the methylation of RASSF1A and WIF-1 and their value in prediction of

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Table I. Primer sequences of genes.

Genes	Sequences	Length (bp)
RASSF1A (U)	F: 5'-GGGGGTTTTGTGAGAGTGTGTTT-3'	
	R: 5'-CCCAATTAAACCCATACTTCACTAA-3'	204
RASSF1A (M)	F: 5'-CGAGAGCGCGTTTAGTTTCGTT-3'	
	R: 5'-CGATTAAACCCGTACTTCGCTAA-3'	192
WIF-1 (U)	F: 5'-GGGTGTTTTATTGGGTGTATTGT-3'	
	R: 5'-AAAAAAACTAACACAAACAAAATACAAAC-3'	154
WIF-1 (M)	F: 5'-CGCTCCACTGGGCGCACCGC-3'	
	R: 5'-TCGCACCTCGCTCGCGCCAGC-3'	145
RASSF1A	F: 5'-CAGATTGCAAGTTCACCTGCCACTA-3'	
	R: 5'-GATGAAGCCTGTGTAAGAACCGTCCT-3'	249
WIF-1	F: 5'-GTCTAAACGGGAACAGCCCT-3'	
	R: 5'-GCTGGCATTCTCTGTTGTGC-3'	354
β-actin	F: 5'-AAAGACCTGTACGCCAACAC-3'	
	R: 5'-GTCATACTCCTGCTTGCTGAT-3'	219

RASSF1A, Ras association domain family 1A; WIF-1, Wnt inhibitory factor-1; F, forward; R, reverse. U, unmethylated primers; M, methylated primers.

clinical outcome of neoadjuvant chemotherapy in patients with locally advanced breast cancer.

Patients and methods

Patient profile. We continuously selected 126 female patients, diagnosed as locally advanced breast cancer in the Affiliated Union Hospital of Fujian Medical University from January 2013 to January 2016.

Inclusion criteria. a) Patients who were confirmed with no distant metastasis by molybdenum target radiography, CT, MRI and ultrasound and whole body bone imaging; b) patients with no contraindications of surgery and anesthesia; c) patients who were diagnosed as locally advanced breast cancer for the first time with no therapy history such as surgeries, chemotherapy, radiotherapy or endocrine therapy; and d) patients with Karnofsky Performance Status (KPS) score >90 points.

Exclusion criteria. a) Patients with metastatic breast tumor; b) patients with the primary malignant tumor in other parts; c) patients who were unable to accomplish four cycles of chemotherapy; d) patients whose clinical data were incomplete. The study was approved by the Ethics Committee of the Affiliated Union Hospital of Fujian Medical University, and patients or their family provided signed written informed consent.

Methods. Patients agreed to accept TAC scheme, cyclophosphamide 600 mg/m^2, intravenous drip, the first day; pirarubicin 50 mg/m^2, intravenous drip, the first day; docetaxel 75 mg/m^2, intravenous drip, the first day; one cycle consisted of 21 days, and at least 4 cycles were conducted. During the execution of TAC scheme, the liver and kidney function, blood routine and coagulation function were regularly monitored and the symptomatic treatment was conducted for patients with minor complication; for patients with severe complications, drug withdrawal and close observation were performed. We recorded the total effective rate, incidence of complica-

tions, progression-free survival and survival rate. The tumor diameter was measured with mammary gland molybdenum target in two dimensions, and the efficacy was determined by UICC solid tumor response criterion which was divided into complete response (CR), partial response (PR), stable disease (SD) and disease progression (PD). The total effective rate = (CR + PR + SD)/total number of x 100%.

Tumor tissues and peripheral blood samples collected in this study was used to detect methylation positive rate of RASSF1A and WIF-1 by methylation-specific PCR (MSP) method and the relative expression level of RASSF1A and WIF-1 mRNA by reverse transcription PCR (RT-PCR) method.

