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***Basic Study***

**Evaluation of epithelial-mesenchymal transitioned** **circulating tumor cells in** **patients with resectable gastric cancer: Relevance to therapy response**

Li TT *et al*. EMT of CTCs in gastric cancer

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**Abstract**

**AIM:** to evaluate the Epithelial-to-mesenchymal transition (EMT) of circulating tumor cells (CTCs) in gastric cancer patients.

**Methods:** We detected tumor cells for expression of four epithelial (E+) transcripts (keratins 8, 18, and 19 and epithelial cell adhesion molecule) and two mesenchymal (M+) transcripts (Vimentin and Twist) bya quantifiable, dual-colorimetric RNA–*in situ* hybridization assay. Between May 2014 and August 2014, forty-four patients with gastric cancer were recruited for CTCs evaluation. Blood samples were obtained from selected patients during the treatment course [before surgery, after surgery and at the 6th cycle of XELOX based chemotherapy (about 6 mo postoperatively)].

**Results:** We found the EMT phenomenon that there were a few biphenotypic E+/M+ cells in primary human gastric cancer specimens. Of the 44 patients, the presence of CTCs was reported in 35 (79.5 %) patients at baseline. Five types of cells including from exclusively E+ CTCs to intermediate CTCs and exclusively M+ CTCs were identified (4 patients with M+ CTCs and 10 patients with M+ or M+ > E+ CTCs). Further, the chemotherapy patient undergoing progressive disease showed a proportional increase of mesenchymal CTCs in the post-treatment blood specimens. We used NCI-N87 cells to analyze of the linearity and sensitivity of CanPatrolTM system and the correlation coefficient (R2) was 0.999.

**Conclusion:** The findings suggest that the EMT phenomenon was both in a few cells of primary tumors and abundantly in CTCs from the blood of gastric cancer patients, which might be used to monitor therapy response.

**Key words:** gastric cancer; Epithelial-to-mesenchymal transition; circulating tumor cells; Chemotherapy; Therapy response

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**Core tip:** Epithelial-to-mesenchymal transition has been thought to play a critical role in tumor metastatic progression in preclinical model, however, characterizing the epithelial *vs* mesenchymal phenotypes of circulating tumor cells has been challenging. In this study, we aimed to evaluate epithelial-to-mesenchymal transition phenomenon in circulating gastric tumor cells by a combination of physical and biological method. Our findings have provided evidence of the phenomenon both in rare cells within primary tumors and more abundantly in circulating tumor cells. Furthermore, we demonstrated that the evaluation of the mesenchymal circulating tumor cells in peripheral blood can be used to monitor therapy response in gastric cancer patients.

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**INTRODUCTION**

Gastric cancer is a serious public health concern in East Asia, South America and Eastern Europe, accounting for more than 950000 new cases per year (China alone accounts for 42% of new cases worldwide) and it is the third cause of cancer death all around the world (GLOBOCAN 2012)[[1](#_ENREF_1)]. Because mass screening is rarely practiced worldwide except Japan and Korea, gastric cancer is often diagnosed in an advanced stage. Like common cancers, most gastric cancer-related deaths are resulted from metastasis[[2](#_ENREF_2)], which is rarely predictable by standard imaging work-ups like positron emission tomography/computed tomography scans or tumor markers tests.　Circulating tumor cells (CTCs) originating from solid tumors are related with the course of hematogenous metastatic spread to the distant sites[[3](#_ENREF_3)], exemplifying the switch from localized to systemic disease. Therefore, evaluating CTCs has clinical relevance in the monitoring and the outcomes of metastatic tumor. The recent CTCs discoveries demonstrate how these cells are related with hematogenous metastasis, with an opinion on the epithelial-mesenchymal transition (EMT) phenomenon[[4](#_ENREF_4)]. The investigation by Yu　*et al*[[5](#_ENREF_5)]　found dynamic changes in the epithelial and mesenchymal number of CTCs in breast cancer patients as well as the potential of monitoring therapy response.

