

ORIGINAL ARTICLE

Clinical relevance of EMT and stem-like gene expression in circulating tumor cells of metastatic colorectal cancer patients

Y Ning¹, W Zhang¹, DL Hanna¹, D Yang², S Okazaki¹, MD Berger¹, Y Miyamoto¹, M Suenaga¹, M Schirripa¹, A El-Khoueiry¹ and H-J Lenz^{1,2}

Using approved methods, circulating tumor cells (CTCs) are only isolated from blood in 30%–50% of metastatic colorectal cancer (mCRC) patients. We previously validated a technique to isolate circulating tumor cells (CTCs) in a cohort of mCRC patients by combining immunomagnetic enrichment of EpCAM⁺/CD45⁻ cells with qRT-PCR amplification of *CK20* and *survivin* expression. Here, we examined the prognostic utility of CTC epithelial–mesenchymal transition (EMT) and stem cell gene expression. An 8 ml blood sample was collected from 78 consecutive mCRC patients before treatment with investigational and standard chemotherapeutics. The mRNA expression of EMT (*PI3Ka*, *Akt-2*, *Twist1*) and stem cell (*ALDH1*) markers was measured. Associations between CTC gene expression and progression-free survival (PFS) and overall survival (OS) were determined using Cox regression models. Among patients without *CK20* or *survivin*-expressing CTCs ($n = 17$), 55% had expression of *ALDH1*, *PI3Ka* and/or *Akt-2*. Patients with positive CTC *Akt-2* expression had a significantly shorter median PFS (3.0 versus 4.0 months) compared with those without CTC *Akt-2* expression in univariable (hazard ratio (HR) = 1.61; log-rank $P = 0.034$) and multivariable analyses (HR = 1.70; adjusted $P = 0.041$). In univariable analysis, CTC *ALDH1* expression was associated with shorter OS (10.0 versus 38.6 months; HR = 2.04, $P = 0.021$). Patients with CTCs expressing *ALDH1*, *PI3Ka* and/or *Akt-2* had a significantly inferior PFS (3.0 versus 7.7 months; HR = 1.88, $P = 0.015$) and OS (10.0 versus 26.8+ months; HR = 2.25, $P = 0.050$) in univariable, but not multivariable, analysis. Conclusions: CTC *Akt-2* expression may serve as a clinically useful prognostic marker in mCRC patients and warrants further evaluation in prospective trials.

The Pharmacogenomics Journal (2018) 18, 29–34; doi:10.1038/tpj.2016.62; published online 9 August 2016

INTRODUCTION

Circulating tumor cells (CTCs) have been isolated from metastatic colorectal cancer (mCRC) patients,^{1,2} and CTC count has been associated with survival.^{3–5} However, circulating cancer cells are rare within the bloodstream and exhibit a phenotypic diversity, which may impede their reliable detection. Using the CellSearch assay, CTCs are detected in only 30%–50% of mCRC patients.^{1–5} CTCs can undergo the epithelial–mesenchymal transition (EMT)^{6–9} and assume a stem cell-like⁶ phenotype. Owing to such heterogeneity, current CTC isolation methods that emphasize epithelial traits may not capture their plasticity through activation of EMT and stem cell pathways.¹⁰ Refining the molecular characterization of CTCs may improve the clinical utility of CTC detection and provide insight into mechanisms directing the metastatic process as well as therapeutic resistance. To this end, several CTC markers^{3–5,11–17} have been explored in an attempt to improve diagnostic yield and predict outcomes, but the optimal CTC gene expression signature remains to be defined.

We recently validated a technique combining immunomagnetic enrichment with quantitative real-time PCR (qRT-PCR) to isolate EpCAM⁺/CD45⁻ CTCs, which express *CK20* and/or *survivin* mRNA in mCRC patients receiving standard and experimental chemotherapy regimens.¹⁸ However, within the cohort of patients with *CK20* and/or *survivin*-expressing CTCs, there was still considerable variability with regards to survival. Furthermore, we hypothesized that a proportion of epithelial–mesenchymal transitioned CTCs

may not express *cytokeratin* or *survivin* and, therefore, evade detection. The *PI3Ka/Akt-2/mTOR* pathway helps regulate the EMT transition by repressing cell adhesion proteins, therefore facilitating cancer cell migration as well as survival and apoptosis.¹⁹ In preclinical studies, *Akt-2* has been shown to be critical to the development of colorectal metastasis.²⁰ Clinically, CTC expression of *PI3Ka*, *Akt-2*, *Twist1* has been significantly associated with outcomes in cancer patients.⁸ Similarly, circulating cancer cells may acquire a stem cell phenotype,¹⁹ marked by aldehyde dehydrogenase 1 (*ALDH1*) upregulation, and CTC *ALDH1* expression has demonstrated prognostic value in patients undergoing chemotherapy.^{8,21}

Here, we aim to refine the utility of our approach by measuring the mRNA expression of EMT (*PI3Ka*, *Akt-2*, *Twist1*) and stem cell (*ALDH1*) markers in circulating cancer cells. We found a proportion of *CK20*⁻ and *survivin*⁻ cells express *PI3Ka*, *Akt-2* or *ALDH1*, and that this gene expression panel may prognostically stratify subgroups of mCRC patients receiving chemotherapy.

