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ORIGINAL ARTICLE

Factors influencing the presence of circulating differentiated thyroid cancer cells in the thyroidectomy perioperative period*

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Abstract

Objective The aim of the study was to detect circulating differentiated thyroid cancer (DTC) micrometastasis and to investigate the factors influencing their presence in the perioperative thyroidectomy period.

Methods DTC micrometastases in the peripheral blood were detected with flow cytometry, and patient clinical and pathological factors were analyzed in 327 DTC patients.

Results Circulating blood micrometastases were present in the peripheral circulation at a higher rate 1 week postoperatively than preoperatively and at 4 weeks postoperatively ($P < 0.05$). The preoperative presence of circulating micrometastasis was associated with the size of the tumor and the presence of lymph node metastasis ($P < 0.05$), but was not related to the degree of tumor differentiation ($P > 0.05$). At 4 weeks postoperatively, the presence of circulating micrometastasis was not associated with tumor size or lymph node stage ($P > 0.05$), but was associated with poorly differentiated tumors ($P < 0.05$).

Conclusion The presence of circulating DTC micrometastases correlates to tumor size, lymph node stage, and operative manipulation. The differentiation degree of the tumors were associated with the persistent presence of micrometastasis in the circulating blood.

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Key words: thyroid cancer; flow cytometry; circulating tumor cell

Differentiated thyroid cancer (DTC), including thyroid papillary carcinoma and follicular carcinoma, is a slowly growing low-grade malignancy with a 10-year survival of > 85%. However, the occurrence of metastases significantly influences the prognosis of DTC [1]. Currently, there are no biomarkers for the detection of the early recurrence and metastasis of thyroid carcinoma. Thyroglobulin (Tg) and thyroglobulin antibodies (TgAb) are used to monitor for postoperative recurrence and metastasis, but their accuracy is limited, and in patients with incomplete resection Tg and TgAb levels are less valuable. In this study, flow cytometry (FCM) was used to detect micro metastasis in peripheral blood samples from 327 patients with DTC in the perioperative period, and factors influencing their presence in the circulation were analyzed.

Materials and methods

Patients

A total of 327 patients with DTC treated at The Cancer Hospital of Gansu Province from 2008 to 2013 were enrolled in the study. There were 76 men and 251 women, with an age range of 14–72 years (median, 38.7 years). All cases were confirmed by pathology based on the universal standard [2]. Before obtaining blood samples, all patients provided written informed consent. No patients underwent chemotherapy or radiotherapy. Patient follow-up was 6–72 months, with a median follow-up time of 42 months.

Clinical and pathological characteristics

The 327 DTC patients were separated into 3 groups based on tumor size: 86 cases with a ≤ 2 cm tumor diameter, 178 cases with a 2–4 cm tumor diameter, and 63 cases with a > 4 cm tumor diameter.

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Patients were also classified according to pathological subtype determined in accordance with the universal standard [3]. Well-differentiated tumors were diagnosed in 248 cases including 106 cases of common follicular carcinoma, 32 cases of general papillary carcinoma, 67 cases of papillary microcarcinoma, and 43 cases of the follicular variant of papillary carcinoma. Intermediate tumors were diagnosed in 79 cases including 46 cases of high cell variant carcinoma, 4 cases of columnar cell variant carcinoma, 2 cases of diffuse sclerosing variant carcinoma, 5 cases of island cell carcinoma, and 22 cases of Hurthle cell carcinoma. One hundred twenty-six cases were lymph node stage N0, and 201 cases were lymph node stage N1. All tumors were completely removed via thyroidectomy, and there was no residual tumor tissue.

Equipment and reagents

The FCM instrument used was FACSCalibur (BD Bioscience, USA). The antibody anti-cytokeratin 19 (CK19_ / FITC and the corresponding negative control IgG1-FITC were provided by eBioscience, USA. Fluorescent monoclonal antibodies, rupture of membrane agent CD45-PerCP-Cy5.5 (No. 641776), and FACS hemolysin (No. 349202) were obtained from BD Biosciences, USA. Mucin 1 (Muc1) / CD227 antibody was obtained from Abcam, USA. FACS hemolysin was diluted with deionized water tenfold before use.

Blood sample collection

All samples were collected by trained staff. Five milliliter of fasting venous blood was collected (with 10% EDTA-Na2 anticoagulant) 1 day before thyroidectomy, during thyroidectomy, 1 week postoperatively, and 4 weeks postoperatively. FCM was carried out within 24 hours of sample collection.

Antibody labeling

Anti-CK19

Five hundred microliter of anticoagulated blood was transferred into two tubes, one as a control and the other as the test sample. Then, 10 μL of CD45-PerCP antibody was added to each tube and incubated at room temperature for 15 min in the dark. Next, 100 μL of the membrane burst reagent A was added and placed in the dark at room temperature for 10 min. Then 5 mL of 1:10 diluted hemolytic reagent was added and incubated for 5 min, and then centrifuged at 800 rpm for 5 min. After discarding the supernatant, membrane burst reagent II and CK19 antibody were added to the test sample and the membrane burst reagent II and negative reagent were added to the control sample. The tubes were incubated at room temperature for 25 min in the dark. After washing with 1 mL phosphate buffer saline (PBS), detection was carried out using FCM.

