# Isolation of circulating tumor cells in patients undergoing surgery for esophageal cancer and a specific confirmation method

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**Abstract.** The clinical significance of circulating tumor cells (CTCs) in patients with esophageal squamous cell carcinoma (ESCC) who have undergone radical surgery was investigated. A novel confirmation method for identifying CTCs or circulating tumor microemboli (CTM) in ESCC was also investigated. Blood samples from 55 patients with ESCC were collected 1-3 days prior to surgery and 7 days post-surgery. All patients underwent curative thoracic esophagectomy and lymphadenectomy. Blood samples from 20 healthy volunteers were obtained as controls. Isolation by size of epithelial tumor cells was also performed. The overall CTC detection rate was 52.7% preoperatively and 49.1% postoperatively. The presence of CTCs correlated with the Tumor-Node-Metastasis stage and the Log odds of positive lymph nodes. No significant difference in perioperative CTC transformation was discovered between the thoracoscopic and laparoscopic approach, and the open approach. The $P40^+$/cluster of differentiation (CD)$45^-$ phenotype was confirmed in the CTCs and CTM. The isolation by size of epithelial tumor cells method appeared to have high sensitivity for detecting CTCs within ESCC patients. Immunofluorescence staining for CD45 and P40 was a specific, accurate and convenient method for confirming the presence of CTCs or CTM in patients with ESCC, and is strongly recommended as a supplement to morphological analysis.

## Introduction

Over the past 20 years, the overall survival of patients with esophageal cancer has remained poor (1). More than two-thirds of patients who undergo radical resection of this type of cancer will eventually succumb as a result of relapse and distant metastasis (2). Esophageal squamous cell carcinoma (ESCC) accounts for the majority of cases (>90%) of esophageal cancer in Asia (3), and the 5-year survival is 15-20% (4). The detection of circulating tumor cells (CTCs) in the peripheral circulation has been demonstrated to serve as a prognostic factor that may offer novel strategies for cancer treatment (5). Studies have reported that the detection of high CTCs by the CellSearch® system in metastatic breast cancer is associated with poor overall survival (6-8). The CellSearch® system has been authorized by the US Food and Drug Administration for the follow up of patients with breast, colonic and prostate metastases. However, this system has its limits; direct and indirect methods have been proposed for detecting CTCs, but these methods vary in specificity, sensitivity and cost (9-16). Among the direct methods, detecting CTCs according to the size of the epithelial tumor cells has been associated with good specificity and sensitivity, and has performed well in esophageal cancer (17). A low cost technique, it allows for the cytomorphological analysis and characterization of CTCs. Although there has been research into the clinical significance of CTCs detected in patients with ESCC, isolation by size of epithelial tumor cells is still not widely used, and the detection of CTCs is not closely associated with surgical or neoadjuvant therapies (18,19). As a preliminary study for the clinical trial no. NCT03005314 (ClinicalTrials.Gov ID)/ChiCTR-OON-17010807 (Chinese Clinical Trial Registry), isolation by size of epithelial tumor cells technology was used in the present study to isolate CTCs and circulating tumor microemboli (CTM) from patients with ESCC who had undergone surgery with curative intent.

## Patients and methods

*Patients*. A total of 55 patients with ESCC were enrolled in this single institution study conducted at the Second Hospital of Shandong University (Jinan, China) between July 2016 and June 2017. The study was approved by the Ethics Committee of the Second Hospital of Shandong University. Where blood samples were obtained, patients provided informed consent. Blood samples from 20 healthy volunteers were used as controls. All the patients who enrolled in the study underwent esophagectomy with 2- or 3-field lymph node dissection.

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*Key words:* circulating tumor cells, esophageal cancer, surgery, cell isolation, laboratory diagnosis

*Peripheral blood samples*. Peripheral blood samples were drawn from the median cubital vein into a tube with K2-EDTA, followed by adequate mixing. The first 2 ml of blood was discarded to prevent epithelial contamination, and the remaining 5 ml was immediately processed (within 2 h). Blood samples were harvested in the morning, 1-3 days prior to surgery and 7 days post-surgery.

*Surgical procedure*. All patients underwent curative thoracic esophagectomy and lymphadenectomy. Patients underwent either right or left thoracotomy, and the thoracoscopic and laparoscopic approaches were encouraged. Additionally, patients who underwent cervical anastomosis (McKeown) (20) or thoracic anastomosis (Ivor-Lewis) (21) were accepted. The incised margin was ≥5 cm from the superior border of the tumor.

