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All circulating EpCAM+CK+CD45− objects predict overall survival in castration-resistant prostate cancer

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Background: Presence of five or more circulating tumor cells (CTC) in patients with metastatic carcinomas is associated with poor survival. Although many objects positive for epithelial cell adhesion molecules and cytokeratin (EpCAM+CK+) are not counted as CTC, they may be an important predictor for survival. We evaluated the association between these objects and survival in patients with prostate cancer.

Patients and methods: Included in this follow-up study were 179 patients with castration-resistant prostate cancer. CellSearch was used to isolate EpCAM+ objects and to stain DNA, cytokeratin and CD45. All EpCAM+CK+ objects were subdivided into seven classes on the basis of predefined morphological appearance in 63 independent samples. Association of each class with survival was studied using Kaplan–Meier and Cox regression analyses.

Results: Each EpCAM+CK+CD45− class showed a strong association with overall survival ($P < 0.001$). This included small tumor microparticles (S-TMP), which did not require a nucleus and thus are unable to metastasize. A higher number of objects in any class was associated with decreased survival. A good prediction model included large tumor cell fragments (L-TCF), age, hemoglobin and lactate dehydrogenase. Models with S-TMP or CTC instead of L-TCF performed similarly.

Conclusion: EpCAM+CK+CD45− that do not meet strict definitions for CTC are strong prognostic markers for survival.

Key words: castration-resistant prostate cancer, circulating tumor cell, morphological appearance, tumor microparticle

introduction

Tumor cells that shed into the blood during metastasis have the potential to become generic biomarkers for many cancers. Considerable technological efforts have been made over the past two decades to develop platforms that reliably identify and count circulating tumor cells (CTC). The CellSearch system has demonstrated in multicenter prospective studies that the presence of five or more CTC identified using the CellSearch system was associated with reduced overall survival in pre- and on-treatment patients with metastatic breast cancer, metastatic colorectal cancer, and castration-resistant prostate cancer (CRPC) [1–8]. In these studies, a CTC was defined as a nucleated object that was positive for epithelial cell adhesion molecule (EpCAM+) and cytokeratin 8, 18 or 19 (CK+) and negative for CD45 (CD45−), was at least $4 \times 4\ \mu m^2$ in size and had specific morphological and immunological features identified by trained operators [9, 10]. Enriched were all objects with ≥2000 EpCAM antigens [11]. The frequency of objects classified as CTC ranged from 0 to 10 000/7.5 ml of blood and approximately a third of patients with advanced breast, colorectal or prostate cancer did not have any circulating objects that met these criteria. High CTC heterogeneity was observed and could result in interoperator error in CTC counting [10, 12, 13]. Although only intact and viable CTC have the potential to form metastases, the presence of apoptotic CTC or other epithelial objects in blood could have independent prognostic relevance. Alternative technologies utilizing less stringent criteria for counting CTC have reported substantially larger numbers of CTC [14–24]. As an increasing number of clinical studies now incorporate the evaluation of CTC in their design, it has become critical that the definition of what constitutes a CTC is clarified and that the different EpCAM+ objects that are detected using CellSearch are more thoroughly studied. To address this issue, images of all EpCAM+CK+ objects previously acquired during a CTC study in CRPC patients were reanalyzed to evaluate the relationship between different EpCAM+CK+ classes and overall survival.

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patients and methods

patients and data collection

Samples were collected from CRPC patients enrolled in a prospective multicenter clinical trial, IMMC38 [8]. The primary aim of this study was

to investigate the association between the presence of five or more CTC identified using CellSearch before the start of first or later lines of cytotoxic chemotherapy and overall survival in advanced CRPC patients. Patients with histologically confirmed prostate cancer that was metastatic and progressing despite castrate levels of testosterone (<50 ng/ml) and who were commencing a new cytotoxic therapy were eligible. Other eligibility criteria included a prostate-specific antigen (PSA) level of ≥5 ng/ml, Eastern Cooperative Oncology Group performance status of zero to two, a 4-week (6 weeks for nilutamide and bicalutamide) washout after discontinuation of an active treatment and no radiation or radionuclide therapy within 30 days of entry into the study. Patients with brain metastases or a history of other malignancies within the last 5 years were excluded. Sixty-five clinical centers in the United States and Europe participated.

