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Models and Technologies

Molecular Cancer Therapeutics

# Cytokeratin-20 and Survivin-Expressing Circulating Tumor Cells Predict Survival in Metastatic Colorectal Cancer Patients by a Combined Immunomagnetic qRT-PCR Approach

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## Abstract

Circulating tumor cells (CTC) express epithelial and stem cell–like genes, though current approved detection methods mainly use epithelial markers. We optimized a CTC isolation method that could capture their molecular heterogeneity and predict overall survival (OS) in metastatic colorectal cancer (mCRC) patients receiving various chemotherapy regimens. We combined immunomagnetic enrichment of CD45-negative, EpCAM-positive circulating cancer cells with qRT-PCR amplification of *CK20* and *survivin* expression in 88 mCRC patients and 20 healthy controls. We then evaluated the prognostic value of baseline CTC *CK20* and *survivin* expression in mCRC patients. The presence of elevated CTC *CK20* or *survivin* expression distinguished mCRC patients from controls with sufficient sensitivity (79.6%) and specificity (85%). In univariate analysis, patients with high CTC-*CK20* expression (9 vs. 33.2+ months, log-rank $P < 0.001$) or high CTC-*survivin* expression (10 vs. 33.2+ months, log-rank $P = 0.032$) had a significantly worse median OS than those with low expression of either marker. In multivariable analysis, the high CTC-*CK20* group had significantly shortened OS (HR, 3.11; adjusted $P = 0.01$), and there was a trend toward inferior OS in the high CTC-*survivin* group (HR, 1.76; adjusted $P = 0.099$). Patients with either high CTC *CK20* or *survivin* expression had inferior OS compared with those with low expression of both markers (HR, 4.39; 95% confidence interval, 1.56–12.35; adjusted $P = 0.005$). Colorectal cancer CTCs can be reliably isolated using epithelial and stem cell markers. CTC *CK20* and *survivin* expression may effectively predict OS in mCRC patients receiving chemotherapy. *Mol Cancer Ther; 14(10); 2401–8. ©2015 AACR.*

## Introduction

Colorectal cancer is the leading cause of death from gastrointestinal malignancy in the United States (1), and mortality is invariably linked to metastatic disease. The development of metastases is largely mediated by circulating tumor cells (CTC) that are shed by the primary tumor and survive within the circulation to home to distant organs (2, 3). CTC detection provides a noninvasive liquid biopsy that heralds the onset of metastases before conventional radiographic imaging and predicts therapeutic response and clinical outcomes once metastases have formed. Among colorectal cancer patients, CTC enumeration and biomarker expression have been associated with clinicopathologic stage (4, 5), surgical resection of metastases (6), tumor recurrence (4) and response (7), as well as overall survival (OS; refs. 7–9).

Although definitions vary between studies, it is widely accepted that CTCs lack CD45 and express epithelial cell adhesion molecule (EpCAM) and cytokeratins. In gastrointestinal tumors, *CK20* is a sensitive and specific marker for circulating cancer cells (10, 11), with prognostic utility in colorectal cancer patients (8, 12). Recent data suggest that CTCs share characteristics of cancer stem cells (13–18), and the canonical Wnt pathway is integral to both stem cell function and colorectal carcinogenesis (19). Survivin (20, 21), a downstream signaling target of Wnt activation, is highly conserved in colorectal tumors (22–24) and rarely detected in normal tissue (25). Histologic (26–29) and CTC *survivin* expression have been shown to predict disease stage (11) and survival (30) in colorectal cancer.

Current CTC detection platforms, including the immunomagnetic-based CellSearch assay (31), primarily use epithelial markers and may not fully capture the stemness of CTCs (19, 32). Moreover, studies have shown that quantitative real-time PCR (qRT-PCR) affords improved sensitivity compared with immunomagnetic enrichment techniques alone (33, 34). Constructing an optimized CTC isolation method with sufficient sensitivity, specificity, and efficiency has the potential to better inform therapeutic decisions.

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We hypothesized that isolation of CTCs that coexpress epithelial and stem cell–like genes may predict clinical outcomes in metastatic colorectal cancer (mCRC) patients. Using commercially available kits, we coupled immunomagnetic enrichment of CD45-negative, EpCAM-positive circulating cancer cells with qRT-PCR amplification of epithelial (*CK20*) and stem cell (*survivin*) markers in mCRC patients. We validated our approach by comparing mRNA expression of *CK20* and *survivin* in healthy controls and cancer patients, using four different colon cancer cell lines (HT29, SW480, HCT116, and Caco2). We then used our hybrid platform to determine the prognostic value of baseline CTC *CK20* and *survivin* gene expression in mCRC patients receiving different chemotherapy regimens.