The range of lymphadenectomy included the periesophageal lymph nodes, subcarinal lymph nodes, left and right recurrent laryngeal nerve lymph nodes, hilar lymph nodes, and lesser omentum (specifically the left gastric vessel region) and any suspicious lymph nodes next to the common hepatic arteries, and cervical lymph nodes were selectively dissected according to tumor location and ultrasound examination. Jian-Hui *et al* (22) established that the log odds of positive lymph nodes (LODDS) exhibited improved prognostic performance compared with either the number of lymph node metastases (LNMs) or the positive lymph node ratio (LNR) in patients with gastric cancer. Cao *et al* (23) considered the LODDS a more accurate index compared with the LNMs or LNR for evaluating the survival of patients undergoing resection for esophageal cancer. LODDS is classified as follows: LODDS1≤-2.6, -2.6<LODDS2≤-1.5, -1.5<LODDS3≤-0.5 and LODDS4>-0.5. As the index increases, the 5-year cancer-specific survival decreases.

*Isolation by size of epithelial tumor cells assay*. The procedure was performed as previously described by Vona *et al* (10). The filtration module was kindly provided by Wuhan YZY Medical Science and Technology Co., Ltd. (Wuhan, China). A total of 5 ml whole blood was diluted to 8 ml with buffer containing 0.2% formaldehyde, and filtered through an 8 $\mu$m membrane. The assay was performed according to the manufacturers' protocol. The cells were classified as CTCs if they met ≥4 of the following criteria: i) A markedly enlarged nucleus (>2-3 calibrated pore sizes); ii) a high nucleocytoplasmic ratio (ratio >0.8); iii) hyperchromasia and nonhomogeneous staining; iv) irregularity of the nuclear membrane; v) anisonucleosis (ratio >0.5) and the presence of three dimensional sheets; vi) the presence of nuclear chromatin side-shift or large nucleoli; and vii) the presence of abnormal mitotic figures. Cells with no cytoplasm were not analyzed. All images were recorded and reviewed independently by 6 cytopathologists from different institutions, and CTCs were confirmed by agreement between ≥4 cytopathologists.

*Confirmation of CTCs*. Immunofluorescence staining for cluster of differentiation (CD)45 and P40 was conducted for preliminary confirmation. The expression of CD45 was observed to distinguish between CTCs and leukocytes, particularly megakaryocytes and large monocytes. The expression of P40 indicated the squamous origin of cells. Lung squamous cell carcinoma cells were used as a $P40^+$ control, and the harvested lymphocytes were used as a $CD45^+$ control. A total of 5 ml/blood sample was separated by CTC biopsy machine. The cells were fixed for 5 min with 200 $\mu$l paraformaldehyde (2%) added to the filter at room temperature (18-26˚C). The cells were rinsed with PBS for 3x2 min. Subsequently, 200 $\mu$l methanol was added to the filter and allowed to stand for 1 min, the filter film was removed, placed on one side of a glass slide, dried for 4-5 min at room temperature, and transferred to the center of the slide, where it was mounted with 2 $\mu$l adhesive [transparent reagent (BASE BA-7002B) and mountant (BASE BA-7004)1/4 (Baso Biotechnology Co., Ltd., Wuhan, China)]. A circle was drawn around the filter film with a PAP pen. The sample was subsequently treated with 200 $\mu$l 0.5% Triton X-100 for 5 min and rinsed with PBS for 3x2 min. Subsequently, 100 $\mu$l 10% goat serum (Jackson ImmunoResearch Europe, Ltd., Newmarket, UK) in PBS was added to the filter film and allowed to stand for 30 min at room temperature; the excess serum was removed. The samples were incubated at 4˚C overnight with 100 $\mu$l primary antibody (anti-CD45; Santa Cruz Biotechnology, Inc., Dallas, TX, USA; cat no. sc-70699; or anti-P40; Abcam, Cambridge, UK; cat no. ab137691), diluted 1:500 and 1:200, respectively, with 10% goat serum. Thee samples were rinsed with PBS for 3x3 min, 100 $\mu$l secondary antibody (Alexa Fluor 488-conjugated goat anti-rat; cat no. A11006; or Alexa Fluor 647-conjugated goat anti-rabbit; cat no. A21245; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) diluted 1:500 with 10% goat serum was added, and the slides were incubated for 50 min at room temperature. Following washing with PBS 3x2 min, the films were sealed with DAPI and observed by fluorescence microscopy (magnification, x40). When images of the slides had been captured, Wright-Giemsa staining was performed (10,17) for comparison with the immunofluorescence results. The slides were stained with 100 $\mu$l diff A (Eosin; YZY Medical Science and Technology Co., Ltd., Wuhan, China; catalog no. YZY-CTC-P100) for 1 min at room temperature. Following rinsing with PBS for 1 min, 100 $\mu$l diffB (Methylthioninium Chloride; YZY Medical Science and Technology Co., Ltd.; catalog no. YZY-CTC-P100) was added for 90 sec at room temperature. The slides were then rinsed with deionized water three times for 30 sec each time and dried for 30 min at 50˚C. Following mounting with permanent mounting medium (Baso Ultra-Clear Advanced Mounting Resin; Baso Biotechnology Co., Ltd.; catalog no. BASE BA-7004), the slides were dried for 1 h at 50˚C. Finally, the cells were observed using an optical microscope (magnification, x40).