It was thought that EMT phenomenon played a critical role in tumor metastatic progression in preclinical model[[6](#_ENREF_6),[7](#_ENREF_7)], however, characterizing the epithelial *vs* mesenchymal phenotypes of CTCs has been challenging. Increasing evidence came from clinical setting of CTCs supports the phenomenon of the EMT in human tumor. Accordingly, we are exploring the methods to identify the unique stem CTCs subpopulation[[7](#_ENREF_7)], and its significance is further emphasized by recent findings suggesting the occurrence of mesenchymal markers in tumor tissues as poor prognostic factor in many cancers[[5](#_ENREF_5),[8](#_ENREF_8),[9](#_ENREF_9)]. Furthermore, sequential analysis of CTCs, so called ‘liquid biopsy’, may provide clinical significance on the effectiveness and progression of systemic therapies and consequently would facilitate ‘tailor-made’ therapeutic strategies[[10](#_ENREF_10),[11](#_ENREF_11)].

To date, the CellSearch System is the only FDA-cleared CTCs enumeration assay, which defines a CTC according to its size, positivity for epithelial cell adhesion molecule (EpCAM) and CK, and negativity of CD45 expression[[12](#_ENREF_12)]. The current techniques besides the CellSearch System are able to isolate CTCs by epithelial markers, however, these epithelial markers based methods most likely overlook a subpopulation of CTCs undergoing EMT[[13](#_ENREF_13),[14](#_ENREF_14)]. Thus, the new CTCs capture systems should be essential to isolate the cell subpopulation with mesenchymal phenotype. To our knowledge, there have been few reports regarding the detecting methods and clinical significance of mesenchymal CTCs in cancer patients, specifically gastric cancer.

In the present study, we adopted two mesenchymal transcripts, Vimentin and Twist, to detect mesenchymal phenotypes of CTCs and tumor tissues in advanced gastric cancer, which have been reported as sensitive markers to detect them[[12](#_ENREF_12)]. Accordingly, the EMT phenomenon of CTCs in advanced gastric cancer and its relationship with chemotherapy response were evaluated as well.

**MATERIALS AND METHODS**

***Gastric cancer cell line***

We used the NCI-N87 cell line for the analysis. NCI-N87 cells were cultured in DMEM medium which supplemented with 10% FBS, 100 U/mL of penicillin and 100 μg/mL of streptomycin, and 37 ℃, 5% CO2 and a humidified atmosphere.

***Patients and study design***

Between July 2014 and October 2014, 44 patients with newly diagnosed gastric cancer in our institution, without receiving neoadjuvant chemo- or radiotherapy, were prospectively enrolled in the study. The blood samples from our selected patients were acquired during the treatment course [before surgery, after surgery and at the 6th cycle of XELOX based chemotherapy (about 6 months postoperatively)[[15](#_ENREF_15)]] and analyzed with CanPatrolTM System (Surexam Biotech, Guangzhou, China)[[16](#_ENREF_16)]. The blood of ten healthy volunteers were used as controls.

***Isolation and enumeration of CTCs (CanPatrolTM system)***

For the gastric cancer patients, 5 ml peripheral blood samples were collected with EDTA tubes from the venipuncture and filtered by a 8-μm diameter pores calibrated membrane (Millipore, Billerica, MA, United States)[[16](#_ENREF_16)]. The required filtration system included a filtration tube containing the membrane (SurExam, Guangzhou, China), a manifold vacuum plate with valve settings (SurExam, Guangzhou, China), an E-Z 96 vacuum manifold (Omega, Norcross, GA, United States), and a vacuum pump (Auto Science, Tianjin, China). Before filtration, red blood cell lysis buffer was used to remove erythrocyte, then PBS with 4% formaldehyde (Sigma, St. Louis, MO, United States) was used to resuspend the remaining cells for 5 min. The pumping pressure is 0.08 MPa.

We established three groups of nucleic acid probes to identify and examine the expression levels of epithelial and mesenchymal genes in CTCs by multiplex mRNA *in-situ* hybridization (RNA-ISH) assay. Group 1 probes contained four pooled epithelial transcripts [keratins (KRT) 8, 18 and 19; EpCAM]. Group 2 probes had two mesenchymal transcripts (Vimentin and Twist). The last group only contained a CD45 transcript which was used to discriminate white blood cells and CTCs. The detail hybridization assay procedure followed the published literature[[16](#_ENREF_16)]. Briefly, the cells retained on the filter were permeabilized and digested with protease. And then, the cells were subjected to a serial of hybridization reactions with a cocktail probe specific to the intended examined genes described above. Finally, we use 4', 6-diamidino-2-phenylindole (DAPI) to stain the cell nucleus. The samples were analyzed in fluorescent microscope. The red and green dots of fluorescent signal observed in the cells represented the epithelial and mesenchymal genes expression respectively. The purple fluorescent dots represented the *CD45* gene expression (the markers of the white blood cells). The assays were applied in both selected primary human gastric cancer specimens and all blood specimens.

