

Clinical Cancer Research



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Clin Cancer Res 2012;18:5711-5718. Published OnlineFirst September 25, 2012.

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Challenges in the Enumeration and Phenotyping of CTC

Frank A.W. Coumans¹, Sjoerd T. Ligthart¹, Jonathan W. Uhr², and Leon W.M.M. Terstappen¹

Abstract

Purpose: Presence of circulating tumor cells (CTC) in metastatic carcinoma is associated with poor survival. Phenotyping and genotyping of CTC may permit "real-time" treatment decisions, provided CTCs are available for examination. Here, we investigate what is needed to detect CTC in all patients.

Experimental Design: CTCs enumerated in 7.5 mL of blood together with survival from 836 patients with metastatic breast, colorectal, and prostate cancer were used to predict the CTC concentration in the 42% of these patients in whom no CTCs were found and to establish the relation of concentration of CTCs with survival. Influence of different CTC definitions were investigated by automated cell recognition and a flow cytometric assay without an enrichment or permeabilization step.

Results: A log-logistic regression of the log of CTC yielded a good fit to the CTC frequency distribution. Extrapolation of the blood volume to 5 L predicted that 99% of patients had at least one CTC before therapy initiation. Survival of patients with EpCAM+, cytokeratin+, CD45– nucleated CTCs is reduced by 6.6 months for each 10-fold CTC increase. Using flow cytometry, the potential three-fold recovery improvement is not sufficient to detect CTC in all patients in 7.5 mL of blood.

Conclusions: EpCAM+, cytokeratin+, CD45– nucleated CTCs are present in all patients with metastatic breast, prostate, and colorectal cancer and their frequency is proportional to survival. To serve as a liquid biopsy for the majority of patients, a substantial improvement of CTC yield is needed, which can only be achieved by a dramatic increase in sample volume. *Clin Cancer Res*; 18(20); 5711–8. ©2012 AACR.

Introduction

In recent years, a variety of technologies have emerged for the detection of circulating tumor cells (CTC; refs. 1–9). The CTC phenotype used by these technologies varies greatly, and as a result, the reported CTC frequencies can vary up to a few orders of magnitude in similar patient groups. At present, CellSearch is the only validated method for enumeration of CTCs in which the presence of CTCs detected has been related with poor clinical outcome in multicenter prospective studies (10–12). Definition of a CTC in this system was set before these studies were initiated. They were defined as EpCAM-enriched objects from 7.5 mL of blood that express cytokeratin (CK) 8, 18, or 19, lack CD45, are >4 μm in size, and have cell-like morphology including a

nucleus, determined by staining with 4',6-diamidino-2-phenylindole (DAPI). In these studies, 20% to 50% of the processed samples were found to have 0 CTC. The prognosis of these patients is much better than the prognosis of patients with CTCs.

The majority of the emerging CTC technologies report that higher frequencies of CTCs contributed to either a more efficient capture and detection or based on other immunologic or physical properties (2–9). Retrospective analysis of recorded fluorescence images captured with the CellSearch system using a variety of different phenotypic and morphologic criteria showed that different definitions of CTCs resulted in a wide range of "CTC" frequencies with varying degrees of clinical significance (13). These observations explained some of the discrepancies between technologies. An important question with regard to the 20% to 50% of patients who did not have CTCs in 7.5 mL of blood is whether they had no EpCAM+CK+CD45–DNA+ CTCs or they had such CTCs but they were not detected. Absence of EpCAM+CK+CD45–DNA+ CTCs in patients with carcinoma could be explained by either lack of expression by the primary tumor or loss of the antigens during the epithelial–mesenchymal transition (14–19). In this study, we could only test 2 alternative hypotheses, namely, either insufficient sample volume or insufficient assay sensitivity that caused patients with CTCs to have no CTC in the CellSearch test. The sample volume is addressed by means of a distribution function fit to the CTC frequency distribution of patients in whom CTCs were detected. This fit can be used to

Authors' Affiliations: ¹Department of Medical Cell BioPhysics, MIRA institute, University of Twente, Enschede, The Netherlands; and ²Cancer Immunobiology Center, University of Texas Southwestern Medical Center, Dallas, Texas

Note: Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

Clinical trials registered: IMMC01: Not registered, started before March 2002; IMMC06: NCT00133913; IMMC38: NCT00133900.