A total of 276 CRPC patients were enrolled into the original study [8]. Forty-two patients were excluded as they did not meet one or more of the listed eligibility criteria. Three patients met the eligibility criteria, but the CDs with image data were unreadable. For 179 patients, samples from baseline and a first follow-up were available and a bone scan was carried out. The baseline data of these 179 patients were used as a validation dataset. The 55 remaining patients were missing a baseline sample (22), a follow-up sample (25) or a bone scan (8). Patients missing a bone scan supplied two samples. The 63 samples from these patients were used for development of the classification.

The study design for the original study has been described in detail elsewhere [8]. Briefly, blood (7.5 ml) was collected into a CellSave™ tube (Veridex LLC, Raritan, NJ) before and every 4 weeks after starting a new cytotoxic treatment and shipped to a central laboratory where isolation and enumeration of CTC was carried out using CellSearch. Samples were also analyzed for serum PSA, lactate dehydrogenase (LDH), alkaline phosphatase (ALP), testosterone, albumin and hemoglobin. Samples from 67 healthy individuals used in previous studies [5] were also analyzed to assess the background level for each of the classes of objects studied. All patients consented to trial protocols that had been approved by ethics review committees at the participating centers.

isolation of EpCAM+ objects

Isolation and image capture of EpCAM+ objects was carried out using the CellSearch system (Veridex LLC). The system consists of a CellTracks™ Autoprep for sample preparation and a CellTracks Analyzer II for sample analysis [9, 10, 12]. The CellTracks Autoprep immunomagnetically enriches epithelial cells from 7.5 ml of blood using ferrofluids coated with epithelial cell-specific EpCAM antibodies and stains the enriched samples with phycoerythrin-conjugated antibodies directed against cytokeratins 8, 18 and 19, an allophycocyanin-conjugated antibody to CD45 and the nuclear dye 4′,6-diamidino-2-phenylindole (DAPI). The CellTracks Analyzer II is a four-color semiautomated fluorescence microscope that captures digital images of the entire surface of the cartridge for four different fluorescent dyes. From the captured images, a gallery of objects is presented to a trained operator who makes a final interpretation for each object.

reanalysis of captured images

Images were imported into the Linux software of the CellTracks Analyzer II. The automated algorithm in this software was used to identify objects that were positive for CK and/or DAPI. The algorithm identified objects at least 2×2 μm^2 in size and of medium-to-high contrast in the DAPI and/or CK channels. If more than one channel was selected for analysis, objects in the selected channels had to be connected in order to be selected. Two sets of analysis were carried out. In one set, objects with CK and DAPI staining were presented to the reviewer; in the other set, objects with only CK staining were presented. Monochrome images revealed staining in DAPI, CK and CD45 channels as well as a false-color overlay of DAPI and CK to show degree of overlap between channels. A single operator assigned objects to the seven classes presented in this study for all samples; the CellSearch CTC numbers were taken from the IMMC38 study. Interoperator variability could not be established but is expected to be comparable with the interoperator variability for CTC [12]. The human reviewer was blinded to all sample information. After a total of 150 objects were assigned to a class, the results were extrapolated to calculate the total number of objects in the sample for each class.

statistical analysis

The primary end point was overall survival, measured as the time from the date of the first CTC blood draw (baseline) to the date of death from any cause. Patients who were lost to follow-up or still alive at the end of study were censored at the last date they were known to be alive or at the end of study date.

Sixty-three samples were selected for use as an independent dataset. Seven EpCAM+CK+ classes were defined using this dataset. Every EpCAM+CK+ class was then categorized into four groups of similar number to optimize the power of this analysis [25]. The group boundaries for each class were set on the 25, 50 and 75 percentile of the independent dataset [Group 1 (G1) was 0%–25%; G2, 26%–50%; G3, 51%–75%; G4, 76%–100%]. The group (G1) with the lowest number of objects (lowest density) was used as the reference for calculation of hazard ratios (HRs).