***Statistical Analysis***

Data are presented as median and range (or mean and SD) for continuous variables and as frequencies and proportions for categorical variables. Mann-Whitney *U* test and the Kruskal-Wallis *H* test were used to analyze the relationship of CTCs counts at baseline with tumor stage. The relationship between CTCs counts and the molecular classification of primary tumor -Her2 was examined by Spearman’s rank correlation coefficient. All statistical calculations were performed with SPSS for Windows release 18.0. It is considered that *P* value of < 0.05 was statistically significant.

**RESULTS**

***Patient characteristics***

Totally 44 patients with gastric cancer (median age 56 years, range 25-87 years) were enrolled in this study. These patients were consisted of 6 (13.6%) patients with gastric signet-ring cell carcinoma (SRCC) and 38 (86.4%) patients with gastric adenocarcinoma. Of these, 11 patients (7 men and 4 women) had early gastric cancer diagnosed pathologically as American Joint Committee on Cancer stage IA and IB. All 37 patients underwent laparoscopic gastrectomy with D2 lymphadenectomy; the remaining 7 patients with unresectable tumors were treated with laparoscopic exploration or palliative surgery. Thirty-six patients had no detectable overt metastasis (stage M0), and 5 patients presented with existing synchronous metastases in peritoneal carcinosis (Table 1). CTCs were detected in 35 patients (79.5%) from gastric cancer group and the average count was 7.25 CTCs. No CTCs were detected in all of of the samples from healthy volunteers. (Figure 1). With regard to clinicopathological features, 11 (37.1%) patients had lymphatic emboli and elevated Her2 levels (+++ or ++) were detected in 9 (22%) gastric cancer patients.

***Detection of EMT markers in primary tumors and draining lymph nodes***

We first applied the RNA-ISH assays to primary human gastric cancer specimens. There are a small number of biphenotypic E+/M+ cells among the majority of E+ cancer cells with clear epithelial histology of primary tumors. (Figure 2A) However, we only found E+ cancer cells in draining lymph nodes. (Figure 2B)

***Correlation of CTCs detection to clinico-histopathologic risk factors***

We also analyzed blood samples from 44 gastric cancer patients. Using the RNA-ISH assays, we defined five types of cells including from exclusively epithelial (E+) CTCs to intermediate (E+ > M+, E + = M+, M + > E+) CTCs and exclusively mesenchymal (M+) CTCs from blood samples (Figure 3A). Of 35 patients, four patients with M+ CTCs and ten patients with M+ or M > E CTCs were observed. CTCs had been captured in 35 patients (79.5%), with EMT features varying according to histological subtype (SRCC and adenocarcinoma) and molecular classification (Her2- and Her2+) (Figure 3B). Among them, nine (25.7%) patients were detected with Her2+ gastric cancer, and 26 (74.3%) patients with Her2- gastric cancer. The CTCs from patients with both SRCC and adenocarcinoma were predominantly mesenchymal phenotypes. Furthermore, we compared the CTCs counts from patients with Her 2+ *vs* Her 2- in primary tumors but observed no statistically significant correlations to the number of E+/M+ cells.

***EMT of CTCs and Therapeutic Response***

To test the possibility of the ratio of mesenchymal (M) to epithelial (E) content served as an indicator of therapeutic response, we combined both the CTCs counts and mesenchymal features of CTCs (Figure 4). Sequential blood samples were obtained from four patients with adjuvant chemotherapy (XELOX regimen, capecitabine plus oxaliplatin). Three patients showed a decrease in CTCs counts and/or a proportional decrease in mesenchymal feature after a post-treatment samples at the 6th cycle adjuvant chemotherapy, compared with those in the post-surgery samples (Figure 4A-C). In contrast, one patient who had progressive disease evaluated by CT scan showed an increased number or proportion of mesenchymal CTCs in the post-treatment specimen (Figure 4D).

***Sensitivity of the CanPatrolTM system with cell line***

We used NCI-N87 cells to analyze of sensitivity and linearity of the CanPatrolTM system. We spiked 10, 50, 100 and 200 NCI-N87 cells into 5 ml of blood to get the recovery of the cells (Figure 5). And the correlation coefficient (R2) was 0.999.

