Supplemental Materials and Methods

Blood Collection and Sample Processing

Following enrollment, 10-mL of venous blood was collected into an ACD solution A tube (BD Pharmigen, Franklin Lakes, NJ) after a 5-mL waste to prevent epithelial cell contamination. Blood was inverted at least 10 times following draw and stored at 4°C until being processed, always within 24 hours of collection. Each NanoVelcro chip can process 2 mL of venous blood, and all samples were run on 2 chips in parallel. Thus, CTC counts are reported per 4 mL of blood throughout the manuscript. Any additional blood was cryopreserved. In each 2 mL VB sample, red blood cells (RBCs) were lysed in ≤ 10 minutes using a 0.15 M Tris-ammonium chloride solution. Cold phosphate buffered saline (PBS) (Gibco, Gaithersburg, MD) was then added for termination of lysis and samples were subsequently centrifuged at 300 x g for 10 minutes at 4^{o}C. After removal of supernatant, cell pellets were re-suspended in cold RPMI-1640 (Cellgro, Manassas, VA) supplemented with 5% cell-free fetal bovine serum (FBS) (Gibco). Following a second, identical centrifugation step and removal of supernatant, cell pellets were re-suspended in PBS supplemented with 2% donkey serum (DS) (Jackson Immunoresearch, West Grove, PA). Biotinylated goat anti-EpCAM (R&D Systems, Minneapolis, MN) was then added to the samples and allowed to incubate at room temperature for 30 minutes with gentle agitation to prevent cell clumping. Following antibody incubation, samples were washed in PBS and then re-suspended in 200 μL PBS for chip loading. All experiments were carried out on parallel duplicate chips.

NanoVelcro Chip Preparation, Immunocytochemistry and Chip Mounting

NanoVelcro chips were assembled and operated as previously described.[17] Briefly, prepared samples were injected into the device at the optimized flow rate of 1.0 mL/h, followed by 4% paraformaldehyde (PFA, Sigma, St. Louis, MO) injected at the same rate for fixation. The chips were then removed from the NanoVelcro platform and washed in PBS for 15 minutes. Chips were blocked and permeabilized using PBS + 2% donkey serum (DS, Jackson Immunoresearch) + 0.1% Triton X-100 (Sigma) for 15 minutes. Chips were subsequently incubated with a cocktail of primary antibodies including two mouse anti-CD45 antibodies (BD Pharmigen), two rabbit anti-cytokeratin (CK) antibodies (Invitrogen, Carlsbad, CA; Abcam, Cambridge, UK) and 1 chicken anti-CEA antibody (Abcam). Primary antibody incubation occurred for 1 hour at room temperature in PBS + 2% DS. Following a wash step using PBS, secondary antibody incubation was performed with a cocktail of AlexaFluor-488 donkey anti-rabbit (Invitrogen), AlexaFluor-647 goat anti-chicken (Invitrogen), and AlexaFluor-555 donkey anti-mouse (Invitrogen). Secondary antibody incubation was carried out in PBS + 2% DS for 1 hour at room temperature. Chips were washed a final time with PBS and then attached to microscope slides (Fisher Scientific, Waltham, MA). A DAPI mounting solution (Life Technologies, Grand Island, NY) was used to mount cover slips, and mounted slides were dried for 1 hour prior to fluorescent imaging.

Chip Scanning and CTC Enumeration

Chips were first scanned at 40x power by an automated chip scanning protocol using the NIS Elements 4.1 software (Nikon, Tokyo, Japan) on an Eclipse 90i fluorescent microscope to identify candidate cells. Higher magnification manual imaging of candidate cells was then performed at 400x power to verify results. When analyzing the resulting multi-channel ICC

image, WBCs were defined as round/ovoid, DAPI+, CD45+ and CK-. CTCs were defined as round/ovoid, size ≥ 6-µm, DAPI+, CD45-, and CK+. Any CD45 positivity greater than 2x background discounted a cell as being a CTC. Final CTC counts are represented as a total count per 4 mL VB.

Supplemental Table and Figure Legends

Supplemental Table 1. Univariate and multivariate analysis for predictors of recurrence-free survival.

Supplemental Table 1. Univariate and multivariate analysis for predictors of recurrence-free survival.

All Patients (n = 40)	***Univariate Analysis***			***Multivariate Analysis***		
Predictor	**Hazard Ratio**	**95% CI**	**P-value**	**Hazard Ratio**	**95% CI**	**P-value**
Clinical predictors						
Age, per year	1.03	1.0 – 1.06	0.12	1.10	1.04 – 1.17	0.002
Male gender	0.99	0.53 – 1.88	0.99	–	–	–
Pre-operative predictors						
Radiographic						
Mesenteric vessel involvement	3.34	1.79 – 6.23	0.001	6.98	2.18 – 22.37	0.001
Tumor Location						
Tail	–	–	–	–	–	–
Head	1.09	0.38 – 3.17	0.87	–	–	–
Tumor Diameter, per cm*	1.10	0.91 – 1.32	0.32	–	–	–
Laboratory						
CA19-9, per log unit	1.08	0.88 – 1.32	0.46	–	–	–
CTC count, per log unit	2.36	1.17 – 4.78	0.017	1.5	0.71 – 3.17	0.29
Early Stage Only (n = 26)	***Univariate Analysis***			***Multivariate Analysis***		
Predictor	**Hazard Ratio**	**95% CI**	**P-value**	**Hazard Ratio**	**95% CI**	**P-value**
Clinical predictors						
Age, per year	1.09	1.01 – 1.17	0.02	1.08	1.00 – 1.16	0.04
Male gender	1.25	0.42 – 3.76	0.69	-	-	-
Pre-operative predictors						
Radiographic						
Tumor Location						
Tail	-	-	-	-	-	-
Head	0.91	0.25 – 3.31	0.89	-	-	-
Tumor Diameter, per cm	1.15	0.92 – 1.44	0.22	-	-	-
Laboratory						
CA19-9, per log unit	1.30	0.95 – 1.78	0.10	1.19	0.81 – 1.74	0.37
CTC count, per log unit	2.57	0.88 – 7.46	0.08	1.76	0.48 – 6.48	0.40

* - Stage III patients downstaged prior to surgery (n = 13)