Original Paper

Circulating Tumor Cells Correlate with Clinicopathological Features and Outcomes in Differentiated Thyroid Cancer

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Key Words

Circulating tumor cells • Differentiated thyroid cancer • Refractory to radioiodine • Distant metastases • NE-iFISH

Abstract

Background/Aims: As biomarkers, circulating tumor cells (CTCs) from solid tumors can predict metastases and prognoses, and help monitor treatment efficacy. However, conventional CellSearch methods have low sensitivity to differentiated thyroid cancer (DTC) CTCs. In this study, for the first time, we used negative enriching (NE) immunofluorescence-in situ hybridization (iFISH) of chromosome 8 to capture and identify CTCs in DTC patients; and investigated how CTCs correlate with clinicopathological factors and prognosis in DTC patients with distant metastases (DM). **Methods:** In this prospective study, we enrolled 72 patients with DTC before they underwent ^{131}I treatment, and 30 healthy controls (HC). Their CTCs were measured in 7.5 ml peripheral blood using the NE-iFISH technique. CTC was defined by the aneuploidy. **Results:** We detected CTCs in 62 (86.1%) of the 72 subjects with DTC. The mean number of CTCs in patients with DTC with DM (DM⁺) was significantly higher than in the HC group ($P < 0.001$) and DTC patients without DM (DM⁻; $P = 0.0016$). We found CTCs ≥ 5 was significantly associated with DM⁺ DTC ($P = 0.009$; sensitivity: 64.3%; specificity: 83.8%); CTCs ≥ 7 was related to poor response to ^{131}I treatment (sensitivity: 73.7 %; specificity: 69.6 %), and was also associated with worse prognosis in DM⁺ DTC ($P < 0.001$). **Conclusion:** We found CTCs ≥ 5 to be a potential predictive index for DM⁺ DTC; and CTCs ≥ 7 as a possible indicator of poor response to ^{131}I treatment and worse prognosis in DM⁺ DTC.

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Introduction

Although the incidence of differentiated thyroid cancer (DTC) has increased worldwide, it has an excellent prognosis, with a ~90% 10-year overall survival rate [1]. However, for DTC patients with distant metastases (DMs), quality of life and survival time are seriously diminished [2, 3]. DTC with DM is commonly treated with radioiodine (^{131}I), but about 30% of such patients have radioiodine-refractory DTC (RR-DTC) [4], which has a 10-year survival rate of only ~10% from DM detection [5]. Obviously, patients with RR-DTC do not significantly benefit from ^{131}I treatment, and may suffer adverse effects from high serum TSH stimulation after thyroxine withdrawal [6]. Although detection of serum thyroglobulin (Tg) levels was a reliable predictor of DTC DMs, this method is limited by metastatic lesions' ability to release detectable amounts of Tg, loss of the ability of secreting Tg with preserved capability of ^{131}I trapping, structural changes in Tg, or Tg level decreased by elevated circulating Tg antibodies (TgAb) [7, 8]. Also, Tg levels do not predict RR-DTC [9]. Therefore, tumor markers that can help detect DTC DMs and predict RR-DTC are sorely needed.

Circulating tumor cells (CTCs) are rare tumor cells that escape from solid tumors, travel into peripheral blood circulation, and seed DMs. They have been widely used for early DM detection, monitoring prognoses and treatment efficacy, and risk stratification for some solid tumors [10, 11]. The CellSearch system is the most widely used assay for CTCs, and depends on expression of CTC-surface epithelial cell adhesion molecule (EpCAM) [12]. However, the CellSearch system has a low detection rate for DTC CTCs, probably due to their low EpCAM expression during epithelial-mesenchymal transition (EMT) [13]. Negative enrichment (NE) combined with immunofluorescence and in situ chromosomal hybridization (NE-iFISH) is reportedly effective in detecting CTCs from gastric or pancreatic cancers [14] [15], but its use in detecting CTCs from metastatic DTC has not been reported.

We hypothesized that CTCs in DTC patients are associated with DMs, response to ^{131}I treatment, and prognosis. In this study, we detected the numbers of CTCs in DTC patients using the NE-iFISH and explored whether CTC levels were related to clinicopathological factors or outcomes in patients with DM⁺ DTC.

Materials and Methods

Ethical approval

This study was performed in accordance with the ethical standards of our hospital.

Informed consent

Informed consents were obtained from all individual participants was used in the study.

Patients

The study was performed at the Shanghai Sixth People's Hospital, a major ^{131}I treatment center in China, and was approved by its ethics committee. We enrolled 42 patients with DM⁺ DTC treated between August 2014 and August 2017, and 30 patients with DM⁻ DTC patients treated during August 2017. The inclusion criteria were (a) patients with histologically proven DTC (follicular thyroid cancer [FTC] or papillary thyroid cancer [PTC]); and (b) treatment with total or near-total thyroidectomy and postoperative ^{131}I ablation. Patients with histories of other malignancies or other adjuvant therapy were excluded. The DTC patients were classified as having intermediate high risk [16]. We also included 30 healthy volunteers, with no illness or past cancer history, as a healthy control (HC) group. All participants gave informed written consent.

