A novel combined fluorescent probe staining method for circulating tumor cell identification

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Background: To develop a novel highly accurate circulating tumor cell (CTC) identification method and to validate its application in cancer diagnostics and/or prognostics.

Methods: We verified and validated the combined fluorescent probe staining protocol (combination of three fluorescent probes: Dil, Hoechst 33342, and PY) through CTC and non-CTC (white blood cell) morphological comparison of five tumor cell lines (THP-1, HEC, HEPG2, Eca-109, HeLa) in vitro and 32 patient tumor samples from the Shandong Cancer Hospital and Institute. Wright’s Giemsa staining and cluster differentiation 45 (CD45) immunocytochemistry (ICC) staining were used as reference control methods. The association between the developed method and clinicopathology was also investigated.

Results: We successfully developed and optimized the protocol, and validated the use of combined fluorescent probe staining for the identification of CTCs in the peripheral blood (PB) of tumor cell lines and tumor patients. Comparable CTC and non-CTC morphologies were observed for combined fluorescent probe staining and Giemsa staining methods in vitro. However, in vivo comparison between the three staining methods revealed that the identified CTCs differed in cell diameter and nucleo-cytoplasmic ratio. In addition, a higher CTC detection rate of 14/32, lower standard deviation (SD), and higher area under the receiver operating characteristic (ROC) curve (AUC) value of 0.844 were noted for combined fluorescence staining. Clinicopathological analysis revealed that CTCs were correlated with platelet levels (P=0.031), but not with age, gender, drinking history, or granule ratio.

Conclusions: We developed a combined fluorescent probe staining method with higher CTC identification accuracy than Wright’s Giemsa staining, and propose this technique as a novel clinical diagnostic/prognostic tool.

Keywords: Fluorescent probe; circulating tumor cell (CTC); immunocytochemistry (ICC); identification

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Introduction

Increased rates of morbidity and mortality have made cancer the leading cause of death in China (1), with more than 90% of cancer deaths being due to tumor metastasis (2). Pathological diagnosis has been the traditional method of cancer diagnosis and prognosis. In 1889, the British pathologist, Paget (3), proposed that circulating tumor cells (CTCs) act as “seeds” in tumorigenesis and metastasis. CTCs refer to tumor cells that enter the peripheral blood (PB) circulatory system from the primary tumor or metastatic lesions either spontaneously or due to diagnosis and treatment (4,5). Only a few tumor cells are metastatic. CTCs are the key cells of cancer metastasis. CTCs can serve as biomarkers to assist in the physician’s assessment of the likelihood of disease recurrence and survival prognosis of a patient (6-9). Monitoring the change trend of CTC type and quantity contributes to real-time individual treatment.

CTC identification is a complicated multi-step process involving cell separation, enrichment, and detection. The challenge of CTC detection and identification is primarily due to the low quantity of CTCs in PB relative to the high numbers of blood cells and platelets (10). Because the content of CTC in blood is very low, the detection is relatively difficult, and different detection methods have their own advantages and disadvantages. At present, there is no “gold standard” method for CTC identification (11-14). The only commercially available CTC identification technology is the FDA-approved CellSearch® system (Menarini Silicon Biosystems, Huntingdon Valley, PA, USA), which is coupled with proprietary immunomagnetic detection technology. There are many studies about the fluorescent probe staining method for CTC identification. However, the primary issue with the current CTC identification methods is the lack of a fully automated classification system that supports accurate consensus comparison across different medical establishments. Thus, CTC detection and assignment is subjective. The key challenge of CTC detection is establishing the consensus criteria for CTC assignment to increase the false positive rejection rate and successfully separate trapped CTCs (15-17). In previous studies, we developed the isolation by size of epithelial tumor cells-immunocytochemistry (ISET-ICC) detection system, which identifies CTCs via physical isolation and enrichment, followed by subsequent morphological identification using the “negative exclusion” method (18-22). To further improve and simplify CTC identification following ISET isolation, we explored a novel direct CTC identification method in the present study using a staining method comprising the combination of three fluorescent probes: Dil, Hoechst 33342, and PY.

To validate the feasibility of identifying CTCs using the combined fluorescent probe staining method, we compared its performance against Wright’s Giemsa staining and cluster differentiation 45 (CD45) ICC staining methods. We selected five tumor cell lines (HEC, EC109, THP-1, HEPG2, and HeLa) and recruited 32 patients with malignant tumors of various cancers who were treated at the Shandong Cancer Hospital and Institute (China) to establish the combined fluorescent probe staining method protocol as well as the CTC identification criteria. We assessed the accuracy of combined fluorescent probe staining relative to Wright’s Giemsa staining in CTC identification by calculating the area under the receiver operating characteristic (ROC) curve (AUC). In addition, we investigated the relationship between CTC identification by combined fluorescent probes and cancer clinicopathology.

We present the following article in accordance with the MDAR reporting checklist (available at https://atm.amegroups.com/article/view/10.21037/atm-21-6476/rc).

Methods

Cell lines and culture

Five tumor cell lines (cute monocytic leukemia THP-1, human endometrial adenocarcinoma HEC, HEPG2, esophageal carcinoma Eca-109, and cervical cancer HeLa) were used in the present study. The HeLa cell line (at the logarithmic growth phase) was provided by Professor Yu Xiaoqiang from the Functional Crystal Materials Laboratory of Shandong University, China. The remaining four cell lines (THP-1, HEC, HEPG2, and Eca-109) were a gift from Wang Xingwu from the Central Laboratory of Shandong Cancer Hospital, China. Single cell suspensions were prepared by 0.25% trypsin digestion at 37 °C.

Patient information and grouping

Thirty-two patients with malignant tumors admitted into Shandong Cancer Hospital and Institute (China) from May, 2017 to September, 2017 were enrolled in the present study based on the inclusion and exclusion criteria. The inclusion criteria were as follows: (I) patients with a clear clinical or pathological diagnosis; (II) patients aged ≥18 years old; and (III) signed consent from both patients and their families.
The exclusion criteria were as follows: (I) patients with a secondary malignant tumor; (II) patients with a history of skin diseases; or (III) those with severe vascular diseases, such as vasculitis. All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013). The necessary approval was obtained from the Ethics Committee of Shandong Cancer Hospital and Institute, China (No. 201702019). Written consent was obtained from all eligible subjects before enrollment into the study. The inclusion criteria for patient selection and recruitment were decided based on the results of the in vitro comparison between the combined fluorescence staining and Wright's Giemsa staining methods.