Corresponding Author: Leon W.M.M. Terstappen, Department of Medical Cell BioPhysics, University of Twente, Carre, Room C4437, P.O. Box 217, 7500 AE Enschede, The Netherlands. Phone: 31-0-53-489-2425; Fax: +31-0-53-489-1105; E-mail: l.w.m.m.terstappen@utwente.nl

doi: 10.1158/1078-0432.CCR-12-1585

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Translational Relevance

Circulating tumor cells (CTC) may reflect the current state of a patients' tumor and have the potential to serve as a liquid biopsy to guide treatment decisions and provide insights into pathways involved in tumor progression and those underlying development of clinical cancer dormancy. Basic requirements to use CTC as a real-time liquid biopsy are their presence in the tested sample volume and that they can be isolated in sufficient condition to detect the presence of treatment targets. In this study, we show a 6.6-month decrease in survival chances for patients with metastatic breast, colorectal, and prostate cancer for each 10-fold increase in EpCAM+, cytokeratin+, CD45- nucleated CTCs. All these metastatic patients have CTCs with this phenotype; however, to detect and probe these cells in all patients, a technology leap is needed to examine 5 L of blood.

predict the frequency distribution in patients in whom no CTCs were detected in 7.5 mL of blood. The sensitivity is addressed by 2 means; first the definition of CTC is varied to determine whether a different CTC definition may yield higher number of cells while maintaining prognostic value and second that CTCs were determined on patients' blood with both CellSearch and a flow assay detecting all EpCAM+DNA+CD45- CTCs in the blood sample, albeit with lower specificity than CellSearch.

In the past, most CellSearch CTC studies dichotomized patients into high and low CTC groups. To test whether the absolute number of CTCs counted by CellSearch is useful, the continuous survival function as a function of CTC count was determined.

Materials and Methods

Patients and clinical trials

Blood samples (7.5 mL) were collected in CellSave vacutainers (Veridex) in prospective, multicenter clinical trials in patients with breast (IMMC-01), colorectal (IMMC-06), and prostate cancer (IMMC-38; refs. 10–12). Included were 177 patients with breast, 428 with colorectal, and 231 with prostate cancer. All participants provided written informed consent. Samples were taken before commencement of a new line of therapy and at first follow-up after initiation of therapy. The primary aim of these studies was to investigate the association between the presence of CTCs and progression-free and overall survival. The primary study endpoint was death of any cause. As control samples for CTC enumeration by the automated classifier—stored images from CellSearch, data from healthy volunteers from the IMMC-06 and IMMC-01 studies ($n = 68$, $n = 136$, respectively) and patients with benign neoplasm from the IMMC-01 study ($n = 190$) were used. For comparison between CTC enumeration by flow cytometry and CellSearch, 186 blood samples were collected in EDTA vacutainers (BD) from 140

patients with metastatic carcinoma from a study reported earlier and were analyzed (20). Of the 140 patients, 65 (46%) had breast cancer, 24 (17%) lung cancer, 16 (11%) colorectal cancer, 12 (9%) ovarian cancer, 7 (5%) prostate cancer, and 16 (11%) had other carcinomas. Patient inclusion for all studies, detailed inclusion criteria, study characteristics, and patient demographics are detailed in Supplementary Material S1.

CTC enumeration by CellSearch

The CellSearch system uses immunomagnetic enrichment targeting the epithelial cell adhesion molecule (EpCAM) and immunofluorescence labeling and detection for enumeration of CTCs (2). Fluorescence images of the enriched cells are recorded and a trained operator identifies CTCs defined as EpCAM-enriched cells larger than 4 μm , meeting morphologic criteria of cells, expressing CK 8, 18, or 19, lacking the leukocyte-specific antigen CD45 and staining with the nucleic acid DAPI from thumbnail images generated by a computer algorithm containing objects staining with both DAPI and CK (2). The trained operator is blinded to patient information at the time of review.

Automated CTC classifier

An automated classifier of CTC was used to identify CTCs with different characteristics in the archived images from the multicenter prospective studies. This classifier compares each found object to a predefined CTC phenotype to determine whether this object is a CTC or not. The phenotype is constructed of 4 parameters; size and signal in CK, CD45, and DNA channels; and the CTC definition was optimized using patient overall survival (21). To test the impact of other phenotypes on both prognostic value and the frequency of found objects, we retrained the classifier using different EpCAM+ phenotypes; DNA+, CK+, DNA+CK+, DNA+CK+CD45-, CK+CD45- CK+CD45+, DNA+CD45-. Size was included in each classifier to allow filtering of excessively large or small objects. The dataset for training included the baseline samples of the entire population of eligible patients from breast, prostate, and colorectal studies, whose archive images could be read, in the breast ($n = 163$), colorectal ($n = 72$), and prostate ($n = 185$) studies. Controls were healthy volunteers from the colorectal and breast studies ($n = 68$ and $n = 136$) and patients with benign neoplasm from the breast study ($n = 190$).