## Patients and Methods

### Patient population and study design

We conducted a feasibility study of a combined immunomagnetic qRT-PCR method to determine the prognostic significance of CTC *CK20* and *survivin* gene expression in patients with histologically confirmed mCRC, defined as metastatic disease at initial presentation or measureable tumor recurrence after curative surgical resection. Patients consented solely for peripheral blood collection and received standard FDA-approved therapies (including varying combinations of fluoropyrimidines, oxaliplatin, irinotecan, bevacizumab, cetuximab, panitumumab, and regorafenib) or received experimental agents being tested in phase I or II clinical trials and consented for molecular correlate studies. Patients were enrolled at the Norris Comprehensive Cancer Center–University of Southern California (NCCC-USC) or the Los Angeles County–USC (LAC-USC) Medical Center, between June 2009 and April 2014. Before treatment initiation, all patients underwent baseline serum measurements of carcinoembryonic antigen (CEA) and lactate dehydrogenase (LDH) levels, as well as contrast-enhanced CT scans of the chest, abdomen, and pelvis to determine extent of metastatic disease. The Institutional Review Board at USC approved the study. All study participants signed informed consent for the analysis of molecular correlates in accordance with the Declaration of Helsinki. Twenty-four healthy blood donors (ages $\geq$ 18 years), who had no known medical illness or history of malignant disease, served as control subjects. Each patient and control subject provided two sets of peripheral blood to confirm reproducibility. All CTC studies were performed without knowledge of patients' clinical status.

### Sample collection and peripheral blood mononuclear cell isolation

A total of 16 mL of blood was drawn from each patient into two Vacutainer CPT Tubes (8 mL/tube) with sodium citrate (BD Biosciences). All samples were maintained at room temperature and centrifuged within 2 hours of collection. Blood samples were centrifuged at room temperature (18°C–25°C) in a horizontal rotor (swing-out head) for a minimum of 30 minutes at 2,700 to 2,800 RPM, and peripheral blood mononuclear cells were then collected.

### Positive and negative immunomagnetic separation using $CD45^+$ depletion and $EpCAM^+$ tumor cell enrichment

Negative immunomagnetic selection using anti–CD45-specific antibodies (Dynabeads M-450 CD45 pan Leukocyte; Invitrogen) was performed to enrich for tumor cells following the manufacturer's instructions. The CD45-negative ($CD45^-$) supernatant was transferred to 15-mL tubes for immune separation using Dynabeads (Dynabeads Epithelial Enrich, #161.02; Invitrogen). Using Dynabeads coated with a monoclonal antibody toward human EpCAM, tumor cell selection was performed following the manufacturer's instructions.

### Isolation of poly(A) mRNA and cDNA synthesis

mRNA was isolated from enriched cell fractions using the Dynabeads mRNA DIRECT Micro Kit (Life Technologies). Following the manufacturer's instructions, highly purified and intact mRNA was isolated. cDNA was reverse transcribed with 12.5 μL mRNA (total 25 μL), using the qScript cDNA Synthesis Kit (Bio-Rad #170-8891; Quanta Biosciences) according to the manufacturer's instructions.

### qRT-PCR and multiplex-PCR analysis

*CK20* and *survivin* mRNA expression levels were analyzed by the iTaq Fast SYBR Green Supermix (Bio-Rad #172-5101; Bio-Rad) and an Applied Biosystems 7500 PCR Detection System (Applied Biosystems, Inc.). Gene expression levels were determined by normalization against the reference, β-actin, using the $2^{-\Delta\Delta Ct}$ method [yielding the fold expression compared with the average $\Delta C_q$ of control subjects; $\Delta C_q = C_q$ (target) $- C_q$ (β-actin)]. Values are presented as the mean $\pm$ SD.

### Analysis of mRNA expression

The forward primer, 5′-AGAACTGGCCCTTCTTGGAGG-3′, and reverse primer, 5′-CTTTTTATGTTCCTCTATGGGGTC-3′, were used to measure *survivin* expression. The forward primer, 5′-CTGAATAAGGTCTTTGATGACC-3′, and reverse primer 5′-ATGCTTGTGTAGGCCATCG-3′ were used to measure *CK20* expression. The forward primer, 5′-CAACTGGGACGACATGGA-3′, and reverse primer, 5′-GTTGGCCTTGGGGTTCAG-3′ were used to measure β-actin expression. Primers were validated by standard curves with an $R^2 > 0.95$, and PCR efficiency at 100% $\pm$ 2% was confirmed for each primer. Threshold cycle values ($C_t$) were determined from three independently isolated RNA samples run in triplicate.

### Cell spiking experiments

The sensitivity and specificity of *CK20* and *survivin* expression for CTC detection were investigated using whole blood from healthy controls and human colon cancer cell lines: HT29, SW480, HCT116, and Caco2. All cell lines were purchased from the ATCC in 2007 (HCT116) and 2011(HT29, SW480, and Caco2; no authentication was done by the authors). HT29, SW480, HCT116, and Caco2 cell lines were maintained in McCoy's 5A and DMEM media, respectively, and supplemented with 10% FBS (Lonza), 5% penicillin/streptomycin, sodium pyruvate, and L-Glutamine (Mediatech, Inc.). We tested whether *CK20* and *survivin* could be detected from live-captured cancer cells by spiking normal blood with HT29, SW480, HCT116, and Caco2 cancer cells. After using trypsin to dissociate the cells, the number of colon cancer cells was counted three times and their mean was determined. Predetermined numbers of cells (10, 100, and 1,000) were spiked in 8 mL peripheral blood samples from healthy controls to test our enrichment method with Dynabeads. After mRNA isolation, *CK20* and *survivin* expressions were analyzed by RT-PCR and q-RT-PCR.

