High-Precision Quantitative Analysis Reveals Carcinoembryonic Protein Expression Differs Among Colorectal Cancer Primary Foci and Metastases to Different Sites

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Abstract
The expression of carcinoembryonic protein (CEA) is an important biological marker and therapeutic target in colorectal cancer (CRC). CEA expression heterogeneity confers resistance to CEA-targeting immunotherapy antibodies. Thus, quantification of the CEA-positive cell ratio among all tumor cells would be important in identifying patients that would benefit from CEA-targeted therapies. However, the proportion of tumor cells that express CEA within primary and metastasized tumors at different sites has not been studied. Therefore, the present study aimed to determine CEA positive cell proportion in paired CRC primary foci, liver metastases, and lymph node (LN) metastases, and whether proportion of CEA positive cell differs among colorectal cancer primary foci, liver metastases, and LN metastases from 26 patients. The CEA expression was detected by immunohistochemical assay. Then we set up a quantification approach to quantify the proportion of CEA-positive cells based on the TissueGnostics (TG) system. Then the proportion of CEA positive cells were measured and compared among primary foci, liver metastases, and LN metastases. As a result, the proportion of CEA positive tumor cells was slightly higher in liver metastases than in primary foci (89.8% ± 2.71% vs 82.1% ± 5.05%, P < 0.001). The proportion of CEA-positive cells was significantly lower in LN metastases than in primary foci (82.3% ± 4.32% vs 70.28% ± 5.04%, P < 0.001). In 8 cases with matched CRC primary foci, liver metastases, and LN metastases, the proportions of CEA proportion in liver metastasis was the highest, followed by primary foci and LN metastasis. In conclusion, this study provided an new approach for quantification of CEA positive cell in tumors and proved the percentage of CEA-positive cells varied in different metastases.

Keywords
carcinoembryonic protein, colorectal cancer, lymph node metastases, liver metastases, primary foci

Abbreviations
CRC, colorectal cancer; CEA, carcinoembryonic antigen; TG, tissue Gnostics; LN, lymph node.

Introduction
Colorectal cancer (CRC) is the third most common malignant tumor worldwide. Statistics from 2018 show over 180,000 new diagnoses of CRC globally, over 860,000 deaths due to CRC, and the mortality and incidence of CRC rank second and third, respectively, among all cancers.1 All tumors were heterogeneous at all sites. The same tumors in the same and different patients can be heterogeneous.2 Drug-resistant relapse in most patients is closely associated with the
heterogeneity of CRC tumors.\(^3\)\(^-\)\(^6\) Carcinoembryonic antigen (CEA, also known as CEACAM5 or CD66e) is a popular tumor marker for CRC and a novel therapeutic target.\(^7\) Levels of CEA expression differ in primary foci of CRC among patients\(^8\) and between CRC primary foci and liver metastases.\(^9\) Studies have analyzed the heterogeneity of CEA in the primary foci of CRC. Compared with the corresponding CEA-positive cells, CEA-negative cells showed higher tumorigenic and metastatic abilities. However, the proportion of CEA-positive cells have not been compared between tumor cells in paired primary foci, liver metastases, and LN metastases at the same time. In addition, whether CEA expression associated with a tendency toward LN metastasis remains unknown.

Cancer immunotherapy is the most exciting advancement in cancer therapy. Several immunotherapies targeting CEA-positive tumors are currently under clinical trials, such as chimeric antigen receptor T (CAR-T) cells\(^10\) and bispecific antibodies (BsAbs).\(^8\)\(^,\)\(^11\) However, it has also been reported that CEA expression heterogeneity and plasticity conferred resistance to the CEA-targeting bispecific immunotherapy antibody cibisatamab.\(^8\) Thus, quantification of the CEA-positive cells would be essential in identifying patients who would not benefit from these novel treatments. However, most studies on CEA expression relied on qualitative and semi-quantitative pathological analyses, and most could only express results as negative, weakly positive, or strongly positive, and not the precise ratio of CEA-positive cells. Flow cytometric evaluation showed concomitant CEA-negative and CEA-positive cells in the same tumor-derived organoids.\(^8\) However, flow cytometry is usually complex and time-consuming. The advanced technique of flow cytometric evaluation is not available in every hospital, which limited its application.

Therefore, we developed a method based on the TissueGnostics (TG) system to measure the ratios of CEA-positive to CEA-negative cells in tumor sections based on traditional immunohistochemical staining, and evaluated the heterogeneity of CEA expression in paired CRC primary foci, liver metastases, and LN metastases using this method.