MSP process
DNA extraction process. Tissue DNA extraction process: 50 mg of cancer tissue was ground and later procedures were performed according to the tissue DNA extraction kit (Beyotime Institute of Biotechnology, Jiangsu, China) instructions; 500 µl of tissue lysate buffer was added and heated at 50˚C water bath for 1 h. Then the mixture was diluted with proteinase K until the concentration reached 100 µg/ml. After water bath for 3 h at 50˚C, extraction was performed using equal volume of saturated phenol, phenol-chloroform (volume ratio, 1:1) and chloroform-isoamyl alcohol (volume ratio, 24:1), respectively. Then 1/10 volume of sodium ethoxide and 2 times the volume of absolute ethanol was added to precipitate the DNA which was later dissolved by a certain volume of TE solution. Then the DNA concentration and purity were detected using ultraviolet spectrophotometer (Applied Biosystems Life Technologies, Foster City, CA, USA).

Peripheral blood DNA extraction process: 5 ml venous blood was collected and centrifuged at 2,500 x g for 20 min; the supernatant was then preserved at -80˚C; 200 µl of the supernatant was for later procedures according to the instruc-

Table II. Baseline data comparison.

Groups	Effective group (n=100)	Ineffective group (n=26)	t/χ^2	P-value
Age (years)	56.7±14.5	55.9±16.3	0.152	0.932
Invasive ductal carcinoma, case (%)	68	19	0.249	0.618
Invasive lobular carcinoma	32	7		
Maximum diameter of tumor (cm)	3.8±1.2	3.9±1.4	0.163	0.879
Phase II, case (%)	46	12	0.000	0.989
Phase III	54	14	0.002	0.966
Lymphatic metastasis, case (%)	38	10		
Chemotherapy cycle	4.9±0.8	4.6±0.5	0.232	0.824
Follow-up time (months)	15.5±3.4	13.6±3.8	0.356	0.763

tion of plasma DNA extraction kit (Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., Beijing, China) using paramagnetic particle method.

Sulfite modification. Relevant procedures were performed according to the instruction of DNA methylation modification kit (Sigma, St. Louis, MO, USA); 1 µg of DNA was dissolved in 45 µl of TE solution. Then it was diluted by 5 µl of NaOH (3 mol/l) until the concentration reached 0.3 mol/l. After 20 min of denaturation at 37℃, 30 µl of hydroquinone (10 nmol/l) and 520 µl of sodium bisulfite (pH 5.0, 3 mol/l) were added and heated in 50℃ water bath for 16 h. For purification by columns, DNA purification agent was added in 45 µl of deionized water. Then it was diluted by 5 µl of NaOH (3 mol/l) until the concentration reached 0.3 mol/l. After desulfurization as well as precipitation by ethanol, it was dissolved in the 20 µl of TE solution and preserved at -20℃. DNA of the lymphocyte in human umbilical cord blood treated by bisulphite was set as the non-methylation positive control, DNA of the lymphocyte in human umbilical cord blood modified by SssI methyltransferase and treated by bisulphite as methylation positive control, and the distilled water as negative control; the DNA of the same sample was amplified by non-methylated primers and methylation-specific primers.

MSP. SYBR-Green I method was applied. Primers were synthesized by Invitrogen (Carlsbad, CA, USA) and the primer sequences are shown in Table I. The reaction system included: 10X buffer 2.5 µl + Mg^2 + l (25 mol/l) 1.5 µl + dNTP (2.5 mol/l) 1.8 µl + DNA 50 ng + upstream and downstream primers (10 µmol/l) 1 µl + Taq polymerase (5 U/µl) 0.25 µl. The reaction system was diluted by water to the total volume of 25 µl. The reaction was performed for 40 cycles under the conditions of 95℃ for 5 min, 95℃ for 1 min, 56℃ for 1 min, and 72℃ for 1 min and the product was extended at 72℃ for 10 min; 2% agarose gel electrophoresis and analysis by gel imaging analysis system (Media Cybernetics, Inc., Rockville, MD, USA) were performed on the MSP product. The production with results of positive methylation were selected for control to calculate the copies of DNA. The production treated by 10 times gradient dilution was chosen for standard substance to perform PCR amplification. Specimens to be detected with more than 500 copies/ml were considered as positive gene methylated.