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### Statistical analysis

A descriptive analysis was used to determine the precision of mRNA levels of *CK20* and *survivin* in blood samples. The mean, SD, and coefficient of variation of the mRNA levels of *CK20* and *survivin* in blood samples among healthy controls were calculated per level of tumor cells added.

The distribution of the mRNA levels of *CK20* and *survivin* in the blood samples of mCRC patients and healthy controls was summarized with medians and ranges. The difference in the mRNA level of *CK20* and *survivin* between patients with mCRC and healthy controls was tested using the AUC of the ROC curve. With 20 healthy controls and 88 mCRC patients, we had 98% power to detect a difference of 0.25 between the area under the ROC curve (AUC) under the null hypothesis of 0.5 and an AUC under the alternative hypothesis of 0.75 using a two-sided $z$ test at a significance level of 0.05. The cutoff values of *CK20* and *survivin* were determined in the blood samples of healthy donors with and without added tumor cells using the maximal $\chi^2$ method. $P$ values were adjusted from multiple testing using 2000 bootstrap-like simulations.

OS was defined as the period from the date of CTC collection to the date of death. The relationships between mRNA levels of *CK20* and *survivin* and OS in mCRC patients were assessed using the cutoff values determined in the previous step by Kaplan–Meier curves, the log-rank test in the univariable analysis, and by Cox regression model in multivariable analysis adjusting for the baseline patient characteristics and treatment.

## Results

### Patient and tumor characteristics

Patient demographics and clinical characteristics are summarized in Table 1. Among the 88 mCRC patients, the median duration of follow-up was 23.5 months (range, 1.3–44.1 months), and median OS was 11.0 months [95% confidence interval (CI), 9.0–17.2 months]. Patients received a median of three prior lines of therapy for metastatic disease (range, 0–5). The majority of patients had received fluoropyrimidines (96.6%), oxaliplatin (89.8%), irinotecan (72.7%), and bevacizumab (86.3%) before CTC collection. After CTC collection, 64.8% of patients received experimental therapies on clinical trials. There was an even distribution of primary tumor site between patients, and most did not have liver-limited metastases. There was a modest positive correlation between CTC *CK20* expression and baseline CEA level (Spearman correlation coefficient = 0.25, $P = 0.023$).

**Table 1.** Patient demographics and clinical characteristics ($N = 88$)

| | *n* | Median OS (95% CI), months | HR (95% CI), univariable analysis | *P* | HR (95% CI), multivariable analysis[a] | *P*[a] |
|---|---|---|---|---|---|---|
| Age, y | | | | 0.14 | | 0.31 |
| <45 | 14 | 38.6 (4.7–44.1+) | 1 (reference) | | 1 (reference) | |
| 45–64 | 51 | 10.3 (8.4–19.3) | 1.95 (0.82–4.60) | | 1.35 (0.54–3.38) | |
| ≥65 | 23 | 10.0 (7.6–17.4) | 2.26 (0.92–5.56) | | 1.92 (0.75–4.92) | |
| Sex | | | | 0.62 | | 0.86 |
| Male | 48 | 10.3 (8.0–16.9) | 1 (reference) | | 1 (reference) | |
| Female | 40 | 14.5 (8.6–21.5) | 0.88 (0.52–1.49) | | 1.05 (0.61–1.79) | |
| Race | | | | 0.096 | | 0.43 |
| White | 56 | 15.6 (10.0–26.5) | 1 (reference) | | 1 (reference) | |
| African American | 3 | 8.9 (7.7–10.2) | 1.66 (0.38–7.16) | | 1.71 (0.37–7.96) | |
| Asian | 11 | 9.2 (3.6–14.5) | 2.28 (1.11–4.67) | | 1.55 (0.74–3.23) | |
| Hispanic | 18 | 8.0 (5.1–21.1) | 1.54 (0.80–2.93) | | 1.57 (0.80–3.09) | |
| Primary tumor site | | | | 0.41 | | 0.49 |
| Right colon | 24 | 16.7 (7.2–21.5) | 1 (reference) | | 1 (reference) | |
| Left colorectal cancer | 61 | 11.4 (8.4–19.3) | 0.88 (0.50–1.56) | | 0.93 (0.52–1.65) | |
| Unspecified | 3 | 10.0 (5.4–10.0) | 1.87 (0.54–6.54) | | 1.91 (0.55–6.65) | |
| Liver-only metastases | | | | 0.20 | | 0.092 |
| Yes | 16 | 16.9 (6.4–33.2+) | 1 (reference) | | 1 (reference) | |
| No | 72 | 10.0 (8.0–16.7) | 1.61 (0.76–3.41) | | 1.93 (0.90–4.15) | |
| ECOG | | | | 0.49 | | 0.086 |
| 0 | 32 | 14.5 (7.7–23.8) | 1 (reference) | | 1 (reference) | |
| 1–2 | 56 | 10.4 (8.6–17.4) | 1.20 (0.69–2.07) | | 1.63 (0.93–2.85) | |
| Number of prior treatments | | | | 0.001 | | 0.11 |
| 0–1 | 20 | 33.3 (16.7–38.6) | 1 (reference) | | 1 (reference) | |
| 2 | 23 | 6.6 (4.5–11.0) | 4.45 (1.90–10.43) | | 3.09 (1.13–8.44) | |
| 3 | 25 | 10.0 (7.8–21.1) | 2.63 (1.11–6.23) | | 2.23 (0.84–5.91) | |
| 4–5 | 20 | 10.3 (7.7–14.5) | 2.72 (1.12–6.61) | | 1.74 (0.61–5.00) | |
| Therapy initiated after CTC collection | | | | <0.001 | | <0.001 |
| Standard therapy | 31 | 33.3 (15.6–38.6) | 1 (reference) | | 1 (reference) | |
| Experimental | 57 | 8.6 (6.9–10.3) | 3.65 (1.93–6.89) | | 4.47 (2.28–8.76) | |
| Baseline CEA | | | | 0.000 | | 0.016 |
| ≤36 | 41 | 21.1 (10.3–35.5) | 1 (reference) | | 1 (reference) | |
| >36 | 42 | 8.0 (6.9–10.0) | 2.59 (1.45–4.64) | | 2.16 (1.15–4.05) | |
| Baseline LDH | | | | 0.001 | | 0.048 |
| ≤300 | 53 | 17.4 (10.0–33.3) | 1 (reference) | | 1 (reference) | |
| >300 | 15 | 8.0 (3.6–11.0) | 3.20 (1.43–7.18) | | 2.44 (1.01–5.88) | |

