

ORIGINAL ARTICLE

Value of circulating tumor cells positive for thyroid transcription factor-1 (TTF-1) to predict recurrence and survival rates for endometrial carcinoma

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Summary

Purpose: This study was conducted to analyze the predictive value of circulating tumor cells (CTC) expressing thyroid transcription factor-1 (TTF-1) on the recurrence and survival rates of endometrial carcinoma patients treated with laparoscopic surgery.

Methods: 78 patients were recruited, diagnosed with endometrial carcinoma and measured CTC expressing TTF-1 using flow cytometry in blood and tissues. Then, the patients were distributed into TTF-1-positive (N=42) and -negative (N=36) groups. The levels of HE4 were determined by ELISA, the levels of cancer antigen CA125 and CA15.3 by chemiluminescent immunoassay, and the levels of mRNA expression of survivin, β -catenin, miR-15a, and PTEN by RT-PCR assay from endometrial carcinoma samples.

Results: Patients in TTF-1-positive group had mainly TNM stages II and III-IV, whereas the TTF-1-negative group stages I and II predominated. The rates of vascular infiltration and lymphatic metastasis in the TTF-1-positive group were higher compared with the TTF-1-negative group ($p < 0.05$). The serum levels of CA125, CA15.3, and HE4 were significantly high-

er in the TTF-1-positive group than in the TTF-1-negative group ($p < 0.05$). The levels of survivin and β -catenin mRNA expression in endometrial carcinoma in the TTF-1-positive group was higher than in the TTF-1-negative group. In contrast, the levels of miR-15a and PTEN mRNA expression were lower in the TTF-1-positive group ($p < 0.05$). The median follow-up duration was 25 months for both groups. At that time, progression-free survival (PFS) and the median survival time decreased in the TTF-1-positive group compared with the TTF-1-negative group. Additionally, the recurrence rate increased in the TTF-1-positive group.

Conclusion: The rate of TTF-1-positive CTC was strongly correlated with TNM staging, vascular infiltration, lymphatic metastasis, and the levels of CA125, CA15.3, and HE4 in endometrial carcinoma. The levels of survivin, β -catenin, miR-15a, and PTEN mRNA also contributed to predict survival rates after laparoscopic surgery.

Key words: circulating tumor cells, endometrial carcinoma, laparoscopic surgery, thyroid transcription factor-1

Introduction

Endometrial carcinoma is a malignancy of the female reproductive system with high mortality. Its atypical clinical features, lack of sensitive and specific markers, and delay of imaging examinations are factors contributing in poor therapeutic

results and prognosis [1,2].

Thyroid transcription factor-1 (TTF-1) is a specific marker for the diagnosis of thyroid and lung carcinoma [3] as well as carcinomas of the reproductive system [4] and plays an important

role in the diagnosis and prognosis of these carcinomas. CTC in the bloodstream play an important role in metastasis and have been detected in the peripheral blood in patients with poor prognosis [5]. Recently, intense efforts have been undertaken to identify prognostic and predictive biomarkers in CTC because they are easily accessible with minimally invasive procedures.

Herein, we examined the presence of TTF-1 in CDC by fluorescence labeling in patients with laparoscopically operated endometrial carcinoma. The goal was to determine the value of TTF-1 presence and levels in CTC for evaluating the disease prognosis. We also analyzed the correlation between TTF-1-positive CTC and tumor staging as well as its malignant biological behavior. Our results support the value of TTF-1 in CDC for predicting poor outcomes for patients with endometrial carcinoma.

Methods

Study subjects

We recruited 78 patients diagnosed with endometrial carcinoma in our hospital from January 2013 to January 2016. Exclusion criteria: Other primary carcinomas, pregnant or lactating women, metastatic endometrial carcinoma, and lack of indications for laparoscopic excision. The same surgical and nursing teams conducted the entire study, following standard medical procedures. Standard therapeutic methods included surgical excision, chemoradiotherapy, etc. The study was approved by the Ethics Committee of our hospital. Patients or relatives read and signed the informed consent form prior to being accepted in the study.

Flow cytometry

Flow cytometry was used to determine the rate of CTC marked by TTF-1. (1): Suspension of mononuclear cells: 5 ml of peripheral venous blood was drawn and placed into 4 centrifuge tubes with 5 ml of neutrophil separating medium added into each tube. After centrifugation at 3,000 x g for 15 min, the buffy coat was removed. The pellet was washed twice and the supernatant was removed. (2): TTF-1 positive marking: 10 μ l of TTF-1-FITC and anti-FITC were added into the centrifuge tubes for cell marking and the tubes were incubated at 4-8°C for 10 min. Twenty volumes of buffer solution were added to wash the cells, the tubes were centrifuged at 2,000 x g for 10 min, and the supernatant was removed. The washing step was repeated once. (3): Preparation and image capture: The positive cells obtained were added in 1 ml of buffer solution and centrifuged at 1500 x g for 5 min. The supernatant was removed, and 10 μ l of PBS were added and the mixture was centrifuged. Two ml of the mixture were smeared

on the flow cytometer (Beckman Coulter) and the rate of positive cells was calculated.