**Combined fluorescence staining**

Combined fluorescent probe staining was performed after ISET isolation of CTCs. The mixture of three fluorescent probes [Hoechst 33342 (Molecular Probes, molecular probe is a technology based on molecular hybridization, which uses probes to detect nucleic acid sequences with complementary sequences), PY (provided by Professor Yu Xiaqiang), and Dil (Molecular Probes, molecular probe is a technology based on molecular hybridization, which uses probes to detect nucleic acid sequences with complementary sequences); 5 μM each] were diluted with phosphate-buffered saline (PBS). The coverslip was incubated with 100 μL of probe mixture at room temperature for 30 min before the solution was removed and the coverslip was washed three times with PBS. Cell imaging was performed using a fluorescence microscope. Three channels were used for cell imaging to obtain a tri-colored fluorescent image: first channel for Hoechst 33342 at 405 nm excitation and 420–470 nm emission wavelengths; second channel for PY at 405 nm excitation and 500–560 nm emission wavelengths; and the third channel for Dil at 543 nm excitation wavelength and 560–600 nm emission wavelengths. The nuclei were stained blue (first channel), nucleoli green (second channel), and cell membrane red (third channel). We determine the dose of fluorescent probe according to previous studies (18-22). Three random fields were selected for each slide, and three intact cells were randomly selected in each field for separate measurement. ImageJ (National Institutes of Health, Bethesda, MD, USA) was used for image analysis.

**Wright's Giemsa staining**

The same samples that were subjected to combined fluorescent probe staining were subsequently subjected to Giemsa staining. The filter was soaked with 300 μL of Diff-A stain for 1 min before dilution with 100 μL of PBS. Excess stain was blotted and removed before staining for 2 min with Diff-B solution (about 300 μL), which was also diluted with PBS before removal. The filter was then rinsed with distilled water. Diff-B residue on the filter was cleared to ensure that the color of Diff-B remains on the filter. The stained filter was then transferred to a slide and dried for 30 min in a 50 °C dry box. Sealing of the coverslip over the slide was then performed by drying for 30 min. Glycerin was then added dropwise before visualization under an optical microscope. Three random fields were selected for each slide, and three intact cells were randomly selected in each field for separate measurement. ImageJ (National Institutes of Health, Bethesda, MD, USA) was used for image analysis.

**CTC assignment criteria**

The morphological characteristics of CTCs, including cell diameter, nuclear diameter, surface area, and nucleocytoplasmic ratio, were used for assessment. The morphological criteria for CTC assignment for both combined fluorescence staining and Giemsa staining are as follows: (I) large variation in cell size (ratio >0.5); (II) large nuclear diameter >24 μm; (III) irregular shape of nucleus; (IV) >3 large nucleoli; and (V) high nuclei quality ratio. A cell is considered a CTC if it fulfills at least four of the above criteria. The consistency of the assessed morphological characteristics between the two staining methods was calculated as part of the verification of the protocol for combined fluorescent probe staining.

**CD45 ICC staining**

The same samples that were subjected to Giemsa staining were subsequently subjected to CD45 ICC staining. Glycerin-sealed slides with coverslips were rinsed with distilled water before being immersed in 100% ethanol for 1 min, followed by 95% ethanol for 1 min, and finally 75% ethanol for 20 min. After ensuring sufficient removal of the dye, the sample slide was immersed in a dye bath containing distilled water and rinsed for 5 min. Triton X-100 (100 μL; 0.1%) was added dropwise to the slide and was incubated for 15 min at room temperature before washing for 2 min × 3 times with distilled water. Hydrogen peroxide (100 μL; 0.3%) was added dropwise and incubated...
for 10 min at room temperature to perforate the cell membrane before washing for 2 min × four times with PBS. CD45 primary antibody (100 μL) was added dropwise and incubated for 1 h at room temperature to block endogenous peroxidase before washing for 2 min × four times with PBS. Diaminobenzidine (DAB) dye (100 μL) was added to the sample simultaneously with the primary antibody and was incubated at room temperature till visualization of color development under the microscope. The DAB dye was removed upon completion of color development and was rinsed with running water for 5 min before hematoxylin staining for 5 min. The sample slide was then incubated with 100 μL of horseradish peroxidase (HRP)-conjugated goat anti-rabbit/mouse secondary immunoglobulin G (IgG) for 15 min at room temperature before rinsing with PBS for 2 min × four times. For nuclear staining, the sample was dehydrated with hydrochloric acid for 8 seconds, rinsed with distilled water for 5 min, and then subjected to gradient alcohol dehydration (75% ethanol for 1 min, 95% ethanol for 1 min, 100% ethanol for 1 min), air-drying, and finally resin sealing. Cell imaging was performed using a light microscope.

The current standard CD45 ICC morphological criteria for CTC identification are as follows: (I) large variation in size of nucleus relative to size of cell (ratio >0.5); (II) large nuclear diameter >24 μm; (III) irregular shape of nucleus; (IV) three-dimensional chromatin staining; and (V) high nucleo-cytoplasmic ratio. A cell is assigned as a CTC if it fulfills at least four of the above criteria.

Statistical analysis

Statistical analysis was performed using the SPSS v2.0 software (IBM, Chicago, IL, USA). The paired t-test was used for morphological comparison of the cell diameter, nuclear diameter, cell surface area, and nucleo-cytoplasmic ratio between the three staining methods. The Chi-squared test was performed for comparisons between the three paired datasets. P<0.5 was considered not statistically significant.

Results

In vitro confirmation of CTC identification protocol via morphological comparison between combined fluorescent probe staining and Giemsa staining in five cell lines

The SOP of the proposed novel combined fluorescent probe staining method was validated by comparing with cells subjected to Wright’s Giemsa staining. Five tumor cell lines were respectively stained with either combined fluorescent probes or Giemsa stain, and their morphological characteristics were compared.

As we intended to use the THP-1 and HEC cell lines for preliminary tests to aid in the execution of downstream experiments, we only collected one data set for the combined fluorescent probe staining of THP-1 cells and one set for the Wright’s Giemsa staining of HEC cells (Tables 1,2). Thus, comparison within groups could not be performed. We found no statistically significant differences in the mean values of cell diameter, nuclear diameter, cell surface area, and nucleo-cytoplasmic ratio of the HEPG2 (Table 3, Figures 1,2), Eca-109 (Table 4), and HeLa (Table 5) cell lines between the Wright’s Giemsa staining and combined fluorescent probe staining methods.

Comparison of the mean values of the cell morphological characteristics examined for the two groups of five tumor cell lines subjected to either Wright’s Giemsa staining or combined fluorescent probe staining showed consistent morphological characteristics (Table 6). These results demonstrate the feasibility of using the proposed novel combined fluorescent probe staining method on tumor cell lines. The morphological characteristics (cell diameter, nuclear diameter, surface area and nucleo-cytoplasmic ratio) of CTC (P=0.826, 0.901, 0.560, and 0.750) and white blood cells (P=0.157, 0.466, 0.446, and 0.475) were consistent for both staining methods.

Optimization of combined fluorescent probe staining protocol for CTC and non-CTC identification

The inclusion criteria used for patient recruitment were based on the results of the comparison between the combined fluorescent probe staining and Giemsa staining methods. Patients who were excluded from this study had malignant tumors with a secondary malignancy (five cases), a history of dermatosis, suffered from severe vascular disease, such as vasculitis (four cases of esophageal cancer and one case of liver cancer), or had undergone a clinical validation experiment that used combined fluorescent probe staining or Wright’s Giemsa staining.