*Statistical analysis*. Stata 12.0 (StataCorp LP, College Station, TX, USA) was used for statistical evaluation. Quantitative data are presented as the mean ± standard deviation. Any associations between CTC detection and clinicopathological parameters, lymph node metastasis and surgical procedure were ascertained by $\chi^2$ test or Fisher's exact test. Student's t-tests were used to analyze continuous variables when samples were from a population with a normal distribution and homogeneous variance; otherwise, two-sample Wilcoxon rank-sum tests were used. Cox proportional hazards regression analysis was employed to evaluate the clinical factors for survival. $P<0.05$ was considered to indicate a statistically significant difference.

Table I. Associations of clinicopathological parameters with CTC detection.

| | | Pre-surgery, n | | Post-surgery, n | |
|---|---|---|---|---|---|
| Variable | All | CTC-positive | P-value | CTC-positive | P-value |
| Age | | | 0.606 | | 0.864 |
| ≥60 | 34 | 17 | | 17 | |
| <60 | 21 | 12 | | 10 | |
| Sex | | | 0.721 | | 0.469 |
| Male | 46 | 25 | | 24 | |
| Female | 9 | 4 | | 3 | |
| Location | | | 0.825 | | 0.645 |
| Upper | 7 | 4 | | 3 | |
| Middle | 25 | 14 | | 14 | |
| Lower | 23 | 11 | | 10 | |
| Size, cm | | | | | |
| >5 | 16 | 11 | 0.127 | 11 | 0.062 |
| ≤5 | 39 | 18 | | 16 | |
| Differentiation | | | 0.188 | | 0.936 |
| Well | 3 | 2 | | 1 | |
| Moderate | 13 | 4 | | 6 | |
| Poor | 37 | 21 | | 19 | |
| Other | 2 | 2 | | 1 | |
| T stage | | | 0.584 | | 0.193 |
| Tis | 1 | 0 | | 0 | |
| T1 | 4 | 2 | | 0 | |
| T2 | 4 | 3 | | 3 | |
| T3 | 42 | 21 | | 22 | |
| T4 | 4 | 3 | | 2 | |
| Venous invasion | | | 0.205 | | 0.322 |
| Positive | 15 | 10 | | 9 | |
| Negative | 40 | 19 | | 18 | |
| Lymphatic invasion | | | 0.475 | | 0.295 |
| Positive | 9 | 6 | | 6 | |
| Negative | 46 | 23 | | 21 | |
| TNM stage | | | 0.051 | | 0.148 |
| I | 3 | 2 | | 0 | |
| II | 18 | 5 | | 8 | |
| III | 32 | 21 | | 19 | |
| IV | 1 | 1 | | 0 | |
| Combination | | | 0.017 | | 0.163 |
| I+II | 21 | 7 | | 8 | |
| III+IV | 33 | 22 | | 19 | |

CTC, circulating tumor cells; TNM, Tumor-Node-Metastasis.

## Results

*Patient clinicopathological parameters and CTC detection*. In all, 55 patients were enrolled between July 2016 and June 2017, of which 46 were male and 9 female, with an age range of 49-86 years. All patients achieved R0 resection (tumor was resected and no pathological residual was observed) on their first treatment; 2 patients received neoadjuvant chemotherapy. Baseline clinical data included age, sex, primary tumor location, tumor size, differentiation, Tumor (T) stage, lymph node metastasis, stage, venous invasion, lymphatic invasion and Tumor-Node-Metastasis (TNM) stage. The associations between the clinicopathological parameters and CTC detection are displayed in Table I.