[a]On the basis of the multivariable Cox regression model adjusting for the current treatment (experimental vs. standard therapy) and the primary tumor site (right colon vs. left colorectal cancer vs. unspecified).

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**Validation of immunomagentic enrichment followed by RT-PCR/qRT-PCR in healthy controls with colon cancer cells**

We optimized the capture of colorectal cancer CTCs by using the following protocol sequence: Negative immunomagnetic selection of $CD45^-$ cells, enrichment for EpCAM positive cells using Dynabeads, and selection of *CK20* and *survivin* mRNA-positive cells by qRT-PCR amplification.

We first determined the sensitivity, specificity, and accuracy of using *CK20* and *survivin* mRNA expression for CTC detection by immunomagnetic RT-PCR and qRT-PCR. Spiking experiments were carried out using four colorectal cancer cell lines (HCT116, SW480, HT29, and Caco2) in healthy control samples to determine the limit of detection and optimal cutoff values of *CK20* and *survivin* mRNA expression.

To validate the *CK20* and *survivin* primers, 1 μg mRNA was isolated from Caco2 cells. The amount of Caco2 mRNA was then serially diluted (1 μg, 100 ng, 10 ng, 1 ng, 100 pg, 10 pg, and 0.1 pg) in 1xPBS, and qRT-PCR was performed. Primers were validated by standard curves, and PCR efficiency at 100% ± 2% was confirmed for each primer (Fig. 1A). Next, we tested whether *CK20* and *survivin* could be detected from live-captured cancer cells by spiking healthy donor blood with HT29, SW480, HCT116, and Caco2 cancer cells. After using trypsin to dissociate the cells, the number of colon cancer cells was counted three times, and their mean was determined. Predetermined numbers of HCT116 (5, 10, 20, 50, 100, and 1,000; qRT-PCR) and HT29 (10, 20 50, and 100; RT-PCR) were spiked in 8 mL peripheral blood from healthy donors to optimize tumor cell enrichment using Dynabeads. After mRNA isolation, *CK20* and *survivin* were analyzed by RT-PCR and qRT-PCR (Fig. 1B and C). All colorectal cancer cell lines expressed both *CK20* and *survivin*, whereas neither marker was detected in the peripheral blood from healthy controls. The limit of detection for each was 0 to 10 colon cancer cells per 8 mL of healthy donor peripheral blood. As shown in Fig. 1D, after varying numbers (0, 10, 100, and 1,000) of HT29, HCT116, SW480, and Caco2 cells were spiked into the whole blood of 4 healthy donors, *CK20* and *survivin* gene expressions were measured by immunomagnetic bead-based qRT-PCR. On the basis of these experiments, the optimal cutoff values for the level of mRNA gene expression were as follows: *CK20*, 0.14; *survivin*, 0.092.

**Validation of CTC detection by immunomagnetic qRT-PCR in mCRC patients**

Next, we determined the sensitivity and specificity of using *CK20* and *survivin* mRNA expression, as measured by

