Chinese-German J Clin Oncol
DOI 10.1007/s10330-314-0009-5
November 2014, Vol. 13, No. 11, P518–P522

Clinical value of cancer cells joint detection in peripheral blood plasma of thyroid cancer patients*

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Received: 24 September 2014 / Revised: 23 October 2014 / Accepted: 5 November 2014

Abstract ***Objective:*** We aimed to detect cytokeratin 19 (CK19) and polymorphic epithelial mucin 1 (MUC1) expression in peripheral blood of thyroid cancer patients, and investigate the clinical value of it as a diagnostic marker for circulating blood micrometastases. ***Methods:*** The flow cytometry (FCM) was used to detect and analyze CK19 and MUC1-expressing cells in peripheral blood of 491 thyroid cancer patients. ***Results:*** CK19 and MUC1 expression showed no statistically significant difference with gender and age in thyroid cancer patients ($P > 0.05$), while had statistically significant difference with tumor size, lymph node stage and distant metastasis ($P < 0.01$). The expression of CK19 and MUC1 were positively correlated ($r = 0.628$, $P = 0.00$). ***Conclusion:*** CK19 is closely related to MUC1 expression, tumor size, extent of invasion and distant metastasis in peripheral blood of thyroid cancer patients. The circulating blood CK19 and MUC1 tests can help predict thyroid cancer micrometastases and prognosis.

Key words thyroid cancer; flow cytometry; circulating tumor cells; cytokeratin; polymorphic epithelial mucin

Thyroid cancer is a common endocrine malignancy in the clinical practice. Its development and malignancy degree is relatively slow, the prognosis is relatively better than the other malignancies. However, if the metastasis occurs, the prognosis may be significantly affected, the survival rate will significantly decrease [1]. Therefore, the proper monitoring and the effective treatment for thyroid cancer are very necessary. In this paper, the flow cytometry (FCM) was used to detect and analyze the cytokeratin (CK) and polymorphic epithelial mucin 1 (MUC1) expressing cells in peripheral blood of 491 cases of thyroid cancer patients, so the status of free thyroid cancer tumor cells in peripheral blood can be better understood.

Materials and methods

Subjects

From February 2008 to October 2013, 491 cases of thyroid cancer patients were enrolled in Gansu Provincial Tumor Hospital, China. There were 117 males and 374 females, age ranged from 14 to 72 years old, with a median age of 41.6 years. All patients were pathologically diagnosed according to uniform standards for diagnosis and treatment [2]. Patients did not receive chemotherapy, radiation therapy and radionuclide therapy before the blood sample collection. A total of 376 cases of thyroid nodular goiter patients were enrolled as controls during the same period.

Main equipments and reagents

FACS Calibur flow cytometer was from BD Biosciences Corporation (USA). Anti-CK19/FITC and corresponding negative control IgG1-FITC were purchased from eBioscience Company (USA). CD45-PerCP-Cy5.5 fluorescent monoclonal antibodies, breaking agent (No. 641776) and FACS hemolysin (No. 349202) were purchased from BD Biosciences Corporation (USA). FACS hemolysin (No. 349202) was diluted as 1:10 with deionized water before use. Muc1 / CD227 antibody was from Abcam Company (USA). Other conventional equipments were produced in China.

Specimen collection

Five mL of venous blood were collected 1 week after surgery, 10% EDTA-Na2 was used for anticoagulation. The venous blood in patients receiving radionuclide therapy was collected 3 days before the treatment. The collected samples were sent for testing within 24 h, all samples were collected by specific medical staffs.

Antibody labeling

Anti-CK19: 500 μL of the anticoagulated whole blood

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* Supported by a grant from the Gansu Province Key Traditional Chinese Medicine Project (No. GZK-2010-Z9).

were added into the control tube and experimental tube. Ten μL of CD45-PerCP antibody was added in each tube and set at room temperature in the dark for 15 min, the breaking agent A 100 μL was added, they were placed in dark place at room temperature for 10 min. Five mL of 1:10 hemolytic agents were added, then they were placed still for 5 min and then for 800 rpm/xg centrifugation for 5 min. Supernatant was discarded, the breaking agent-II and CK19 were added in positive tubes. The breaking agent-II and negative controls were added in control tube. They were set at room temperature in the dark for 25 min, after adding 1 mL of PBS, the samples were set on machine for testing.

MUC1/CD227: 10 μL of MUC1 / CD227 antibody and 10 μL of CD45-PerCP antibody were added in the experimental tube, 10 μL of CD45-PerCP antibody was added in the control tube. 500 μL of anticoagulanted whole blood were added to the control tube and the detecting tube, they were set at room temperature in the dark for 15 min. Five mL of 1:10 FACS hemolysin were added and placed still for 5 min, after 800 rpm/xg centrifugation for 5 min, the supernatant was removed, 1 mL of PBS solution was added for washing, the samples were set on machine for testing.