Serum data were also categorized into four groups using the data for the samples in the independent set. For all classes, overall survival was calculated using the Kaplan–Meier method and survival plots were generated. Log-rank tests were used to compare survival between groups. Cox regression models were used to determine HRs of death for the different groups within each class with the appropriate 95% confidence intervals (CIs). Age was included as a continuous variable in all Cox regression models.

Correlation between EpCAM+CK+ classes was determined using a two-tailed Spearman's rho test. As several classes were highly correlated (see 'Results' section), the final multivariate model was built by starting with a full model including all prognostic variables from the univariate analyses. In each successive step, one variable was deleted, and the new model compared with the former model, using the likelihood ratio test with the appropriate degrees of freedom to obtain a parsimonious model. The final model was confirmed by using forward conditional Cox regression analyses ($p_{in} = 0.05$ and $p_{out} = 0.10$). In this final model, the variable with the highest impact was replaced by all other variables to test whether any could replace the highest impact variable without degrading the model.

Race and processing site were also available but did not contribute to survival and therefore were not included in any model. Statistical analysis was carried out in SPSS Windows version 16.0 (SPSS Inc., Chicago, IL).

results

patient characteristics

For the 179 patients in the validation set, baseline blood draw was carried out from October 2004 to February 2006. If possible, patients were followed up to a visit with the oncologist in the 35th–39th month. Median duration of follow-up was 16 months (range 0.2–38.7 months). Overall, 134 (75%) patients died during follow-up. Their average age was 70 years (range 49–92 years); 138 (77%) were treated in the United States [41 (23%) in Europe]. Most patients (120, 67%) were starting first-line cytotoxic therapy after baseline, while 29 (16%) were going on second-line and the remaining 30 (17%) started third- to sixth-line therapy. The cytotoxic therapy included docetaxel for 129 (72%) patients and mitoxantrone for 17 (9%) patients. Metastasis to the bones was found in 158 (88%) patients.

Descriptive statistics for serum markers can be found in Table 1. CellSearch identified different classes of circulating EpCAM+ objects.

Samples from the 63 patients assigned to the independent set were used to define the criteria for classification of EpCAM+ classes. We assumed that only a few CTC had the ability to form metastasis, and the majority of CTC were at different stages of disintegration. We did not investigate, before this classification, whether the defined EpCAM+CK+ objects were observed in blood of healthy individuals. Seven different classes of EpCAM+CK+ objects with different morphological and size criteria were outlined. Intact CTC, granular CTC, large tumor cell fragments and small tumor cell fragments (L-TCF and S-TCF) required the presence of a nucleus or DNA staining (see Figure 1 panel A1–A4), while the large tumor microparticles and small tumor microparticles (L-TMP and S-TMP) did not require the presence of DNA (Figure 1 panel B1, B2). Intact CTC had an intact nucleus, entirely surrounded by intact CK. Granular CTC had at least three higher intensity CK dots connected to a nucleus. Intact CTC and granular CTC required a nucleus of at least 4×4 μm^2. The L-TCF, S-TCF, L-TMP and S-TMP were assigned on the basis of whether the size of the CK stain was smaller or larger than 4×4 μm^2. This size cut-off was chosen because a larger than 4 μm requirement is applied in the CellSearch CTC definition [1, 5, 8, 10]. All classes were negative for CD45, except CK+/CD45+ (Figure 1 panel B3), which represents white blood cells staining nonspecifically with CK and/or CTC staining nonspecifically with CD45. The CK+CD45+ class was not expected to correlate with survival and was used as control.

An object could be assigned to only one class in each review or not assigned at all. Since objects identified with the CK/DAPI filter and the CK filter were analyzed separately, it is probable that objects assigned to one of the four classes in the CK/DAPI set were also assigned as S/L-TMP in the CK-alone set.

less stringent CTC definitions increase object counts in CRPC patients but increase false-positive object counts in healthy individuals

Figure 2 shows the distribution of CTC identified with the original CellSearch definition and the number of objects within each of the EpCAM+CK+ classes for the CRPC patients at baseline and the control group of healthy individuals. For every class except CK+CD45+, a clear separation is found between the control healthy subject group and the samples from cancer patients. Only the CellSearch CTC and intact CTC classes had zero objects in all samples from healthy individuals. The lowest counts were for intact CTC and granular CTC, followed by CellSearch CTC, L-TCF and S-TCF. The highest counts were