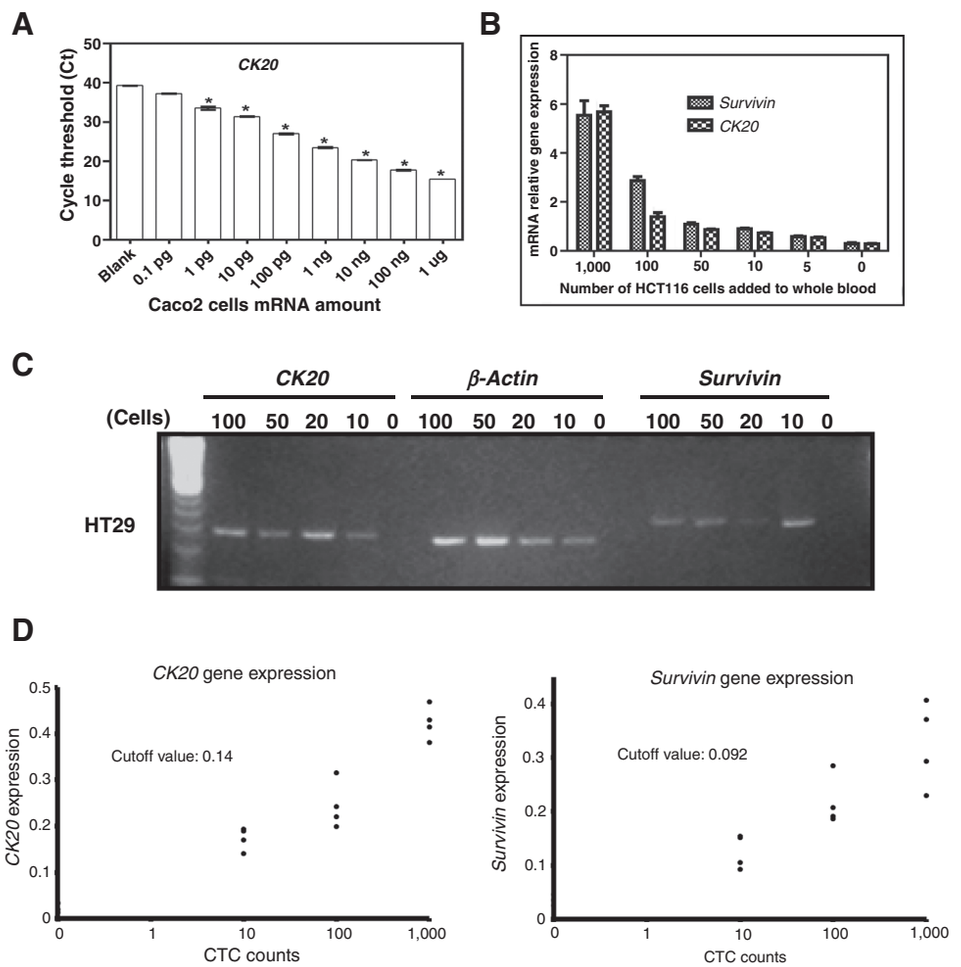


**Figure 1.**
Cell spiking experiments. A, *CK20* expression level detected from serial dilutions of Caco2 cells mRNA. All histogram results are means of triplicate independent experiments ($P < 0.05$). B, *CK20* and *survivin* gene expression were measured by immunomagnetic qRT-PCR by adding varying numbers of HCT 116 colon cancer cells (5, 10, 50, 100, and 1,000) into the whole blood of one healthy donor. C, *CK20* and *survivin* gene expression on 2% agarose gel from HT29 colon cancer cells (10, 20, 50, and 100) by immunomagnetic RT-PCR. D, the cutoff values for the level of mRNA gene expression were: *CK20*, 0.14; and *survivin*, 0.092.

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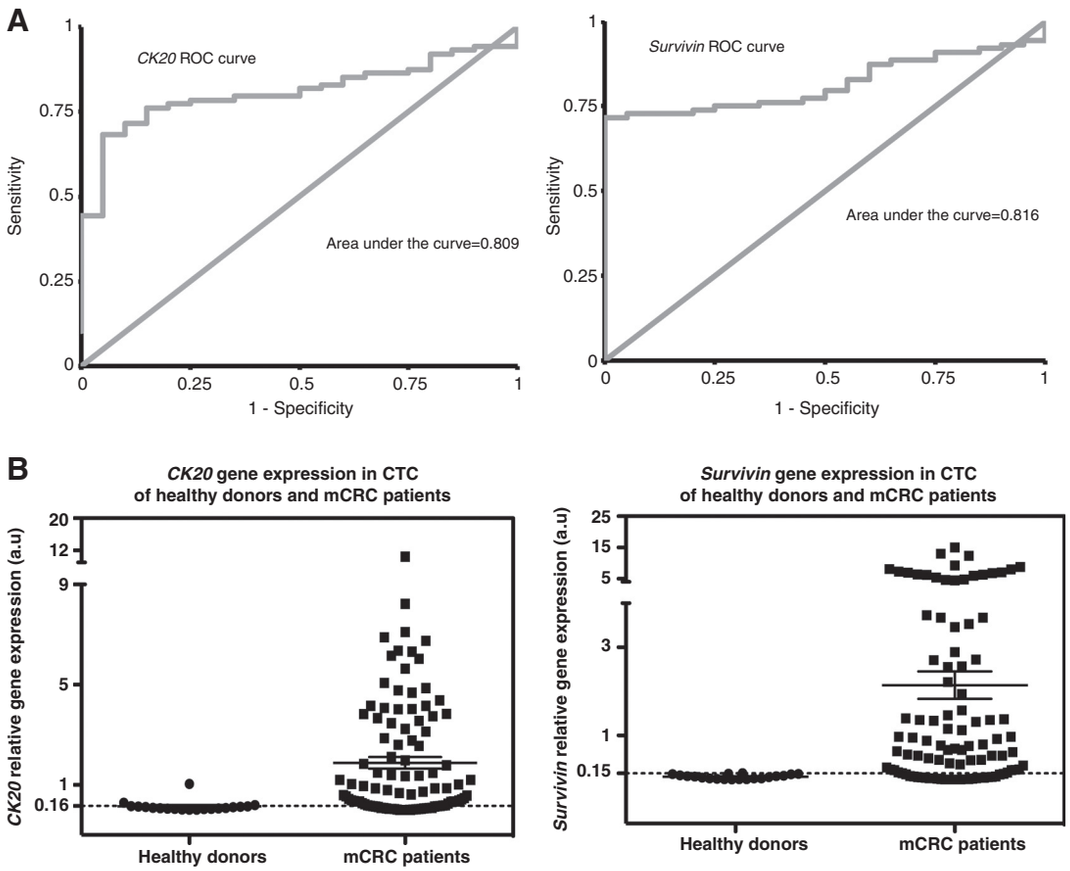