CTC enumeration by flow cytometry

The frequency of CTCs was determined using flow cytometry and compared with CellSearch CTC enumeration using blood from 186 patients with metastatic cancer. We wanted to have approximately 50 samples with at least 1 CTC/90 μL of blood. To achieve this, we needed to run 200 comparisons assuming (i) 25% of patients have 11 or more CTC/mL blood, (ii) the flow assay has at least comparable recovery to CellSearch, and (iii) 10% of samples fail. An aliquot of 100 μL of blood was stained with 10 μL each of EpCAM-PE (Veridex LLC), CD45-PerCP (BD Biosciences), and the nucleic acid dye used in procount (BD Biosciences).

After 15 minutes of incubation, 0.5 mL of FACSLyse (BD Biosciences) was added. The samples were analyzed on a FACSCalibur flow cytometer (BD Biosciences), and CTCs were defined as EpCAM+DNA+CD45- as described elsewhere (20).

Statistical analysis

All statistical analysis was conducted in MATLAB 2010b with statistics toolbox (Mathworks). The CTC data from patients in the breast, prostate, and colorectal studies at baseline and first follow-up were used to generate empirical cumulative distribution functions (CDF). The percentage of patients with >0 CTC was highest with prostate cancer; therefore, various functions were fit to the prostate cancer CDF via maximum likelihood estimation to the empirical curve, including the logistic function, Weibull CDF, normal CDF, and exponential CDF. As input variables, all measurements with CTC >0 and the log thereof were tested. The best fitting distribution was fit to the empirical CDF of each study for the baseline (before commencement of chemotherapy) and first follow-up samples (2–8 weeks after baseline). Each fit was extrapolated to a blood volume of 5 L. The 95% confidence interval (CI) of each fit was determined from the covariance matrix.

All studies were designed to follow patients for up to 36 months; survivals recorded beyond 36 months were censored at 36 months. Cox regression analysis was conducted to determine the impact of different automatically counted phenotypes on survival. Patients were dichotomized on the median number of found objects. To show the relationship

between survival time and CTCs, we produced smoothed Kaplan–Meier estimates of the median survival conditional (22, 23), both by study and with all studies grouped together. A 95% CI for the all study relationship was determined by bootstrap aggregation of the dataset 400 times.

Results

99% of patients are predicted to have at least one CTC in circulation

The empirical CDF using data from a previously reported metastatic castration-resistant prostate cancer study (10) was used to test the fit of various distribution functions; the CDF and the tested fits are shown Fig. 1. The best function was selected on the basis of log likelihood ratio (LLR). Only distributions fit to the log of CTC had a good fit, with the log-logistic fitting (LLR = 1.002) slightly better than the log Weibull and log normal distribution (LLR = 1.004 for both). The regular logistic function (LLR = 1.078) and the exponential function (LLR = 2.080) fit poorly. The log-logistic function was fit to all studies for samples before initiation of therapy (baseline) and after initiation of therapy (first follow-up) and extrapolated to 5 L in Fig. 2. Included were patients with metastatic cancer originating from the breast (177), prostate (231), and colon or rectum (428). CIs for the follow-up samples for breast and especially colorectal cancer are large due to the large fraction of samples with 0 CTC. At baseline, 99% of patients in each study are predicted to have at least 1 CTC in 5 L of blood (breast 98.7%, colorectal 99.1%, prostate 99.5%) whereas

