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

YUNPENG ZHAO1, SHUKANG ZHAO1, YINGJIE CHEN1, XIAOPENG DONG1, CHUANLIANG PENG1, QIFENG SUN1, LEI SHAN1, ZHENDAN WANG2 and XIAOGANG ZHAO1

1Department of Thoracic Surgery, The Second Hospital of Shandong University, Jinan, Shandong 250033; 2Department of Thoracic Surgery, Shandong Cancer Hospital and Institute (HL), Jinan, Shandong 250177, P.R. China

Received April 29, 2018; Accepted December 6, 2018

DOI: 10.3892/ol.2019.10017

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 (ISET) was performed also. 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. ISET 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 cyt morphological 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 (ISET) 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), ISET 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.

Correspondence to: Professor Xiaogang Zhao, Department of Thoracic Surgery, The Second Hospital of Shandong University, 247 Beiyuan Street, Jinan, Shandong 250033, P.R. China E-mail: zhaoxiaogang@sdu.edu.cn

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.

ISET assay. The procedure was performed as previously described by Vona et al (10). The filtration module was kindly provided by Wuhan ZYZ 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 µm 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 µl paraformaldehyde (2%) added to the filter at room temperature (18-26°C). The cells were rinsed with PBS for 3x2 min. Subsequently, 200 µl 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 µl adhesive [transparent reagent (BASE BA-7002B) and mountant (BASE BA-7004)]/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 µl 0.5% Triton X-100 for 5 min and rinsed with PBS for 3x2 min. Subsequently, 100 µl 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 µl 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. The samples were rinsed with PBS for 3x3 min. 100 µl 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 µl diff A (Eosin; ZYZ Medical Science and Technology Co., Ltd., Wuhan, China; catalog no. ZYZ-CTC-P100) for 1 min at room temperature. Following rinsing with PBS for 1 min, 100 µl diff B (Methylthioninium Chloride; ZYZ Medical Science and Technology Co., Ltd.; catalog no. ZYZ-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 χ² 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.
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.
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) was conducted on portions of the samples. The P40<sup>+</sup>/CD45<sup>−</sup> 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 is destroyed by RNA extraction, and benign cells are present in the peripheral circulation (26). ISET is considered to be a suitable method for application in ESCC (17). The present study was the first to use ISET 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.

Table V. Associations of surgical procedures with CTC detection.

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

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

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 thorascopic 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 IIB, IICC 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_{12} 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 test 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 thorascopic 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,

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

| Prognostic factors | χ^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.
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 ISET (10). In the present study, immunofluorescence staining demonstrated for the first time that the CTCs or CTM harvested by ISET 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.

Acknowledgements

The authors would like to thank Dr Chengke Zhang (Second Hospital of Shandong University (Shandong, China) and Dr Shili Han (Wuhan YZY Medical Science and Technology Co., Ltd., Wuhan, China) for their technical support.

Funding

The present study was funded by the National Key Research and Development Program of China (grant no. 2016YFC0106005).

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
The authors declare that they have no competing interests.

References

1. Allum WH, Stenning SP, Bancewicz J, Clark PL and Langlee RE: Long-term results of a randomized trial of surgery with or without preoperative chemotherapy in esophageal cancer. J Clin Oncol 27: 5062-5067, 2009.

2. Jemal A, Siegel R, Ward E, Hao Y, Xu J and Thun MJ: Cancer statistics, 2009. CA Cancer J Clin 59: 25-29, 2009.

3. Kamangar F, Liao J and Anderson WF: Patterns of cancer incidence, mortality, and prevalence across five continents: Defining priorities to reduce cancer disparities in different geographic regions of the world. J Clin Oncol 24: 2137-2150, 2006.

4. Pennathur A, Gibson MK, Jobe BA and Luketich JD: Oesophageal carcinoma. Lancet 381: 400-412, 2013.

5. Paterlini-Brechot P and Benali NL: Circulating tumor cells (CTC) detection: Clinical impact and future directions. Cancer Lett 253: 180-204, 2007.

6. Cristofanilli M, Budd GT, Ellis MJ, Stopeck A, Matera J, Miller MC, Reuben JM, Doyle GV, Allard WJ, Terstappen LW and Hayes DF: Circulating tumor cells, disease progression, and survival in metastatic breast cancer. N Engl J Med 351: 781-791, 2004.