Baseline data were recorded when peripheral blood samples were collected for CTC detection, including age, sex, maximum tumor diameter, DTC pathology, extrathyroidal invasion, numbers of primary tumors, N stage, serum stimulated thyroglobulin (sTg), and TgAb (Table 1); after each ^{131}I therapy and during the follow-up, results of sTg, TgAb, ^{131}I whole-body scan (^{131}I -WBS) combined with SPEC/CT, other medical imaging such as CT and ^{18}F -FDG PET/CT, courses ^{131}I treatment, cumulative ^{131}I activity, sites and numbers of DMs and follow-up time were recorded.

Table 1. Patient demographics. Tumor size (mm)*: maximum diameter of tumor; DTC: differentiated thyroid cancer; FTC: follicular thyroid carcinoma; PTC: papillary thyroid carcinoma; DM: distant metastases

Characteristic	NO. of patients			Healthy control (N=30)
	All (N=72)	DTC without DM (N=30)	DTC with DM (N=42)	
Sex				
Male	31	14	17	11
Female	41	16	25	19
Mean age at baseline (range)	44.4(12-71)	46.1 (16-68)	43.2 (12-71)	44.5(23-71)
Pathological of DTC				
PTC	61	28	33	-
FTC	11	2	9	-
Extrathyroidal invasion				
Yes	31	9	22	-
No	41	21	20	-
Multifocality				
1	51	20	31	-
>1	21	10	11	-
Tumor size(mm)*				
≤30	43	27	16	-
>30	29	3	26	-
N stage				
N0	19	9	10	-
N1a	15	10	5	-
N1b	22	8	14	-
Nx	16	3	13	-
Mean sTg level prior to ¹³¹ I Treatment, ng/ml(range)	1431 (1.3-17221)	59 (1.3-532.6)	2412 (41.4-17221)	-
Mean TgAb level prior to ¹³¹ I Treatment, IU/ml(range)	37(10-231)	14(10-31)	54(10-231)	-

Diagnostic criteria for DTC DMs

Diagnosis of DMs from DTC depended on Tg, TgAb under T4 withdrawal for 2–3 weeks, ¹³¹I-WBS combined with SPECT/CT after ¹³¹I therapy, ¹⁸F-FDG PET/CT, CT, magnetic resonance imaging (MRI) or fine needle biopsy results; and was based on the following three criteria: (a) pathological results showed DMs; (b) ¹³¹I-WBS demonstrated distant lesions with high ¹³¹I uptake, with at least one imaging method (CT, MRI, or ¹³¹I-SPECT/CT) showing DMs; or (c) ¹³¹I-WBS reveals no ¹³¹I uptake, but the ¹⁸F-FDG-PET/CT scans are positive for DM lesions with elevated serum Tg.

¹³¹I empiric treatment

Each patient stopped taking L-T4 and undertook a low-iodine diet for 2–3 weeks before ¹³¹I therapy. Subsequently, the patients received oral ¹³¹I after routine measurements that included FT3, FT4, TSH, Tg, TgAb, neck ultrasonography (US), and CT scans. The first oral dose of 3.7 GBq (100 mCi) of ¹³¹I was administered to ablate thyroid remnants. Oral doses of ¹³¹I with standard activities of 5.55–7.4 GBq (150–200 mCi) were administered for subsequent treatment of DTC DMs. Patients underwent ¹³¹I-WBS combined with ¹³¹I-SPECT/CT imaging 3–5 days after ¹³¹I oral administration. Treatment intervals ranged from 4–6 months; treatments were repeated 1–6 times. Median follow-up period was 1.2 years (range: 0.2–3 years).

Definition of Radioiodine-Refractory DTC

According to the 2015 American Thyroid Association management guideline [16], radioiodine-refractory structurally evident DTC in patients with DMs who were under appropriate TSH stimulation and iodine preparation is classified by 4 mechanisms: (i) metastatic lesions do not ever uptake ¹³¹I after successful remnant thyroid ablation; (ii) tumor tissue loses the ability to concentrate ¹³¹I after previous evidence of RAI-avid disease (in the absence of stable iodine contamination), (iii) ¹³¹I is concentrated in some lesions but not in others; and (iv) metastatic disease progresses despite significant concentration of ¹³¹I in the course of ¹³¹I treatment.

Evaluation criteria for progression-free survival (PFS)

Progressive disease (PD), stable disease (SD), partial response (PR), and complete response (CR) to ¹³¹I treatment for DTC lung and bone metastases were evaluated based on the Response Evaluation Criteria in Solid Tumors (RECIST 1.1) [17] and MDA criteria [18] respectively.

RECIST 1.1: complete response (CR), disappearance of all target lesions, any pathological lesions (target or non-target) must have a reduction in short axis to <10 mm; partial response (PR), ≥30% decrease in the diameters of target lesions; progressive disease (PD), ≥20% increase in the diameters of target lesions, combined with an absolute increase of ≥5 mm in the sum of diameters (the appearance of one or more new lesions was also considered progression); stable disease (SD), neither sufficient shrinkage to qualify for PR nor sufficient increase to qualify for PD.