**Figure 2.**
ROC analysis of CTC *CK20* and *survivin* gene expression. A, ROC curves of *CK20* and *survivin* expression in mCRC patients compared with healthy donors. B, CTC *CK20* and *survivin* gene expression using immunomagnetic qRT-PCR approach were analyzed following the cutoff values established with sufficient sensitivity and specificity from 20 healthy donors and 88 mCRC patients. Left, an optimal cutoff value of 0.16 for *CK20* yields a sensitivity of 76% and a specificity of 85%; right, an optimal cutoff value of 0.15 for *survivin* yields a sensitivity of 72% and a specificity of 100%.

immunomagnetic qRT-PCR, for CTC detection by using peripheral blood samples from 20 healthy donors and 88 mCRC patients. Cutoff values for *CK20* and *survivin* gene expression were established to obtain sufficient sensitivity and specificity. With regard to *CK20* expression, ROC curves showed that a cutoff value of 0.16 for *CK20* yielded a sensitivity of 76% and a specificity of 85% (Fig. 2A). Among 88 mCRC patients, 67 had high *CK20* expression (>0.16) compared with 3 of 20 healthy donors ($P < 0.005$; Fig. 2B). Similarly, a cutoff value of 0.15 for *survivin* was found to yield a sensitivity of 72% and a specificity of 100% (Fig. 2A). Within the mCRC cohort, 63 of 88 patients had high levels of *survivin* expression (>0.15), compared with 0 of 20 healthy donors ($P < 0.001$; Fig. 2B). Sixty-eight percent ($N = 60$) of mCRC patients had elevated levels of both *CK20* and *survivin* expression, and 79% ($N = 70$) had elevations in either marker. Taken together, using either elevated CTC *CK20* or *survivin* expression yielded a sensitivity of 79.6% and a specificity of 85% for CTC detection in mCRC patients (Supplementary Fig. S1). Twenty percent of mCRC patients (18 of 88) had decreased expression of both markers compared with 85% (17/20) of healthy donors (Table 2).

### Prognostic utility of CTC *CK20* and *survivin* expression in mCRC patients by immunomagnetic qRT-PCR

We determined the association between CTC *CK20* and *survivin* gene expression and OS in our cohort of mCRC patients. In univariate analysis, patients with high CTC *CK20* (>0.16) or *survivin* (>0.15) gene expression had a significantly worse median OS than those with low expression of either marker (*CK20*: HR, 4.01; 95% CI, 1.73–9.27, log-rank $P < 0.001$; *survivin*: HR, 1.99; 95% CI, 1.03–3.85, log-rank $P = 0.032$; Table 3).

**Table 2.** Detection of CTC biomarkers in mCRC patients and healthy controls by immunomagnetic qRT-PCR

| | Control group ($N = 20$) | mCRC patients ($N = 88$) |
|---|---|---|
| Biomarker | | |
| *CK20* | 3 (15%) | 67 (76%) |
| *Survivin* | 0 | 63 (71%) |
| Numbers of markers detected | | |
| 0 | 17 (85%) | 18 (20%) |
| ≥1 | 3 (15%) | 70 (79%) |
| 2 | 0 | 60 (68%) |

NOTE: Cutoff points, *CK20* > 0.16 and *survivin* > 0.15.

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**Table 3.** Association between CTC gene expression levels and OS in mCRC patients

| Gene | *N* | Median OS (95% CI), months | HR (95%CI), univariable analysis | *P* | HR (95%CI), multivariable analysis[a] | *P*[a] |
|---|---|---|---|---|---|---|
| *CK20* | | | | <0.001 | | 0.010 |
| ≤0.16 | 21 | 33.2+ (15.6–33.2+) | 1 (reference) | | 1 (reference) | |
| >0.16 | 67 | 9.0 (7.8–11.4) | 4.01 (1.73–9.27) | | 3.11 (1.31–7.41) | |
| *Survivin* | | | | 0.032 | | 0.099 |
| ≤0.15 | 25 | 33.2+ (8.4–33.2+) | 1 (reference) | | 1 (reference) | |
| >0.15 | 63 | 10.0 (8.0–16.7) | 1.99 (1.03–3.85) | | 1.76 (0.90–3.44) | |
| *CK20/Survivin* | | | | <0.001 | | 0.005 |
| ≤0.16 and ≤0.15 | 18 | 33.2+ (9.2–33.2+) | 1 (reference) | | 1 (reference) | |
| >0.16 or >0.15 | 70 | 9.4 (7.8–13.1) | 5.16 (1.89–14.09) | | 4.39 (1.56–12.35) | |

[a]On the basis of the multivariable Cox regression model adjusting for the current treatment (experimental vs. standard therapy) and the primary tumor site (right colon vs. left colorectal cancer vs. unspecified).