For optimization of the combined fluorescent probe staining protocol for CTC identification, we used the selection (inclusion and exclusion) criteria as a reference, coupled with the PB samples of five tumor patients. Wright’s Giemsa staining and CD45 ICC staining were
used as experimental controls for finalization of the combined fluorescent probe staining method. The cell morphologies of both CTCs and non-CTCs (white blood cells) were assessed in terms of cell diameter, nuclear diameter, cell surface area, and nucleo-cytoplasmic ratio. We measured all CTCs, including cells that were suspected to be CTCs. Three random fields and three cells (CTCs or non-CTCs) from each field (total of nine cells) for each sample were selected and measured separately, followed by ImageJ analysis.

Comparison of combined fluorescent probe staining vs. Giemsa staining or CTC identification in the PB of five tumor patients (YG01 to YG05; one case of liver cancer and four cases of esophageal cancer) revealed that 2/5 cases (YG04 and YG05) were detected by combined fluorescent probe staining, while no cases were identified by both Giemsa staining and CD45 ICC staining (Table 7). Thus, the relatively higher CTC detection rate of the developed combined fluorescent probe staining method verified the protocol and selection criteria used for clinical identification of CTCs in the PB of tumor patients.

To assess the non-CTC (white blood cell) detection rate of the combined fluorescent probe staining method, only the white blood cells in the PB of patient YG04 Li × Cheng were photographed and measured. Comparisons between the three staining methods were made by comparing between pairs (pair 1: Giemsa staining-combined fluorescent probe staining; pair 2: combined fluorescent probe staining-CD45 ICC staining; pair 3: Wright’s Giemsa staining-CD45 ICC staining). No significant differences

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**Table 1** Comparison of the morphological characteristics of THP-1 acute monocytic leukemia cells subjected to either combined fluorescent probe staining or Wright’s Giemsa staining

| Staining method                    | Cell ID | Cell diameter (μm) | Nuclear diameter (μm) | Cell surface area (μm²) | Nucleo-cytoplasmic ratio |
|------------------------------------|---------|--------------------|-----------------------|------------------------|--------------------------|
| Wright’s Giemsa staining           | 1       | 15.840             | 14.918                | 197.16                 | 0.7498                   |
|                                    | 2       | 16.802             | 14.629                | 187.40                 | 0.7797                   |
|                                    | 3       | 17.228             | 15.900                | 233.88                 | 0.7156                   |
|                                    | 4       | 16.890             | 15.302                | 193.66                 | 0.8036                   |
|                                    | 5       | 15.393             | 14.365                | 162.21                 | 0.7930                   |
|                                    | 6       | 16.368             | 14.994                | 217.74                 | 0.8104                   |
|                                    | 7       | 19.740             | 16.565                | 302.07                 | 0.7376                   |
|                                    | 8       | 16.824             | 14.748                | 212.22                 | 0.7954                   |
| Average                            |         | 16.886             | 15.178                | 212.2925               | 0.7274                   |
| Combined fluorescent probe staining| 1       | 16.936             | 14.262                | 203.06                 | 0.7780                   |

ID, identification.

**Table 2** Comparison of the morphological characteristics of HEC endometrial adenocarcinoma cells subjected to either combined fluorescent probe staining or Wright’s Giemsa staining

| Staining method                    | Cell ID | Cell diameter (μm) | Nuclear diameter (μm) | Cell surface area (μm²) | Nucleo-cytoplasmic ratio |
|------------------------------------|---------|--------------------|-----------------------|------------------------|--------------------------|
| Wright’s Giemsa staining           | 1       | 11.774             | 9.148                 | 118.240                | 0.5828                   |
|                                    | 2       | 12.074             | 9.844                 | 110.717                | 0.5215                   |
|                                    | 3       | 10.740             | 9.197                 | 100.128                | 0.5602                   |
| Average                            |         | 11.5293            | 9.3963                | 109.6950               | 0.5548                   |
| Combined fluorescent probe staining| 1       | 11.703             | 10.215                | 102.915                | 0.6755                   |

ID, identification.
in morphological characteristics were observed among the three staining methods (Table 8).

### Clinical validation of the combined fluorescent probe CTC identification method with 32 patients

We confirmed the feasibility of the combined fluorescent probe staining method and finalizing the protocol in both in vitro (five tumor cell lines) and in vivo (five cancer patients) samples, and also confirmed the cell morphological consistency with both Wright’s Giemsa staining and CD45 ICC staining methods. Next, we validated and assessed the performance of the developed combined fluorescent probe staining method in 32 cases of malignant tumor patients that were admitted into Shandong Cancer Hospital and Institute from May, 2017 to September, 2017 (including liver cancer, esophageal cancer, prostate cancer, kidney cancer, bladder cancer, and lung cancer; Table 8).

We had initially recruited 49 tumor patients for the present study and collected the PB samples from these patients. The patients were given a patient identity (ID) tag from YG01–YG49 based on the chronological order of PB collection. The samples of patients YG01–YG30, YG34, and YG42 were used for the subsequent performance assessment of CTC and non-CTC identification compared to Wright’s Giemsa staining and CD45 ICC staining. Staining by all three methods was not performed for the PB samples of 17 patients YG31–YG33, YG35–YG41, and

| Staining method                          | Cell ID | Cell diameter (μm) | Nuclear diameter (μm) | Cell surface area (μm²) | Nucleo-cytoplasmic ratio |
|-----------------------------------------|---------|--------------------|-----------------------|------------------------|-------------------------|
| Wright’s Giemsa staining                | 1       | 14.338             | 12.117                | 154.560                | 0.6918                  |
|                                         | 2       | 15.190             | 11.381                | 183.074                | 0.6202                  |
|                                         | 3       | 13.586             | 11.011                | 140.465                | 0.6823                  |
|                                         | 4       | 11.688             | 9.907                 | 119.863                | 0.7124                  |
|                                         | 5       | 13.152             | 11.391                | 137.153                | 0.6737                  |
|                                         | 6       | 10.535             | 8.732                 | 90.230                 | 0.6977                  |
|                                         | 7       | 12.730             | 9.148                 | 114.280                | 0.6738                  |
|                                         | 8       | 14.938             | 12.414                | 177.101                | 0.6300                  |
|                                         | 9       | 13.773             | 10.755                | 140.916                | 0.6716                  |
|                                         | Average | 13.3556            | 10.7617               | 139.7378               | 0.6726                  |
| Combined fluorescent probe staining     | 1       | 11.391             | 9.946                 | 106.312                | 0.6320                  |
|                                         | 2       | 13.370             | 9.718                 | 135.077                | 0.5653                  |
|                                         | 3       | 9.858              | 7.428                 | 83.71                  | 0.5945                  |
|                                         | 4       | 16.080             | 10.236                | 201.815                | 0.4970                  |
|                                         | Average | 12.6747            | 9.332                 | 131.7285               | 0.5722                  |
| P value                                 |         | 0.681              | 0.165                 | 0.631                  | 0.069                   |

ID, identification.
YG43–YG49 (Figures 3-5). The CTC detection rates in the PB of the 32 tumor samples for the three staining methods were as follows: 16/32 for combined fluorescent probe staining; 8/32 for Wright’s Giemsa staining; and 6/32 for CD45 ICC staining.