MDA criteria: 1) CR: complete sclerotic fill-in of lytic lesions on X-ray or CT; normalization of bone density on X-ray or CT; normalization of signal intensity on MRI. 2) PR: development of a sclerotic rim or partial sclerotic fill-in of lytic lesions on X-ray or CT; interval visualization of lesions with sclerotic rims or new sclerotic lesions in the setting of other signs of PR and absence of progressive bony disease; ≥50% decrease in measurable lesions on X-ray, CT, or MRI; ≥50% subjective decrease in the size of ill-defined lesions on X-ray, CT, or MRI. 3) SD: no change; <25% increase or <50% decrease in size of measurable lesions; <25% subjective increase or <50% decrease in size of ill-defined lesions; no new bone metastases. 4) PD: ≥25% increase in size of measurable lesions on X-ray, CT, or MRI; ≥25% subjective increase in the size of ill-defined lesions on X-ray, CT, or MRI; new bone metastases.

PFS was measured from the date of baseline blood sample to the date of confirmed clinical progression or death was censored at last follow-up.

Subtraction enrichment of CTCs

Enrichment of CTCs was performed according to NE-iFISH kit instruction (Majorbio, Shanghai, China) and according to a previously published protocol [14, 15]. In brief, peripheral blood samples (7.5 ml) were collected into ACD anticoagulant tubes when all patients ceased thyroid hormone medication and began low-iodine diets, more than 2–3 weeks before the first ¹³¹I therapy. The supernatant was discarded after centrifuging the tubes within 24 hours after sample collection. Samples were transferred to centrifuge tubes containing 3 ml of lymphocyte separation media (Majorbio). After centrifuging for 10 min at 450 g, the cell suspension was collected from the buffy-coat layer. Immunomagnetic particles conjugated anti-CD45 antibody (Majorbio) were added into the cell suspension, which was incubated at room temperature for 10 min and then placed on a magnetic stand (Majorbio) until the liquid became clear. The supernatant was pipetted off the magnetic field to remove leukocytes by centrifuging at 900 g for 5 min.

Identification of aneuploidy CTCs

The identification of CTCs was performed according to mi-FISH kit instruction (Majorbio). To identify aneuploid CTCs, fluorescence in situ hybridization (FISH) and immunocytochemistry were used in combination. The supernatant was rinsed with wash buffer, and then monoclonal antibody anti-CD45 conjugated to Alexa Fluor 594 (Majorbio) and anti-anti-EpCAM Antibody conjugated to Alexa Fluor 488 (Majorbio) were added before incubation at room temperature for 30 min. The supernatant was rinsed with wash buffer. To the obtained cellular precipitation, we immediately added cell fixatives-1 (Majorbio) before making smears. The cell smears were dried at 32 °C overnight. We added 200 µl of cell fixatives-2 (Majorbio) to the cell smears and let stand for 10 min. After rinsing with wash buffer, the mixture was put into in a 100% alcohol after washing and let stand for 1 min. After the slide was air-dried, 10 µl of probe solution containing fluorescence-labeled alpha-satellite probes for Centromere Probe 8 (CEP8) SpectrumOrange (Vysis, Abbott Laboratories, Abbott Park, IL, USA) was added and then covered with a coverslip and sealed with neutral resin (rubber). The hybridization procedure was as follows: degeneration at 75 °C for 5 min, followed by hybridization at 37 °C for 4 hours. After rinsing with PBS, the slides were mounted with mounting medium containing DAPI and photographed with a fluorescence microscope (Zeiss, German). CTCs were confirmed to be negative for CD45 and either positive for EpCAM staining or aneuploidy chromosome 8. CTCs were defined as EpCAM+/CD45-/DAPI+/CEP8≥2 or EpCAM-/CD45-/DAPI+/CEP8>2, whereas EpCAM-/CD45+/DAPI+/CEP8=2 was defined as white blood cells (WBCs) [14, 15]. All CTC numbers in this paper are the numbers of CTCs counted in 7.5-ml samples of subjects' peripheral venous blood.

Statistical analysis

Student's *t*-test was used to test for significant differences in numbers of CTCs among the DM⁺ DTC, DM⁻ DTC and HC groups. Receiver operating characteristic (ROC) curves were constructed, and area under curve (AUC) was calculated to evaluate specificity and sensitivity of predicting DMs from DTC or RR-DTC. The chi-square test or Fisher's exact test and multivariate logistic regression analyses were used to evaluate CTC numbers with respect to clinicopathological factors. The Kaplan-Meier method was used for PFS analysis; differences in PFS were examined using the log-rank test. Multivariate analysis was performed to assess relationships between PFS and several variables simultaneously using Cox's proportional hazards model. *P* < 0.05 was considered significant. MedCalc software version 17.0 (MedCalc, Mariakerke, Belgium) and SPSS17.0 (SPSS Inc., Chicago, IL, USA) were used for statistical analyses.

Results

Distribution of CTC counts in each classification

The CTC counts for the HC, DM⁻ DTC and DM⁺ DTC groups are shown in Fig. 1 and Fig. 2a. The CTCs were defined as EpCAM⁺/CD45⁻/DAPI⁺/CEP8 ≥ 2, which weren't be detected in the 7.5ml blood. The numbers of detected CTCs (per 7.5 ml blood) in the DTC patients were median: 4; range: 0–72; mean: 7.31, total: 527; and were found in 62 of the 72 patients (86.1%). In the DM⁺ DTC group, CTCs were detected in 92.9% (39/42) of patients (mean: 10.69; range: 0–72). In the DM⁻ DTC group, CTCs were in 76.7% (23/30) of patients (mean: 2.6; range: 0–10). In the HC group, CTCs were found in 26.7% (8/30) of subjects (mean: 0.4; range: 1–3, including 1 CTC: *n*=5; 2 CTCs: *n*=1; 3 CTCs: *n*= 2). Mean CTC numbers were significantly higher in the DM⁺ DTC than in the HC