**DISCUSSION**

Because of the minimally invasive feature of obtaining sequential blood specimens from cancer patients as well as potential clinical implications of the CTCs, seeking for methods to isolate and analyze CTCs for diagnosis, prognosis, and monitoring of cancer patients has been highlighted in recent years[[17](#_ENREF_17)]. There are several underlying benefits for successful CTCs /circulating tumor DNA-based diagnostics, due to the ability to obtain sequential blood samples from cancer patients throughout the treatment course[[18](#_ENREF_18)]. That is, characterizing CTCs may potentially provide clinicians with: (1) biomarkers predictive of therapy resistance[[19](#_ENREF_19),[20](#_ENREF_20)]; (2) an independent biomarker of prognosis[[3](#_ENREF_3),[21-23](#_ENREF_21)]; and (3) an indicator for early relapse[[24](#_ENREF_24)], as well as materials for the evaluation of therapy resistance[[17](#_ENREF_17),[25](#_ENREF_25),[26](#_ENREF_26)]. Currently, CTCs enumerations have been widely admitted as an independent prognostic biomarker for a few tumors, however, tentative roles as predictive biomarkers that may influence treatment decisions have been highly elusive and challenging to carry out, partially attributing to the extreme exiguity of CTCs in the peripheral circulation.

***CTCs enumeration***

CellSearch System was used in the majority of published studies, which depends on epithelial tumour cell expression of KRT 8, 18, and 19 and EpCAM presence of an intact nucleus (DAPI), absence of the leukocyte marker CD45. However, because of the heterogeneity of CTCs, most current platforms overlooked mesenchymal-like CTCs, on which mesenchymal markers, such as Vimentin and N-cadherin are upregulated[[4](#_ENREF_4)]. Like a recent published report[[5](#_ENREF_5)], a novel technology with a combination of epithelial and mesenchymal markers was used in our study, resulting in an increase of ten percent (4 patients with mesenchymal CTCs) in gastric CTCs enumeration as well as identifying 10 more patients with intermediate phenotypes (biphenotypic E+/M+ cells). The approaches of CTCs isolation or enrichment can be mainly categorized into two groups: physical methods and biological methods[[14](#_ENREF_14)]. Perhaps using more than one technique, limited isolation may be improved by the inclusion of physical methods-size based isolation of CTCs, which may help to specifically target CTCs. Besides the biological method (immunological antibody-based capture of CTCs), we simultaneously used a filtration-based approach (physical method, an 8 µm filtration tube in present study)[[16](#_ENREF_16)] for CTCs isolation, further increasing sensitivity and specificity.

***Mesenchymal CTCs detection and the EMT phenomenon***

The vital technical challenge for CTCs research comes from the rarity of tumor cells in blood. Most CTCs technologies rely on the expression of epithelial markers (EpCAM-positive and keratins-positive cells) by tumor cells for their capture. But considerable disparity exists between the numbers of ‘epithelial’ CTCs detected in different cancer types, perhaps because of a subpopulation of CTCs undergoing EMT, linked to their stemness[[27](#_ENREF_27)]. Studies aiming to research the EMT phenomenon of CTCs have high expression levels of mesenchymal markers such as AKT2, TWIST, PIK3α, N-cadherin and Vimentin[[28](#_ENREF_28)]. Mesenchymal CTCs were correlated with cancer prognosis and therapy resistance in several cancer types[[5](#_ENREF_5),[9](#_ENREF_9),[29](#_ENREF_29)]. While there exist few literatures of the EMT phenomenon in CTCs specifically in gastric cancer, which is currently a distinct research focus. In present study, we attempted at detecting and characterizing the EMT phenomenon in CTCs and gastric tumor tissue in clinical settings by using mesenchymal CTCs markers (Vimentin and Twist). We found that human primary gastric tumor tissues contain scarce tumor cells that express epithelial and mesenchymal markers, but not in lymph node metastasis. In addition, the presence of CTCs bearing a mesenchymal phenotype has also been detected in the present study, which highlights the heterogeneity present in the circulating gastric tumor cells. Although there was an obvious increase in the numbers of mesenchymal CTCs in late-stage gastric cancer (data now shown), these data did not show a statistically significant difference in our analysis, which might be due to the relative small sample size. A large scale trial with higher statistic power is warranted.