7. Cristofanilli M, Hayes DF, Budd GT, Ellis MJ, Stopeck A, Reuben JM, Doyle GV, Matera J, Allard WJ, Miller MC, et al: Circulating tumor cells: A novel prognostic factor for newly diagnosed metastatic breast cancer. J Clin Oncol 23: 1420-1430, 2005.

8. Hayes DF, Cristofanilli M, Budd GT, Ellis MJ, Stopeck A, Miller MC, Matera J, Allard WJ, Doyle GV and Terstappen LW: Circulating tumor cells at each follow-up time point during therapy of metastatic breast cancer patients predict progression-free and overall survival. Clin Cancer Res 12: 4218-4224, 2006.

9. Sabile A, Louha M, Bonte E, Poussin K, Vona G, Mejean A, Chretien Y, Bougas L, Lacour B, Capron F, et al: Efficiency of Ber-EP4 antibody in isolating circulating epithelial tumor cells before RT-PCR detection. Ann J Clin Pathol 112: 171-178, 1999.

10. Vona G, Sabile A, Louha M, Sitrauk V, Romana S, Schütte K, Capron F, Franco D, Pazzagli M, Vekemans M, et al: Isolation by size of epithelial tumor cells: A new method for the immunomorphological and molecular characterization of circulating tumor cells. Am J Pathol 156: 57-63, 2000.

11. Anker P, Mulcahy H and Stroun M: Circulating nucleic acids in plasma and serum as a noninvasive investigation for cancer: Time for large-scale clinical studies? Int J Cancer 103: 149-152, 2003.

12. Clarke LE, Leitzel K, Smith J, Ali SM and Lipton A: Epidermal growth factor receptor mRNA in peripheral blood of patients with pancreatic, lung, and colon carcinomas detected by RT-PCR. Int J Oncol 22: 425-430, 2003.

13. Chen TF, Jiang GL, Fu XL, Wang LJ, Qian H, Wu KL and Zhao S: CK19 mRNA expression measured by reverse-transcription polymerase chain reaction (RT-PCR) in the peripheral blood of patients with non-small cell lung cancer treated by chemoradiation: An independent prognostic factor. Lung Cancer 56: 105-114, 2007.

14. Guo J, Xiao B, Jin Z, Qin L, Chen J, Chen H, Zhang X and Liu Z: Detection of cytokeratin 20 mRNA in the peripheral blood of patients with colorectal cancer by immunomagnetic bead enrichment and real-time reverse transcription-polymerase chain reaction. J Gastrointest Oncol 20: 1279-1284, 2005.

15. Hayes DC, Seerist H, Bangur CS, Wang T, Zhang X, Harlan D, Goodman GE, Houghton RL, Persing DH and Zhenthener BK: Multigene real-time PCR detection of circulating tumor cells in peripheral blood of lung cancer patients. Anticancer Res 26: 1567-1575, 2006.

16. Gervasoni A, Monasterio Muñoz RM, Wengler GS, Rizzi A, Zamboni A and Parolini O: Molecular signature detection of circulating tumor cells using a panel of selected genes. Cancer Lett 263: 267-279, 2008.

17. Li H, Song P, Zou B, Liu M, Cui K, Zhou P, Li S and Zhang B: Circulating tumor cell analyses in patients with esophageal squamous cell carcinoma using epithelial marker-dependent and independent approaches. Medicine (Baltimore) 94: e1565, 2015.

18. Kaganoi J, Shimada Y, Kobata M, Okamura T, Watanabe G and Imamura M: Detection of circulating oesophageal squamous cancer cells in peripheral blood and its impact on prognosis. Br J Surg 91: 1055-1060, 2004.

19. Matsushita D, Uenosono Y, Aragami T, Yanagita S, Nishizono Y, Hagihara T, Hirata M, Haraguchi N, Arima H, Kijima Y, et al: Clinical significance of circulating tumor cells in peripheral blood of patients with esophageal squamous cell carcinoma. Ann Surg Oncol 22: 3674-3680, 2015.