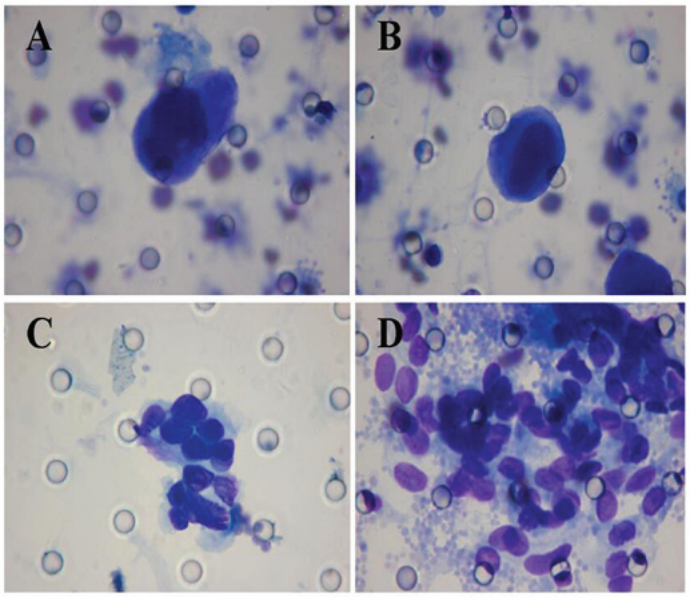


Figure 1. Morphological analysis of CTCs/CTM in patients with esophageal squamous cell carcinoma. Giemsa stain, Diff-Quik staining method (magnification, x1,000), isolated by CTCBIOPSY®. (A) The diameter of the CTC was >40 $\mu$m, the shape of the CTC was irregular, the nuclear membrane was thickened, the nucleus was stained deeply, and a large amount of cytoplasm was present and was stained blue. (B) The diameter of the CTC was >40 $\mu$m, there was a relatively high ratio of nucleus to cytoplasm, the nucleolus was irregularly shaped and not stained equally, thick chromosomes were apparent, the nuclear membrane was thickened, abnormal mitosis of the nucleus was present. (C) CTM was apparent in 11 CTCs, the nucleoli were irregularly shaped, the nuclear membranes were thickened and folded, nucleoli were present, a large volume of cytoplasm was present and appeared pale blue. (D) the nucleoli CTM were irregularly shaped and different in size, the nuclear membrane was thickened and folded. CTC, circulating tumor cell; CTM, circulating tumor microemboli.

The overall CTC detection rate was approximately 52.7% preoperatively and 49.1% postoperatively (data not shown). The CTC and CTM are presented in Fig. 1. No significant differences were observed for CTC positivity in terms of age, sex, location, size, differentiation, T stage, venous invasion, or lymphatic invasion prior to or following surgery. However, a difference in CTC positivity prior to surgery was observed for TNM stage (P=0.051). Additionally, following the combination of stages I and II, and stages III and IV, a significant difference was observed despite 2 out of 3 stage I patients being positive for CTCs, which was considered to be a sampling error (P=0.017).

As for the number of CTCs or CTM, another issue was encountered during detection. There were 18 samples from 6 patients, with three samples from each patient to test for consistency during repeated detection; all samples were drawn before 09:00 am on three separate preoperative days under almost exactly the same conditions. The results are presented in Table II. Although the first detection results indicate the presence of CTM or CTCs, the numbers were highly variable, which was considered attributable to the instantaneous sampling of the peripheral circulation and the uncertainty of the internal physiological environment. Repeated CTC detection for clinical stages III and IV is suggested if there is special consideration of the count; however, further studies are required to determine whether the mean or the maximal value should be used.

*Systemic inflammatory response, platelet count and CTC detection*. CTC/CTM detection was not significantly associated with the preoperative neutrophil-to-lymphocyte ratio (NLR), the preoperative platelet-to-lymphocyte ratio (PLR) or the platelet count, as displayed in Table III. Nonetheless, the platelet count remained closely associated with CTCs and CTM.

*Lymph node metastasis and CTC detection*. There were no LODDS4 patients in the present study. A single patient was excluded from the analysis due to a large difference in the lymph node dissection between the surgical procedure and the pathological report. There were no significant differences in CTC positivity between lymph node metastasis-positive and negative patients prior to or following surgery. However, there was a significant difference among the LODDS1, LODDS2 and LODDS3 groups (P=0.033) prior to surgery (Table IV). CTC positivity significantly increased from LODDS1 to LODDS2 (P=0.027), but there was no significant difference between LODDS2 and LODDS3 (P=0.063) (Table IV).

*Pre- and postoperative CTC detection and surgical procedures*. Liu *et al* (24) established a quantitative system for evaluating the role of CTCs in patients with esophageal cancer who had undergone surgery. It was postulated that surgery for esophageal cancer results in tumor cell dissemination and a significant increase in the number of CTCs in the peripheral blood, which is associated with the development of metastasis. In the present study, the number of CTCs detected prior to and following different surgical procedures was compared: The thoracoscopic and laparoscopic approach; and left thoracotomy or thoracotomy and laparotomy (Table V). CTC detection revealed that: i) CTCs or CTM declined following surgery, or in particular cases, CTM disappeared entirely; or ii) CTCs or CTM increased following surgery, or in particular cases, CTM developed after previously being absent.