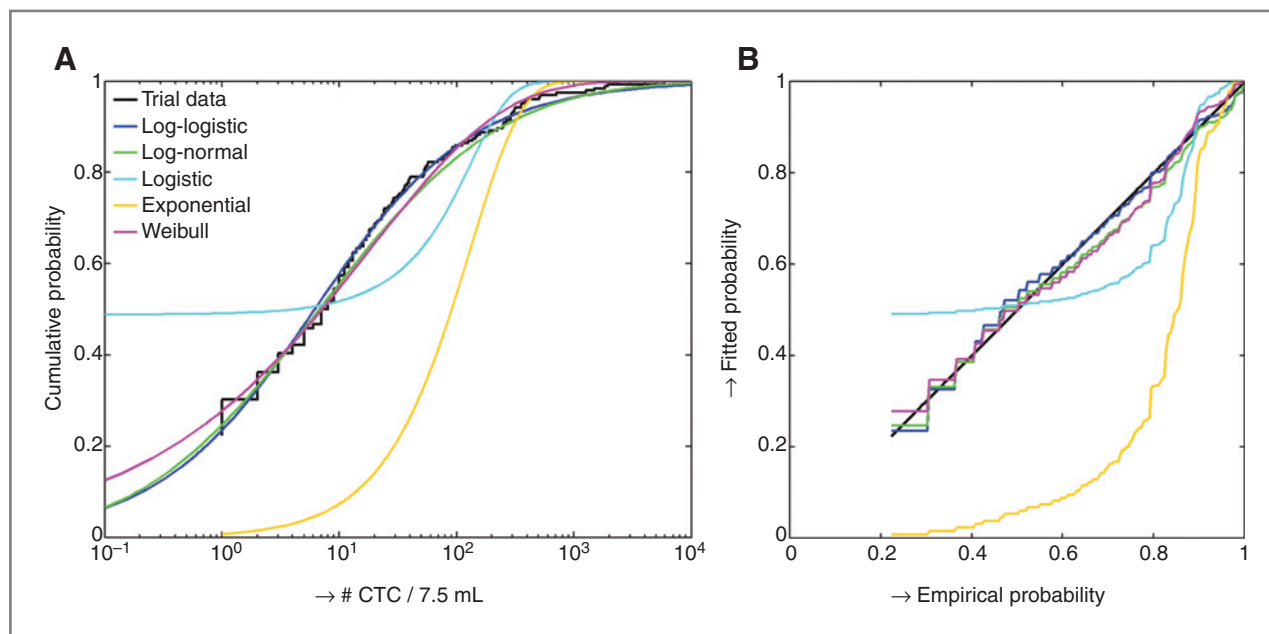


Figure 1. Fits to the empirical CDF of the prostate study baseline samples ($n = 231$). Fits were conducted using all non-zero CTC counts. Log-logistic regression on the log of CTC yields the closest fit (LLR = 1.002). A log-normal distribution and Weibull distribution on the log of CTCs also yields an acceptable fit (LLR = 1.004 for both). Log-binomial regression on the number of CTCs and a fit of an exponential distribution both are poor fits (LLR = 1.076 and 2.079, respectively). A, the CDF functions. B, the probability–probability plot where a line with slope of 1 is a perfect fit.