MATERIALS AND METHODS

Patient population and study design

Patients consented for peripheral blood collection and received standard FDA-approved therapies (including fluoropyrimidines, oxaliplatin, irinotecan, bevacizumab, cetuximab, panitumumab, regorafenib), or received experimental agents being tested in three phase I or II clinical

¹Division of Medical Oncology, Sharon A. Carpenter Laboratory, Norris Comprehensive Cancer Center, Keck School of Medicine, University of Southern California, Los Angeles, CA, USA and ²Department of Preventive Medicine, Norris Comprehensive Cancer Center, Keck School of Medicine, University of Southern California, Los Angeles, CA, USA. Correspondence: Professor H-J Lenz, Norris Comprehensive Cancer Center, Keck School of Medicine, University of Southern California, 1441 Eastlake Avenue, Suite 3456, Los Angeles, CA 90089, USA.

E-mail: lenz@usc.edu

Received 3 February 2016; revised 3 May 2016; accepted 15 June 2016; published online 9 August 2016

trials examining 5-FU plus brivanib (NCT01046864), PRI-724 (NCT01302405) and celecoxib plus EPO906 (NCT00159484). Patients were enrolled at the Norris Comprehensive Cancer Center-University of Southern California or the Los Angeles County-University of Southern California Medical Center, between June 2009 and April 2014.¹⁸ The Institutional Review Board at the University of Southern California approved the study. Twenty healthy blood donors (age ≥ 18 years), who had no known medical illness or history of malignant disease, served as control subjects. All the CTC studies were performed without knowledge of patients' clinical status.

Sample collection and tumor cell enrichment

The sample collection and tumor cell enrichment were performed as previously described.¹⁸ An 8 ml blood sample was drawn from each patient into one Vacutainer CPT Tube with Sodium Citrate (BD, Franklin Lakes, NJ, USA). Negative immunomagnetic selection using anti-CD45 specific antibodies (Dynabeads M-450 CD45 pan Leukocyte, Invitrogen, Waltham, MA, USA) 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 towards human EpCAM, tumor cell selection was performed following the manufacturer's instructions.

qRT-PCR and multiplex-PCR

CK20 and *survivin* mRNA expression levels were analyzed by the iTaq Fast SYBR Green Supermix (Bio-Rad #172-5101; Bio-Rad, Hercules, CA, USA) and an Applied Biosystems 7500 PCR Detection System (Applied Biosystems, Foster City, CA, USA). *ALDH1*, *Akt-2* and *PI3Ka* mRNA expression levels were analyzed using the HotStarTaq Master Mix (Qiagen, Qiagen GmbH, Germany, #203443) and a thermocycler, and determined by using the Agilent 2100 Bioanalyzer on a DNA LabChip (Agilent Technologies, Santa Clara, CA, USA).

Analysis of mRNA expression

Primers for EMT markers and stem cell markers were provided by AdnaGen (AdnaGen, Langenhagen, Germany). The analysis of tumor cell-derived mRNA was performed by qRT-PCR for the following transcripts: *PI3Ka*, *Akt-2*, *Twist1* and *ALDH1*. Thermal profiles were used as per the recommendations of the supplier (AdnaGen). The AdnaTest EMT-2/StemCell Detect Kit (AdnaGen), containing oligo(dT)₂₅-coated beads, was used to isolate mRNA from tumor cells. The PrimerMix StemCell was used to amplify *ALDH1*, and the PrimerMix EMT-2 was used to amplify three EMT-related genes (*Akt-2*, *PI3Ka*, *Twist1*) and one control gene (*Actin*). Visualization of PCR fragments was carried out with the Agilent 2100 Bioanalyzer, with a limit of detection of ≥ 0.15 ng μl^{-1} .

Statistical analysis

The mRNA levels of CTC *ALDH1*, *PI3K* and *Akt-2* were categorized into low and high values at the cutoff values provided by AdnaGen. The associations between the three markers and progression-free survival (PFS) and overall survival (OS) were analyzed by Kaplan–Meier curves and log-rank test in the univariable analysis and Cox regression model in the multivariable model, adjusting for baseline characteristics and treatment administered after CTC collection that were associated with PFS or OS at a significance level of 0.1.

RESULTS

Patient and tumor characteristics

Patient and tumor characteristics are described in Table 1. Among 78 mCRC patients, the median duration of follow-up was 23.5 months (range: 2.0, 38.6 months), median PFS was 3.1 months (95% confidence interval: 2.1, 5.0 months) and median OS was 10.4 months (95% confidence interval: 8.9, 17.2 months). Patients received a median of three prior lines of therapy (range 0–5). Most patients had received fluoropyrimidines (97.4%), oxaliplatin (89.7%), irinotecan (75.6%) and bevacizumab (88.5%) before CTC collection. After CTC collection, 62.8% of patients received experimental therapies on clinical trials. There was an even distribution of primary tumor site across all the patients, and most did not have liver-limited metastases.