MUC1/CD227

Five hundred microliter of the anticoagulated blood sample was transferred into two tubes. Ten microliter of MUC1/CD227 antibody and 10 μL of CD45-PerCP antibody were added to the test sample, but only 10 μL of CD45-PerCP antibody was added to the control tube. Both tubes were incubated at room temperature for 15 min in the dark and then 5 mL of 1:10 diluted FACS hemolysin was added and incubated at room temperature for 5 min. The samples were then centrifuged at 800 rpm for 5 min and, after the supernatant was removed, the pellet was washed with 1 mL of PBS and subjected to FCM.

Flow cytometry detection

FACSCalibur FCM, with a 15 mW argon ion laser excitation source and 300 μw of output power, was employed for the detection of labeled cells. The device setup was as follows. The excitation wavelength was 488 nm, and forward scatter and side scatter settings were used to remove the small particles of debris in the sample and aqueous solution to avoid interference with the cells. The data collection in the test and control tubes used a single parameter histogram. The abscissa represents the relative fluorescence intensity and the unit is number of tracks. The ordinate represents cells count events, which represent the relative cell number.

Data processing

The statistical software SPSS 17.0 (IBM, USA) was used to analyze the data.

Results

In this study, patients with only CK19 or MUC1 positive cells present in the circulating blood were excluded, and cases with both CK19 and MUC1 positive cells present in the circulating blood were considered cases with micrometastasis.

Comparison of circulating blood micrometastasis before and after thyroidectomy

Thyroidectomy was performed in 327 patients with DTC. Circulating micrometastases were detected in 93 (28.4%) patients on the day before surgery. One week postoperatively, blood micrometastases were detected in 108 out of 286 patients (37.8%). The difference between these two groups was statistically significant ($\chi^2 = 6.01$, $P = 0.014$). Four weeks postoperatively, 35 cases were positive for micrometastases (20.1%) in 174 patients who were negative for micrometastases 1 week postoperatively. Compared with the preoperative results, this difference was statistically significant ($\chi^2 = 4.17$, $P = 0.041$). This difference was also significant compared to 1 week

Table 1 Relationship between circulating blood micro metastasis and clinicopathological characteristics in patients with DTC

The parameters of the sample	Before the operation			Four weeks after operation		
	n	Micro metastasis (+)	*P*	*n*	Micro metastasis (+)	*P*
The maximum diameter of the tumor (cm)			0.028			0.178
≤ 2	86	17		41	7	
2–4	178	51		98	17	
> 4	63	25		35	11	
Lymph node staging			0.048			0.177
N0	126	28		67	10	
N1	201	65		107	25	
The histologic subtype classification			0.062			0.016
High differentiation type	248	64		131	21	
Intermediate differentiated	79	29		43	14	

postoperatively ($\chi^2 = 15.65$, $P = 0.000$).

Association of preoperative circulating blood micrometastasis with clinicopathological characteristics

Before surgery, in 327 cases of DTC, we detected 93 cases with circulating blood micrometastasis (28.4%). Analysis of the 93 patients with circulating micrometastasis preoperatively showed that tumor size was significantly related to the presence of circulating micrometastasis ($\chi^2 = 7.13$, $P = 0.028$). Lymph node stage was also significantly associated with micrometastasis ($\chi^2 = 3.88$, $P = 0.048$). The presence of micrometastasis was not related to the degree of tumor differentiation ($\chi^2 = 3.47$, $P = 0.062$; Table 1).

Association of postoperative circulating micrometastasis with clinicopathological characteristics

In 174 patients who had no circulating micrometastases at 1 week postoperatively, 35 patients (20.1%) subsequently demonstrated circulating micrometastases at 4 weeks postoperatively. The micrometastases in these cases were related to the degree of tumor differentiation ($\chi^2 = 5.62$, $P = 0.016$), but were not associated with tumor size or lymph node stage ($\chi^2 = 3.45$, $P = 0.178$, and $\chi^2 = 1.82$, $P = 0.176$, respectively; Table 1).

Follow-up results

Out of 327 patients with differentiated thyroid carcinoma, 152 cases completed follow-up. In these patients, 3 patients had lung metastasis, 1 patient had bone metastasis, and 2 patients had tumor recurrence in the neck. No patient deaths were recorded during the follow-up period.

Discussion

Hematogenous metastasis is one of the principle methods of malignant tumor spread. It is important to detect circulating micrometastasis in the subclinical period for tumor staging, treatment selection, and patient prognosis. In thyroid cancer, the distant metastasis reflects a more aggressive biological behavior, and is the leading cause of death in patients with thyroid carcinoma [4]. Circulation of tumor cells through the blood allows the development of distant metastasis. However, the mechanisms of metastasis are complicated and influenced by a multitude of factors that remain obscure [5]. The specific molecular mechanisms involved in thyroid carcinoma metastasis are unknown. The discovery of biomarkers for DTC invasion and metastasis at an early stage would allow for highly effective therapy and improved prognosis and survival rates.