RT-PCR process. DNA was extracted using conventional TRIzol method, concentration and purity of RNA were determined by ultraviolet spectrophotometric method, and cDNA was synthesized by reverse transcription kit. Primers were synthesized and designed by Takara Bio, Inc. (Otsu, Japan) as shown in Table I. Reaction system: 5X 2.5 µl buffer l + 1.5 µl $MgCl_2$ l + 0.5 µl dNTP + GAP-43 and upstream and downstream primers for internal reference each 1 + 0.3 µl Taq polymerase + 2 µl cDNA template. The reaction system was diluted by water to the total volume of 25 µl. Reactions were performed for 35 cycles under the conditions of 95℃ for 5 min, 95℃ 30 sec, 62℃, 30 sec, 30 sec 72℃ for 30 sec and the product was extended at 72℃ for 10 min. We composed the dissolution curve and used $2^{-\Delta\Delta Cq}$ method (7) to calculate the relative expression level of mRNA.

Statistical analysis. We used SPSS 20.0 software (IBM SPSS, Armonk, NY, USA) for statistical analysis. Measurement data are presented as mean ± standard deviation. Independent sample t-test was performed in intergroup comparison. Countable data are presented as the cases or (%). Chi-square (χ^2) test was performed in intergroup comparison; the specificity of clinical outcome prediction using RASSF1A and WIF-1 gene was analyzed by receiver operating characteristic (ROC) curve, and the accuracy was presented in the area under the curve (AUC). P<0.05 was considered to indicate a statistically significant difference.

Results

Chemotherapy outcome analysis. The 126 studied cases constituted 18 cases of CR, 32 cases of PR, 50 cases of SD and 26 cases of PD. The total effective rate was 79.37%. There was no difference in comparison of baseline data between effective group and ineffective group (P>0.05) (Table II).

The major adverse reactions, including gastrointestinal reaction, bone marrow suppression, cardiac toxicity, and hair loss, were divided into 0-IV levels, according to the World Health Organization (WHO) classification standard for adverse reaction of chemotherapy. Comparison on adverse reactions occurrence between the two groups showed no difference (P>0.05); compared with the ineffective group, the effective group had longer progress-free survival and higher survival rate and the differences between the groups were statistically significant (P<0.05) (Table III).

Table III. Survival outcome analysis.

Groups	Effective group (n=100)	Ineffective group (n=26)	χ^2	P-value
Gastrointestinal reaction, case (%)				
I-II	50	11	0.489	0.783
III-IV	20	6		
Bone marrow suppression, case (%)				
I-II	30	8	0.134	0.935
III-IV	10	2		
Cardiotoxicity, case (%)				
I-II	15	5	0.405	0.817
III-IV	6	2		
Hair loss, case (%)				
I-II	35	9	0.101	0.951
III-IV	13	4		
Progression-free survival time (months)	6.5±1.2	3.4±0.9	6.532	0.013
Survival rate, case (%)	78 (78.0)	12 (46.2)	8.064	0.005

Comparison on methylation positive rates of RASSF1A and WIF-1 of tissue and serum. In comparison on methylation positive rates of RASSF1A and WIF-1 of tissue and serum, the effective group was significantly lower than the ineffective group (P<0.05) (Table IV).

Comparison on relative expression level of RASSF1A and WIF-1 mRNA in tissue and serum. The relative expression level of RASSF1A and WIF-1 mRNA in tissue and serum of the effective group was obviously higher than the ineffective group (P<0.05) (Table V).