Fig. 1. Identification of CTCs in patients with differentiated thyroid cancer (DTC) by NE-iFISH assay. CTCs were defined as EpCAM⁺/CD45⁻/DAPI⁺/CEP8 ≥ 2; white blood cells (WBCs) were defined as EpCAM⁻/CD45⁺/DAPI⁺/CEP8 = 2. a: EpCAM⁻/CD45⁻/DAPI⁺/CEP8 = 3 (white dotted arrow); b: EpCAM⁻/CD45⁻/DAPI⁺/CEP8 = 4 (white dotted arrow); c: EpCAM⁻/CD45⁻/DAPI⁺/CEP8 = 5 (white dotted arrow), EpCAM⁻/CD45⁺/DAPI⁺/CEP8 = 2 (white solid arrow); d: EpCAM⁺/CD45⁻/DAPI⁺/CEP8 ≥ 5 (white dotted arrow).

Fig. 2. a: CTC count in healthy controls (A), patients with differentiated thyroid cancer (DTC) without distant metastases (B) and DTC patients with distant metastases (C). b: CTC count in RR-DTC patients (A) and non-RR-DTC patients (B). c: ROC curve shows sensitivity and specificity at 4.5 CTCs in 7.5 ml of blood in predicting distant metastases in patients with DTC. d: ROC curve shows sensitivity and specificity at 6.5 CTCs in 7.5 ml of blood in predicting distant metastases in RR-DTC patients.

group ($P < 0.001$) and DM⁻ DTC group ($P = 0.0016$); and higher in the DM⁻ DTC group than in the HC group ($P < 0.001$).

Correlation of CTCs with clinicopathological characteristics between DM⁺ DTC and DM⁻ DTC groups

We plotted a ROC curve to compare the sensitivity and specificity of CTC numbers with respect to DM⁺ DTC and DM⁻ DTC, using 3.5 CTCs (71.4% sensitive; 73.3% specific), 4.5 CTCs (64.3% sensitive; 83.8% specific) and 5.5 CTCs (61.9% sensitive; 90% specific) as possible cut-off values (Youden's index). We therefore defined the cutoff at 4.5 CTCs (AUC: 0.789; 95% CI: 0.685–0.893; Fig. 2c). In the DM⁺ DTC group, 27 (64.3%) had more, and 15 had fewer, than 4.5 CTCs, whereas in the DM⁻ DTC group, 5 (16.7%) had more CTCs and 25 had fewer ($P < 0.001$). Therefore, CTCs ≥ 5 was considered predictive of DMs.

Patient age at baseline, sex, DTC pathology, tumor size, extrathyroidal invasion, multifocality and N stage were not significantly associated with CTCs ≥ 5 . In univariate regression, CTCs ≥ 5 was significantly associated with sTg > 200 ng/ml (odds ratio [OR]: 3.667, 95% CI: 1.370–9.810) and DM⁺ DTC (OR: 0.111, 95% CI: 0.035–0.351) in binary logistic regression, only DM⁺ DTC was independently associated with CTCs ≥ 5 ($P = 0.009$; OR compared with DM⁻ DTC: 0.028 (95% CI: 0.002–0.403, Table 2).

Table 2. Comparison of Clinical and Pathologic Factors between DTC patients with CTCs $\geq 5/7.5$ ml and CTCs $< 5/7.5$ ml blood using the Univariate and Multivariate Analysis. Tumor size (mm): maximum diameter of tumor; DTC: differentiated thyroid cancer; DM: distant metastases

Variations	All	No. of patients CTCs ≥ 5	CTCs < 5	Univariate analysis OR(95%CI)	P	Multivariate analysis OR(95%CI)	P
Sex					0.287		0.193
Male	41	16	25	1		1	
Female	31	16	15	1.667[0.649-4.281]		2.223[0.667-7.405]	
Age at baseline					0.289		0.992
< 45 y	32	12	20	1		1	
≥ 45 y	40	20	20	1.667[0.647-4.296]		1.011[0.304-3.331]	
Pathological of DTC					0.464		0.701
FTC	61	26	35	1		1	
PTC	11	6	5	1.615[0.444-5.873]		1.415[0.243-8.239]	
Extrathyroidal invasion					0.915		0.359
No	41	18	23	1		1	
Yes	31	14	17	1.052[0.412-2.690]		1.747[0.530-5.754]	
Multifocality					0.584		0.594
1	54	23	31	1		1	
> 1	18	9	9	1.348[0.462-3.924]		1.453[0.369-5.720]	
Tumor size(mm)					0.043		0.485
≤ 30	41	14	27	1		1	
> 30	31	18	13	2.670[1.020-6.989]		1.585[0.435-5.773]	
N stage					0.792		0.804
N1	37	17	20	1		1	
N0,x	35	15	20	0.882[0.348-2.238]		0.842[0.217-3.266]	
sTg (ng/ml)					0.008		0.277
≤ 200	35	10	25	1		1	
> 200	37	22	15	3.667[1.370-9.810]		4.278[0.311-58.86]	
DTC with DM					< 0.001		0.009
Yes	42	27	15	1		1	
No	30	5	25	0.111[0.035-0.351]		0.028[0.002-0.403]	

Correlation of CTCs with RR-DTC in DTC patients

Clinical characteristics of the DM⁺ DTC group ($n=42$) are shown in Table 3 and Table 4. Their median age was 43.2 years (range 12–71 years); they included 17 males and 25 females; 33 had PTC and 11 had FTC; 5 had only bone metastases, 24 had only lung metastases, 11 had synchronous bone and lung metastases; 1 had synchronous bone and brain metastases, and 1 had synchronous bone and kidney metastases.