Comparison of non-CTC clinical detection accuracy between the three staining methods

The lower standard deviation (SD) of each morphological characteristic for the combined fluorescence staining method compared to the other two staining methods (cell diameter: 1.01652 vs. 1.16724 and 1.12383 μm for Giemsa and CD45 ICC staining respectively; nuclear diameter: 0.34397 vs. 0.64424 and 0.62349 μm; cell surface area: 12.79116 vs. 13.37422; and 13.13872 μm; nucleo-cytoplasmic ratio: 0.06341 vs. 0.07474 and 0.07231) indicated a higher accuracy for the developed combined fluorescence staining method (Tables 9-11). The degree of skewness between 0.05 and 0.35 signified that the data was statistically significant.

The non-CTCs identified and selected for the combined fluorescence staining method were as follows: three cells in YG03; two cells in YG04; one cell in YG08; and one cell
in YG09. Eight cells in YG04 were identified for Wright's Giemsa staining method and five cells in YG04 were identified for CD45 ICC staining method. Comparison of the morphological parameters (cell diameter, nuclear diameter, cell surface area and nucleo-cytoplasmic ratio) of non-CTCs revealed no significant difference in any parameter among the three staining methods (Table 12).

**Comparison of CTC clinical detection accuracy between combined fluorescent probe staining and Giemsa staining**

The morphological characteristics of CTCs in the PB of 32 tumor samples were assessed and compared between combined fluorescent probe staining and Giemsa staining. The CD45 ICC staining method was not included in the comparison of the CTC morphological parameters. The lower SD of each morphological characteristic for the combined fluorescent probe staining method compared to Giemsa staining indicated a higher accuracy for the developed combined fluorescence staining method (Tables 13, 14). The degree of skewness between 0.05 and 0.35 signified that the data was statistically significant. Comparison of the morphological parameters between the two staining methods revealed no significant differences.

| Staining method                        | Cell ID | Cell diameter (μm) | Nuclear diameter (μm) | Cell surface area (μm²) | Nucleo-cytoplasmic ratio |
|----------------------------------------|---------|--------------------|-----------------------|------------------------|--------------------------|
| Wright's Giemsa staining               | 1       | 25.843             | 17.889                | 504.981                | 0.5509                   |
|                                        | 2       | 29.059             | 25.677                | 632.300                | 0.6761                   |
|                                        | 3       | 22.535             | 19.164                | 386.499                | 0.6036                   |
|                                        | 4       | 23.110             | 18.056                | 391.709                | 0.6308                   |
|                                        | 5       | 22.301             | 18.457                | 353.553                | 0.7188                   |
|                                        | 6       | 23.415             | 19.817                | 414.725                | 0.6979                   |
|                                        | 7       | 21.995             | 19.205                | 385.119                | 0.5529                   |
|                                        | 8       | 22.274             | 17.205                | 374.129                | 0.5932                   |
|                                        | Average | 23.8165           | 19.4337               | 430.3768               | 0.6280                   |
| Combined fluorescent probe staining    | 1       | 25.303             | 22.368                | 523.658                | 0.7103                   |
|                                        | 2       | 22.878             | 18.853                | 419.439                | 0.7373                   |
|                                        | 3       | 24.583             | 19.435                | 449.982                | 0.5813                   |
|                                        | 4       | 25.289             | 20.557                | 449.515                | 0.5923                   |
|                                        | 5       | 22.925             | 18.788                | 403.934                | 0.6369                   |
|                                        | 6       | 23.598             | 19.593                | 452.430                | 0.6446                   |
|                                        | 7       | 29.720             | 24.528                | 643.031                | 0.6702                   |
|                                        | 8       | 23.519             | 20.217                | 421.421                | 0.6320                   |
|                                        | 9       | 22.917             | 17.626                | 402.069                | 0.5871                   |
|                                        | 10      | 25.589             | 20.892                | 492.765                | 0.6815                   |
|                                        | 11      | 26.137             | 21.382                | 465.953                | 0.7060                   |
|                                        | 12      | 27.148             | 19.474                | 506.288                | 0.6451                   |
|                                        | Average | 24.9166           | 20.3094               | 469.2070               | 0.6520                   |

P value

0.559

0.436

0.402

0.428

ID, identification.
Table 6 Comparison of the cell morphological characteristics between the five cell lines for the two staining methods, Wright's Giemsa staining vs. combined fluorescent probe staining, respectively

| Staining method                      | Cell line ID | Cell diameter (μm) | Nuclear diameter (μm) | Cell surface area (μm²) | Nucleo-cytoplasmic ratio |
|--------------------------------------|--------------|--------------------|-----------------------|-------------------------|--------------------------|
| Wright's Giemsa staining             | THP-1        | 16.886             | 15.178                | 212.2925                | 0.7274                   |
|                                       | HEC          | 11.5293            | 9.3963                | 109.6850                | 0.5548                   |
|                                       | Eca-109      | 18.7212            | 13.4425               | 223.7822                | 0.6682                   |
|                                       | HEPG2        | 13.3556            | 10.7617               | 139.7378                | 0.6726                   |
|                                       | HeLa         | 23.8165            | 19.4337               | 430.3768                | 0.6280                   |
| Combined fluorescent probe staining  | THP-1        | 16.936             | 14.262                | 203.06                  | 0.7780                   |
|                                       | HEC          | 11.703             | 10.215                | 102.915                 | 0.6755                   |
|                                       | Eca-109      | 17.635             | 13.7823               | 238.6053                | 0.6371                   |
|                                       | HEPG2        | 12.6747            | 9.332                 | 131.7285                | 0.5722                   |
|                                       | HeLa         | 24.9166            | 20.3094               | 469.2070                | 0.6520                   |
| P value                              | 0.826        | 0.901              | 0.560                 | 0.750                   |

ID, identification.

in cell diameter and cell surface area (P=0.308 and 0.147); however, significant differences in nuclear diameter and nucleo-cytoplasmic ratio (P=0.013 and 0.004; Table 15) were observed. Notably, the difference was larger for the combined fluorescent probe staining method.

In a previous study, we established the ISET-ICC (ISET + CD45 ICC staining) method for the identification of CTCs in PB, which enabled accurate determination of false positives using the following formula: specificity = true negative number/(true negative number + false positive number) × 100%. In this study, we compared the specificity of the developed ISET + combined fluorescent probe staining method with that of the ISET + Wright's Giemsa staining method by plotting the ROC curve and obtaining the AUC values. A higher AUC value of 0.844 was obtained for the combined fluorescent probe staining method compared to that of Wright's Giemsa staining (0.750). This indicates that the combined fluorescent probe staining method has higher CTC detection specificity than the Giemsa staining method.