*Confirmation of CTCs/CTM*. To further confirm the presence of CTCs/CTM, immunofluorescence staining for CD45 (leukocytes) and P40 (cells of squamous epithelial origin)

Table II. Samples (n=18) from 6 patients for CTC detection.

| | 1st detection | | 2nd detection | | 3rd detection | |
|---|---|---|---|---|---|---|
| Patient, n | CTM | CTCs | CTM | CTCs | CTM | CTCs |
| 1 | 4 | - | - | 2 | 18 | 1 |
| 2 | 1 | 1 | - | 1 | - | - |
| 3 | - | - | - | - | - | - |
| 4 | 8 | 2 | 2 | 2 | 15 | 7 |
| 5 | 1 | 4 | 16 | - | 6 | 1 |
| 6 | 4 | 1 | 2 | 3 | - | |

CTC, circulating tumor cells; CTM, circulating tumor microemboli.

Table III. Associations of the NLR, PLR, and platelet count with CTC detection.

| Item | CTC-positive | CTC-negative | P-value |
|---|---|---|---|
| NLR (median ± SD) | 3.12±1.54 | 2.96±1.32 | 0.3479 |
| PLR (median ± SD) | 182.53±96.68 | 178.46±87.73 | 0.4364 |
| Platelet count ($x10^9$/l, median ± SD) | 249.82±68.88 | 224.88±87.71 | 0.1282 |

CTC, circulating tumor cells; NLR, neutrophil-to-lymphocyte ratio; PLR, platelet-to-lymphocyte ratio; SD, standard deviation.

Table IV. Associations of lymph node metastasis with CTC detection.

| | | Pre-surgery, n | | Post-surgery, n | |
|---|---|---|---|---|---|
| Variable | All | CTC-positive | P-value | CTC-positive | P-value |
| Lymph node metastasis | | | 0.083 | | 0.186 |
| Positive | 33 | 21 | | 18 | |
| Negative | 22 | 9 | | 8 | |
| LODDS | | | 0.033 | | 0.918 |
| LODDS1 | 11 | 5 | | 6 | |
| LODDS2 | 16 | 14 | | 10 | |
| LODDS3 | 5 | 2 | | 3 | |

CTC, circulating tumor cells; LODDS, log odds of positive lymph nodes.

was conducted on portions of the samples. The $P40^+/CD45^-$ phenotype was confirmed in CTCs and CTM (Fig. 2). In particular cases, cells suspected to be CTCs or CTM were confirmed to be leukocytes (Fig. 3).

*Survival analysis*. Cox proportional hazards regression analysis was performed under the condition of inadequate follow up time. A total of 9 parameters was analyzed, including sex, age, preoperative CTC detection, postoperative CTC detection, surgical procedure (minimally invasive esophagectomy or open surgery), differentiation degree (poor or middle-high), tumor cutting area (≥5 or <5 cm), infiltration depth (T1, T2, T3) and the number of positive lymph nodes. A log-rank test was first used to filter the prognostic factor (Table VI). As a result, the factors of preoperative CTC detection, postoperative CTC detection, differentiation degree (poor or middle-high), tumor cutting area, and the number of positive lymph nodes were subjected to Cox proportional hazards regression analysis. The results (including hazard ratio) are displayed in Table VII. The Kaplan-Meier survival curve is illustrated in Fig. 4.

## Discussion

Various methods have been used to detect CTCs, including those depending on tumor cell size, tumor-associated markers, or reverse transcription-quantitative polymerase chain reaction (RT-qPCR)-based assays. The CellSearch® system depends on tumor-associated markers and has been demonstrated to have an extremely low detection rate in ESCC (17). Although RT-qPCR has been widely used in the past few years (25), the cell integrity

Table V. Associations of surgical procedures with CTC detection.

| Variable | CTCs or CTM increase | CTCs or CTM decline | P-value |
|---|---|---|---|
| Thoracoscopic and Laparoscopic Approach | 9 | 16 | 0.864 |
| Left thoracotomy or thoracotomy and laparotomy | 5 | 10 | |

CTC, circulating tumor cells; CTM, circulating tumor microemboli.