We classified 19 DM⁺ DTC patients as RR-DTC and 23 as non-RR-DTC (2015 ATA guidelines). Mean numbers of CTCs in the RR-DTC group (16.8) were significantly higher than in the non-RR-DTC group (5.60; $P=0.0049$; Fig. 2b).

To determine the CTC count cutoff that would be most indicative of RR-DTC, we tested 4.5 CTCs (sensitivity: 84.2%; specificity: 52.2%), 5.5 CTCs (sensitivity: 78.9%; specificity: 52.2%) and 6.5 CTCs (sensitivity: 73.7%; specificity: 69.6%), and finally used 6.5 CTCs as the cut-off number (AUC=0.781; 95%CI 0.635–0.928; Fig. 2d). In the DM⁺ RR-DTC group ($n=19$), 14 (73.6%) patients had more CTCs and 5 had fewer than the 6.5 CTC cut-off; whereas in the DM⁻ non-RR-DTC group ($n=23$), 7 (30.4%) had more CTCs and 16 had fewer than the cut-off ($P<0.001$). Therefore, CTCs ≥ 7 was considered indicative of RR-DTC.

Univariate analysis of factors considered likely to be associated with RR-DTC (or specifically, CTCs ≥ 7) are shown in Table 4. Only RR-DTC was significantly associated with CTCs ≥ 7 ($P=0.005$; OR compared with non-RR-DTC: 0.156, 95% CI: 0.040–0.605). In multivariate logistic regression analysis, patient age at baseline, sex, DTC pathology, tumor

Table 3. Clinical Data for Each Patient with Distant Metastases from Differentiated Thyroid Cancer. Age *: age at baseline; F: female; M: male, sTg: Stimulated thyroglobulin; FTC: follicular thyroid carcinoma; PTC: papillary thyroid carcinoma; Lungs: multiple lung metastases; bones: multiple bone metastases

Patient number	Sex	Age* (years)	Diameter _{max} of primary tumor (cm)	Pathology	Sites of DM	sTg (ng/ml)	No. of CTCs	Courses ¹³¹ I treatment (n)	Cumulative ¹³¹ I activity	Refractory to radioiodine
1	F	32	4.8	PTC	Lungs	811.4	72	5	37.0	Yes
2	M	25	3.9	PTC	Lungs	883	37	2	14.8	Yes
3	F	27	3.2	PTC	Lungs, bone	981	33	2	12.95	Yes
4	F	56	3.5	PTC	Bones, brain	458.4	30	3	22.2	Yes
5	M	28	7.0	PTC	Lungs	369.7	28	2	11.1	Yes
6	M	23	6.1	PTC	Lungs, bones	808	23	6	44.4	No
7	M	59	4.5	FTC	Bones	574	18	2	7.4	Yes
8	M	62	1.1	PTC	Bones	3659	17	2	12.95	No
9	M	36	2.1	PTC	Lungs	807	15	3	22.2	Yes
10	F	59	3.2	FTC	Bones	519.5	13	2	7.4	Yes
11	M	64	3.7	PTC	Lungs	1141	11	1	7.4	Yes
12	F	45	4.0	FTC	Lungs, Bone	17221	11	2	11.1	Yes
13	M	42	2.8	FTC	Lungs	571.2	10	4	20.35	Yes
14	F	43	3.2	PTC	Lungs	268	10	3	18.5	No
15	M	71	2.1	PTC	Lungs, bones	3023	9	1	14.8	Yes
16	M	31	4.2	PTC	Lungs, bones	532.6	9	2	12.95	Yes
17	F	34	2.5	PTC	Lungs	118	9	4	25.9	No
18	F	53	4.0	PTC	Lungs	215.2	7	1	7.4	Yes
19	F	66	4.5	PTC	Lungs	415	7	2	11.1	No
20	M	54	2.4	PTC	Lungs	85	7	1	7.4	No
21	F	12	1.8	PTC	Lungs	41.4	7	2	11.1	No
22	M	41	1.8	PTC	Lungs	127	6	3	22.2	No
23	M	29	3.2	PTC	Lungs	1303	6	2	12.95	Yes
24	F	22	5.0	PTC	Lungs	1523	6	5	35.15	No
25	F	69	3.8	PTC	Lungs, bones	12785	6	3	22.2	No
26	F	62	4.3	FTC	Lungs, bones	1578	6	2	14.8	No
27	F	29	4.0	FTC	Lungs	110	5	5	25.9	Yes
28	F	33	2.2	PTC	Lungs	4186	4	3	16.65	No
29	F	66	2.8	PTC	Lungs, bones	9676	4	3	22.2	Yes
30	M	39	4.7	PTC	Lungs	15060	4	1	7.4	No
31	F	45	3.2	FTC	Lungs	917	3	2	14.8	No
32	F	36	2.5	PTC	Lungs	4673	3	3	22.2	No
33	F	33	3.2	PTC	Lungs, bone	724	3	2	12.95	No
34	F	23	2.3	PTC	Lungs	899.5	2	3	22.2	Yes
35	M	14	3.0	PTC	Lungs	5100	2	4	20.35	No
36	F	28	2.5	PTC	Lungs, bone	105	2	3	22.2	No
37	F	63	5.0	FTC	bones kidney	2311	2	6	44.4	No
38	F	37	3.0	PTC	bones	3493	1	6	44.4	No
39	F	65	3.4	FTC	bones	611.7	1	3	22.2	No
40	M	20	4.5	PTC	Lungs, bones	1397	0	3	18.5	Yes
41	F	60	1.1	PTC	Lungs	740	0	4	27.75	No
42	M	58	3.2	PTC	Lungs	497	0	3	16.65	No