Table 1. Metastatic colorectal cancer patient baseline characteristics (n = 78)

Characteristic	n (%)
<i>Age, years</i>	
Median (range)	57.5 (28-81)
<i>Sex</i>	
Male	42 (53.8)
Female	36 (46.2)
<i>Race</i>	
White	51 (65.4)
African American	3 (3.8)
Asian	9 (11.5)
Hispanic	15 (19.2)
<i>Primary tumor site^a</i>	
Right colon	21 (26.9)
Left colon	31 (39.7)
Rectum	23 (29.5)
Unspecified CRC	3 (3.8)
<i>Liver-only metastases</i>	
Yes	14 (18.0)
No	64 (82.0)
<i>ECOG performance status</i>	
0	24 (30.8)
1	48 (61.5)
2	6 (7.7)
<i>Number of prior lines of therapy</i>	
0–1	17 (21.8)
2	20 (25.6)
3	23 (29.5)
4–5	18 (23.1)
<i>Prior lines of therapy</i>	
Fluoropyrimidine	76 (97.4)
Oxaliplatin	70 (89.7)
Irinotecan	59 (75.6)
Bevacizumab	69 (88.5)
Cetuximab, Panitumumab	28 (35.9)
<i>Therapy initiated after CTC collection</i>	
Fluoropyrimidine	28 (35.9)
Oxaliplatin	14 (17.9)
Irinotecan	12 (15.4)
Bevacizumab	20 (25.6)
Cetuximab, Panitumumab	2 (2.6)
Experimental Agents	49 (62.8)
<i>Baseline CEA (ng ml⁻¹) (N = 75)</i>	
Median (range)	35.9 (0.9–16 300)
<i>Baseline LDH (units l⁻¹) (N = 61)</i>	
Median (range)	199 (120–1871)

Abbreviations: CEA, carcinoembryonic antigen; CRC, colorectal cancer; CTC, circulating tumor cell; ECOG, Eastern Cooperative Oncology Group; LDH, lactate dehydrogenase. ^aLeft- and right-sided tumors were demarcated by the splenic flexure; transverse colon tumors were considered right-sided.

CTC *ALDH1*, *PI3Ka* and *Akt-2* expression in mCRC patients and healthy donors

We first compared biomarker expression in 78 mCRC patients and 20 healthy donors. As shown in Figure 1a, the expression of *ALDH1* (69 versus 0%), *PI3Ka* (4 versus 0%) and *Akt-2* (49 versus 0%) was significantly higher in the mCRC patients compared with the

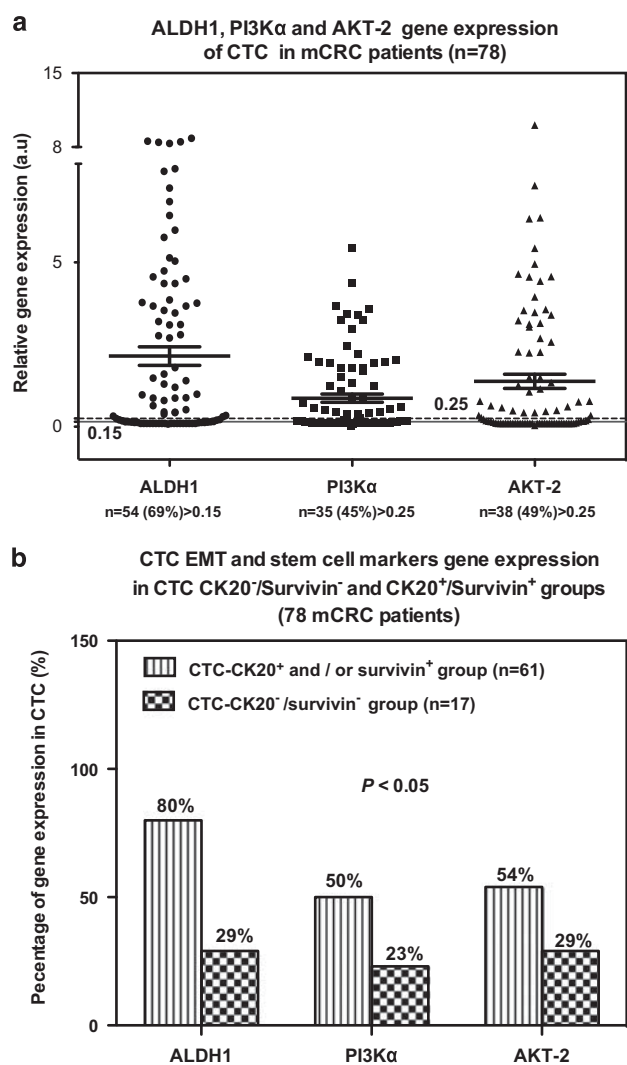


Figure 1. (a) CTC *ALDH1*, *PI3K α* , *Akt-2* gene expression levels in 78 mCRC patients. (b) Comparison of EMT markers, *ALDH1* in mCRC patients. CTC, circulating tumor cell; EMT, epithelial–mesenchymal transition; mCRC, metastatic colorectal cancer.

healthy donors ($P < 0.001$ for all comparisons). Cutoff values for detection were provided by AdnaGen: $0.25 \text{ ng } \mu\text{l}^{-1}$ for *PI3K α* , *Akt-2*, *Twist1* and $0.15 \text{ ng } \mu\text{l}^{-1}$ for *ALDH1*. On the basis of these cutoff points, the sensitivity and specificity for detecting CTCs by the presence of either *ALDH1*, *PI3K α* or *Akt-2* expression was 83% and 100%, respectively. There was no significant *Twist1* gene expression detected in mCRC patients or in healthy donors (data not shown).