**Correlation between CTC clinical detection accuracy of combined fluorescent probe staining method and clinicopathology**

We examined the correlation between CTC detection accuracy of the combined fluorescence staining method and clinicopathology of 32 tumor patients (22 males and 10 females), including 18 cases of esophageal cancer, six cases of liver cancer, four cases of renal cancer, one case of prostate cancer, one case of bladder cancer, one case of penile cancer, and one case of lung cancer. The 16 CTC-positive cases identified included seven cases of esophageal cancer, two cases of liver cancer, two cases of renal cancer, one case of prostate cancer, one case of bladder cancer, and one case of penile cancer. The 16 CTC-negative cases included 11 cases of esophageal cancer, four cases of liver cancer, two cases of renal cell carcinoma, and one case of penile cancer (Table 8). The CTC-positive rate was 11/22 for male patients and 5/10 for female patients (Table 16). CTCs were positively correlated with platelet levels (CTC-positive vs. CTC-negative: 261.71±42.21×10⁹/L vs. 211.73±71.20×10⁹/L; P=0.031) but were not associated with age, gender, drinking history, or granule ratio.

**Discussion**

In this study, we successfully verified the feasibility and established the protocol and CTC identification criteria for the novel combined fluorescent probe staining method. This method is an improvement to our previously established ISET-ICC CTC identification system. The combined fluorescent probe staining method showed consistent cell morphological characteristics (cell diameter,
Table 7 Assessment of the performance of the novel combined fluorescent probe staining vs. Wright's Giemsa staining and CD45 ICC staining methods for *in vivo* CTC identification in 32 cancer patients

| Patient No. | Fluorescence ID | Gender (years old) | Hospital admission ID | Cancer type | Cancer stage | Combined fluorescent probe staining | Wright's Giemsa staining | CD45 ICC staining |
|-------------|-----------------|--------------------|-----------------------|-------------|-------------|--------------------------------------|--------------------------|------------------|
| 1           | YG01            | F 66               | 4335××                | Esophageal carcinoma | cT2N0M0    | 0 0 0                                |                          |                  |
| 2           | YG02            | M 53               | 4338××                | Liver cancer      | cT4N0M0    | 0 0 0                                |                          |                  |
| 3           | YG03            | M 52               | 4274××                | Esophageal carcinoma | cT2N0M0    | 0 0 0                                |                          |                  |
| 4           | YG04            | M 66               | 4318××                | Esophageal carcinoma | cT2N0M0    | 2 0 0                                |                          |                  |
| 5           | YG05            | F 66               | 4335××                | Esophageal carcinoma | cT2N0M0    | 2 0 0                                |                          |                  |
| 6           | YG06            | M 66               | 4318××                | Esophageal carcinoma | cT2N0M0    | 0 0 0                                |                          |                  |
| 7           | YG07            | F 66               | 4335××                | Esophageal carcinoma | cT2N0M0    | 0 0 0                                |                          |                  |
| 8           | YG08            | M 56               | 4335××                | Liver cancer      | cT1bN0M0   | 1 1 0                                |                          |                  |
| 9           | YG09            | M 53               | 4338××                | Liver cancer      | cT4N0M0    | 0 0 0                                |                          |                  |
| 10          | YG10            | F 50               | 4259××                | Renal cancer      | cT2N0M0    | 0 1 1                                |                          |                  |
| 11          | YG11            | M 61               | 4322××                | Prostate cancer   | pTxN0M1b   | 1 1 1                                |                          |                  |
| 12          | YG12            | M 62               | 4338××                | Bladder cancer    | cT2N0M0    | 0 0 0                                |                          |                  |
| 13          | YG13            | M 54               | 4331××                | Renal cancer      | cT2N0M0    | 0 0 0                                |                          |                  |
| 14          | YG14            | M 54               | 4340××                | Penile cancer     | cT2N0M0    | 1 1 0                                |                          |                  |
| 15          | YG15            | M 72               | 4342××                | Renal cancer      | cT2N0M0    | 2 4 4                                |                          |                  |
| 16          | YG16            | F 40               | 4336××                | Renal cancer      | cT4N1M1    | 0 0 0                                |                          |                  |
| 17          | YG17            | F 66               | 4335××                | Esophageal cancer | cT2N0M0    | 1 0 0                                |                          |                  |
| 18          | YG18            | F 68               | 4341××                | Esophageal cancer | cT2N0M0    | 0 0 0                                |                          |                  |
| 19          | YG19            | M 66               | 4342××                | Liver cancer      | cT2N0M0    | 0 0 0                                |                          |                  |
| 20          | YG20            | M 53               | 4319××                | Esophageal cancer | cT2N1M0    | 1 1 1                                |                          |                  |
| 21          | YG21            | M 70               | 4345××                | Liver cancer      | cT2N0M0    | 0 0 0                                |                          |                  |
| 22          | YG22            | M 66               | 4318××                | Esophageal cancer | cT2N0M0    | 2 0 0                                |                          |                  |
| 23          | YG23            | M 53               | 4319××                | Esophageal cancer | cT2N1M0    | 0 0 0                                |                          |                  |
| 24          | YG24            | F 53               | 434690                | Esophageal cancer | cT2N0M0    | 1 1 1                                |                          |                  |
| 25          | YG25            | M 53               | 4319××                | Esophageal cancer | cT2N1M0    | 0 0 0                                |                          |                  |
| 26          | YG26            | M 54               | 1274××                | Lung carcinoma    | cT2N0M0    | 0 0 0                                |                          |                  |
| 27          | YG27            | F 53               | 4346××                | Esophageal cancer | cT2N0M0    | 0 0 0                                |                          |                  |
| 28          | YG28            | F 53               | 4346××                | Esophageal cancer | cT2N0M0    | 0 0 0                                |                          |                  |
| 29          | YG29            | M 79               | 4345××                | Esophageal cancer | cT2N0M0    | 0 0 0                                |                          |                  |
| 30          | YG30            | M 79               | 4345××                | Esophageal cancer | cT2N0M0    | 0 0 0                                |                          |                  |
| 34          | YG34            | M 47               | 4738××                | Esophageal cancer | cT3N1Mx    | 1 1 1                                |                          |                  |
| 42          | YG42            | M 73               | 4326××                | Liver cancer      | cT2N0M0    | 0 0 0                                |                          |                  |

CD45, cluster differentiation 45; ICC, immunocytochemistry; CTC, circulating tumor cell; ID, Identification; F, female; M, male.
Table 8 Comparison of the morphological characteristics of PB white blood cells in patient YG04 Li × Cheng among the three staining methods