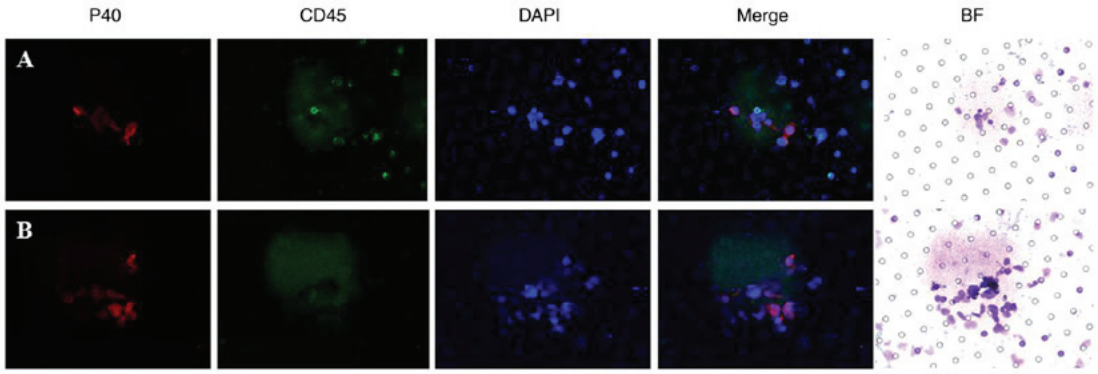


Figure 2. Immunofluorescence staining characteristics of CTCs/CTM (magnification, x40). Specimens were stained with anti-P40 (red for cells of squamous epithelial origin), anti-CD45 (green for leukocytes) and DAPI (blue for nuclei). (A) CTCs, strong expression of P40 and no expression of CD45 ($P40^+/CD45^-$); lymphocytes, strong expression of CD45 and no expression of P40 ($P40^-/CD45^+$); (B) CTCs/CTM, strong expression of P40 and no expression of CD45 ($P40^+/CD45^-$). CTC, circulating tumor cell; CTM, circulating tumor microemboli; CD, cluster of differentiation; BF, bright field.

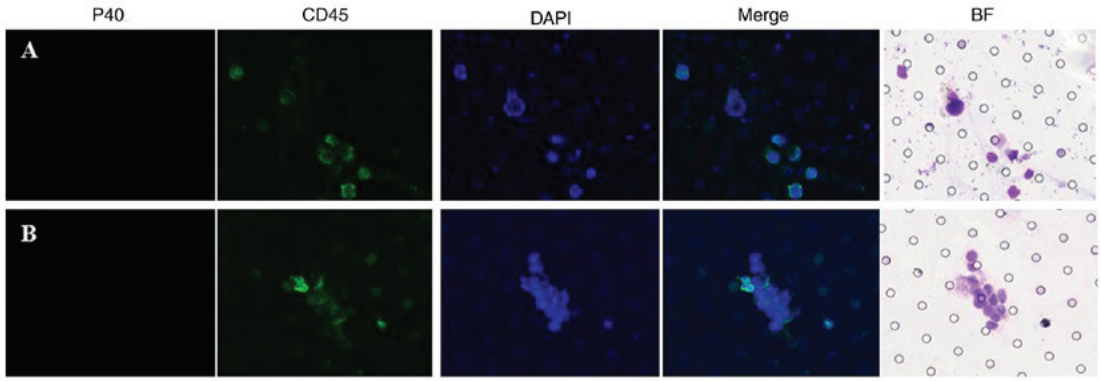


Figure 3. Immunofluorescence staining for confirmation of CTCs/CTM (magnification, x40). Following diagnosis by ≥4 cytopathologists, putative CTCs/CTM were likely to be accurately identified. The specimens were stained with anti-P40 (red for cells of squamous epithelial origin), anti-CD45 (green for leukocytes), and DAPI (blue for nuclei). The panels labeled A and B represent different patients. Strong expression of CD45 and no expression of P40 ($P40^-/CD45^+$); cells were ultimately confirmed as leukocytes. CTC, circulating tumor cell; CTM, circulating tumor microemboli.

is destroyed by RNA extraction, and benign cells are present in the peripheral circulation (26). Isolation by size of epithelial tumor cells is considered to be a suitable method for application in ESCC (17). The present study was the first to use isolation by size of epithelial tumor cells to detect CTCs in patients with ESCC prior to and following surgery, and to use immunofluorescence staining to observe the expression of P40 and CD45 by CTCs or CTM detected in patients with ESCC.

The 7th edition of the American Joint Committee on Cancer (AJCC) Cancer Staging Manual describes stage 0 (Tis) and stages I-IV. In the present study, the CTC detection rate prior to surgery revealed a significant difference between stages I-II and III-IV. This finding indicated that the CTC detection rate was associated with the prognosis of ESCC. Other studies have also used the CellSearch® system to isolate and enumerate CTCs (19). Kaganoi *et al* (18) used RT-qPCR to detect cancer cells using specific mRNAs; patients who were positive for the mRNA encoding squamous cell carcinoma antigen (SCCA mRNA) had a higher recurrence rate compared with those who were negative for the antigen. Also, SCCA mRNA was associated with the depth of the tumor and venous invasion. However, in the present study, there was no significant difference in CTC detection performed prior to and following surgery.