CTC *ALDH1*, *PI3K α* and *Akt-2* expression in mCRC patients

Next, we compared the expression of *ALDH1*, *PI3K α* and *Akt-2* in patients with and without *CK20*/*survivin*-expressing CTCs using the AdnaGen cutoff values. Seventy-eight percent of patients ($n = 61$) had *CK20* and/or *survivin*-expressing CTCs. Among patients without *CK20* or *survivin*-expressing CTCs ($n = 17$), 29%, 23% and 29% had expression of *ALDH1*, *PI3K α* and/or *Akt-2* markers, respectively; 55% had expression of any of three markers. Among patients without *CK20*-expressing CTCs ($n = 21$), 14, 33, 29 and 29% had expression of *survivin*, *ALDH1*, *PI3K α* and *Akt-2*, respectively; 62% had expression of any of four markers. The expression of *ALDH1* (80 versus 29%), *PI3K α* (50 versus 23%) and *Akt-2* (54 versus 29%) was significantly higher in patients with *CK20* and/or *survivin*-expressing CTCs relative to those with CTCs negative for both *CK20* and *survivin* expression ($P < 0.05$ for all comparisons; Figure 1b).

Prognostic utility of CTC *PI3K α* , *Akt-2* and *ALDH1* expression

We examined the prognostic relevance of CTC *ALDH1*, *PI3K α* and *Akt-2* expression in the mCRC patients with and without *CK20*/*survivin*-expressing CTCs.

In univariate analysis, patients with positive CTC *Akt-2* expression had a significantly shorter PFS (3.0 versus 4.0 months, hazard ratio (HR) = 1.63, log-rank $P = 0.034$) compared with those without CTC *Akt-2* expression (Table 2, Figure 2a). This association maintained significance in a multivariate model accounting for baseline performance status and therapy received (standard versus experimental) after CTC measurement (HR = 1.70, log-rank $P = 0.041$). There was no significant association between CTC *Akt-2* expression and OS (Table 3). Patients with positive CTC *ALDH1* expression had a significantly shorter OS (10.0 versus 38.6 months, HR = 2.04, log-rank $P = 0.021$; Figure 2b and Table 3) and trend towards inferior PFS (3.0 versus 4.5 months, HR = 1.50, log-rank $P = 0.079$; Table 2) compared with CTC *ALDH1*-negative patients in

Table 2. CTC marker expression and progression-free survival (PFS) in mCRC patients

Gene	n	Median (95% CI), months	HR (95% CI), univariable analysis	P-value ^a	HR (95% CI), multivariable analysis ^b	P-value ^b
<i>ALDH1</i>	-	4.5 (0.9, 9.1)	1 (reference)	0.079	1 (reference)	0.72
	+	3.0 (2.1, 4.0)	1.50 (0.89, 2.55)		0.90 (0.49, 1.63)	
<i>PI3Kα</i>	-	3.3 (2.1, 5.7)	1 (reference)	0.10	1 (reference)	0.38
	+	2.9 (1.5, 4.4)	1.46 (0.90, 2.37)		1.25 (0.76, 2.05)	
<i>Akt-2</i>	-	4.0 (2.1, 6.4)	1 (reference)	0.034	1 (reference)	0.041
	+	3.0 (1.5, 3.3)	1.61 (0.98, 2.64)		1.70 (1.02, 2.84)	
<i>ALDH1/PI3Kα/Akt2</i>	-	7.7 (0.9, 14.4)	1 (reference)	0.015	1 (reference)	0.34
	+	3.0 (2.1, 4.0)	1.88 (1.01, 3.51)		1.40 (0.70, 2.81)	

Abbreviations: CI, confidence interval; CTC, circulating tumor cell; HR, hazard ratio; mCRC, metastatic colorectal cancer. ^aP-value was based on the log-rank. ^bBased on multivariable Cox regression model adjusting for ECOG (Eastern Cooperative Oncology Group) at baseline and therapy received after CTC measurement. Bold entries mean patients with positive *Akt-2* expression ($P = 0.034$) or any positive CTC marker ($P = 0.015$) had a significant shorter PFS compared with those without CTC *Akt-2* expression or no elevated CTC markers.

univariate analysis. However, CTC *ALDH1* expression was not significantly associated with survival in multivariate analysis. Patients with positive CTC *PI3Ka* expression trended towards a shorter PFS, but this difference was not statistically significant (2.9 versus 3.3 months, HR = 1.46, log-rank $P=0.10$; Table 2) and did

not withstand correction in multivariable analysis. There was no significant association between CTC *PI3Ka* expression and OS (Table 3).