Table 4. Comparison of Clinical and Pathologic Factors between Distant Metastatic DTC Patients with CTCs $\geq$ 7/7.5ml and CTCs $<$ 7/7.5ml using the Univariate and Multivariate Analysis

Variations	All	NO of patients CTCs $\geq$ 7	CTCs $<$ 7	Univariate analysis OR(95%CI)	P	Multivariate analysis OR(95%CI)	P
Sex					0.116		0.107
Female	25	15	10	1		1	
Male	17	6	11	0.364[0.101-1.303]		0.183[0.023-1.439]	
Age at baseline					0.533		0.181
<45 y	24	11	13	1		1	
$\geq$ 45 y	18	10	8	1.477[0.432-5.047]		4.161[0.516-33.528]	
Pathological of DTC					0.707		0.219
PTC	33	17	16	1		1	
FTC	9	4	5	0.753[0.171-3.312]		0.155[0.008-3.027]	
Extrathyroidal invasion					0.217		0.105
Yes	22	13	9	1		1	
No	20	8	12	2.167[0.631-7.442]		6.361[0.678-59.712]	
Multifocality					0.726		0.058
>1	11	5	6	1		1	
1	31	16	15	0.280[0.322-5.088]		0.100[0.009-1.082]	
Tumor size(cm)					0.525		0.420
>3	26	14	12	1		1	
$\leq$ 3	16	7	9	0.677[0.190-2.334]		0.472[0.076-2.928]	
Site of metastases					0.739		0.485
Lung, bone and/or other distant metastases	13	6	7	1		1	
Lung or bone only	29	15	14	1.121[0.565-2.222]		2.216[0.238-20.674]	
Discovery of metastases					0.634		0.686
Delayed distant metastases	37	18	19	1		1	
At presentation	5	3	2	1.583[0.236-10.605]		2.549[0.027-237.397]	
sTg (ng/ml)					0.172		0.044
>500	30	17	13	1		1	
$\leq$ 500	12	4	8	0.382[0.094-1.552]		0.087[0.008-0.932]	
Refractory to radioiodine					0.005		0.007
Yes	19	14	5	1		1	
No	23	7	16	0.156[0.040-0.605]		0.0451[0.0047-0.4345]	

size, extrathyroidal invasion, multifocality, site of metastases, and presence of DM at diagnosis were not associated with CTCs $\geq$ 7; whereas CTCs $\geq$ 7 was associated with RR-DTC ($P=0.007$; OR: 0.0451, 95% CI: 0.0047–0.4345) and sTg level $\leq$ 500 ng/ml ($P=0.044$; OR: 0.087, 95% CI: 0.008–0.932; Table 4).

CTCs correlate with PFS

Three-year PFS rates and prognostic factors in the DM⁺ DTC group (n=42) are listed in Table 5 and Table 6. All 42 DM⁺ DTC patients had completed at least one course of 131I therapy. At the time of analysis, 19 (45.2%) of the 42 patients had PD, 2 achieved CR (4.8%), 4 PR (9.5%), 17 (4.0%) SD. Median PFS was 1.2 years (range: 0.2–3 years). At the time of follow-up, 4 patients had died of metastatic lesions, and the remaining 38 patients were alive.

In univariate analysis, CTCs $\geq$ 7 and RR-DTC were strong predictors of worse 3-year PFS in all DM⁺ DTC patients compared with CTCs $<$ 7 and non-RR-DTC. In this study, the 3-year PFS rates were CTCs $\geq$ 7: 33.3%; CTCs $<$ 7: 76.2% (Fig. 3a); non-RR-DTC group: 78.3%; RR-DTC group: 26.3% (Fig. 3b). In multivariate Cox regression analyses, PFS was significantly associated with CTCs $\geq$ 7 (relative risk [RR]: 5.108, 95% CI: 1.334–19.550), sTg level $\leq$ 500

Table 5. Univariate Analysis of Significant Prognostic Factors Influencing Progression-Free Survival (PFS) in 42 Patients with Differentiated Thyroid Cancer and Distant Metastases. PD*: Progressive disease