| Staining method                  | Cell ID | Cell diameter (μm) | Nuclear diameter (μm) | Cell surface area (μm²) | Nucleo-cytoplasmic ratio |
|----------------------------------|---------|--------------------|-----------------------|------------------------|--------------------------|
|                                  | 1       | 15.065             | 11.347                | 143.011                | 0.6499                   |
|                                  | 2       | 12.102             | 11.853                | 110.431                | 0.7705                   |
|                                  | 3       | 13.774             | 11.516                | 119.447                | 0.5809                   |
|                                  | 4       | 13.475             | 11.482                | 110.281                | 0.7700                   |
|                                  | 5       | 14.043             | 10.889                | 129.766                | 0.7442                   |
|                                  | 6       | 12.358             | 10.120                | 125.628                | 0.6200                   |
|                                  | 7       | 12.249             | 10.539                | 101.202                | 0.6455                   |
|                                  | 8       | 11.714             | 10.252                | 111.991                | 0.6222                   |
| Combined fluorescent probe      | 9       | 14.444             | 10.926                | 154.700                | 0.6057                   |
| staining                         | 12      | 14.581             | 13.592                | 172.664                | 0.6383                   |
| CD45 ICC staining                | 13      | 13.002             | 10.735                | 117.188                | 0.6936                   |
|                                  | 14      | 12.572             | 10.957                | 106.146                | 0.5488                   |
|                                  | 15      | 13.537             | 10.866                | 126.854                | 0.5943                   |
|                                  | 16      | 15.283             | 11.228                | 142.292                | 0.5868                   |
|                                  | 17      | 14.402             | 10.203                | 138.800                | 0.6448                   |
| P value                          |         |                    |                       |                        |                          |
| Pair 1                           | 0.656   | 0.651              | 0.382                 | 0.295                  |
| Pair 2                           | 0.438   | 0.454              | 0.173                 | 0.994                  |
| Pair 3                           | 0.890   | 0.400              | 0.717                 | 0.162                  |
| $\chi^2$ value                   | 0.157   | 0.466              | 0.446                 | 0.475                  |

PB, peripheral blood; ID, identification; CD45, cluster differentiation 45; ICC, immunocytochemistry.

Figure 3 YG34 Chen × Wei (Giemsa staining; ×40).

Figure 4 YG34 Chen × Wei (CD45 ICC staining; ×40). CD45, cluster differentiation 45; ICC, immunocytochemistry.
Figure 5 YG34 Chen x Wei [(A) blue marks the nucleus; (B) green marks the cytoplasmic; (C) red marks the cell membrane; (D) red and blue mark the fluorescent combination ×40)].

Table 9 Cell morphological characteristics of non-CTCs in PB detected by Wright’s Giemsa staining

| Staining method             | Slide ID | Cell ID | Cell diameter (μm) | Nuclear diameter (μm) | Cell surface area (μm²) | Nuclear: cytoplasmic mass ratio |
|-----------------------------|----------|---------|--------------------|-----------------------|------------------------|---------------------------------|
| Wright’s Giemsa staining    | 4        | 1       | 15.065             | 11.347                | 143.011                | 0.6499                          |
|                             | 2        | 1       | 12.102             | 11.853                | 110.431                | 0.7705                          |
|                             | 3        | 1       | 13.774             | 11.516                | 119.447                | 0.5809                          |
|                             | 4        | 1       | 13.475             | 11.482                | 110.281                | 0.7700                          |
|                             | 5        | 1       | 14.043             | 10.889                | 129.766                | 0.7442                          |
|                             | 6        | 1       | 12.358             | 10.120                | 125.628                | 0.6200                          |
|                             | 7        | 1       | 12.249             | 10.539                | 110.202                | 0.6455                          |
|                             | 8        | 1       | 11.714             | 10.252                | 111.991                | 0.6222                          |
| SD                          |          |         | 1.16724            | 0.64424               | 13.37422               | 0.07474                         |
| Degree of skewness          |          |         | 0.2741             | 0.3001                | 0.3076                  | 0.1327                          |

CTC, circulating tumor cell; PB, peripheral blood; ID, identification; SD, standard deviation.

Table 10 Cell morphological characteristics of non-CTCs in PB detected by combined fluorescent probe staining

| Staining method                  | Slide ID | Cell ID | Cell diameter (μm) | Nuclear diameter (μm) | Cell surface area (μm²) | Nucleo-cytoplasmic ratio |
|----------------------------------|----------|---------|--------------------|-----------------------|------------------------|--------------------------|
| Combined fluorescent probe staining | 3        | 1       | 13.554             | 11.804                | 169.202                | 0.7145                   |
|                                  | 2        | 1       | 14.423             | 13.315                | 165.439                | 0.6909                   |
|                                  | 3        | 1       | 15.595             | 14.275                | 171.311                | 0.5898                   |
|                                  | 4        | 9       | 14.444             | 10.926                | 154.700                | 0.6057                   |
|                                  | 12       | 9       | 14.581             | 13.592                | 172.664                | 0.6383                   |
|                                  | 8        | 1       | 14.663             | 11.107                | 146.060                | 0.6516                   |
|                                  | 9        | 1       | 15.949             | 11.385                | 206.625                | 0.5635                   |
| SD                              |          |         | 1.01652            | 0.34397               | 12.79116               | 0.06341                  |
| Degree of skewness              |          |         | 0.1342             | 0.3253                | 0.3141                 | 0.2317                   |

CTC, circulating tumor cell; PB, peripheral blood; ID, identification; SD, standard deviation.
Table 11 Cell morphological characteristics of non-CTCs in PB detected by CD45 ICC staining

| Staining method    | Slide ID | Cell ID | Cell diameter (μm) | Nuclear diameter (μm) | Cell surface area (μm²) | Nucleo-cytoplasmic ratio |
|--------------------|----------|---------|--------------------|-----------------------|------------------------|--------------------------|
| CD45 ICC staining  | 4        | 13      | 13.002             | 10.735                | 117.188                | 0.6936                   |
|                    | 14       |         | 12.572             | 10.957                | 106.146                | 0.5488                   |
|                    | 15       |         | 13.537             | 10.866                | 126.854                | 0.5943                   |
|                    | 16       |         | 15.283             | 11.228                | 142.292                | 0.5868                   |
|                    | 17       |         | 14.402             | 10.203                | 138.800                | 0.6448                   |
| SD                 |          |         |                    |                       |                        |                          |
| Degree of skewness |          |         |                    |                       | 1.12383                | 0.62349                  |

CTC, circulating tumor cell; PB, peripheral blood; CD45, cluster differentiation 45; ICC, immunocytochemistry; ID, identification; SD, standard deviation.

Table 12 Comparison of morphological characteristics of non-CTCs in PB among the three staining methods