In univariate analysis, patients with any positive CTC marker (*ALDH1*, *PI3Ka* or *Akt-2*) had a significantly inferior median PFS (3.0 versus 7.7 months, HR = 1.88, log-rank $P=0.015$; Table 2) and OS (10.0 versus 26.8+ months, HR = 2.25, log-rank $P=0.050$; Table 3) compared with those with no elevated CTC markers (Figure 3). These associations, however, were not significant in multivariate analysis.

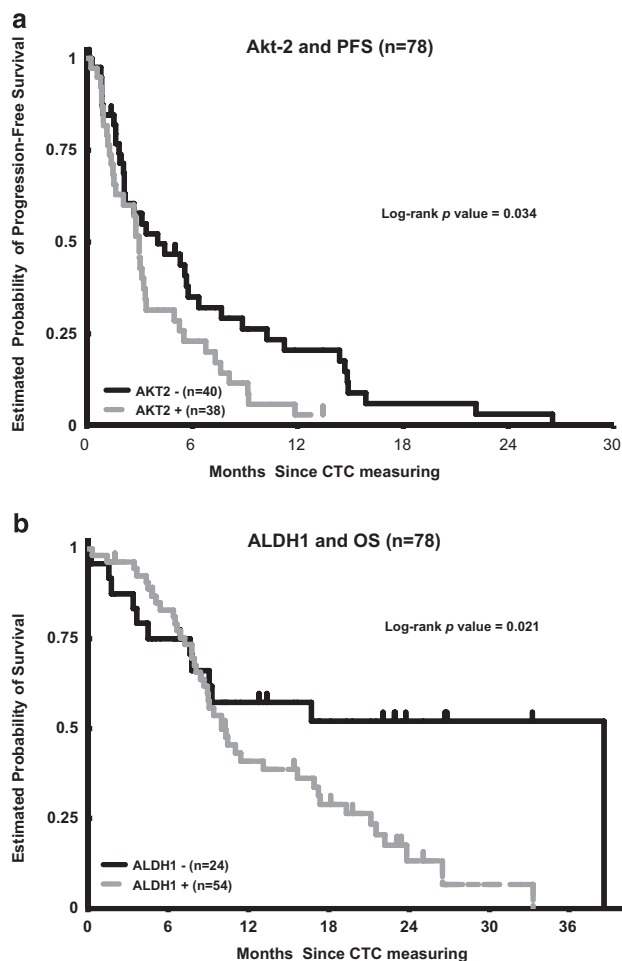


Figure 2. Kaplan–Meier cumulative probability of survival based on (a) CTC *Akt-2* and (b) CTC *ALDH1* gene expression. CTC, circulating tumor cell; OS, overall survival; PFS, progression-free survival.

DISCUSSION

We evaluated the clinical relevance of *PI3Ka*, *Akt-2* and *ALDH1* gene expression in the colorectal circulating cancer cells. To our knowledge, we are the first to report a significant association between CTC *Akt-2* expression and PFS in mCRC patients receiving different standard and experimental therapeutics. Moreover, we found that patients whose CTCs do not express *CK20* or *survivin* may express *PI3Ka*, *Akt-2* and/or *ALDH1* and that this gene expression signature may serve as a clinically useful prognostic marker.

Although the EMT program is thought to be integral to the ability of CTCs to form metastases, CTCs may also revert back to an epithelial state through the mesenchymal to epithelial transition.²² The position along which a CTC resides within the epithelial–mesenchymal axis is malleable, influenced by disease progression and cancer-directed therapy.¹⁰ Furthermore, it has been postulated that a subpopulation of CTCs exists in a semi-mesenchymal state, exhibiting both epithelial and mesenchymal traits.²³

On the basis of these observations, we hypothesized that incorporating both epithelial and mesenchymal genes into a multimarker model would optimize the prognostic value of CTC detection. In our cohort, 62% of patients without *CK20*-expressing CTCs were found to have expression of *survivin*, *PI3Ka*, *Akt-2* or *ALDH1*. Over half of patients whose CTCs did not express *CK20* or *survivin* expressed *PI3Ka*, *Akt-2* or *ALDH1*.

PI3Ka and *Akt-2* expression have been implicated in EMT and stem cell renewal²⁴ and have been reliably detected in CTCs.⁸ In cancer cell lines, *PI3Ka*-*Akt-2* signaling has been shown to promote cell motility, invasiveness and metastatic potential,²⁵ partly through its interaction with *Twist1* and downstream effectors. In a landmark study by Ryachahou *et al.*,²⁰ the *Akt-2* isoform, in particular, was found to have sustained overexpression across all stages of CRC. In *Akt-2* knockout CRC mouse models, the formation of liver tumors was greatly repressed, further