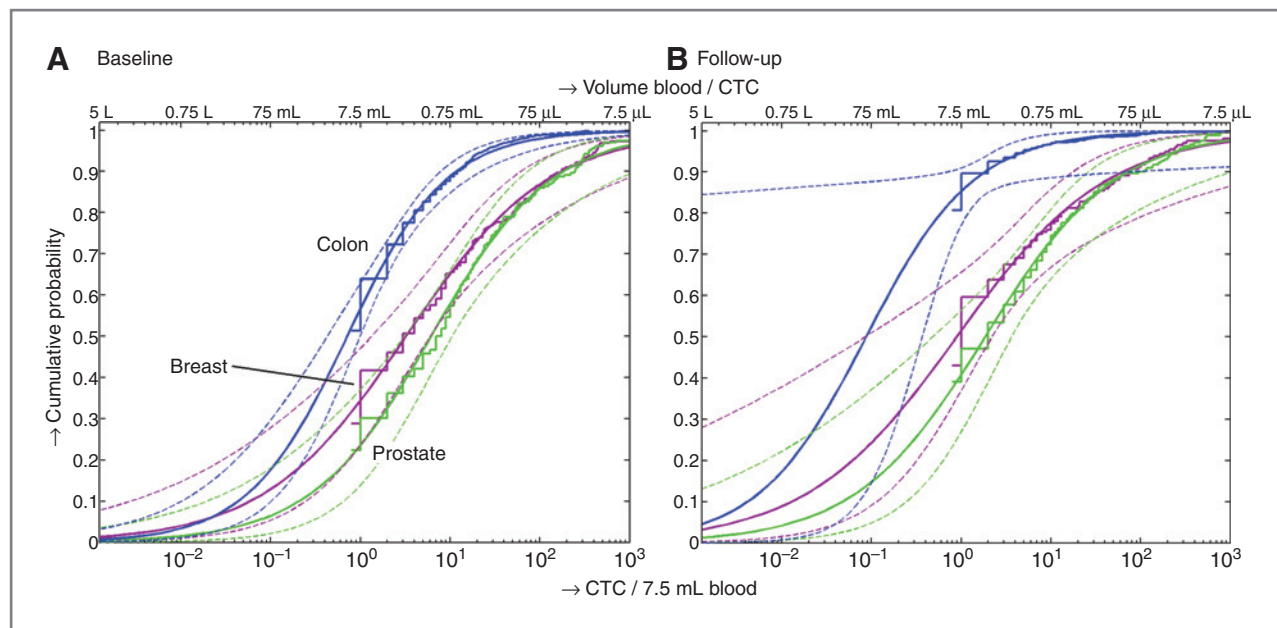


Figure 2. Extrapolation of log-logistic fit curves for all studies at baseline and first follow-up. Stair plots show the empirical CDF, the continuous line the fit, and the dashed lines outline the 95% CI for patients with breast ($n = 177$), prostate ($n = 231$), and colorectal ($n = 428$). Extrapolation predicts that >99% of patients in these studies had CTCs at baseline (A) and >95% of patients still had CTC at first follow-up (B). The 95% CIs are much wider for follow-up because a smaller part of the empirical CDF is available for fitting.

after 1 month of chemotherapy, 96% to 99% of patients still have 1 CTC in 5 L of blood (breast 95.8%, colorectal 99.0%, prostate 98.7%).

Survival chance decreases with increasing CTCs in metastatic cancer patients

Survival versus CTC number was plotted in a scatter plot in Fig. 3 and a smoothed median survival function was estimated for each study. For this estimate, patients with 0 CTC were included as a single group, with the number of CTCs equal to the median as predicted from the log-logistic fit. For visualization purposes, the survival data of patients with 0 CTC were randomly spread out between 10^{-3} and 0.9 CTC/7.5 mL with a distribution as estimated by the log-logistic fit. Survival monotonically declines with increasing CTC number for each study, with the exception of patients with breast cancer with 0 CTC, who had median survival at 1 month less than the 22.4 months of patients with 1 CTC. The slope of the curve for patients with prostate cancer is much steeper than the other slopes. The survival graph for all patients shows that survival chances are reduced by 6.6 months by each 10-fold increase in CTC in a 7.5-mL blood volume. The 95% CI by bootstrapping was 4.2 to 8.2 months for an increase from 100 to 1,000 CTCs and 4.5 to 8.1 months for an increase from 10 to 100 CTCs. The shape of the survival curves suggests that this can be extrapolated to the higher blood volumes with less CTC.

Increased concentration of CTC can be detected at the cost of clinical significance

Alternative definitions of EpCAM+ objects enriched with the CellSearch method were tested using an automated

classifier (21) on all available archived images from the 3 studies (420 patients, 204 healthy controls, 190 patients with benign disease). The frequency of the alternative CTC definitions and the influence of HR are shown in Table 1. Manual review of the images using the CellSearch definition resulted in an HR of 2.5 at baseline and 3.4 at follow-up. For the automated classifier, the EpCAM+, DNA+, CK+, CD45- and EpCAM+, DNA+, CK+ phenotypes had the highest HR of 2.7 at baseline and 3.2 at follow-up. However, these definitions also had the lowest frequency. Whereas, EpCAM+ DNA+ cells were approximately 500 times more frequent than EpCAM+ DNA+ CK+ CD45- cells, the HR was only 1.5 at baseline and 1.3 at follow-up. Loosening the criteria that define that a CTC increases the frequency of counted objects not only in patients but also in controls and thereby reduces the HR.

A 3.3-fold loss of EpCAM+ CTCs can be contributed to the enrichment and staining procedure

To evaluate the potential loss of CTCs through the immunomagnetic enrichment and staining procedure, a comparison was made between CTC detected with the CellSearch system and flow cytometry. For flow cytometric analysis, 100 μ L of blood was stained with EpCAM-PE, CD45-PerCP, and a nucleic acid dye (20). Cytokeratin was not stained to avoid potential tumor cell loss due to permeabilization. CTCs were detected in blood samples from 186 patients with metastatic carcinoma using both methods. To correct for the different blood volumes, the CellSearch results were divided by 75. 105 (55%) samples were negative by both methods. Figure 4 shows the comparison. Both methods correlated with an R^2 of 0.6, and from the