| Staining method          | Slide ID | Cell ID | Cell diameter (μm) | Nuclear diameter (μm) | Cell surface area (μm²) | Nucleo-cytoplasmic ratio |
|--------------------------|----------|---------|--------------------|-----------------------|------------------------|--------------------------|
| Wright’s Giemsa staining | 4        | 1       | 15.065             | 11.347                | 143.011                | 0.6499                   |
|                          | 2        |         | 12.102             | 11.853                | 110.431                | 0.7705                   |
|                          | 3        |         | 13.774             | 11.516                | 119.447                | 0.5809                   |
|                          | 4        |         | 13.475             | 11.482                | 110.281                | 0.7700                   |
|                          | 5        |         | 14.043             | 10.889                | 129.766                | 0.7442                   |
|                          | 6        |         | 12.358             | 10.120                | 125.628                | 0.6200                   |
|                          | 7        |         | 12.249             | 10.539                | 101.202                | 0.6455                   |
|                          | 8        |         | 11.714             | 10.252                | 111.991                | 0.6222                   |
| Combined fluorescent probe staining | 3 | 1     | 13.554             | 11.804                | 169.202                | 0.7145                   |
|                          | 2        |         | 14.423             | 13.315                | 165.439                | 0.6909                   |
|                          | 3        |         | 15.595             | 14.275                | 171.311                | 0.5898                   |
|                          | 4        | 9       | 14.444             | 10.926                | 154.700                | 0.6057                   |
|                          | 8        | 1       | 14.663             | 11.107                | 146.060                | 0.6516                   |
|                          | 9        | 1       | 15.949             | 11.385                | 206.625                | 0.5635                   |
| CD45 ICC staining        | 4        | 13      | 13.002             | 10.735                | 117.188                | 0.6936                   |
|                          | 14       |         | 12.572             | 10.957                | 106.146                | 0.5488                   |
|                          | 15       |         | 13.537             | 10.866                | 126.854                | 0.5943                   |
|                          | 16       |         | 15.283             | 11.228                | 142.292                | 0.5868                   |
|                          | 17       |         | 14.402             | 10.203                | 138.800                | 0.6448                   |
| P value                  |          |         |                    |                       |                        |                          |
| Pair 1                   |          |         | 0.612              | 0.421                 | 0.184                  | 0.119                    |
| Pair 2                   |          |         | 0.521              | 0.320                 | 0.221                  | 0.219                    |
| Pair 3                   |          |         | 0.773              | 0.213                 | 0.200                  | 0.174                    |
| χ² value                 |          |         | 0.172              | 0.423                 | 0.421                  | 0.439                    |

CTC, circulating tumor cell; PB, peripheral blood; ID, identification; CD45, cluster differentiation 45; ICC, immunocytochemistry.
Table 13 Cell morphological characteristics of CTCs in PB that were subjected to Wright’s Giemsa staining

| Staining method       | Slide ID | Cell ID | Cell diameter (μm) | Nuclear diameter (μm) | Cell surface area (μm²) | Nucleo-cytoplasmic mass ratio | Detection by CD45 ICC staining |
|-----------------------|----------|---------|--------------------|-----------------------|--------------------------|--------------------------------|--------------------------------|
| Wright’s Giemsa staining | 8        | 1       | 24.665             | 20.254                | 441.236                  | 0.7251                         | Negative                       |
|                       | 10       | 1       | 27.320             | 22.512                | 441.672                  | 0.7251                         | Negative                       |
|                       | 11       | 1       | 27.320             | 22.512                | 441.672                  | 0.7251                         | Negative                       |
|                       | 14       | 1       | 27.120             | 23.712                | 442.632                  | 0.8124                         | Not found                      |
|                       | 15       | 1       | 23.413             | 17.952                | 295.134                  | 0.7134                         | Not found                      |
|                       | 20       | 1       | 23.751             | 20.651                | 419.345                  | 0.8264                         | Negative                       |
|                       | 3        | 2       | 21.732             | 19.991                | 417.821                  | 0.8521                         | Negative                       |
|                       | 3        | 3       | 22.311             | 20.231                | 401.278                  | 0.8011                         | Negative                       |
|                       | 24       | 1       | 26.100             | 24.671                | 441.134                  | 0.8100                         | Negative                       |
|                       | 34       | 1       | 26.116             | 20.290                | 475.372                  | 0.7241                         | Negative                       |
| SD                    |          |         | 2.10237            | 2.01976               | 57.23151                 | 0.05336                        |                                |
| Degree of skewness    |          |         | 0.1275             | 0.0952                | 0.1421                   | 0.0691                         |                                |

CTC, circulating tumor cell; PB, peripheral blood; ID, identification; CD45, cluster differentiation 45; ICC, immunocytochemistry; SD, standard deviation.

nuclear diameter, cell area, and nucleo-cytoplasmic ratio) for both CTCs and non-CTCs as Wright’s Giemsa staining for in vitro CTC identification and similarly as Giemsa staining and CD45 ICC staining for in vivo CTC identification for the same samples examined. The newly developed combined fluorescent probe staining method exhibited higher CTC detection accuracy, as evident from the lower SD in the measurements for the cell morphological parameters examined and the higher AUC value compared to other reference control methods used. Investigation of the association between CTC detection by combined fluorescent probe staining and clinicopathology revealed a positive correlation with platelet count, but not in other assessed parameters. This suggests that platelet count may represent a potential biomarker for the presence of CTCs and tumor metastasis.

In the verification stage of the feasibility of the newly developed combined fluorescent probe staining method, the staining of cell morphological features in in vitro tumor cell lines by this method was compared to Giemsa staining. The absence of significant difference in the nucleo-cytoplasmic ratio in the five tumor cell lines between these two staining methods may be due to the following: (I) the lower fluorescence ratio of the combined fluorescent probe staining method used (4,970) than the fluorescence intensities of cell 1 (0.6320), cell 2 (0.5653), and cell 3 (0.5945), as well as the average value of Wright’s Giemsa staining (0.6726); and (II) only four cells in the combined fluorescent probe staining method met the measurement criteria, thus there was insufficient number of cells available for comparison.

Comparison between the combined fluorescent probe staining and Giemsa staining methods for the same 32 tumor samples revealed no significant differences in cell diameter and surface area, but significant differences in nuclear diameter and nucleo-cytoplasmic ratio. Moreover, the differences were more significant for the former method. This larger difference in CTC nuclear diameter and nucleo-cytoplasmic ratio may be due to the following: (I) variation in cell morphology and nuclear size; (II) both staining methods failed to detect CTCs; and (III) consecutive staining of the same sample with the combined fluorescent probe method followed by Giemsa staining may have affected CTC morphology. Collecting duplicate PB samples from the same tumor patients for in vivo experiments or performing the staining on two lots of the same tumor cell line for in vitro experiments instead of using the same samples for both combined fluorescent probe staining and Giemsa staining can clarify whether a significant difference exists. Another limitation that prevented a fair comparison between the two staining methods is the use of different microscopes for visualizing and imaging cell morphology;
A fluorescence microscope was used for cells stained with combined fluorescent probes, while a light microscope was used for Giemsa-stained cells.

As part of the comparison of the CTC detection rate and accuracy among the staining methods, we also compared the performance of these methods in identifying non-CTCs. Although we observed many non-CTCs in the random fields selected, we only selected three random cells for cell morphology measurements. Considering the large variation in measurement values of the assessed parameters for non-CTCs, we decided only to compare the morphological features of non-CTCs among the staining methods used.