Table VI. Log-rank test for filtering the prognostic factors.

| Prognostic factors | $\chi^2$ | P-value |
|---|---|---|
| Sex | 0.67 | 0.4122 |
| Age | 0.30 | 0.5864 |
| CTCpre | 6.41 | 0.0113 |
| CTCpost | 4.20 | 0.0403 |
| Therapy | 0.18 | 0.6694 |
| Differentiation | 9.49 | 0.0021 |
| Size | 6.72 | 0.0095 |
| Depth | 3.84 | 0.2788 |
| Node | 36.10 | 0.0002 |

CTC, circulating tumor cells; CTCpre, CTC detection pre-operation; CTCpost, CTC detection post-operation.

Measuring the systemic inflammatory response is another method for assessing the outcome of malignancies; this method is simple and reliable and may be incorporated into current staging procedures as Roxburgh and McMillan demonstrated in a review that preoperative measures of the systemic inflammatory response predict cancer survival (27,28). Tumors interact with inflammatory cells, and the tumor-associated inflammatory response may promote metastasis by upregulating inflammatory mediators, inhibiting apoptosis, damaging DNA and enhancing angiogenesis (29). An elevated NLR was associated with significantly decreased disease-free survival and overall survival (adenocarcinomas accounted for 68% of these tumors) (30). However, another study has reported that neither NLR nor PLR is an independent prognostic factor in ESCC (31). Similarly, the present study did not indicate a significant correlation between CTC detection with NLR, PLR or platelet count, but these three parameters displayed a positive trend for association with CTC positivity compared with CTC negativity.

Notably, a single patient who accepted the left thoracotomy surgery approach, was CTC positive, with postoperative pathological staging of T1bN0M0, IB, while another patient, who accepted the thoracoscopic and laparoscopic approach with thoracic anastomosis (Ivor-Lewis), was CTM-positive with the same postoperative pathological stage. This may reflect the current staging system, or be due to a correlation with the platelet count. The 7th Union for International Cancer Control/AJCC TNM edition acknowledges the LNM as the current standard for N staging (32), and the minimum suggested number of lymph nodes harvested ranges from 12 to 18 (33,34). The former patient had 21 lymph nodes sampled, while the latter patient had 22. Theoretically, these numbers are sufficient for accurate staging; however, as a result of the surgical approach the superior mediastinal lymph nodes were not adequately sampled. In general, two- or three-field lymph node dissection may increase the staging accuracy. Additionally, there was a significant difference in the CTC detection rate between stages I and II and stages III and IV, while the platelet count did not reveal a significant difference. Nevertheless, there was no significant difference in the CTC detection rate for stages IIIB, IIIC and IV compared with stages I, II and IIIA; though the platelet count did highlight a significant difference. These findings suggest that an elevated platelet count may promote cancer cell extravasation and increase the number of CTCs in peripheral blood. Schumacher *et al* (35) demonstrated that platelets promote cancer cell transendothelial migration via the $P2Y_2$ receptor to facilitate tumor cell survival and dissemination. The two previously mentioned patients had relatively high platelet counts of $2.91x10^{11}$/l and $3.38x10^{11}$/l, respectively, yet were not independently statistically analyzed due to the small sample size. Platelet counts still revealed an obvious trend when the presence of CTM was observed, using two-sample Wilcoxon rank-sum tests due to the small sample size), although there was no significant difference. From another point of view, these results corroborate past research suggesting that a high platelet count is associated with tumor progression and poor survival in patients with ESCC.

Regarding the lymph node staging system, LODDS has gained attention as a novel indicator and is defined as the log of the ratio of the number of positive lymph nodes to the number of negative lymph nodes. LODDS has been suggested as a powerful system for predicting survival in gastric (22) and esophageal cancer (23). One study (23) of esophageal cancer illustrated that LODDS predicts survival more accurately compared with the present system of LNMs, and may serve as another indicator of the LNR. Furthermore, LODDS is a factor that does not depend on the number of lymph nodes sampled. Indeed, there are situations in which surgeons cannot perform adequate lymph node resection due to extensive pleural adhesions or advanced patient age. The present study revealed that CTC positivity was associated with LODDS group prior to surgery. While the connection between the presence of CTCs and the LODDS group requires further research, the parameters hold value for evaluating prognosis in esophageal cancer and reflecting the tendency toward tumor cell metastasis.

An earlier study (24) demonstrated that surgery for esophageal cancer results in cancer cell dissemination, which is associated with metastasis. In the current study, CTCs were used to investigate the effect of different surgical approaches which may enhance CTC dissemination to different extents. No significant difference was illustrated between the thoracoscopic and laparoscopic approach and the open approach. Such research is not easy to perform due to the uncertainty of the backflow of the vein from the ESCC tumor body. In lung cancer, it has been demonstrated that the pulmonary vein CTC count significantly increases at the time of lobectomy completion. Additionally, the number of CTCs in preoperative peripheral blood or intraoperative pulmonary venous blood is an independent risk factor for tumor-free survival and overall survival in patients with resected non-small-cell lung cancer (36,37). The backflow of the esophagus may pass through the azygos vein, inferior phrenic vein or left gastric vein, and these veins could not be sampled at the same time. As a result, direct evidence is difficult to obtain; therefore, the increase in CTCs in peripheral blood may be attributable to narcotism or stress.