### Materials and Methods

This study included 26 patients with surgically treated, advanced CRC that had been pathologically diagnosed postoperatively at the Cancer Hospital Affiliated to Guangxi Medical University between January 2014 and December 2019. Primary CRC foci were surgically removed from all patients, and paired LN and liver metastases were obtained from 21 and 13 patients, respectively, and both were obtained from 8 patients. Table 1 presents the clinical data of the patients. The ethics review committee of the Affiliated Cancer Hospital of Guangxi Medical University approved this study. Written informed consent for this research was obtained from the patients prior to surgery.

We purchased reagents from the following suppliers: CEA mouse anti-human cancer embryo antigen monoclonal antibodies (Item No: ZM-0062; Beijing ZSGB Biotechnology Co., Ltd., Beijing, China), ready-to-use rapid immunohistochemistry MaxVision™test kit (Item No: KIT-5920), immunohistochemical antigen repair buffer (EDTA method) (Item No: MVS-0098; both from Fuzhou Maxim Biotechnology Co., Ltd., Fuzhou, China), phosphate buffered saline (Item No: P-1010-2L), modified Lillie-Mayer hematoxylin stain (Item No: G4070; both from Beijing Solarbio Science & Technology Co., Ltd., Beijing, China), and Ready-to-Use Quick immunohistochemistry MaxVision™Detection Kit (MaxVision Biosciences Inc., Bothell, WA, USA).

Specimens in wax blocks were cut into 10 3-μm sections, placed in an oven at 65°C for 2 h, dewaxed using a graded series of xylene (numbered I, II, III, IV, V, VI) for 3 min, hydrated with a graded series of 100%, 95%, 85%, 75% ethanol, and rehydrated with antigen repair buffer (pH 9.0) using the EDTA method. Endogenous antigens were blocked using 3% hydrogen peroxide. The sections were washed with PBS and incubated with CEA mouse anti-human carcinoembryonic antigen monoclonal antibody overnight at 4°C. The sections were heated for 30 minutes to bring them to room temperature and incubated with secondary antibodies for 20 minutes using the Ready-to-Use Quick immunohistochemistry MaxVision™Detection Kit. Staining was visualized using DAB color developing solution, as described by the manufacturer. The sections were stained with hematoxylin for 30 s, washed 3 times with running water, differentiated in 1% hydrochloric acid in alcohol for 3 s, immersed in PBS, and dehydrated in a series of

| Table 1. Clinical Features of Patients. |
|----------------------------------------|
| Age (Years)                            |
| ≥ 60                                   |
| < 60                                   |
| Sexuality                              |
| Male                                   |
| Female                                 |
| Primary sites                          |
| Rectum                                 |
| Colon                                  |
| Size of primary foci                   |
| ≥ 5cm                                  |
| < 5cm                                  |
| Differentiation                        |
| High                                   |
| Medium-low                             |
| Pathological type                      |
| Adenocarcinoma                         |
| Positive lymph node                    |
| ≥ 3                                    |
| < 3                                    |
| TNM stage                              |
| III                                     |
| IV                                      |

| CRC patients with liver metastases (N = 13) | CRC patients with lymph node metastases (N = 21) |
|---------------------------------------------|--------------------------------------------------|
| Age (Years)                                |
| ≥ 60 (46%)                                 |
| < 60 (54%)                                 |
| Sexuality                                  |
| Male (62%)                                 |
| Female (38%)                               |
| Primary sites                              |
| Rectum (8%)                                |
| Colon (92%)                                |
| Size of primary foci                       |
| ≥ 5cm (77%)                                |
| < 5cm (23%)                                |
| Differentiation                            |
| High (8%)                                  |
| Medium-low (92%)                           |
| Pathological type                          |
| Adenocarcinoma (100%)                     |
| Positive lymph node                        |
| ≥ 3 (46%)                                  |
| < 3 (54%)                                  |
| TNM stage                                  |
| III (0%)                                   |
| IV (100%)                                  |