There are few studies that have investigated the association between the CTC detection rate and cancer clinicopathology. In the present study, we found that CTC detection by combined fluorescent probe staining was positively correlated with platelet count. Our finding is consistent with two previous studies by our research group, which showed a correlation between platelet count and CTC detection rate in esophageal cancer (1,2). Further research is necessary to better understand the implications and potential clinical application of the association between platelet count and CTC detection rate, as well as to determine whether this can be applied in tumor metastasis

| Table 14 Cell morphological characteristics of CTCs in PB that were subjected to combined fluorescent probe staining |
|---------------------------------------------------------------|
| Staining method                      | Slide ID | Cell ID | Cell diameter (μm) | Nuclear diameter (μm) | Cell surface area (μm²) | Nucleo-cytoplasmic ratio |
|--------------------------------------|----------|---------|--------------------|-----------------------|------------------------|--------------------------|
| Combined fluorescent probe staining  | 4        | 10      | 24.262             | 21.905                | 421.438                | 0.8469                   |
|                                      | 11       | 10      | 28.730             | 27.771                | 513.918                | 0.9519                   |
|                                      | 5        | 9       | 39.538             | 36.854                | 1227.419               | 0.8430                   |
|                                      | 12       | 10      | 31.762             | 32.716                | 708.316                | 0.8950                   |
|                                      | 7        | 1       | 42.508             | 38.009                | 915.802                | 0.7708                   |
|                                      | 2        | 10      | 35.016             | 34.035                | 612.053                | 0.9614                   |
|                                      | 8        | 1       | 21.770             | 20.544                | 338.973                | 0.8107                   |
|                                      | 10       | 2       | 23.478             | 22.789                | 362.559                | 0.8107                   |
|                                      | 3        | 10      | 24.650             | 23.823                | 409.600                | 0.8973                   |
|                                      | 11       | 2       | 23.478             | 22.789                | 362.559                | 0.8107                   |
|                                      | 3        | 10      | 24.650             | 23.823                | 409.600                | 0.8973                   |
|                                      | 14       | 2       | 29.853             | 27.489                | 538.733                | 0.8080                   |
|                                      | 15       | 2       | 44.195             | 42.352                | 1342.288               | 0.8741                   |
|                                      | 4        | 10      | 27.608             | 26.495                | 523.006                | 0.8942                   |
|                                      | 17       | 1       | 37.294             | 34.358                | 794.345                | 0.9135                   |
|                                      | 20       | 4       | 27.786             | 26.763                | 549.013                | 0.8123                   |
|                                      | 5        | 10      | 38.375             | 32.338                | 711.124                | 0.8326                   |
|                                      | 21       | 1       | 29.641             | 28.512                | 513.71                 | 0.9051                   |
|                                      | 22       | 1       | 28.134             | 24.231                | 598.543                | 0.8174                   |
|                                      | 24       | 2       | 28.672             | 27.011                | 595.861                | 0.8541                   |
|                                      | 26       | 1       | 27.753             | 26.763                | 519.456                | 0.8234                   |
|                                      | 34       | 2       | 27.672             | 24.835                | 562.866                | 0.7510                   |
| SD                                  |          |         |                    |                       |                        |                          |
| Degree of skewness                  | 1.9321   | 1.1021  | 73.1451            | 0.1243                |                        |                          |

CTC, circulating tumor cell; PB, peripheral blood; ID, identification; SD, standard deviation.
### Table 15 Comparison of the cell morphological characteristics of CTCs in PB between Wright's Giemsa and combined fluorescent probe staining methods

| Staining method                  | Slide ID | Cell ID | Cell diameter (μm) | Nuclear diameter (μm) | Cell surface area (μm²) | Nucleo-cytoplasmic ratio |
|----------------------------------|----------|---------|--------------------|-----------------------|------------------------|--------------------------|
| Wright's Giemsa staining         | 8        | 1       | 24.665             | 20.254                | 441.236                | 0.7251                   |
|                                  | 10       | 1       | 27.320             | 22.512                | 441.672                | 0.7251                   |
|                                  | 11       | 1       | 27.320             | 22.512                | 441.672                | 0.7251                   |
|                                  | 14       | 1       | 27.120             | 23.712                | 442.632                | 0.8124                   |
|                                  | 15       | 1       | 23.413             | 17.952                | 295.134                | 0.7134                   |
|                                  | 20       | 1       | 23.751             | 20.651                | 419.345                | 0.8264                   |
|                                  |          | 2       | 21.732             | 19.991                | 417.821                | 0.8521                   |
|                                  |          | 3       | 22.311             | 20.231                | 401.278                | 0.8011                   |
|                                  | 24       | 1       | 26.100             | 24.671                | 441.134                | 0.8100                   |
|                                  | 34       | 1       | 26.116             | 20.290                | 475.372                | 0.7241                   |
| Combined fluorescent probe staining | 4    | 10      | 24.262             | 21.905                | 421.438                | 0.8469                   |
|                                  |          | 11      | 28.730             | 27.771                | 513.918                | 0.9519                   |
|                                  | 5        | 9       | 39.538             | 36.854                | 1227.419               | 0.8430                   |
|                                  |          | 12      | 31.762             | 32.716                | 708.316                | 0.8950                   |
|                                  | 7        | 1       | 42.508             | 38.009                | 915.802                | 0.7708                   |
|                                  |          | 2       | 35.016             | 34.035                | 612.053                | 0.9614                   |
|                                  | 8        | 1       | 21.770             | 20.544                | 338.973                | 0.8107                   |
|                                  | 10       | 2       | 23.478             | 22.789                | 362.559                | 0.8107                   |
|                                  |          | 3       | 24.650             | 23.823                | 409.600                | 0.8973                   |
|                                  | 11       | 2       | 23.478             | 22.789                | 362.559                | 0.8107                   |
|                                  |          | 3       | 24.650             | 23.823                | 409.600                | 0.8973                   |
|                                  | 14       | 2       | 29.853             | 27.489                | 538.733                | 0.8080                   |
|                                  | 15       | 2       | 44.195             | 42.352                | 1342.288               | 0.8741                   |
|                                  |          | 4       | 27.608             | 26.495                | 523.006                | 0.8942                   |
|                                  | 17       | 1       | 37.294             | 34.358                | 794.345                | 0.9135                   |
|                                  | 20       | 4       | 27.786             | 26.763                | 549.013                | 0.8123                   |
|                                  |          | 5       | 38.375             | 32.338                | 711.124                | 0.8326                   |
|                                  | 21       | 1       | 29.641             | 28.512                | 513.71                 | 0.9051                   |
|                                  | 22       | 1       | 28.134             | 24.231                | 598.543                | 0.8174                   |
|                                  | 24       | 2       | 28.672             | 27.011                | 595.861                | 0.8541                   |
|                                  | 26       | 1       | 27.753             | 26.763                | 519.456                | 0.8234                   |
|                                  | 34       | 2       | 27.672             | 24.835                | 562.866                | 0.7510                   |
| P value                         |          |         | 0.308              | 0.013                 | 0.147                  | 0.004                    |

CTC, circulating tumor cell; PB, peripheral blood; ID, identification.
prognosis. In addition, clinical validation of the newly developed combined fluorescent probe staining method with a larger patient cohort and stratifying patients based on cancer types can further provide insights into the true clinical applicability of this novel technique.

In this study, we successfully developed and validated the new combined fluorescent probe staining method for CTC identification. Notably, we demonstrated its higher CTC detection accuracy compared to the reference control, Giemsa staining method. Our study proposes the potential application of the novel staining method in clinical diagnostics and/or prognostics.

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013). The necessary approval was obtained from the Ethics Committee of Shandong Cancer Hospital and Institute, China (No. 201702019). Written consent was obtained from all eligible subjects before enrollment into the study.

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