Detection of serum tumor markers

The human epididymal protein (HE4) tumor marker was detected in the peripheral blood by double-antibody sandwich ELISA assay. CA125 and CA15.3 were detected by chemiluminescent immunoassay. Three ml of venous blood were drawn with the patient standing at room temperature. The blood was centrifuged at 4,000 x g for 30 min. The supernatant was frozen at -70°C until assayed. The HE4 ELISA kit was purchased from Sigma Corporation (St. Louis, MO, USA) and the microplate reader from Bio-Rad Corporation (Hercules, California, USA). We detected CA125 and CA15.3 by Western blot following the manufacturer's instructions. The signal produced by CA125 and CA15.3 immunodetection was developed with the Elecsys 2010 analyzer using Roche Corporation (Switzerland) reagents and calibration.

RT-PCR

The mRNA expression levels of survivin, β -catenin, miR-15a, and PTEN were detected by RT-PCR assay. Tissue samples from endometrial carcinoma were collected during the laparoscopy and frozen. Samples were later thawed, homogenized, and centrifuged. The supernatant was combined with trichloromethane (chloroform) and dimethyl carbinol to extract RNA. Then, the mixture was centrifuged to precipitate the RNA. Total RNA was resuspended in water and used as template to synthesize cDNA using the inverse transcription kit (American Fermentas, K1622). Survivin, β -catenin, miR-15a, and PTEN mRNA were amplified by fluorogenic quantitative PCR kit (Shanghai GenePharma). The reaction conditions were: 95°C for 15 sec, primer-specific annealing temperature for 20 sec, and 72°C for 25 sec. The cycle was repeated 40 times. β -actin mRNA was used as internal reference for normalization, and the results were presented as $2^{-\Delta\Delta Ct}$.

Follow-up indexes

The duration of follow-up ranged from 5 to 40 months (median 25). The PFS, median survival, recurrence rate, and survival rate were counted.

Statistics

The SPSS 20.0 software package was used for statistical analysis of the experimental data. Data were presented as mean \pm standard deviation. T-test was used for comparisons between groups and enumeration data were presented by cases (%). Chi-square (χ^2) test was applied for comparisons between groups. Kaplan-Meier method and log-rank test were applied for the analysis of survival time. $P < 0.05$ suggested that the differences were statistically significant.

Results

Correlation between TTF-1-positive CTC and clinical tumor features

We first examined the expression of TTF-1 in CTC in blood in 78 endometrial carcinoma patients. Forty-two patients showed TTF-1-positive CTC and 36 were TTF-1-negative. We next divided the 78 endometrial carcinoma samples according to TTF-1 presence/absence in CTC. The two groups showed comparable basic clinical parameters, including age and time since menopause onset (Table 1). Only 10% of the patients in the TTF-1-positive group were diagnosed as TNM stage I and 38% were in stages III-IV (Table 1). In contrast, 50% of the TTF-1-negative patients were in stage I and only 16% were in stages III-IV (Table 1). The rates for vascular infiltration and lymphatic metastasis were significantly higher in the TTF-1-positive group (Table 1). These data show strong correlation of TTF-1-positive CTC with clinical relevant features.

Levels of serum tumor markers

Next, we tried to confirm the relevance of the previous findings by detecting tumor markers in serum in the TTF-1-positive and -negative groups (Table 2). We found that the levels of CA125, CA15.3, and HE4 were significantly higher in the TTF-1-positive group compared with the TTF-1-negative group (Table 2). These data support the association of malignancy with TTF-1 presence in CTC.

Survivin, β -catenin, miR-15a and PTEN expression in carcinoma

Next, we examined the levels of survivin, β -catenin, miR-15a, and PTEN mRNA in the carcinoma tissues and we found that the levels of survivin and β -catenin mRNA in the TTF-1 positive patients were significantly higher than in the TTF-1-negative patients (Table 3). In contrast, the expression of miR-15a and PTEN was lower in the TTF-1-positive patients compared with the TTF-1-negative patients (Table 3).