Table 3. CTC marker expression and overall survival (OS) in mCRC patients

Gene	n	Median (95% CI), months	HR (95% CI), univariable analysis	P-value ^a	HR (95% CI), multivariable analysis ^b	P-value ^b
<i>ALDH1</i>				0.021		0.30
–	24	38.6 (7.6, 38.6)	Reference		Reference	
+	54	10.0 (8.4, 15.6)	2.04 (1.05, 3.97)		1.45 (0.72, 2.92)	
<i>PI3Ka</i>				0.25		0.74
–	43	15.6 (9.4, 23.8)	Reference		Reference	
+	35	8.9 (7.7, 17.4)	1.36 (0.78, 2.34)		1.10 (0.63, 1.93)	
<i>Akt-2</i>				0.72		0.69
–	40	13.1 (8.4, 19.3)	Reference		Reference	
+	38	10.3 (7.8, 21.1)	1.10 (0.64, 1.89)		0.88 (0.48, 1.62)	
<i>ALDH1/ PI3Ka/Akt2</i>				0.050		0.41
–	15	26.8+	Reference		Reference	
+	63	10.0 (8.4, 16.9)	2.25 (0.96, 5.27)		1.46 (0.60, 3.55)	

Abbreviations: CI, confidence interval; CTC, circulating tumor cell; HR, hazard ratio; mCRC, metastatic colorectal cancer. ^aP-value was based on the log-rank test for the positive (+) or negative (–) groups of CTC *ALDH1*, *PI3Ka* and *Akt-2* expression. ^bBased on multivariable Cox regression model adjusting for ECOG (Eastern Cooperative Oncology Group) at baseline and therapy received after CTC measurement. Bold entries mean patients with positive *ALDH1* expression ($P=0.021$) or any positive CTC marker ($P=0.05$) had a significant shorter OS compared with those without CTC *ALDH1* expression or no elevated CTC markers.

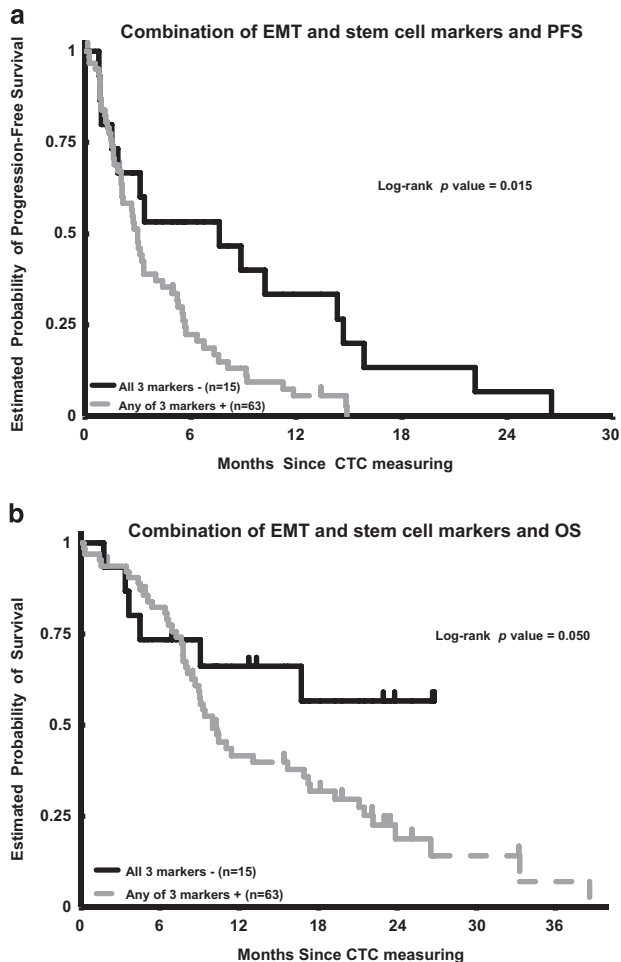


Figure 3. Kaplan–Meier cumulative probability of (a) progression-free survival (PFS) and (b) overall survival (OS) based on circulating tumor cell (CTC) *ALDH1*, *PI3Ka*, *Akt-2* gene expression. EMT, epithelial–mesenchymal transition.

supporting an essential role for Akt-2, in conjunction with PI3K, in the development and growth of CRC metastases.²⁰

Clinically, evidence from breast cancer patients supports the use of *PI3Ka*, *Akt-2* and *Twist1* as sensitive markers for CTC identification,⁸ as well as predictors of response to endocrine and cytotoxic chemotherapy.⁸ In our study, 47%–51% of mCRC ($n=78$) and 52%–57% patients ($n=70$) with *CK20/survivin*-expressing CTCs also overexpressed *PI3Ka* and/or *Akt-2*, respectively, which is comparable to findings in other metastatic cancer patients.⁸ CTCs also frequently overexpress stem cell markers²⁴ and acquire many of their stem cell properties during the EMT, allowing them to re-seed distant organs. In CRC patients, the stem cell marker, *ALDH1*, has been associated with EMT signaling.²⁶ In our study, 70% of mCRC patients ($n=78$) and 80% patients ($n=70$) with *CK20/survivin*-expressing CTCs had *ALDH1* overexpression.