***Mesenchymal CTCs and monitoring chemotherapy***

CTCs may be the promise of serving as ‘liquid biopsies’ for tumors with the potential for providing information on predictive of response and chemotherapy resistance. Several reports have demonstrated the ratio of epithelial to mesenchymal markers on CTCs can be used to monitor the likeliness of therapy response. Yu and colleagues[[5](#_ENREF_5)] found a subpopulation of CTCs with a mixed epithelial-mesenchymal phenotype at baseline and the mesenchymal phenotype was observed at stages of disease progression (suppressed at stages of treatment response), further implicating mesenchymal CTCs in the metastatic progression. Additionally, Satelli *et al*[[9](#_ENREF_9)] suggested that CTCs enumeration from a combination of EpCAM and Vimentin-based methods appeared to be a strong and reliable predictor for therapeutic outcomes in metastatic breast cancer with chemotherapy. Likewise, we compared CTCs features in serial blood samples from four patients underwent D2 gastrectomy (pre-operative, post-operative and post-adjuvant chemotherapy). One case who had progressive disease after 6 cycles XELOX regimen showed the phenotypic changes in post-adjuvant chemotherapy specimen, compared with pre-treatment, showing an increased numbers of mesenchymal CTCs (Twist and Vimentin upregulated). The remaining three cases who responded to therapy showed a decrease in CTCs counts and/or a proportional decrease in mesenchymal CTCs. Notably, one case underwent curable resection surgery showed an increased numbers of mesenchymal CTCs in post-operative samples compared with pre-operative one, suggesting that surgical operation may play an critical role in the detachment of primary tumor cells to the peripheral circulation[[30](#_ENREF_30)]. Therefore, adjuvant therapy should be highlighted to reduce the risk of hematogenous metastasis even after curable resection in selected patients.

***Limitations***

A caveat has to be noted for the present study as well as all other studies which do not confirm the tumor cell identity by genomic markers. Because markers for mesenchymal-like CTCs are mostly not tumor-specific[[31](#_ENREF_31)]. Furthermore, small sample size is another drawback when evaluating therapy response through obtaining sequential blood specimens.

In conclusion, our findings have provided evidence of the EMT phenomenon in human gastric cancer specimens, both in rare cells within primary tumors and more abundantly in CTCs by a combination of physical and biological method. Furthermore, we demonstrated that the evaluation of the mesenchymal CTCs in peripheral blood can be used to monitor therapy response in gastric cancer patients. Clinical relevance of mesenchymal CTCs as a potential biomarker of therapeutic resistance and as a potential drug target in gastric cancer warrants further investigation.

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**COMMENTS**

***Background***

Like common cancers, most gastric cancer-related deaths are resulted from metastasis, which is rarely predictable by standard imaging work-ups like positron emission tomography/computed tomography scans or tumor markers tests. Circulating tumor cells (CTCs) originating from solid tumors are related with the course of hematogenous metastatic spread to the distant sites, exemplifying the switch from localized to systemic disease. Therefore, evaluating CTCs has clinical relevance in the monitoring and the outcomes of metastatic tumor.

***Research frontiers***

The recent CTCs discoveries demonstrate how these cells are involved in hematogenous metastasis, with a focus on the epithelial-mesenchymal transition (EMT). The investigation by Yu and colleagues found that dynamic changes in the epithelial and mesenchymal number of CTCs in breast cancer patients as well as the potential of monitoring therapy response.

***Innovations and breakthrough***

So far, there have been few reports regarding the detecting methods and clinical significance of mesenchymal CTCs in cancer patients, specifically gastric cancer. This is the first study evaluating the mesenchymal CTCs in peripheral blood and therapy response in gastric cancer patients.

***Applications***

The EMT phenomenon in human gastric cancer specimens, both in rare cells within primary tumors and more abundantly in CTCs by a combination of physical and biological method was found. Furthermore, the evaluation of the mesenchymal CTCs in peripheral blood can be used to monitor therapy response in gastric cancer patients. Mesenchymal CTCs maybe is a potential biomarker of therapeutic resistance or a potential drug target in gastric cancer.

***Terminology***

CTCs in peripheral circulation originating from solid tumors are involved in the process of hematogenous metastatic spreading to distant sites, exemplifying the switch from localized to systemic disease. Mesenchymal-to-epithelial transition (MET), a crucial physiologic event converts mesenchymal cells to epithelial cells. There were increasing evidences suggesting that MET maybe also regulate epithelial carcinogenesis.