20. McKeown KC: Total three-stage oesophagectomy for cancer of the oesophagus. Br J Surg 63: 259-262, 1976.

21. Lewis I: The surgical treatment of carcinoma of the oesophagus; with special reference to a new operation for growths of the middle third. Br J Surg 34: 18-31, 1946.

22. Jian-Hui C, Shi-Rong C, Hui W, Si-le C, Jian-Bo X, Er-Tao Z, Chuang-Qi C and Yu-Long H: Prognostic value of three different lymph node staging systems in the survival of patients with gastric cancer following D2 lymphadenectomy. Tumor Biol 37: 1105-1113, 2016.

23. Cao J, Yuan P, Ma H, Ye P, Wang Y, Yuan X, Bao F, Lv W and Hu J: Log odds of positive lymph nodes predicts survival in patients after resection for esophageal cancer. Ann Thorac Surg 102: 424-432, 2016.

24. Liu Z, Jiang M, Zhao J and Ju H: Circulating tumor cells in perioperative esophageal cancer patients: Quantitative assay system and potential clinical utility. Clin Cancer Res 13: 2992-2997, 2007.

25. Pelkey TJ, Frierson HP Jr and Bruns DE: Molecular and immunological detection of circulating tumor cells and micrometastases from solid tumors. Clin Chem 42: 1369-1381, 1996.

26. Hofman VJ, Ilie MI, Bonnetaud C, Selva E, Long E, Molina T, Vignaud JM, Fléjou JF, Lantuejoul S, Piaiton E, et al: Cytopathologic detection of circulating tumor cells using the isolation by size of epithelial tumor cell method. Am J Clin Pathol 135: 146-156, 2011.

27. Roxburgh CS and McMillan DC: Role of systemic inflammatory response in predicting survival in patients with primary operable cancer. Future Oncol 6: 149-163, 2010.

28. DeNardo DG, Johansson M and Coussens LM: Immune cells as mediators of solid tumor metastasis. Cancer Metastasis Rev 27: 11-18, 2008.

29. Balkwill F and Mantovani A: Inflammation and cancer: Back to Virchow? Lancet 357: 539-545, 2001.

30. Shiraishi RZ, Halazonetis TJ, Mizra S, Fort JL, Lee PC, Neugut AI, Alford NK and Abrams JA: Elevated preoperative neutrophil: Lymphocyte ratio as a predictor of postoperative disease recurrence in esophageal cancer. Ann Surg Oncol 18: 3362-3369, 2011.

31. Xiaoleti W: Clinical study on the prognostic value of preoperative neutrophil lymphocyte ratio and platelet lymphocyte ratio in patients of esophageal squamous cell carcinoma. Jian, Shandong University, 2013.

32. Edge SB, Byrd DR, Compton CC, Fritz AG, Greene FL and Trotti A: AJCC Cancer Staging Manual, 7th edition. Springer, New York, NY, 2010.

33. Dutkowski P, Hommel G, Böttger T, Schlick T and Junginger T: How many lymph nodes are needed for an accurate pN classification in esophageal cancer? Evidence for a new threshold value. Hepatogastroenterology 49: 176-180, 2002.

34. Rizk NF, Ishwaran H, Rice TW, Chen LQ, Schipper PH, Kesler KA, Law S, Lerut TE, Reed CE, Salo JA, et al: Optimum lymphadenec- tomy for esophageal cancer. Ann Surg 251: 46-50, 2005.

35. Schumacher M, Strilic B, Sivaraj KK, Wetschereck N and Offermanns S: Platelet-derived nucleotides promote tumor-cell transendothelial migration and metastasis via P2Y2 receptor. Cancer Cell 24: 130-137, 2013.

36. Li Y, Cheng X, Chen Z, Liu Y, Liu Z and Xu S: Circulating tumor cells in peripheral and pulmonary venous blood predict poor long-term survival in resected non-small cell lung cancer patients. Sci Rep 7: 4971, 2017.

37. Hashimoto M, Tanaka F, Yoneda K, Takuwa T, Matsumoto S, Okamura Y, Kondo N, Tsabota N, Tsujimura T, Tabata C, et al: Significant increase in circulating tumor cells in pulmonary venous blood during surgical manipulation in patients with primary lung cancer. Interact Cardiovasc Thorac Surg 18: 775-783, 2014.

This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) License.