Table 1. Descriptive statistics for patients at baseline showing mean, SD, median and range for several serum markers

	Patients at baseline			
	N	Mean ± SD	Median	Range
PSA (ng/ml)	179	452 ± 1213	126	1.9–13 246
LDH (IU/ml)	170	301 ± 248	224.5	114–2092
Testosterone (ng/ml)	173	0.20 ± 0.17	0.14	0.10–1.44
ALP (IU/ml)	173	223 ± 233	137	39–1801
Hemoglobin (g/dl)	176	12.3 ± 1.6	12.3	8.2–15.7
Albumin (g/dl)	173	4.2 ± 3.9	3.8	2.1–41

Cox hazard ratios of groups 2–4 relative to the reference group (G1) are shown on the right.

SD, standard deviation; PSA, prostate-specific antigen; LDH, lactate dehydrogenase; ALP, alkaline phosphatase.

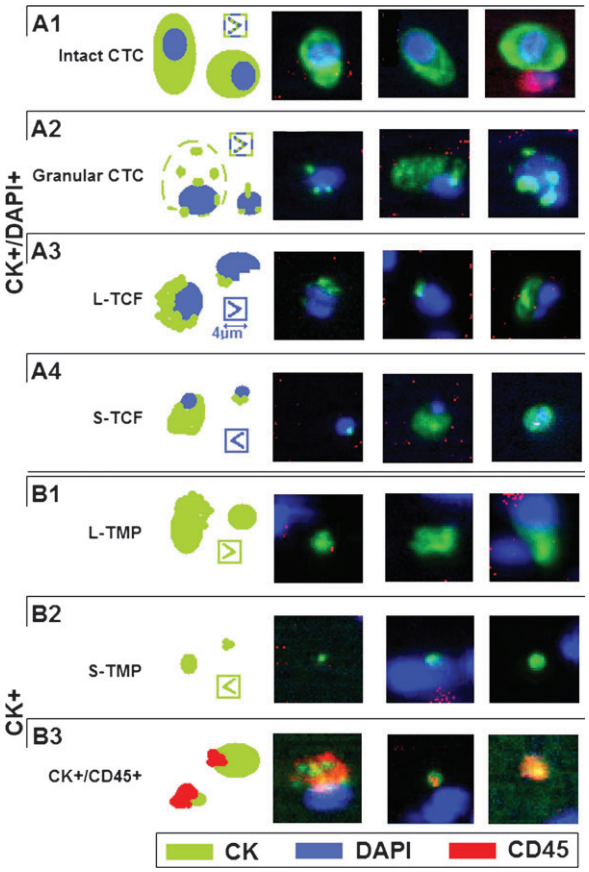

Figure 1. Representation and three typical false-color images of the seven CTC classes. Green represents the cytokeratin-phycoerythrin staining; the blue, the DAPI staining and the red, the CD45 staining. The cartoons show the classification rules explained in the text. All classes except 'CK+/CD45+' were CD45 negative. In the first analysis (panel A) that included both CK and DAPI staining, the four distinct classes of EpCAM+CK+CD45− objects were as follows—A1: intact CTC with a round or ellipsoid nucleus (DAPI) entirely surrounded by a uniform CK stain at least 4×4 μm^2 in size and negative staining for CD45; A2: granular CTC with nucleus of any shape connected to at least three higher intensity dots of CK, at least 4×4 μm^2 in size; A3: L-TCF with a nucleus of at least 4×4 μm^2 with any size CK; A4: S-TCF with a nucleus smaller than 4×4 μm^2 with any size CK. In the second analysis (panel B) that considered just CK+ objects, the three distinct classes of EpCAM+CK+ objects were as follows: B1: L-TMP with a CK staining area larger than 4×4 μm^2 and with or without a nucleus; B2: S-TMP with a cytokeratin staining area smaller than 4×4 μm^2, with or without a nucleus; B3: CK+/CD45+ object with or without a nucleus (control). CTC, circulating tumor cells; L-TCF, large tumor cell fragment; S-TCF, small tumor cell fragment; L-TMP, large tumor microparticles; S-TMP, small tumor microparticles; EpCAM+CK+, positive for epithelial cell adhesion molecules and cytokeratin.

found for S-TMP and L-TMP, but these also had the highest counts in healthy individuals. The median number of S-TMP was 17 times the median CellSearch CTC count.

all EpCAM+CK+CD45− objects predict survival in CRPC

All EpCAM+CK+CD45− classes were associated with survival (all log-rank $P < 0.001$). Figure 3 shows the Kaplan-Meier curves for all classes and the number of patients assigned to each group. For intact CTC, there were too few different values and only three groups were created.