| CRC patients with liver metastases (N = 13) | CRC patients with lymph node metastases (N = 21) |
|---------------------------------------------|--------------------------------------------------|
| Age (Years)                                |
| ≥ 60 (43%)                                 |
| < 60 (57%)                                 |
| Sexuality                                  |
| Male (43%)                                 |
| Female (57%)                               |
| Primary sites                              |
| Rectum (19%)                               |
| Colon (81%)                                |
| Size of primary foci                       |
| ≥ 5cm (52%)                                |
| < 5cm (48%)                                |
| Differentiation                            |
| High (10%)                                 |
| Medium-low (90%)                           |
| Pathological type                          |
| Adenocarcinoma (100%)                     |
| Positive lymph node                        |
| ≥ 3 (62%)                                  |
| < 3 (38%)                                  |
| TNM stage                                  |
| III (14%)                                  |
| IV (86%)                                   |
75%, 85%, 95%, and 100% alcohol. Thereafter, the sections were dried naturally in a fume hood, transparentized with xylene, and sealed with neutral resin. The negative control contained PBS instead of the primary antibody, and a proven positive section served as the positive control.

The expression of CEA was considered positive in cells with brownish yellow granules in the membrane or cytoplasm and negative in those without brownish yellow granules but with blue-stained nuclei. The sections were scanned to obtain tissue images using TissueFAXS imaging software (TissueGnostics). Thereafter, positive/negative cells were identified in 3-5 representative areas (0.23 mm$^2$ each) using HistoQuest software (TissueGnostics), with manual assistance for judgment. Rates of positive cells were calculated in the representative areas, and the average was taken as the positive cell rate for each specimen.

Data were statistically analyzed using SPSS 23.00 software (IBM Corp., Armonk, NY, USA), and numerical data are expressed as ratios $r_{lb}\%$. Inter-group comparisons were assessed using one-way ANOVA, and values with $P < 0.05$ were considered statistically significant.

Results

The 26 patients with advanced CRC comprised 13 men and 13 women (mean age, 53±15 years). Most had moderately or poorly differentiated stage IV tumors, all of which were adenocarcinomas. Thirteen patients with liver metastases comprised 8 men and 5 women (average age, 54±18 years). The 21 patients with LN metastasis of CRC comprised 9 men and 12 women (average age, 54±16 years).

Analysis of 13 pairs of matched liver metastasis samples revealed that CEA was mainly expressed in the cytoplasm and cell membrane of CRC primary foci and liver metastases, but not in nuclei. Scattered glandular cavity-like cells were infiltrated by lymphocytes (Figure 2A). Statistical analysis of the proportion of CEA-positive cells in tumor cells from 13 paired CRC primary foci and liver metastases showed a higher rate of CEA-positive cells in 10 pairs of liver metastases than in primary foci, but there was no significant difference among the 3 pairs (Figure 2B). The CEA-positive cell rate was slightly higher in liver metastases than in primary foci (89.8%±2.71% vs 82.1%±5.05%, $P<0.001$; Table 2).

We found that CEA was mostly expressed in the cytoplasm from 21 pairs of LN metastases, which was similar to that in the primary foci (Figure 3A). The proportion of CEA-positive cells in tumor cells from the primary foci and LN metastases in 21 pairs of CRC was analyzed. The results showed a lower rate of CEA-positive cells in 18 of 21 pairs of LN metastases than in primary foci, with no significant difference among the 3 pairs (Figure 3B). The rate of CEA-positive cells was lower in LN metastases than in primary foci (82.3%±4.32% vs 70.57%±5.04%, $P<0.001$; Table 3).

We compared the rates of CEA-positive tumor cells in 8 pairs of CRC primary foci, liver metastases, and LN metastases. The rates were significantly higher and lower in liver and LN metastases than in primary foci (Figure 4 and Table 4).
Although carcinoembryonic antigen is the most prevalent marker of gastrointestinal tumors and is heterogeneously expressed in both CRC primary foci and liver metastases,\(^{13-15}\) most studies relied on traditional qualitative and semi-quantitative methods to compare CEA expression. In addition, rare studies have compared the exact ratio of CEA-positive tumor cells between LN and CRC primary foci metastases in different organs as far as we can ascertain. Besides, a convenient and precisely method to quantified CEA-positive tumor cells could also help to select patients with could benefit the most for anti-CEA therapy. The present study attempted to set up a new quantification approach, which was based on panoramic tissue section imaging, and compare the ratio of CEA-positive cells among CRC primary foci, liver metastasis, and LN metastasis based on this approach.