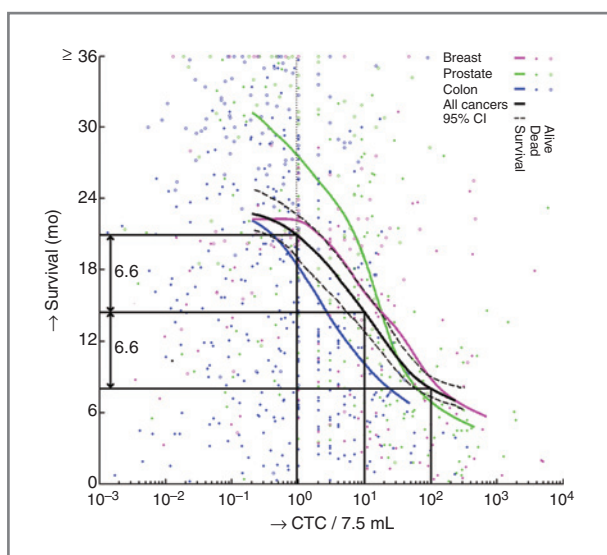


Figure 3. Scatter plot of survival versus number of CTC for breast, colorectal, and prostate cancer. Time from baseline to death is indicated by closed symbols, time from baseline to censoring is indicated with open symbols. Survivals exceeding 36 months are truncated at 36 months. Curves show the estimated relationship between median survival time and CTC count for breast ($n = 177$), prostate ($n = 231$), colorectal ($n = 428$), and all patients ($n = 836$). Samples with 0 CTC per 7.5 mL blood were randomly redistributed to a CTC density between 0.001 and 0.9 CTC/7.5 mL of blood with a distribution as predicted by the CDF fits. Median survival monotonically decreases with increasing CTC count for all studies, except the change from 0 to 1 CTC for patients with breast cancer, where an increase in survival of 1 month was observed. The 95% CI is shown in dashed lines for the overall curve. A decrease of 6.6 months in survival for each 10-fold increase in CTC is indicated.

slope, a higher yield of CTC by the flow cytometric approach can be estimated. The slope is highly dependent on the highest CTC data points, removal of the highest 0 to 3 values results in slopes of 6.5 (0 points removed), 1.0, 2.5, and 3.1

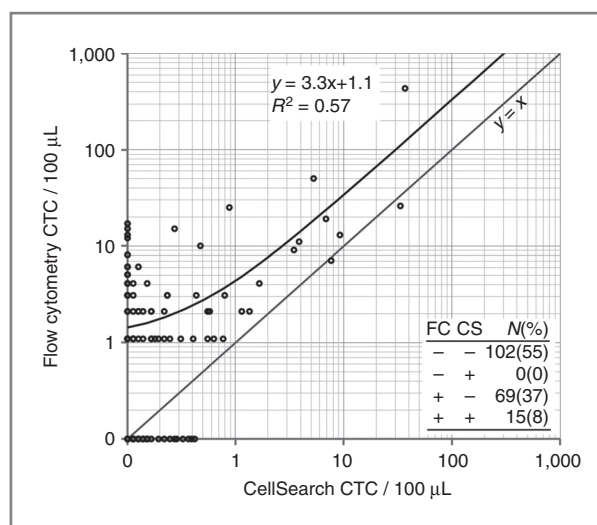


Figure 4. Detection of CTC by flow cytometry (FC) versus CellSearch (CS) per 100 μ L of blood in patients with metastatic carcinoma ($n = 186$). CellSearch was conducted on 7.5 mL of blood and then divided by 75 to obtain results per 100 μ L. The slope between FC and CS is 3.3 indicating that flow cytometry finds more CTCs. The number of samples in which ≥ 1 CTCs are detected in 100 μ L of blood is shown in the bottom right. Samples (55%) were negative by both methods, 37% positive by flow only, and 8% positive by both flow and CellSearch.

(3 points removed); respectively. The mean of these 4 slopes is 3.3, which represents the potential gain in recovery of EpCAM+ DNA+ CD45- cells.

Discussion

Tumor cells circulating in blood of patients with cancer hold the potential to serve as a liquid biopsy and, thereby, to be used to tailor treatment for the individual patient and to gain insights into the cellular pathways underlying tumor

Table 1. Impact on survival and CTC frequency using different CTC definitions in metastatic cancers and CTC frequency in healthy donors and patients with benign disease