Flow cytometry

FACS Calibur flow cytometer was used, the excitation source was 15 mW argon ion laser, the output power was 300 μw, the excitation wavelength was 488 nm. After the specific binding FITC-labeled antibodies and human peripheral blood CK19 or CD227-positive cells, the fluorescence excited by laser irradiation were accurately counted and measured.

The CellQuest software in the instrument was used to obtain 2×10^6–4×10^6 cells, data of the experimental tube and the control tube were collected and compared using the single parameter histograms. The horizontal axis was the logarithm of the fluorescence intensity relative value, the unit was Dao, the vertical axis represented the frequency of appeared cells, which was the relatively number of cells.

Ethics

The study was approved by the Local Ethical Committee to collect thyroid cancer patients tissues and all the patients gave informed consent to the research.

Statistical analysis

The statistical analysis was performed using SPSS 17.0 software (SPSS Inc.: Chicago, IL, USA), data of the two groups were compared using Fisher's exact test or χ^2 analysis. The correlation analysis was performed using *t* test.

Results

CK19, MUC1 positive cells

In peripheral blood samples of 491 thyroid cancer patients, 174 cases had the CK19-positive (35.4%), 161 cases had the MUC1-positive (32.8%). In peripheral blood samples of 376 thyroid nodular goiter patients, 29 cases had the CK19-positive (7.7%), 13 cases had MUC1-positive (3.5%). The CK19 positive rates in peripheral blood samples showed the statistically significant difference between thyroid cancer patients and thyroid nodular goiter patients ($\chi^2 = 91.28$, $P = 0.00$). The difference of MUC1 positive rates between the two groups was also statistically significant ($\chi^2 = 114.21$, $P = 0.00$; Fig. 1 and 2).

Association between CK19, MUC1-positive cells and gender, age in thyroid cancer patients

Difference of CK19, MUC1-positive cells and gender, age in thyroid cancer patients was not statistically significant (Table 1).

Association between CK19, MUC1-positive expression and clinical and pathological characteristics in thyroid cancer patients

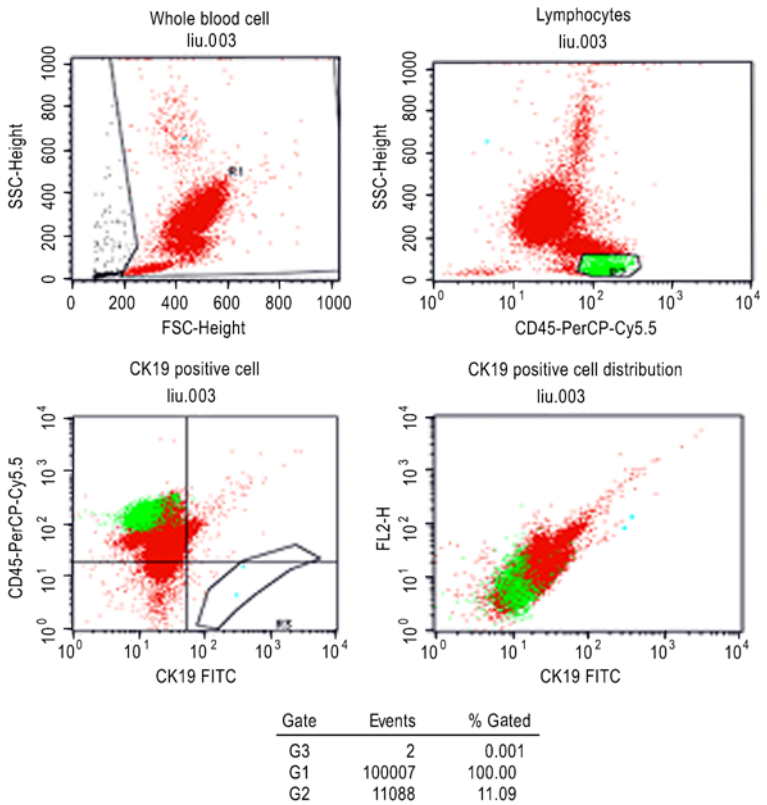
In thyroid cancer patients, difference between CK19, MUC1-positive expression and tumor size, thyroid capsule invasion, lymph node staging and distant metastasis were statistically significant (Table 2).

Correlation between CK19 and MUC1 positive expression in thyroid cancer patients

In peripheral blood samples of thyroid cancer patients, 146 cases had both CK19 and MUC1 positive (29.7%), 302 cases had both CK19 and MUC1 negative (61.5%), CK19 and MUC1 expression was significantly correlated ($r = 0.628$, $P = 0.00$).