Variable	No. of patients	No. of PD*	3-year PFS rate (%)	Log-rank value	P
No. of CTCs (cell/7.5ml)				6.271	0.01
≥7	21	14	33.33		
<7	21	5	76.19		
Sex				0.275	0.600
Female	25	11	56.00		
Male	17	8	52.94		
Age				0.595	0.441
<45 y	24	13	45.83		
≥45 y	18	6	66.67		
Pathological of DTC				0.015	0.903
PTC	33	15	54.55		
FTC	9	4	55.56		
Extrathyroidal invasion				0.669	0.413
Yes	22	8	63.64		
No	20	11	45.00		
Multifocality				0.121	0.782
>1	11	4	63.64		
1	31	15	51.61		
Tumor size(cm)				2.455	0.117
>3	26	13	50.00		
≤3	16	6	62.50		
Site of metastases				0.001	0.972
Lung, bone and/or other distant metastases	13	5	61.54		
Lung or bone only	29	14	51.72		
Discovery of metastases				0.228	0.634
Delayed distant metastases	37	17	54.05		
At presentation	5	2	60.00		
sTg (ng/ml)				1.859	0.173
>500	30	14	53.33		
≤500	12	5	58.33		
Refractory to radioiodine				12.97	<0.001
Yes	19	14	26.31		
No	23	5	78.26		

Table 6. Multivariate Analysis of Prognostic Factors of Progression-Free-Survival from 42 Differentiated Thyroid Cancer Patients with Distant Metastases Using Cox's Proportional Hazards Model

Variable	Risk ratio	95% CI	P
No. of CTCs (cell/7.5ml)			0.021
<7	1		
≥7	5.108	[1.334-19.550]	
Sex			0.400
Female	1		
Male	0.535	[0.125-2.280]	
Age			0.110
<45 y	1		
≥45 y	3.094	[0.779-12.282]	
Pathological of DTC			0.320
PTC	1		
FTC	0.320	[0.083-2.243]	
Extrathyroidal invasion			0.068
No	1		
Yes	4.725	[0.901-24.792]	
Multifocality			0.112
1	1		
>1	3.393	[0.758-15.189]	
Tumor size(cm)			0.902
≤3	1		
>3	1.091	[0.271-4.393]	
Site of metastases			0.555
Lung or bone only	1		
Lung, bone and/or other distant metastases	1.720	[0.287-10.328]	
Discovery of metastases			0.224
At presentation	1		
Delayed distant metastases	0.271	[0.033-2.196]	
sTg (ng/ml)			0.041
>500	1		
≤500	0.216	[0.050-0.936]	
Refractory to radioiodine			0.011
Yes	1		
No	0.093	[0.015-0.575]	

ng/ml (0.216, 95% CI: 0.050–0.936) and non-RR-DTC (RR: 0.093, 95% CI: 0.015–0.575).

Discussion

Circulating biomarkers can potentially provide early and minimally invasive diagnoses help assess response to therapy and predict prognosis of cancer patients. Studies on the utility of circulating biomarkers such as cell-free DNA, miRNA, and mRNA as novel biomarkers for various human diseases have increased exponentially in the last decade [6, 19]. Recent studies have shown CTCs to be prognostic and predictive biomarkers for several solid cancers [20, 21]. CTCs are malignant cells that have escaped from primary or metastatic tumor sites [22]. CellSearch is the only FDA-approved technology for CTC detection, which is based on detecting EpCAM expression [23, 24]. Few studies of the prognostic significance of CTCs from DTC are available. Nonetheless, Xu et al. found that only 1 of 14 patients with DM⁺ DTC had detectable CTCs using CellSearch [12]. Therefore, detecting CTCs in DTC patients requires other approaches.

In the present study, we applied a new capture technique (NE-iFISH) to detect DTC CTCs and to assess their relationships with clinicopathological factors, therapeutic repose to ¹³¹I and prognosis of DTC. Compared with conventional CellSearch, NE-iFISH has demonstrated high sensitivity for CTCs from other cancer types [25, 26].

NE-iFISH has several advantages. On one hand, it applies anti-multiple WBC markers antibodies to ensure both minimum hypotonic injuries to CTCs and maximal removal of WBCs; on the other hand, it can enrich CTCs independently of EpCAM expression and tumor size [27]. We were also able to combine CEP 8, EpCAM, CD45 and DAPI to detect CTCs. Aneuploidy, which includes both numerical and structural chromosomal abnormalities, is a hallmark of cancer, and might be exploited for CTC detection. Numerical abnormalities of CTCs can be divided into different subtypes, such as triploidy, tetraploidy and multiploidy. Previous studies have shown that CEP 8 reflects numerical chromosomal abnormalities, which have been reported in thyroid cancer, and lung, esophageal, gastric and colon cancers [27–29]. Thus, aneuploidy of CEP 8 examined by CEP8-iFISH can be used to detect CTCs. In our study, of 72 DTC patients, EpCAM⁺/CD45[−]/DAPI⁺/CEP8^{>2} cells were detected in 86.11% (62/72) patients (mean: 7.14; range: 0–72 CTCs) compared with the HC group, in whom only 26.7% (8/30) subjects had EpCAM⁺/CD45[−]/DAPI⁺/CEP8^{>2} cells, and none had more than 3 such cells. Obviously, DTC patients had significantly more CTCs than did the HC group. However, EpCAM⁺/CD45[−]/DAPI⁺/CEP8^{≥2} weren't detected in all DTC patients and HC people. Although EpCAM expression has reportedly been found in DTC specimens, those CTCs have not been successfully isolated by means of anti-EpCAM Ab. The reason may be that down-regulated membrane expression and up-regulated nuclear accumulation of EpCAM are associated with EMT during DTC progression and metastasis [13]. This is consistent with the observation of Xu et al. using CellSearch [13].