EpCAM	DNA	CK	CD45	Size	Baseline ($n = 420$)				Follow-up ($n = 364$)				Healthy ($n = 204$)			Benign ($n = 190$)		
					HR	Median	Min	Max	HR	Median	Min	Max	Median	Min	Max	Median	Min	Max
mCTC																		
+	+	+	-	>4 μ m	2.5	4	0	23,618	3.4	4	0	9,864	0	0	1	0	0	12
aCTC																		
+	+	+	-	>4 μ m	2.7	4	0	5,897	3.2	1	0	7,911	0	0	12	0	0	15
+	+	+	-	>4 μ m	2.7	3	0	5,591	2.8	1	0	6,450	0	0	13	0	0	12
+		+	-	>4 μ m	2.4	48	0	18,622	2.4	23	0	15,904	11	0	209	15	1	155
+		+	-	>4 μ m	2.3	23	0	16,236	2.7	8	0	8,628	1	0	61	1	0	70
+	+		-	>4 μ m	1.5	980	0	82,397	1.3	1,263	0	80,810	868	2	70,971	1,094	2	27,302
+	+	+	-	<4 μ m	2.4	49	0	6,679	2.3	25	0	5,759	15	1	280	16	2	140
+		+	-	<4 μ m	2.2	52	0	13,157	2.9	29	0	9,024	9	0	2,079	5	0	124

NOTE: All HRs had $P < 0.0001$ except for EpCAM+ DNA+ CTCs with a $P = 0.02$ for baseline samples and $P = 0.03$ for follow-up samples. DNA, Hoechst staining.

Abbreviations: aCTC, automated CTC count with different definitions; mCTC, manual CTC count using the CellSearch CTC definition.

progression (24) or development of clinical cancer dormancy (25). Requirements for fulfilling this potential include the presence of CTC in the sample volume, their isolation and the maintenance of their molecules pertinent to diagnoses and treatment. In studies using CellSearch, the only clinically validated system for CTC detection, 20% to 50% of blood samples of patients with metastatic cancer were found to have 0 CTC in 7.5 mL of blood and, when CTCs were detected, only a portion of these cells allowed assessment of treatment targets (10–12, 26, 27). The study of RNA, DNA, and protein composition of CTCs, detected by other methods (28–32), also suffers from low numbers of CTCs per sample. The number of CTCs recovered per patient must be increased to yield clinically useful information in all patients. In the present study, we investigated possible approaches to achieve this increase: (i) an increase of sample volume, (ii) increased recovery of CTCs by improved enrichment of cells expressing EpCAM, or (iii) increased recovery by applying a different morphologic definition of a CTC.

The frequency of CTCs detected in past studies was used to predict the CTC concentration in all 5 L of blood of these patients. We initially hypothesized that the driving mechanism behind the number of CTCs found in patients was self-seeding exponential growth with a frequency-independent growth rate and had thus expected a log normal distribution for an optimal fit of the frequency distribution of the CTCs (33). Instead the log-logistic function turned out to better fit the CTC distribution, possibly because the tumor growth rate slows as the tumor increases in size (34–37). Application of this function to the data predicts that before initiation of a new line of therapy, 98.7% to 99.5% of the patients have more than 1 CTC in all 5 L of blood, which decreases to 95.8% to 99% after these patients receive 1 to 2 months of cytotoxic chemotherapy (Fig. 2). The low percentage of patients in which CTCs disappear after treatment is consistent with the low success rate of treatment in metastatic cancer. The extrapolation down to 5 L hinges on the assumption that the distribution of CTCs across patients can be described with a single function. Nevertheless, the close agreement between fit and data shows that an increase in sample volume achieved by drawing more tubes of blood will marginally affect the number of patients in whom CTCs are found.

Our results suggest that almost all metastatic patients have EpCAM+ CK+ CD45–DNA+ tumor cells circulating in their blood at all times. However, a subset of patients lack the EpCAM and CK antigens in their tumor tissue (38, 39). For example, in breast cancer, the reported percentage of patients whose tumor tissue does not express EpCAM antigens ranges from 3% to 10% (20, 40, 41). Possible explanations for this contradiction are that the EpCAM and/or CK expression is below the detection threshold for immunohistochemistry but not for immunofluorescence or that the EpCAM– tumor cells in the primary tumor or the metastatic sites represent too small a fraction to be detected during routine immunophenotyping of the tissues.

The smoothed survival curves shown in Fig. 3 suggest that survival decreases by 6.6 months for each 10-fold increase in CTCs. These EpCAM+ CK+ cells may represent a more aggressive phenotype and, thereby, resemble the concept of tumor stem cells (18, 42, 43). Support for the aggressive phenotype hypothesis is obtained by the monotonic and steep reduction of survival as the number of CTC increases as depicted in Fig. 3. Alternative explanations for the strong correlation between survival and CTC concentration could be that only a small fraction of the EpCAM+ CK+ CTCs are metastasis initiator cells or that these CTCs are directly correlated to these metastasis initiator cells. Xenotransplantation experiments in immunodeficient mice are needed to clarify this point.