Baseline variables associated with OS included number of prior therapies, treatment received after CTC collection, and LDH level. In a multivariate model stratified for these variables, high CTC *CK20*–expressing patients had significantly shortened survival relative to those with low CTC *CK20* expression (HR, 3.11; 95% CI, 1.31–7.41; adjusted $P = 0.01$), and patients with high CTC *survivin* expression had a trend toward inferior OS (HR, 1.76; 95% CI, 0.90–3.44; adjusted $P = 0.099$; Fig. 3, Table 3). Patients with either high CTC *CK20* or *survivin* expression had significantly worse OS than those with low expression of both genes in univariate (HR, 5.16; 95% CI, 1.89–14.09; log-rank $P < 0.001$), and multivariate analyses (HR, 4.39; 95% CI, 1.56–12.35; log-rank $P = 0.005$; Fig. 3, Table 3).

## Discussion

CTC isolation allows for an assessment of cancer recurrence, therapeutic response and resistance, and prognosis. Efforts to molecularly characterize CTCs have advanced our understanding of how metastases develop, but what defines a CTC and the role of stem cell markers in shaping this definition is not fully elucidated. We optimized a combined immunomagnetic qRT-PCR protocol for colorectal CTC characterization based on epithelial and stem cell biomarkers. Using this assay, we evaluated the clinical relevance of pretreatment CTC *CK20* and *survivin* expression in mCRC patients receiving various chemotherapeutic and experimental agents, and found this molecular signature to predict survival.

CTCs are as heterogeneous as the tumors from which they originate, and this has made CTC detection methods vulnerable to limitations in efficiency, sensitivity, and standardization (32). For instance, in the study by Cohen and colleagues (7), which examined the prognostic utility of CTC count using the CellSearch assay in mCRC patients, 52% of patients had no detectable baseline CTCs in a 7.5 mL blood sample, and only 27% of patients with radiographic disease progression had a corresponding unfavorable CTC profile (as defined by ≥3 CTCs). In a subsequent investigation of 836 individuals with metastatic breast, colorectal or prostate cancer, all patients were found to have at least one CTC using the CellSearch method, but only after extrapolating the individual blood volume to 5 L with a logistic regression model (32). These findings underscore the need for improving diagnostic yield, either by modifying the enrichment procedure and/or adjusting the biomarker criteria for CTC detection.

With regard to enrichment techniques, Guo and colleagues (33) compared the additive value of using negative and positive immunomagnetic selection with RT-PCR and found the combination of both separation methods to yield the best sensitivity. Furthermore, qRT-PCR has consistently demonstrated superior sensitivity relative to CellSearch and other immunomagnetic-based tools for CTC detection in colorectal cancer patients (34), and combining this modality with an immunomagnetic enrichment step eliminates a substantial source of false-positive results. With respect to marker selection, the use of multiple genes may lower the detection limit of a particular assay but at the cost of decreased specificity. To circumvent these obstacles and balance diagnostic yield with specificity, we selected *CK20* as a well-established marker for CTC detection in colorectal cancer patients (35) and *survivin*, which is more specific to malignant tissue and has been associated with the development of colorectal cancer metastases (28). In our study, CTC *CK20* and *survivin* expression each showed acceptable sensitivity (*CK20*, 76%; *survivin*, 72%) and high specificity (*CK20*, 85%; *survivin*, 100%). Using both markers together, our combined immunomagnetic qRT-PCR assay demonstrated 79.6% sensitivity and 85% specificity for CTC identification in mCRC patients. This compares more favorably with the only other study (11) examining these biomarkers in mCRC patients using an immunomagnetic qRT-PCR method (sensitivities 47.4%, 57.7% and specificities 83.2%, 80.0% for CTC *CK20* and *survivin* expression, respectively), though the caveats inherent to cross-study comparison and the different cancer cell lines used limit definitive conclusions.

In our cohort of mCRC patients, the presence of CTCs and baseline expression of each measured biomarker independently predicted OS. Specifically, patients whose CTCs had either elevated *CK20* or *survivin* gene expression had an over three times increased risk of death, as compared with patients with low CTC expression of both markers. Our findings are consistent with that of prior investigations examining the prognostic utility of these biomarkers in colorectal cancer patients, though these studies mainly evaluated either *CK20* (10, 12) or *survivin* (30) in isolation, did not use a combined immunomagnetic qRT-PCR assay (12), or did not explicitly evaluate associations with survival (10, 11). To the best of our knowledge, our study represents the largest cohort of colorectal cancer patients with metastatic disease evaluated for both CTC *CK20* and *survivin* expression using a combined enrichment and amplification approach.