High CEA expression is a common feature of CRC, not all CRC primary cells express CEA but various colon cancer cell lines have different abilities to secrete CEA.\(^{16,17}\) Most studies relied on traditional qualitative and semi-quantitative methods to compare CEA expression. In addition, rare studies have compared the exact ratio of CEA-positive tumor cells between LN and CRC primary foci metastases in different organs as far as we can ascertain. Besides, a convenient and precisely method to quantified CEA-positive tumor cells could also help to select patients with could benefit the most for anti-CEA therapy. The present study attempted to set up a new quantification approach, which was based on panoramic tissue section imaging, and compare the ratio of CEA-positive cells among CRC primary foci, liver metastasis, and LN metastasis based on this approach.

High CEA expression is a common feature of CRC, not all CRC primary cells express CEA but various colon cancer cell lines have different abilities to secrete CEA.\(^{16,17}\) Current study by Gonzalez-Exposito reported the ratio of CEA positive tumor cells in organoids derived from CRC patients varied, and very low portion CEA positive cells also indicated the worse response to cibisatamab,\(^8\) an immunotherapeutic agent that targets CEA. They found that around 70% of tumor cells were CEA positive in the primary foci of CRC, determined using flow cytometry. Our study showed that up to ~80% (individual range, 75%-92%) of all tumor cells in CRC primary foci overexpressed CEA, which was inconsistent with the previous study. Gonzalez-Exposito reported a case with 33% CEA positive cells, but we failed to identify a similar case with such low percentage of CEA positive cells. Whatever, the above results suggested that the panoramic tissue section imaging approach we used here could reflect the proportion of CEA positive cells as previous FACS approach.

Previous reported have compared the expression of CEA in normal tissues and colorectal cancers. They showed that significantly higher CEA expression in CRC tissues and liver tumor glands than in normal colon crypt tissues, but no difference in CEA expression between CRC primary foci and liver metastases, which all expressed high levels of CEA.\(^9,18\) All the above results were obtained by pathological scoring of cells that stained negatively, weakly, or strongly positive, but ratios of CEA-positive cells were not precisely quantified. As showed by our results, up to ~80% of cells in paired primary and liver metastasis tissues were CEA-positive (Table 2), but the proportion of CEA-positive cells was significantly higher in liver metastases than in paired primary foci from the same patient (Figures 2B and 4).

A few studies have investigated CEA and LN metastasis, but their significance remains unclear. Using qRT-PCR, Oberg \textit{et al} found that CEA mRNA levels are lower in LN metastases of CRC than in primary foci.\(^{19}\) We found that the proportion of CEA-positive tumor cells in LN metastases from all 21 analyzed patients was 60%-80%. And it surprised that the proportion of CEA-positive cells were lower in LN metastases than those in paired CRC primary foci (Figure 3B and Table 3) and liver metastases (Figure 4).

The different immune microenvironment among primary foci, LN and liver metastasis may be the reason for the varied proportion of CEA positive cell. The LN are critical for initiating anti-tumor responses. Meanwhile, during the process of LN metastasis, polyclonal cancer cells proliferated into the LN and compromised the anti-tumor responses. For example, some immune cells are excluded from the lesion, or unable to eliminate tumor cells.\(^{20-22}\) Thus, the lower percentage of CEA-

**Table 2. Rates of CEA-Positive Cells in Paired Primary Foci and Hepatic Metastases.**

| Groups             | Patient numbers | Rate of CEA positive cells | P-value  |
|--------------------|-----------------|---------------------------|----------|
| CRC primary foci   | 13              | 82.87 ± 5.05              | <0.001   |
| CRC liver metastases | 13           | 89.94 ± 2.71              |          |

**Figure 2.** Comparison of rates of CEA-positive cells in paired colorectal cancer primary foci and liver metastases. A, Presentive CEA-positive staining cells in primary foci and paired liver metastases. Original magnification 200×. B, Rates of CEA-positive cells in 13 pairs of colorectal cancer primary foci and liver metastases tissues, \(P < 0.001\).
positive tumor cells in LN metastasis may be the result of they are more easily recognized by the immune system in LN than CEA-negative tumor cells. The possibility that CEA negative cells were more easily to be recruited or stay in the LN was not excluded.

In conclusion, we set up a new quantification approach based on panoramic tissue section imaging, and discover the differences in CEA-positive cells in different metastases. However, the characteristics of tumor cells that determine CEA expression and metastasis to different sites require further investigation.

Authors’ Note
Written informed consent for this research was obtained from the patients prior to surgery. The datasets used and/or analyzed during the present study are available from the corresponding author upon reasonable request. The ethics review committee of the Guangxi Medical University Cancer Hospital approved this study. Number: LW2021027. Date: April 19, 2021.

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