Analysis of the prediction for clinical outcome using the methylation positive rate of RASSF1A and WIF-1 in tissue and serum. The sensitivity of clinical outcome prediction using tissue RASSF1A methylation was 67.0%, the specificity 15.4%, positive predictive value 69.0% and negative predictive value 31.0%. The above mentioned indexes of tissue WIF-1 were 76.0, 31.4, 72.2 and 27.8%, respectively. These indexes of serum RASSF1A were 85.0, 50.0, 76.2 and 23.8%, respectively, and indexes of serum WIF-1 were 94.0, 75.0, 81.0 and 19.0%, respectively.

ROC analysis of tissue and serum RASSF1A mRNA level in clinical outcome prediction. The tissue and serum RASSF1A and WIF-1 mRNA levels served as the diagnostic index and the clinical outcome as the diagnosis, which were substituted into the ROC for analysis and suggested: The accuracy of clinical outcome prediction using tissue RASSF1A mRNA level was 0.812 (CI=0.756-0.932, P=0.008). The sensitivity 85.2%, the specificity 76.3% and the critical value 0.4256. These indexes of tissue WIF-1 were 0.833 (95% CI=0.721-0.948, P=0.010), 86.7%, 75.4% and 0.3562, respectively. These indexes of serum RASSF1A were 0.864 (CI=0.737-0.964, P=0.003), 88.3%, 77.4% and 0.2564, respectively. These indexes of serum WIF-1 were 0.882 (95% CI=0.727-0.986, P=0.005), 89.4%, 73.5% and 0.1562, respectively (Fig. 1).

Discussion

RASSF1A is highly expressed in almost every normal tissue, through inhibiting tumor formation by regulating the cell cycle, apoptosis and genome stability, and the deficiency of its

Table IV. Comparison on methylation positive rates of RASSF1A and WIF-1 of tissue and serum, case (%).

Groups	Effective group (n=100)	Ineffective group (n=26)	χ^2	P-value
Tissue RASSF1A	33 (33.00)	20 (76.92)	16.335	<0.001
Tissue WIF-1	24 (24.00)	15 (57.69)	10.960	0.001
Serum RASSF1A	15 (15.00)	11 (42.31)	9.396	0.002
Serum WIF-1	6 (6.00)	8 (30.77)	10.433	0.001

RASSF1A, Ras association domain family 1A; WIF-1, Wnt inhibitory factor-1.

Table V. Comparison on relative expression level of RASSF1A and WIF-1 mRNA in tissue and serum.

Groups	Effective group (n=100)	Ineffective group (n=26)	t-test	P-value
Tissue RASSF1A	0.6358±0.1236	0.3256±0.1124	7.532	<0.001
Tissue WIF-1	0.5427±0.1247	0.2142±0.1032	7.123	<0.001
Serum RASSF1A	0.4326±0.1326	0.1213±0.0639	5.629	0.008
Serum WIF-1	0.3598±0.1528	0.0659±0.0124	6.124	<0.001

RASSF1A, Ras association domain family 1A; WIF-1, Wnt inhibitory factor-1.
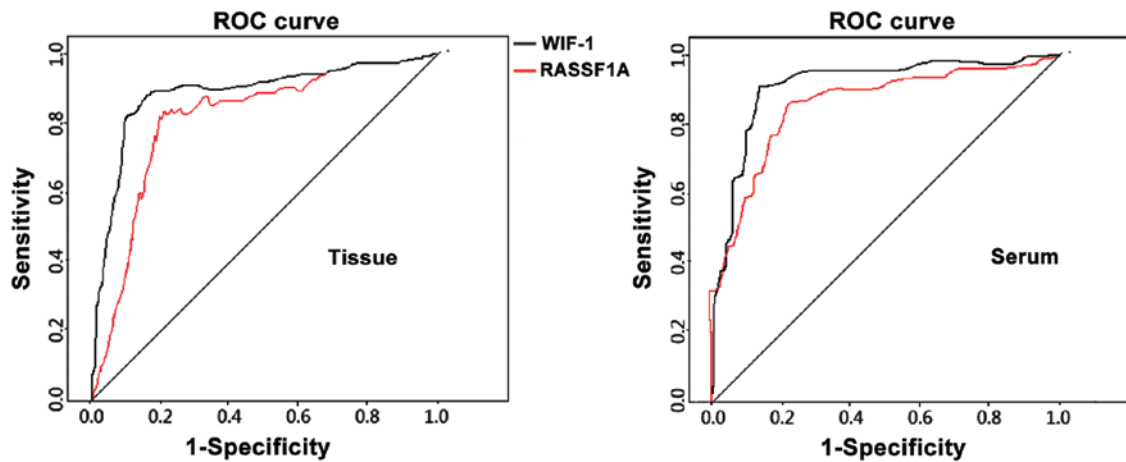
Figure 1. ROC analysis of RASSF1A and WIF-1 mRNA level in clinical outcome prediction. ROC, receiver operating characteristic curve; RASSF1A, Ras association domain family 1A; WIF-1, Wnt inhibitory factor-1.