***Peer-review***

the paper is a good contribution in investigating the role of mesenchymal-to-epithelial transition in circulating tumor cells of gastric cancer. The issue isn't new but every new contribution confirming the feasibility and efficacy of a possible new marker is welcome.

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**Figure 1 Circulating tumor cells were detected in 35 (79.5%) patients from gastric cancer group.** The average number of circulating tumor cells (CTCs) was 7.25 in the group. CTCs were not observed in any samples from healthy volunteers.

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**Figure 2 mRNA *in-situ* hybridization analysis of epithelial-to-mesenchymal transition markers in human gastric tumors.** The probes were constructed as described in the method section. Representative mRNA *in-situ* hybridization analysis of pooled epithelial (E) (red dots, arrowheads) and mesenchymal (M) (green dots, arrows) markers in (A) primary tumor and (B) tumor-infiltrated lymph node of a patient with gastric cancer. Scale bars: (A) to (B), 20 mm; inserts, 10 mm.

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**Figure 3 mRNA *in-situ* hybridization analysis of epithelial-to-mesenchymal transition markers in circulating tumor cells from patients with gastric cancer.** (A) Representative images of five types of CTCs isolated from patients with gastric cancer, based on RNA-ISH staining of E (red dots) and M (green dots) markers. Scale bar, 5 µm; (B) Quantitation of EMT features in CTCs based on E and M RNA-ISH staining of histological subtypes of gastric cancer (SRCC and Adenocarcinoma), along with molecular classification of primary tumors (Her2- and Her2+). CTCs numbers per 10 ml of blood are listed below. RNA-ISH: mRNA *in-situ* hybridization; CTCs: circulating tumor cells; EMT: epithelial-to-mesenchymal transition; DAPI: 4', 6-diamidino-2-phenylindole.

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**Figure 4 Longitudinal monitoring of epithelial-to-mesenchymal transition features in circulating tumor cells from patient with resectable gastric cancer following surgery and adjuvant chemotherapy.** CTC: circulating tumor cell; E: epithelial; M: mesenchymal.

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**Figure 5 Regression analysis of the number of observed tumor cells *vs* the number of expected tumor cells produced a correlation coefficient (R2) of 0.999.** Even a single cell spiked into the samples was detected using this system.

**Table 1 Characteristics of gastric cancer patients (*n* = 44) *n* (%)**

|  |  |  |  |
| --- | --- | --- | --- |
|  |  | **CanPatrol** | |
|  | **All** | **Positive, *n*/total** | ***P* value** |
| Median age, years(range) | 56 (25-87) |  |  |
| Gender |  |  | 0.780 |
| Male | 31 | 25/31 (80.6) |  |
| Female | 13 | 10/13 (76.9) |  |
| Post-TNM stage |  |  |  |
| Tumor |  |  | 0.799 |
| T1 | 11 | 9/11 (81.2) |  |
| T2 | 4 | 3/4 (75) |  |
| T3 | 5 | 4/5 (80) |  |
| T4 | 21 | 19/21 (90.5) |  |
| Node |  |  | 0.710 |
| N0 | 22 | 17/22 (77.3) |  |
| N+ | 19 | 18/19 (94.7) |  |
| Metastatis |  |  | 0.115 |
| M0 | 36 | 30/36 (83.3) |  |
| M1 | 5 | 5/5 (100) |  |
| Site of metastases |  |  |  |
| Peritoneal carcinosis | 5 | 5/5 (100) |  |
| Histology |  |  | 0.400 |
| Adenocarcinoma | 38 | 31/38 (81.6) |  |
| Signet-ring cell carcinoma | 6 | 4/6 (66.7) |  |
| Lymphatic emboli |  |  | 0.461 |
| No | 21 | 17/21 (81) |  |
| Yes | 11 | 10/11 (90.9) |  |
| Her2 |  |  | 0.303 |
| +++ | 2 | 2/2 (100) |  |
| ++ | 7 | 7/7 (100) |  |
| + | 12 | 11/12 (91.7) |  |
| 0 | 20 | 15/20 (75) |  |

T1: Tumor invades lamina propria or submucosa; T2: Tumor invades muscularis propria or subserosa; T3: Tumor penetrates serosa (visceral peritoneum) without invasion of adjacent structures; T4: Tumor invades adjacent structures; N0: No regional lymph node metastasis; N+: Lymph node metastasis; M0: No distant metastasis; M1: Distant metastasis.