EpCAM+CK+CD45+ objects were not associated with survival. Survival prospects decreased with increasing number of objects for all other CTC classes as evidenced by the shortest survival for the group with the highest number of objects (G4) followed by G3, G2 and then G1, with the lowest number of objects. Patients who appear in the highest group of one CTC class tend to appear in the highest groups of the other classes too.

Figure 4 shows the HRs of groups 2–4 (G2–G4) relative to the reference group (G1). The highest HR was found for L-TCF. Age was prognostic for survival with a typical HR of

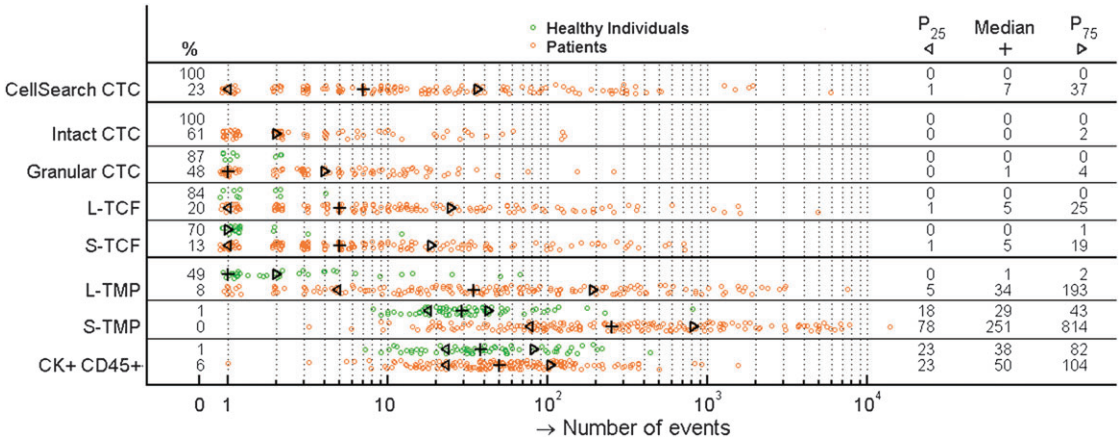

Figure 2. Distribution of the number of objects in each CTC class for healthy normals (green) and CRPC patients (orange). The percentage of samples with zero objects is shown on the left and each data point is given a random amount of jitter (<0.5) to show the density of samples in case of overlap. Markers indicate the 25 percentile, median and 75 percentile. At the right side of the figure, the actual values are shown. CTC, circulating tumor cells; CRPC, castration-resistant prostate cancer.