Our study has its limitations, the first of which is its retrospective nature. Importantly, by examining only baseline peripheral blood samples, we could not explore the predictive utility of this gene signature with each chemotherapeutic regimen. One of the main limitations of qRT-PCR–based techniques is the potential for false-positive results. For instance, circulating epithelial cells from nonmalignant disease states

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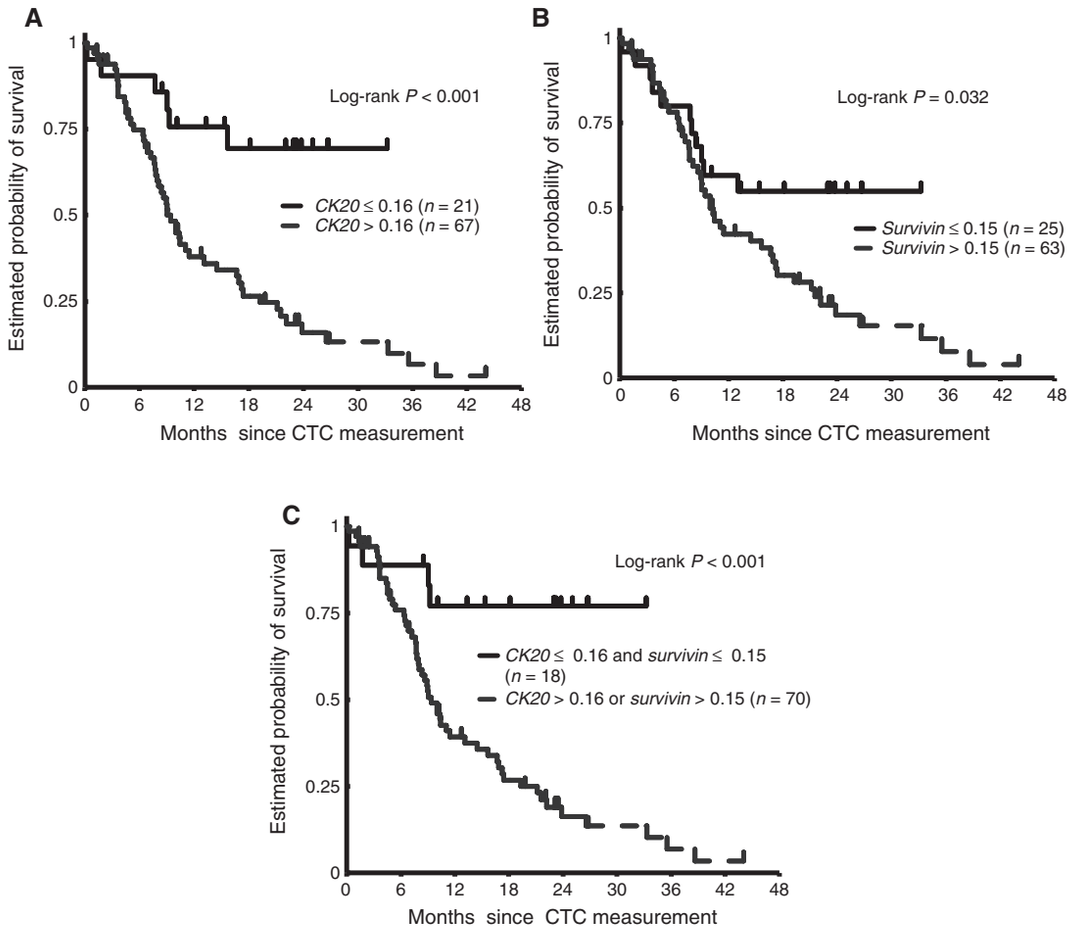


**Figure 3.**
Kaplan–Meier cumulative survival curves stratified by CTC CK20 and survivin expression. OS curves according to CTC *CK20* expression (log-rank $P < 0.001$; A), CTC *survivin* expression (log-rank $P = 0.032$; B), and CTC *CK20* and *survivin* expression (log-rank $P < 0.001$; C).

(such as inflammatory bowel disease, polyps, etc.) may express *CK20*, though previous studies (12) have shown this to not be the case. Moreover, we accounted for this potential limitation by incorporating two immunomagnetic enrichment steps and determining optimal cutoff values to better distinguish cancer cells from nonmalignant cells. Another potential limitation is that a proportion of CTCs may lose their epithelial marker expression as they undergo the epithelial–mesenchymal transition (EMT), and therefore may not be detected by our method. In subsequent studies, we plan to incorporate and compare the predictive and prognostic value of different CTC EMT and stem cell–associated markers.

Predicting the onset of metastatic disease and monitoring response to treatment with a noninvasive, reproducible method remains a fundamental objective in individualizing cancer care. CTC enumeration and biomarker assessment may provide a safe and simple means of achieving this objective by exposing the dynamic molecular alterations driving disease progression and therapeutic efficacy. However, the implementation of CTC technology into clinical decision-making is still in its infancy, as our approach to defining and detecting CTCs continues to evolve. Using an immunomagnetic qRT-PCR platform, we validated a gene expression signature based on epithelial and stem cell markers capable of CTC detection with sufficient sensitivity, specificity, and efficiency. This model effectively predicted prognosis in mCRC patients. Future investigations should determine the predictive utility of this model by incorporating it into biomarker-driven therapeutic trials.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

## Authors' Contributions

**Conception and design:** Y. Ning, H.-J. Lenz
**Development of methodology:** Y. Ning, W. Zhang, H.-J. Lenz
**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** Y. Ning, S. Stremitzer, A. Barzi, H.-J. Lenz
**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** D.L. Hanna, W. Zhang, D. Yang, S. Stremitzer, S. Okazaki, A. Barzi, H.-J. Lenz
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