Although the phenotype of CTCs and CTM harvested from ESCC patients has been previously investigated (17), cytokeratin and vimentin levels were used as indices to explain why CTM could not be detected by the CellSearch® system. SCCA mRNA is detectable by RT-qPCR (18) but with a confirmed lower sensitivity compared with isolation by size of epithelial tumor cells (10). In the present study, immunofluorescence staining demonstrated for the first time that the CTCs or CTM harvested

Table VII. Cox proportional hazards regression analysis.

| Prognostic factor | Hazard ratio | Standard error | z | P>\|z\| | 95% confidence interval |
|---|---|---|---|---|---|
| Preoperative CTC detection | 1.84 | 1.05 | 1.06 | 0.29 | 0.60-5.66 |
| Postoperative CTC detection | 1.19 | 0.70 | 0.30 | 0.77 | 0.38-3.75 |
| Differentiation degree | 7.39 | 7.80 | 1.89 | 0.06 | 0.93-58.53 |
| Tumor cutting area | 2.11 | 1.10 | 1.42 | 0.16 | 0.75-5.88 |
| Positive lymph nodes | 1.12 | 0.06 | 1.94 | 0.052 | 0.99-1.25 |

CTC, circulating tumor cells.

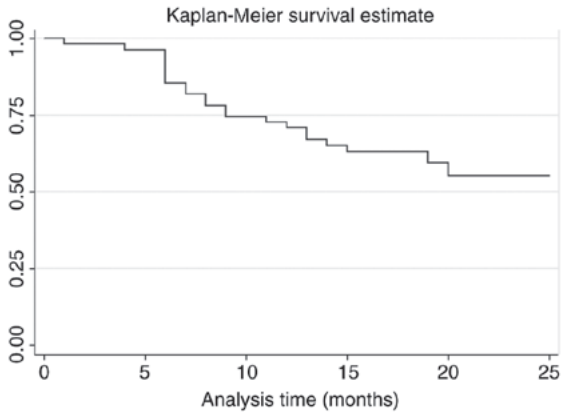


Figure 4. Kaplan-Meier survival curve with the results of preoperative and postoperative circulating tumor cell detection.

by isolation by size of epithelial tumor cells were indeed cells of squamous epithelial origin, and this method was faster and less costly compared with RT-qPCR. The procedure has great clinical significance in that abnormal cells are definitively classified as CTCs and CTM, and different cells of epithelial origin may be distinguished in the future. Furthermore, chemotherapy protocols benefit from this procedure, especially for synchronous cancers. It also revealed that identifying suspected cells by morphology was not completely reliable. Clear morphological standards or abnormal cell aggregates could not be used as definite diagnostic criteria for blood samples in ECSS.

In Cox proportional hazards regression analysis, preoperative CTC detection, postoperative CTC detection, differentiation degree (poor or middle-high), tumor cutting area, and the number of positive lymph nodes had a significant impact on postoperative survival during factors screening. There was no significant correlation between the factors and postoperative mortality risk. Kaplan-Meier survival curves were separate particularly when pre- and postoperative CTC detection were positive. Long-term follow up is suggested to determine the impact of CTC detection on postoperative survival. Circulating DNA evaluated by next generation sequencing may be combined with the aforementioned methods to predict tumor response, or to provide more information on metastasis or survival.

In conclusion, the present study illustrated the value of CTC detection in patients with ESCC, and provides a specific approach for confirming other CTCs of epithelial origin. Testing for the $P40^{+}/CD45^{-}$ phenotype is strongly advocated to ensure accurate identification.

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**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Authors' contributions**

YZ analyzed the patient data and wrote the manuscript. SZ performed the experiments. YC made substantial contributions to quality control of the experiments, and analyzed and described the figures. XD, CP and QS acquired the data and were involved in drafting the manuscript. LS and ZW interpreted the data. XZ conceived and designed the study. All authors read and approved the final manuscript.

**Ethics approval and consent to participate**

The present study was approved by the Ethics Committee of the Second Hospital of Shandong University. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

**Patient consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

## Note added post-publication

Since the publication of this article, note that the nine references to 'ISET' have been expanded to 'isolation by size of epithelial tumor cells'. These changes affect neither the results nor the conclusions reported in this study.

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