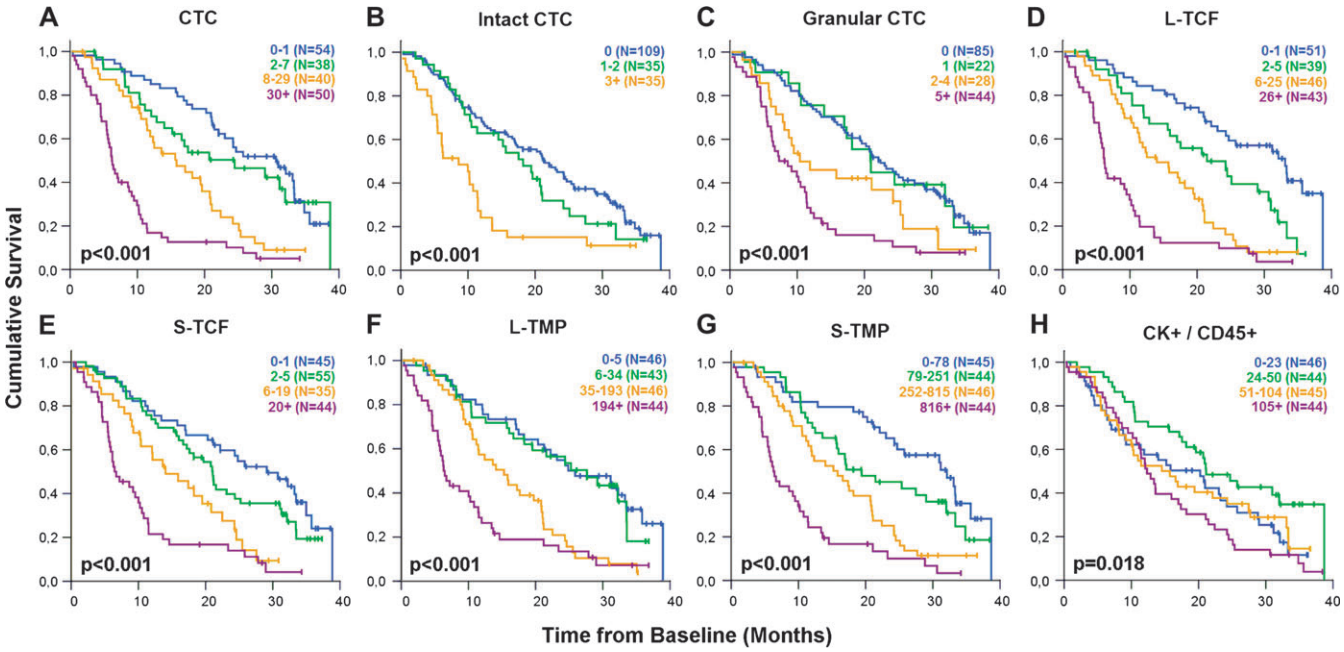

Figure 3. Kaplan–Meier plots of overall survival of CRPC patients for all EpCAM+CK+ objects (panels A–H). The vertical markers represent censored patients. The number of patients in each group and the group boundaries are shown in the top-right corner of each plot. Due to the large number of samples with zero objects for many EpCAM+ classes, the reference group G1 includes a larger than 25% share of the samples. *P* values of the log-rank test are shown in the bottom left of each plot. Units for all EpCAM+CK+ classes are 'number of objects/7.5 ml'. EpCAM+CK+, positive for epithelial cell adhesion molecules and cytokeratin. CRPC, castration-resistant prostate cancer.

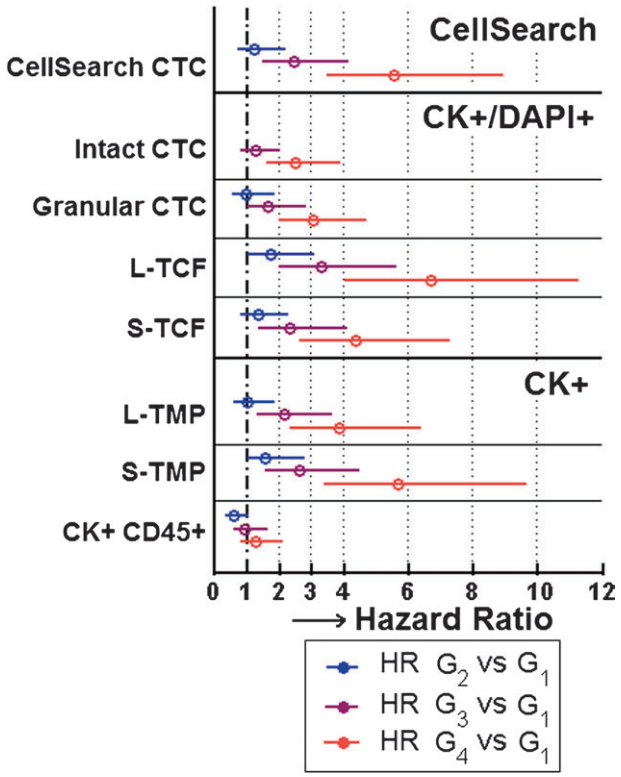

Figure 4. Hazard ratios (HR) of groups 2-4 relative to group 1 with 95% confidence intervals. In the control class (CK + CD45), the confidence interval includes 1 (no change in hazard) for all groups. For all other classes, the HR shows a continuous increase across. While the confidence interval for group 2 (G_2) includes 1 for most classes, the confidence interval of group 4 (G_4) clearly shows high HR and excludes one. Highest HR are found for S-TMP and CellSearch CTC, closely followed by L-TMP, L-TCF and S-TCF.