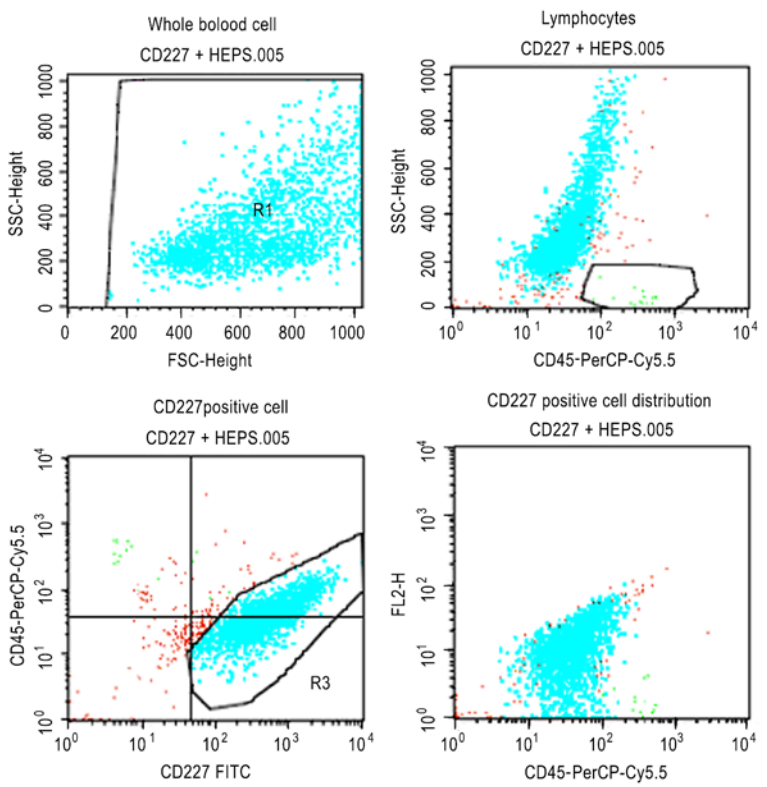
Discussion

In recent years, the role of circulating tumor cells (CTC) in cancer metastasis has received growing concern. CTC is currently defined as tumor cells released from metastases from solid tumors into the peripheral blood circulating spontaneously or because of medical operations. The vast majority of tumor cells into the circulation was dead in the short term, only a very small number of tumor cells with highly dynamic and highly metastatic potential can survive in the circulatory system, they aggregated to form tiny tumor thrombus, and under certain conditions they developed into metastases. Cytokeratin 19 (CK19) is a low molecular weight keratin, it existed in epithelial cells and did not express in mesenchymal tissues, it is a specific marker for micrometastasis detection for epithelial originated tumors [3]. Polymorphic epithelial mucin 1



Gate	Events	% Gated
G3	2	0.001
G1	100007	100.00
G2	11088	11.09

Fig. 1 Detection and analysis of peripheral blood CK19 positive cells



Gate	Events	% Gated
G3	2027	87.71
G2	16	0.69
G1	2311	100.00

Fig. 2 Detection and analysis of peripheral blood MUC1 positive cells

Table 1 Association between CK19, MUC1-positive cells and gender, age in thyroid cancer patients

Indices	CK19		χ^2	P	MUC1		χ^2	P	CK19 & MUC1		χ^2	P
	+	–			+	–			+	Single +/–		
Gender												
Male	40	77	0.02	0.91	37	80	0.09	0.82	32	85	0.42	0.56
Female	134	240			124	250			114	260		
Age (years)												
< 45	102	202	1.24	0.29	97	207	0.28	0.62	90	214	0.01	1.00
≥ 45	72	115			64	123			56	131		

Table 2 Association between CK19, MUC1-positive expression and clinical and pathological characteristics in thyroid cancer patients

Indices	CK19		χ^2	P	MUC1		χ^2	P	CK19 & MUC1		χ^2	P
	+	–			+	–			+	Single +/–		
The size of the tumor (cm)												
≤ 2	27	101			23	105			15	113		
2–4	91	176	33.72	0.00	87	180	30.78	0.00	81	186	42.88	0.00
> 4	56	40			51	45			50	46		
Tumor invasion												
Non-capsule	71	251	73.29	0.00	66	256	64.15	0.00	60	262	55.19	0.00
Capsule	103	66			95	74			86	83		
Lymph node staging												
N0	42	144	21.63	0.00	36	150	24.53	0.00	30	156	26.53	0.00
N1	132	173			125	180			116	189		
Distant metastasis												
M0	138	305	36.39	0.00	127	315	34.94	0.00	115	328	30.92	0.00
M1	36	12			34	14			31	17		

(MUC1) is a high molecular weight glycoprotein, it can inhibit the adhesion among tumor cells, its abnormal expression often indicated tumor invasion and metastasis [4]. It has been reported that MUC1 had varying degrees of abnormal expression in thyroid carcinoma, its sensitivity and specificity to distinguish benign and malignant thyroid tissues were 89% and 53%, the increased or abnormal MUC1 expression often suggested tumor invasion and metastasis [5].