To test whether the number of patients in which CTCs could be detected by increased sensitivity, we first evaluated whether different definitions of CTCs can be used to increase the sensitivity of the assay without loss of specificity. To this end, an automated classifier was applied on the stored image sets of the prospective multicenter clinical studies. An increase in the number of CTCs can be obtained by using less strict criteria to define a CTC but this gain reduced the impact on clinical outcome as signified by the HR in all our attempts (Table 1). This also implies that clinical trials are needed to determine whether the (often higher) CTC number as determined using other CTC enumeration assays is equally or more prognostic for survival than the cells assigned as CTC by the CellSearch method. Next, we evaluated whether significant losses of CTCs could have occurred because of either ineffective immunomagnetic enrichment or loss from the staining procedure using permeabilization to enable intracytoplasmic staining of the cytokeratins. To answer this question, a flow cytometric assay was used to detect cells expressing EpCAM and lacking CD45 in 100 μ L of blood. Because this assay does not permeabilize the cells and a flow cytometer is an extremely sensitive tool to detect cell surface and intracellular antigens, we assume the loss of CTC in this assay to be negligible. Flow cytometry could detect a larger number of CTCs as compared with CTC detection by CellSearch in the same blood volume. The highest estimate of this increase was 6.5-fold but removal of the highest 1 to 3 measurement values from the fit shows that the true potential improvement is probably closer to 3.3-fold. Furthermore, addition of the cytokeratin requirement in the CTC definition as well as the morphologic criteria to the definition will surely reduce this increase in sensitivity. Still even if a 3.3-fold increase in yield of CTCs could be achieved, the CDF fits show that the effect of this increase is limited as the percentage of samples with ≥ 1 CTC will only increase from 61% to 68%. Additional increases in yield may be achieved by including alternative phenotypes for detection (18, 44–47); however, proof will be needed that these CTCs with a different phenotype are also associated with a bad prognosis and can be used to assess treatment targets.

A solution to the issue would be to significantly increase the sample volume. However, the blood volume taken from the patient cannot be sufficiently increased. This apparent

conflict could be solved by *in vivo* detection methods, in which feasibility has been shown (48–51). An alternative approach is to conduct leukapheresis for CTC isolation (52). Although this procedure is more cumbersome compared with a simple blood draw, it is more attractive than taking biopsies from the metastatic sites. The use of a microfluidic device for CTC detection would require a pre-enrichment step to reduce the sample volume.

The key conclusions from the present study are as follows: (i) Statistical analysis of the CTC distribution in 7.5 mL of blood detected by CellSearch in patients with metastatic cancer suggests that virtually all patients including those who do not have detectable CTCs by CellSearch have at least 1 CTC in 5 L of blood. (ii) A potential 3-fold improvement in the yield of EpCAM+ CTCs will not be sufficient to detect CTCs in all patients. Therefore, much larger volumes of blood are needed to obtain intact, nucleated, EpCAM+, CK+, CD45– CTCs. (iii) Although more "CTC" can be detected by loosening the criteria to define CTC, their association with survival decreases. (iv) For every 10-fold increase in the number of intact, nucleated, EpCAM+, CK+, CD45– CTCs, survival decreases by 6.6 months, supporting the notion that this phenotype of tumor cells may be responsible for metastasis and ultimately death of patients.

However, further studies of additional phenotypes are needed.

Disclosure of Potential Conflicts of Interest

F.A.W. Coumans, S.T. Ligthart, and L.W.M.M. Terstappen have received a commercial research grant from Veridex LLC. J.W. Uhr has Ownership Interest (including patents) as a patent used by J. & J. L.W.M.M. Terstappen is a Consultant/Advisory Board member of Veridex LLC.

Authors' Contributions

Conception and design: F.A.W. Coumans, L.W.M.M. Terstappen

Development of methodology: F.A.W. Coumans

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): F.A.W. Coumans, S.T. Ligthart, J.W. Uhr

Writing, review, and/or revision of the manuscript: F.A.W. Coumans, S.T. Ligthart, J.W. Uhr, L.W.M.M. Terstappen

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S.T. Ligthart

Study supervision: L.W.M.M. Terstappen

Grant Support

This work was funded by a research grant from Veridex LLC.

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Received May 17, 2012; revised July 24, 2012; accepted August 6, 2012; published OnlineFirst September 25, 2012.

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