Table 1. Correlation between the positive rate of CTCs marked by TTF-1 and the clinical tumor features

Groups	Cases	Age (years)	Menopause time (years)	Stage			Vascular infiltration	Lymphatic metastasis
				I	II	III-IV		
CTC pos*	42	63.4±10.3	7.5±2.8	10(23.8)	16(38.1)	16(38.1)	25(59.5)	26 (61.9)
CTC neg*	36	64.2±11.5	7.6±3.0	18(50.0)	12(33.3)	6(16.7)	4(11.1)	7 (19.4)
t/ χ^2		0.324	0.432	6.982	4.314	19.452	24.635	22.721
P		0.657	0.527	0.030	0.043	0.000	0.000	0.000

*mean±standard deviation

Table 2. Comparison of the levels of serum tumor markers between groups

Group	CA125 ($\times 10^3$ U/L)	CA15.3 ($\times 10^3$ U/L)	HE4 (pmol /L)
Positive group*	70.7±8.3	46.8±5.2	89.7±9.2
Negative group*	56.4±7.1	31.9±3.5	65.3±7.1
t	5.293	4.723	4.823
P	0.017	0.021	0.019

*mean±standard deviation

Table 3. Comparison of mRNA expression levels of survivin, β -catenin, miR-15a and PTEN in carcinoma tissues between groups

Group	survivin	β -catenin	miR-15a	PTEN
Positive group*	143.8±15.4	152.7±16.8	78.3±8.1	65.8±7.1
Negative group*	100±9.4	100±8.7	100±10.4	100±10.7
t	5.347	5.283	4.793	5.273
P	0.015	0.016	0.020	0.016

*mean±standard deviation

Table 4. Comparison of prognosis between groups

Group	Cases	Progression free survival (mean±SD, months)	Overall survival (mean±SD, months)	Recurrence rate N (%)	Survival rate N (%)
Positive group*	42	8.7±1.5	16.2±3.5	20 (47.6)	25 (59.5)
Negative group*	36	13.8±2.7	27.4±4.6	9 (25.0)	29 (80.6)
χ^2		7.324	8.623	4.246	4.025
P		0.000	0.000	0.039	0.045

*mean±standard deviation

Prognosis value of TTF-1

Finally, we determined the clinical relevance of TTF-1 presence in CTC. PFS, median survival time, and the survival rate in the TTF-1-positive patients was shorter than in the TTF-1-negative patients (Table 4). Additionally, the recurrence rate significantly increased in the TTF-1-positive patients (Table 4). These data support the discrimination of the endometrial carcinoma based on TTF-1 expression in CTC.

Discussion

Hematogeneous metastasis or transfer of cancer to unconnected organs is common in the early stage of endometrial carcinoma. Therefore, tumor cells can be detected in CTC as evidence of micrometastasis [6]. Studies on mammary and prostatic carcinoma unlimited proliferation of CTC in the blood is one of the major reasons for tumor spreading to distant organs and establishing metastasis [7]. Patients with CTC in early stages usually have poor prognosis. Therefore, the detection of CTC in patients with malignancies can be used as a new method for early cancer screening, judging its severity, and evaluating the therapeutic results, thus providing important information for clinical treatment [8].

The TTF-1 in thyroid and lung carcinoma has 65% sensitivity and 90% specificity [9]. In our study, TTF-1-positive CTC correlated with higher TNM stage. The rates of vascular infiltration and lymphatic metastasis in the positive group were also significantly elevated compared with the negative group. Importantly, TTF-1-positive CTC was strongly correlated with the biological behavior of the tumor [10]. Furthermore, the serum levels of CA125, CA15.3, and HE4 in the TTF-1-positive group were higher compared with the negative group. CA125, CA15.3, and HE4 are important serum tumor mark-

ers, and the combined detection of CA125 and CA15.3 in patients with endometrial carcinoma assists in making a definitive diagnosis [11]. HE4 is a tumor marker of the pelvic cavity neoplasias, which is highly expressed in ovarian carcinoma and is abnormally expressed in endometrial carcinoma [12]. Additionally, the levels of survivin and β -catenin mRNA in endometrial carcinoma were higher in the TTF-1-positive group than in the negative group, whereas miR-15a and PTEN were lower. Survivin, β -catenin, miR-15a and PTEN are intimately correlated with malignant behaviors such as proliferation, invasion and antiapoptotic activities. Survivin is the most powerful inhibitor of apoptosis, which is relevant for angiogenesis, invasion, and metastasis of tumors [13]. β -catenin can mediate the aberrant activation of the Wnt pathway and participates in the early stages of tumor development [14]. MiR-15a is an anti-oncogene expressed at low levels in many carcinomas [15]. And PTEN is a gene with high mutation rates in endometrial carcinomas and its expression level correlates with the sensitivity to chemotherapy [16].

The follow-up of the 78 patients indicated that TTF-1-positive CTC is associated with poorer outcomes, suggesting that TTF-1 in CTC is a good indicator of prognosis. Overall, TTF-1-positive CTC correlated strongly with advanced stages and poor outcomes in endometrial carcinomas. These clinical features agreed with several molecular markers indicating that TTF-1 in CTC is a good marker for endometrial carcinoma and metastasis. These studies suggest that TTF-1 in CTC should be considered as a routine marker for several cancers, including endometrial carcinomas, due to its diagnostic and predictive power.

Conflict of interests

The authors declare no conflict of interests.

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