The evidence for a prognostic effect of CTC EMT-related and stem-like markers has been inconsistent, with some^{8,27,28} but not all studies^{29,30} demonstrating a significant relationship with disease recurrence or survival. The prognostic utility of adding stem cell (for example, CD133) to epithelial markers in advanced CRC patients was demonstrated by Linuma *et al.*,¹⁷ though this study did not use a negative enrichment step, which may have limited assay sensitivity. In our study, patients whose CTCs expressed at least one elevated marker (*ALDH1*, *PI3Ka*, *Akt-2*) had inferior survival, suggesting that an increasingly mesenchymal

or stem-like CTC phenotype may serve as a surrogate for tumor invasiveness and refractory disease to affect survival.

Our study has several limitations, the first of which is the small sample size of our retrospective cohort. Furthermore, there was heterogeneity with respect to the type of treatment received and the time point at which blood was drawn from patients, which may have limited the power of our analysis. Notably, we did not detect *Twist1* expression in any of our patients, and this may be due to *Twist1* downregulation in CD45-depleted cells. However, the expression of *PI3Ka*, *Akt-2*, or *ALDH1* was able to distinguish cancer patients from healthy controls and patients with or without *CK20/survivin*-expressing CTCs with high sensitivity and specificity. Importantly, a proportion of CTCs may lose their EpCAM expression during the EMT^{30,31} and may, therefore, not be detected by our method.

In summary, we examined CTC *ALDH1*, *PI3Ka* and *Akt-2* mRNA expression on CTC and found CTC *Akt-2* expression to predict PFS in mCRC patients receiving different standard and experimental regimens. Larger prospective studies are needed to validate this gene expression model and assess its utility to predict disease progression and response to cytotoxic and targeted therapeutics.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

This work was supported by the National Institute of Health (P30CA014089 to H-JL) and the Gloria Borges Wunderglo Project and Dhont Family Foundation (H-JL). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Cancer Institute or the National Institutes of Health. MDB received a grant from the Swiss Cancer League (BIL KLS-3334-02-2014).

REFERENCES

- Allard WJ, Matera J, Miller MC, Repollet M, Connelly MC, Rao C *et al*. Tumor cells circulate in the peripheral blood of all major carcinomas but not in healthy subjects or patients with nonmalignant diseases. *Clin Cancer Res* 2004; **10**: 6897–6904.
- Molnar B, Ladanyi A, Tanko L, Sreter L, Tulassay Z. Circulating tumor cell clusters in the peripheral blood of colorectal cancer patients. *Clin Cancer Res* 2001; **7**: 4080–4085.
- Aggarwal C, Meropol NJ, Punt CJ, Iannotti N, Saidman BH, Sabbath KD *et al*. Relationship among circulating tumor cells, CEA and overall survival in patients with metastatic colorectal cancer. *Ann Oncol* 2013; **24**: 420–428.
- Cohen SJ, Punt CJ, Iannotti N, Saidman BH, Sabbath KD, Gabrail NY *et al*. Relationship of circulating tumor cells to tumor response, progression-free survival, and overall survival in patients with metastatic colorectal cancer. *J Clin Oncol* 2008; **26**: 3213–3221.
- Cohen SJ, Punt CJ, Iannotti N, Saidman BH, Sabbath KD, Gabrail NY *et al*. Prognostic significance of circulating tumor cells in patients with metastatic colorectal cancer. *Ann Oncol* 2009; **20**: 1223–1229.
- Mani SA, Guo W, Liao MJ, Eaton EN, Ayyanan A, Zhou AY *et al*. The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell* 2008; **133**: 704–715.
- Tam WL, Weinberg RA. The epigenetics of epithelial-mesenchymal plasticity in cancer. *Nat Med* 2013; **19**: 1438–1449.
- Aktas B, Tewes M, Fehm T, Hauch S, Kimmig R, Kasimir-Bauer S. Stem cell and epithelial-mesenchymal transition markers are frequently overexpressed in circulating tumor cells of metastatic breast cancer patients. *Breast Cancer Res* 2009; **11**: R46.
- Gazzaniga P, Gradilone A, Petracca A, Nicolazzo C, Raimondi C, Iacovelli R *et al*. Molecular markers in circulating tumour cells from metastatic colorectal cancer patients. *J Cell Mol Med* 2010; **14**: 2073–2077.
- Yu M, Bardia A, Wittner BS, Stott SL, Smas ME, Ting DT *et al*. Circulating breast tumor cells exhibit dynamic changes in epithelial and mesenchymal composition. *Science* 2013; **339**: 580–584.
- Sastre J, Maestro ML, Puente J, Veganzones S, Alfonso R, Rafael S *et al*. Circulating tumor cells in colorectal cancer: correlation with clinical and pathological variables. *Ann Oncol* 2008; **19**: 935–938.