1.018/year (95% CI 0.999–1.037). For CellSearch CTC, S/L-TCF and S/L-TMP, it is clear that a patient in a higher group, with more objects, has a poorer prognosis compared with a patient in a lower group. For granular CTC and intact CTC, there is little difference between G2 and G1.

L-TCF, S-TMP and CellSearch CTC are the best predictors of survival

Parameters associated with survival in univariate analyses were used as input parameters for multivariate analysis (MVA) (Table 1 and Figure 3). The input variables were all CTC classes, LDH, ALP, hemoglobin, albumin and PSA as categorical variables and age as a continuous variable. Table 2 shows the results of this analysis. The final model contained age, hemoglobin, LDH and L-TCF. The correlation between CTC classes was high (mean Spearman's $\rho = 0.70$, range 0.40–0.92). The performance of models containing S-TMP or CellSearch CTC instead of L-TCF was similar, while models with other CTC classes were performing worse.

discussion

Multicenter prospective clinical studies have demonstrated a significant relation between the presence of CTC defined by the CellSearch CTC definition and poor progression-free and overall survival [1–8]. The definition of CellSearch CTC was set before the initiation of these studies and no subsequent analysis has been carried out to determine whether less or more stringent definitions of CTC were similarly associated with worse outcome. Moreover, multiple conflicting CTC reports, utilizing different technologies and describing very different CTC counts, have led to questions being raised as to whether different methods are counting different CTC subclasses. This has resulted in an urgent need for guidelines on what is the best definition of a clinically relevant CTC. Our study is the first to have comprehensively evaluated all EpCAM+CK+ objects isolated by immunomagnetic selection using the CellSearch system, which remains the only technology for isolating CTC to have been studied in multicenter prospective phase III studies. We report that all circulating EpCAM+CK+CD45− objects identified in CRPC patients predict survival. On MVA, the CD45− L-TCF with a nucleus at least $4 \times 4\ \mu m^2$ and any size CK staining (L-TCF) as well as the more strict CellSearch definition for CTC and the less stringent definition of S-TMP that are smaller than $4 \times 4\ \mu m^2$ and do not require DNA staining were equally prognostic.

These observations have very significant implications for future prognostic studies of CTC. Applying the strictest morphological definition of CTC that have the strongest likelihood of being viable tumor cells (intact CTC) is not necessary and even less significant than other classes. There are many patients with no intact CTC found, which could result in a reduction of median survival of the reference group (G1) and thus reduce the HR of the other groups. Tumor microparticles may be better indicators of disease burden in these patients because they occur at higher frequency. The presence of TMP could infer that intact CTC are present albeit at a frequency <1/7.5 ml of blood. Overall, it appears that very different morphological CTC definitions can be applied. Many of the patients who appear in the highest group of one CTC class tend to appear in the highest groups of the other classes too.

To investigate whether the level of CTC in each class matters, we applied a 4-level categorization of the data wherever possible to show the continuous behavior of the classes without estimating a linearization transformation for Cox regression. The ranking within each subclass was on the basis of an independent set of 63 samples. A higher number of EpCAM+CK+ objects resulted in a reduced survival for CellSearch CTC, L/S-TCF and L/S-TMP. We expect this relationship between the number of events and survival to hold true for metastatic breast and colon cancer.