Patients with breast, prostate, or colorectal cancers with hematogenous metastases reportedly have high incidences of CTCs; CTCs ≥ 5 in 7.5 ml peripheral venous blood has been widely used as a cut-off to predict DMs from these cancers [23, 30, 31]. To predict DMs from DTC we plotted a ROC curve (Yourdon's index), which gave an optimal cutoff for CTCs at 4.5 (as measured by NE-iFISH) which was 64.3% sensitive and 83.8% specific for predicting DTC DMs. We therefore used CTCs ≥ 5 to predict DTC DMs, which is consistent with the above studies. Although we also found that CTCs ≥ 5 were associated with sTg level ≥ 200 ng/ml in univariate analysis, in multivariate analysis, only DM⁺ DTC was associated with CTCs ≥ 5 .

Fig. 3. a: Comparison of PFS curves at CTCs $\geq 7/7.5$ ml blood (dotted line) and CTCs $< 7/7.5$ ml blood (solid line) in patients with differentiated thyroid cancer (DTC) and distant metastases. b: Comparison of PFS curves between RR (dotted line) and non-RR (solid line) DTC patients with distant metastases.

For RR-DTC patients, ^{131}I therapy has very limited benefits. Such patients should not undergo unnecessary ^{131}I treatment, which would needlessly expose them to the risk of high serum TSH stimulation after thyroxine withdrawal. Identification of accurate predictive biomarkers is critical to develop novel ^{131}I therapeutic strategies. Specifically, noninvasive biomarkers to detect RR-DTC patients are urgently needed before ^{131}I therapy. However, circulating tumor biomarkers that can predict response to ^{131}I therapy in patients with DM⁺ DTC have not been available. Only our previous study has shown that four circulating long non-coding RNAs have potential utility as diagnostic markers for non- ^{131}I -avid lung metastases from DTC [6]. In a review of CTCs, only Winkens et al. reported that ^{131}I therapy could decrease the number of circulating epithelial cells in the DTC patients [32].

To our knowledge, the present study is the first in which CTCs have been analyzed or compared between non-RR-DTC and RR-DTC patients with DMs. We compared numbers of CTCs between non-RR-DTC and RR-DTC patients using ROC curves and observed whether CTC numbers could predict DM⁺ RR-DTC. Our study found that the optimal cutoff for CTCs was 6.5, which was 73.7% sensitive and 69.6% specific in predicting DM⁺ RR-DTC. Therefore, CTCs in 7.5 ml blood serve as a predictor of DM⁺ RR-DTC. Univariate and multivariate analyses associated CTCs ≥ 7 in 7.5 ml blood with DM⁺ RR-DTC, and (in multivariate analysis only) with sTg level ≥ 500 ng/ml.

Radioiodine-refractory character is associated with poor outcome in DM⁺ DTC. Durante et al. reported that the 10-year survival rate can be 56% in patients with intense radioiodine accumulation, but as low as 10% in those who lose radio-iodine avidity [5]. Previously our group showed that DTC patients with non- ^{131}I -avid disease had a 10-year survival rate of 38.1% compared with 69.2% for those with ^{131}I -avid metastases [2]. As CTCs ≥ 7 was associated with DM⁺ RR-DTC, we speculate that CTCs ≥ 7 may also correlate with DM⁺ DTC prognosis and progression. Nevertheless, due to short follow-up times in our study (median: 1.2 years, range: 0.2–3 years), we did not evaluate overall survival, and only analyzed PFS of DM⁺ DTC patients. In view of this, we compared 3-year PFS rates between non-RR-DTC and RR-DTC patients and found that CTCs ≥ 7 was significantly associated with lower 3-year PFS than CTCs < 7 (33.3% vs 76.2%). Multivariate Cox-regression analyses confirmed that CTC ≥ 7 is a strong independent predictor of DM⁺ RR-DTC.

This study had some limitations. First, the sample size was relatively small, especially for DM⁺ DTC patients. Second, dynamic monitoring of CTCs wasn't performed in the course of ^{131}I treatment; which may have missed some measurement deviation. Third, overall survival wasn't evaluated in these DM⁺ DTC patients because of short follow-up time. Fourth, as our center specializes in ^{131}I treatment for DTC patients, primary thyroid surgeries often occurred at other institutions. Therefore, N stages for 16 DTC patients were unclear for us. We also didn't compare sensitivity and specificity of the CTC detection between NE-iFISH and CellSearch technology in these patients.

Conclusion

For the first time, we have developed a strategy to identify CTCs in DTC patients using NE-iFISH technology. The CTC detection rate was 86.1% in our DTC patients; CTCs ≥ 5 in 7.5 ml blood is correlated with DM⁺ DTC (sensitivity: 64.3%; specificity: 83.8%). Further, CTCs ≥ 7 may predict DM⁺ RR-DTC (sensitivity: 73.7%; specificity 69.6 %), and is associated with worse prognosis in DM⁺ DTC. We found that assessing CTCs can help predict metastasis, response to ^{131}I treatment and prognosis in DTC patients. However, our results need further validation in a larger study.

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Disclosure Statement

The authors have declared that no competing interest existed.

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