- 12 Tol J, Koopman M, Miller MC, Tibbe A, Cats A, Creemers GJ et al. Circulating tumour cells early predict progression-free and overall survival in advanced colorectal cancer patients treated with chemotherapy and targeted agents. *Ann Oncol* 2010; **21**: 1006–1012.
- 13 Steinert G, Scholch S, Niemiets T, Iwata N, Garcia SA, Behrens B et al. Immune escape and survival mechanisms in circulating tumor cells of colorectal cancer. *Cancer Res* 2014; **74**: 1694–1704.
- 14 Yokobori T, Iinuma H, Shimamura T, Imoto S, Sugimachi K, Ishii H et al. Plastin3 is a novel marker for circulating tumor cells undergoing the epithelial-mesenchymal transition and is associated with colorectal cancer prognosis. *Cancer Res* 2013; **73**: 2059–2069.
- 15 Wharton RQ, Jonas SK, Glover C, Khan ZA, Klokouzas A, Quinn H et al. Increased detection of circulating tumor cells in the blood of colorectal carcinoma patients using two reverse transcription-PCR assays and multiple blood samples. *Clin Cancer Res* 1999; **5**: 4158–4163.
- 16 Wong SC, Chan CM, Ma BB, Hui EP, Ng SS, Lai PB et al. Clinical significance of cytokeratin 20-positive circulating tumor cells detected by a refined immunomagnetic enrichment assay in colorectal cancer patients. *Clin Cancer Res* 2009; **15**: 1005–1012.
- 17 Iinuma H, Watanabe T, Mimori K, Adachi M, Hayashi N, Tamura J et al. Clinical significance of circulating tumor cells, including cancer stem-like cells, in peripheral blood for recurrence and prognosis in patients with Dukes' stage B and C colorectal cancer. *J Clin Oncol* 2011; **29**: 1547–1555.
- 18 Ning Y, Hanna DL, Zhang W, Mendez A, Yang D, El-Khoueiry R et al. Cytokeratin-20 and survivin expressing circulating tumor cells predict survival in metastatic colorectal cancer patients by a combined immunomagnetic qRT-PCR approach. *Mol Cancer Ther* 2015; **14**: 2401–2408.
- 19 Thiery JP, Sleeman JP. Complex networks orchestrate epithelial-mesenchymal transitions. *Nat Rev Mol Cell Biol* 2006; **7**: 131–142.
- 20 Rychahou PG, Kang J, Gulhati P, Doan HQ, Chen LA, Xiao SY et al. Akt2 over-expression plays a critical role in the establishment of colorectal cancer metastasis. *Proc Natl Acad Sci USA* 2008; **105**: 20315–20320.
- 21 Ginestier C, Hur MH, Charafe-Jauffret E, Monville F, Dutcher J, Brown M et al. ALDH1 is a marker of normal and malignant human mammary stem cells and a predictor of poor clinical outcome. *Cell Stem Cell* 2007; **1**: 555–567.
- 22 Shook D, Keller R. Mechanisms, mechanics and function of epithelial-mesenchymal transitions in early development. *Mech Dev* 2003; **120**: 1351–1383.
- 23 Barbazan J, Muinelo-Romay L, Vieito M, Candamio S, Diaz-Lopez A, Cano A et al. A multimarker panel for circulating tumor cells detection predicts patient outcome and therapy response in metastatic colorectal cancer. *Int J Cancer* 2014; **135**: 2633–2643.
- 24 Zeuner A, Todaro M, Stassi G, De Maria R. Colorectal cancer stem cells: from the crypt to the clinic. *Cell Stem Cell* 2014; **15**: 692–705.
- 25 Irie HY, Pearline RV, Grueneberg D, Hsia M, Ravichandran P, Kothari N et al. Distinct roles of Akt1 and Akt2 in regulating cell migration and epithelial-mesenchymal transition. *J Cell Biol* 2005; **171**: 1023–1034.
- 26 Kim YH, Kim G, Kwon CI, Kim JW, Park PW, Hahm KB. TWIST1 and SNAI1 as markers of poor prognosis in human colorectal cancer are associated with the expression of ALDH1 and TGF-beta1. *Oncol Rep* 2014; **31**: 1380–1388.
- 27 Deng Y, Zhou J, Fang L, Cai Y, Ke J, Xie X et al. ALDH1 is an independent prognostic factor for patients with stages II-III rectal cancer after receiving radiochemotherapy. *Br J Cancer* 2014; **110**: 430–434.
- 28 Sun YF, Xu Y, Yang XR, Guo W, Zhang X, Qiu SJ et al. Circulating stem cell-like epithelial cell adhesion molecule-positive tumor cells indicate poor prognosis of hepatocellular carcinoma after curative resection. *Hepatology* 2013; **57**: 1458–1468.
- 29 Kasimir-Bauer S, Hoffmann O, Wallwiener D, Kimmig R, Fehm T. Expression of stem cell and epithelial-mesenchymal transition markers in primary breast cancer patients with circulating tumor cells. *Breast Cancer Res* 2012; **14**: R15.
- 30 Mikolajczyk SD, Millar LS, Tsinberg P, Coutts SM, Zomorodi M, Pham T et al. Detection of EpCAM-negative and cytokeratin-negative circulating tumor cells in peripheral blood. *J Oncol* 2011; **2011**: 252361.
- 31 Grover PK, Cummins AG, Price TJ, Roberts-Thomson IC, Hardingham JE. Circulating tumour cells: the evolving concept and the inadequacy of their enrichment by EpCAM-based methodology for basic and clinical cancer research. *Ann Oncol* 2014; **25**: 1506–1516.