CK19 is a low molecular weight keratin and component of the epithelial cell skeleton that is only expressed in epithelial cells and epithelial origin tumor cells. It is a marker for tumor micrometastasis, especially for epithelial origin cancers [6]. Mucin 1 (MUC1) is a high molecular weight protein expressed mainly in epithelial tissues. It plays an important role in the regulation of cell-cell interactions, intercellular signal transduction, cell adhesion function, and the regulation of immune function. The abnormal expression of MUC1 may signal the occurrence of tumor invasion and metastasis. Generally, there are no cells of epithelial origin in the circulating blood. Therefore, the expression of CK19 and MUC1 in the peripheral blood may be a marker for the detection of thyroid tumor cells in the circulating blood.

Numerous studies have evaluated micrometastases in DTC. Lymph node metastasis and circulating tumor cells are pivotal in the formation of distant metastasis, and the detection of tumor cells in the peripheral blood may allow early diagnosis and improve prognostication and therapeutic monitoring [7–8]. It is generally accepted that most circulating tumor cells rarely survive to become distant metastasis; however if tumor cells are continuously present in the peripheral blood after surgical treatment, these patients would be considered as high risk for the development of distant metastasis [9].

In our study population, 37.8% (108/286) of cases demonstrated circulating micrometastasis 1 week after thyroidectomy in DTC patients. The preoperative presence of circulating micrometastasis was detected in 28.4% of all enrolled patients (93/327). The positive rate in the former was significantly higher than in the later ($P = 0.014$). Theoretically, the appearance of tumor cells in the peripheral blood is related to tumor burden and the inherent tendency for tumor metastasis. After surgery, the number of tumor cells in circulating blood should decrease due to tumor burden reduction, tumor cells decreased, immune function enhanced. In our current investigation, there was a higher rate of circulating micrometastasis 1 week postoperatively than preoperatively. The more frequent presence of circulating micrometastasis in the postoperative period may be related to the distraction and extrusion of the tumor the tumor, which may prompt shedding of tumor cells into the blood and lymph circulation.

In some cases, tumor cells may enter the circulation because of local spread with lymph node and capsular infiltration. In our study, 23 blood samples were collected in which 11 cases (47.8%) with circulating blood micro metastasis were detected. This suggests that surgery may allow tumor cells to enter the peripheral blood, and thereby increase the occurrence of metastases. Therefore, it is important to take measures to prevent tumor cell spread during surgery. These measures might include the non-tumor technique and minimizing distraction and compression of tumor body. To minimize cancer cell spread through the lymph circulation it is important to remove the lymph node whole, avoiding damage to the capsule of the lymph node.

In this study, we demonstrated a high frequency of circulating micrometastasis at 4 weeks postoperatively (20.2%, 35/174) and 1 week postoperatively 37.8% (108/286). In these patients, even though the primary thyroid tumor was removed, the continued presence of tumor cells in the circulating blood puts these patients at a very high risk of distant metastasis. Another important finding in this study is the association of circulating micrometastasis with tumor size and lymph node stage. In 63 patients with a tumor > 4 cm in diameter, 25 (39.7%) had circulating micro-metastasis. The tumor cells enter into the peripheral blood, resulted for tumor volume increases, tumor load increases, the internal pressure of the tumor increases and the inherent transfer tendency of tumor cells.

We also found that circulating micrometastases at 4 weeks postoperatively were associated with the degree of tumor differentiation, but were not related to tumor size or lymph node stage. In our opinion, it may be that surgical removal of the tumor and lymph node removal, body's immune regulation, the effects of the operation, tumor size and lymph node metastasis caused by circulating blood cancer cells has been basically eliminated. However, poorly DTCs are fast growing and invasive, and micrometastases from these tumors have a better chance to survive in the blood and cause distant metastasis. In our follow-up group, 2 patients developed lung metastasis and 1 patient developed bone metastasis. These distant tissue metastases suggest that micrometastases in the blood are important in the formation of distant metastases. Monitoring micrometastases in the circulation may play a dynamical role in predicting the prognosis.

In conclusion, it is important to recognize that DTC cannot be completely eradicated by thyroidectomy. The analysis of the molecular pathological profile will aid in the effort to elucidate the mechanisms of thyroid cancer metastasis. As a biomarker, circulating micrometastases can be used as a reference for the early metastasis of cancer cells, and may be a helpful guide for treatment and allow the prevention of metastasis and an improved prognosis. Knowledge of circulating micrometastases and of tumor genomic alterations will allow more individualized therapy of DTC patients with improved survival rates.

Conflicts of interest

The authors indicated no potential conflicts of interest.

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