Determining which class is most suitable for prognostication is difficult. From the univariate Cox regression, we find that CellSearch CTC, L/S-TCF and L/S-TMP all have high HRs. Multivariate regression showed that CellSearch CTC, L-TCF and S-TMP had similar predictive values. Other desirable properties are low counts in healthy volunteers and a quick, objective and reproducible determination. CellSearch CTC determination performs best from the point of view of being generally absent in healthy volunteers. TMP were most prevalent and easier to enumerate, while CellSearch CTC and L/S-TCF required the most operator training. None the less, TMPs are unlikely to have the same potential as intact CTC for

Table 2. Results of the Cox proportional hazard analysis of the markers associated with survival at BL

Variable	Range	HR (95% CI)			
		Univariate[a]	MVA with L-TCF	MVA with CellSearch CTC	MVA with S-TMP
Age (years)	Continuous	1.018 (0.999–1.037)	1.021 (1.000–1.043)	1.023 (1.002–1.044)	1.023 (1.002–1.045)
LDH (IU/ml)	≤173	1 (ref)	1 (ref)	1 (ref)	1 (ref)
	174–228	1.11 (0.63–1.96)	0.87 (0.48–1.57)	0.84 (0.46–1.53)	0.75 (0.41–1.38)
	229–313	1.46 (0.81–2.63)	0.83 (0.44–1.56)	0.76 (0.39–1.45)	0.84 (0.44–1.62)
	314+	3.94 (2.19–7.12)	1.99 (1.03–3.82)	1.9 (0.98–3.65)	1.77 (0.90–3.49)
Hemoglobin (g/dl)	13.5+	1 (ref)	1 (ref)	1 (ref)	1 (ref)
	12.6–13.4	1.26 (0.70–2.27)	1.03 (0.55–1.92)	1.13 (0.60–2.12)	1.07 (0.57–2.02)
	11.3–12.5	3.00 (1.81–5.00)	1.70 (0.97–2.97)	1.92 (1.10–3.36)	1.93 (1.07–3.48)
	≤11.2	4.28 (2.57–7.13)	2.26 (1.26–4.05)	2.25 (1.26–4.04)	2.98 (1.66–5.37)
L-TCF (count/7.5 ml blood)	0–1	1 (ref)	1 (ref)		
	2–5	1.92 (1.12–3.3)	1.52 (0.87–2.69)		
	6–25	3.30 (1.98–5.49)	2.13 (1.19–3.81)		
	26+	6.57 (3.94–10.95)	4.00 (2.20–7.27)		
CTC (count/7.5 ml blood)	0–1	1 (ref)		1 (ref)	
	2–7	1.20 (0.70–2.06)		1.05 (0.60–1.84)	
	8–29	2.39 (1.46–3.91)		1.64 (0.94–2.88)	
	30+	5.20 (3.25–8.32)		3.36 (1.87–6.07)	
S-TMP (count/7.5 ml blood)	0–78	1 (ref)			1 (ref)
	79–251	1.55 (0.91–2.65)			1.26 (0.71–2.26)
	252–815	2.66 (1.59–4.43)			1.58 (0.87–2.85)
	816+	5.50 (3.28–9.24)			3.43 (1.90–6.19)

The beta and HRs for univariate analysis and the three equivalent multivariate models are shown.
BL, baseline; HR, hazard ratio; CI, confidence interval; MVA, multivariate analysis; L-TCF, large tumor cell fragments; CTC, circulating tumor cells; S-TMP, small tumor microparticles; LDH, lactate dehydrogenase.
[a]Univariate analysis including only one variable.

the assessment of treatment targets by FISH [26–29], proteins [24,30] and RNA [31].

Fully automated CTC enumeration is desirable to increase reproducibility in object assignment. Importantly, the fully automated CTC counting with less stringent definitions for CTC will be easier to achieve than enumerating CTC as defined by CellSearch. Finally, these data need to be replicated in other CRPC studies and their broader significance in other tumor types needs to be investigated.

In conclusion, these data have important implications to the future evaluation of CTC. We have investigated the prognostic value of different CTC (DAPI+) or TMP (DAPI+/−) definitions from 179 baseline samples from a CRPC study [8] and have shown that all EpCAM+CK+CD45− classes were prognostic for survival. For CellSearch CTC, L-TCF, S-TCF, L-TMP and S-TMP, we found that the presence of a larger number of objects predicted shorter survival. Overall, CellSearch CTC had the lowest background, while the highest number of objects in a tube of blood was for S-TMP. We recommend that reproducibility of CTC assignment needs to be improved, preferably by the use of automated image analysis.

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disclosure

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