After radical surgery of differentiated thyroid cancer, if there was the presence of cancer cells in the outer periphery circulating blood, the treatment programs should be replaced or the intervention should be given early to make the patients benefit. Currently the use of lymph node micrometastasis detection to guide the surgical treatment of thyroid cancer has been used clinically [6]. However, there is no effective biological indicator to detect and evaluate early distant organ metastasis of thyroid cancer. In this study, we used CK19 and MUC1 monoclonal antibody to label tumor cells in peripheral blood, FCM technique was used for joint detection of the two kinds of antibodies, the two test results showed differences and complements for each other to make the results of tumor cells detection in peripheral blood more accurate, it can also help to improve the detection sensitivity and specificity. The results showed that in peripheral blood of nodular goiter patients, 29 cases had CK19-positive (7.7%), 13 cases had MUC1-positive (3.5%), there was the significant difference compared with thyroid cancer patients (P = 0.00). In peripheral blood of thyroid cancer patients, 146 cases had both CK19 and MUC1 positive (29.7%), in peripheral blood of nodular goiter patients, both CK19 and MUC1-positive cases were not detected, it indicated that the combining the two antibody detection helped to improve specificity. This paper displayed that in thyroid cancer patients, CK19 and MUC1 expression was significantly positively correlated (r = 1.000, P = 0.00), the difference between tumor size, thyroid capsule invasion, the lymph node and distant metastasis staging was significant (P = 0.00). In 96 cases of thyroid cancer patients with tumor diameter of more than 4 cm, 50 cases had both CK19 and MUC1 positive (52.1%). With the increase of the tumor and the tumor load, the pressure increased within the tumor, it resulted in tumor cell outward spill and the chance of metastasis increased. In 169 cases of thyroid cancer patients with capsular invasion, 86 cases had the both CK19 and MUC1 positive (50.9%), the invasion of thyroid cancer tissue capsular membrane was one of the risk factors for metastasis, these patients were prone to lymph nodes and blood metastasis [7]. After the invasion of thyroid capsule, the outer periphery microvascular invasion appeared with the growth of tumor. The probability of tumor cells into the tiny blood vessels increased, the possibility of peripheral blood transferring randomly

increased. In this study, 305 cases had the lymph node metastases, 116 cases had both CK19 and MUC1 positive (38%), in 48 cases of distant metastasis (M1) patients, 31 cases had the both CK19 and MUC1 positive (64.6%), the difference was statistically significant ($P < 0.01$). Distant metastases were transferred through blood, the patients with free circulating tumor cells were certainly more than the patients with lymph node metastasis. It indicated that CTC positive was closely associated with distant metastasis, it further confirmed the important role of CTC in metastatic lesion formation. Some researchers thought that CTC can be used as favorable window to explore the biological behavior of solid tumors, it was expected to become a potential screening indicator of tumor metastasis, it was also an independent prognostic indicator, its value was better than tumor grade and stage [8–9]. The presence of circulating tumor cells in blood did not necessarily mean the formation of solid metastatic tumors, because the ultimate formation of metastases was associated with many factors including the immune status, tumor vasculature generation and biology of tumor itself. However, the release of tumor cells into the peripheral blood circulation was a prerequisite for tumor metastasis, the presence of CTC indicated the existence of possible hematogenous in a certain number of patients. In this paper, 31 cases out of 48 distant metastasis cases had tumor cells in the circulating blood (64.6%). Therefore, if the tumor cells in the peripheral blood were detected, the early stage intervene of tumor metastasis will effectively decrease the potential micrometastases, it will be of great help to improve the survival rate of cancer patients undoubtedly.

In short, the peripheral detection of CTC has the high feasibility and repeatability as a non-invasive diagnostic method, its role in the treatment and prognosis of thyroid cancer metastasis is obvious. As one blood test, it can capture and evaluate circulating tumor cells to determine the prognosis. Meanwhile, as the detection technology continues to improve, the sensitivity and specificity continues to increase, CTC detection will be used widely in the diagnosis and treatment of thyroid cancer.

Conflicts of interest

The authors indicated no potential conflicts of interest.

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DOI 10.1007/s10330-314-0009-5
Cite this article as: Ni YQ, Liu QJ, Tian YX. Clinical value of cancer cells joint detection in peripheral blood plasma of thyroid cancer patients. Chinese-German J Clin Oncol, 2014, 13: 518–522.