expression is closely related to tumor occurrence and development (8). It has been proved (9,10) that the deficiency of its expression is observed in more than 20 tumor types, including the lung, breast, ovarian and prostate cancer, ans its expression could be activated by antitumor drugs to have its biological activity recovered. Abnormal methylation of gene promoter is an important cause for deactivation. Malignantly cloned DNA in the tumor tissues can be continuously released into the peripheral circulating blood and the circulating tumor cells as well as micro-metastases can also be delivered into the blood for detection (11). High levels of methylation of RASSF1A and low level of mRNA expression are closely related with clinical stages, differentiation degree, sensitivity to chemotherapy and survival rate of breast cancer (12). Although levels of RASSF1A methylation and mRNA have been widely applied in the early diagnosis of breast cancer (13), there are few studies assessing the clinical efficacy of neoadjuvant chemotherapy on locally advanced breast cancer. In the present study, we found that the total effective rate of TAC scheme was 79.37% with lower incidence and slight symptoms of adverse reactions, suggesting better safety and efficacy of TAC scheme. The progression-free survival of the effective group was ~6.5 months, and the survival rate was 78.0%. Rates of positive methylation of tissue, and serum RASSF1A and WIF-1 in the effective group were remarkably lower than those in the ineffective group; but, with levels of mRNA significantly higher than those in the ineffective group. Wnt/β-catenin signaling pathway plays a key role in biological behavior of breast cancer occurrence, differentiation, proliferation and invasion (14). C-myc and cyclin D1, as two important downstream target genes, regulate the cell division cycle (15). Not only is E-cadherin an important effector molecular in this signal pathway, but also a key factor in the invasion, metastasis and recurrence of breast cancer (16). As a major tumor suppressor gene of Wnt pathways, WIF-1 which is highly methylated or expresses a low level of mRNA can significantly affect the occurrence and development of breast cancer (17).

Further study found that the sensitivity of clinical outcome prediction using tissue RASSF1A and WIF-1 methylation positive rate was 67.0-76.0%, positive predictive value was 69.0-72.2%, and the specificity and negative predictive value were relatively low. The sensitivity of clinical outcome prediction using the rate of serum RASSF1A and WIF-1 positive methylation was 85.0-94.0%, positive predictive value was 76.2-81.0%, specificity was 50.0-75.0%, and the negative predictive value was relatively low. For tumor suppression genes such as RASSF1A and WIF-1, higher efficacy could be achieved by lowering the methylation positive rate of these two genes; besides, some abnormally methylated genes may be involved in the sensitivity to chemotherapy. Through the analysis of ROC, we found that the mRNA levels of RASSF1A and WIF-1 in tissues and serum show better accuracy, sensitivity and specificity when applied to predict the clinical outcomes. In conclusion, the detection of methylation and mRNA expression levels of RASSF1A and WIF-1 genes in tissues and serum of the patient with locally advanced breast cancer is of great significance in predicting clinical efficacy of the neoadjuvant chemotherapy TAC scheme.

Acknowledgements

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