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ORIGINAL ARTICLE – CANCER RESEARCH

Relationship between post-surgery detection of methylated circulating tumor DNA with risk of residual disease and recurrence-free survival

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Abstract

Purpose Methylation in *IKZF1* and *BCAT1* are common events in colorectal cancer (CRC). They are often detected in blood as circulating tumor DNA (ctDNA) at diagnosis and disappear after surgery in most CRC patients. A prospective study was conducted to determine the relationship between detection of these markers following surgery and risk for residual disease and for recurrence.

Methods ctDNA status with methylated *BCAT1* and *IKZF1* was determined within 12 months of surgical resection of CRC, and was related to presence of or risk for residual disease (margins involved, metastases present or nature of node involvement), and to recurrence-free survival.

Results Blood was collected from 172 CRC patients after surgery and 28 (16%) were ctDNA positive. Recurrence was diagnosed in 23 of the 138 with clinical follow-up after surgery (median follow-up 23.3 months, IQR 14.3–29.5). Multivariate modeling indicated that features suggestive of residual disease were an independent predictor of post-surgery ctDNA status: cases with any of three features (close resection margins, apical node involved, or distant metastases) were 5.3 times (95% CI 1.5–18.4, $p=0.008$) more likely to be ctDNA positive. Multivariate analysis showed that post-surgery ctDNA positivity was independently associated with an increased risk of recurrence (HR 3.8, 1.5–9.5, $p=0.004$).

Conclusions CRC cases positive for methylated ctDNA after surgery are at increased risk of residual disease and subsequently recurrence. This could have implications for guiding recommendations for adjuvant therapy and surveillance strategies. Randomized studies are now indicated to determine if monitoring cases with these biomarkers leads to survival benefit.

Keywords Colorectal cancer (CRC) · Circulating tumor DNA (ctDNA) · Survival · Residual disease · Methylated DNA

Introduction

Worldwide, more than one million people are diagnosed with colorectal cancer (CRC) annually, with approximately 90% of deaths resulting from metastasis (Christofori 2006). Despite deeming patients free of disease at the conclusion of initial therapy, approximately 35% of patients have recurrence. As the majority of these are within 2 years, it suggests the presence of undetected residual disease or micrometastases (Sargent et al. 2009). Five year survival rates vary from 93% with stage I to 8% with stage IV. While the standard practice of staging with depth of tumor invasion, nodal involvement and metastasis (TNM stage) is highly prognostic for stages I and IV, it is less predictive for stages II and III, and consequently, after resection, patients are entered into a follow-up schedule to detect asymptomatic recurrence

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early enough to enable curative therapy. A number of clinicopathological factors are associated with poor prognosis such as tumor grade, lymphovascular invasion, invasion depth, and number of examined lymph nodes (Liu et al. 2016). This helps guide specialists with treatment and surveillance plans; however, recurrence still occurs in patients without these risk factors (Liu et al. 2016). There remains a need for prognostic markers to establish who would (and would not) benefit from therapy. Biomarkers could also be applied during surveillance to allow early detection of recurrence and subsequent curative treatment when lesions are considered treatable.

Carcinoembryonic antigen (CEA) is currently the only blood biomarker included in surveillance guidelines; however, it has an unacceptably low sensitivity for recurrent CRC (Rockall and McDonald 1999; Shinkins et al. 2017; Su et al. 2012; Young et al. 2016). This highlights the need for other blood markers to assess risk for recurrence. This might be achieved by assaying blood for circulating tumor DNA (ctDNA) carrying genetic or epigenetic alterations associated with sporadic CRC (Rasmussen et al. 2016) Personalized ctDNA monitoring tests, which target mutation-specific ctDNA biomarkers identified by genotyping the cancer itself have been reported to inform risk of residual disease and hence, identify patients at risk of recurrence (Tie et al. 2016; Wood et al. 2007). However, none of the common somatic mutations linked to CRC development occurs universally, and all appears with a low frequency (Diaz et al. 2012; Bettegowda et al. 2014).

In addition, this approach might be compromised if the mutation profile becomes more heterogeneous as the cancer evolves over time (Markowitz and Bertagnolli 2009; Vogelstein et al. 2013). This limitation could be overcome through analysing aberrant alterations of DNA methylation which are more common, not affected by tumor heterogeneity and clonal shifts, and frequently precede many mutational changes (Weisenberger 2014). Detection of circulating tumor-derived methylated DNA biomarkers might reflect the presence of residual disease. If this were to be linked to recurrence, then detection of these methylated markers might serve to improve identification of those who might benefit from adjuvant therapy.

We have previously reported methylated ctDNA biomarkers with good sensitivity (62.1–77.0%) and specificity (91.8–94.4%.) for CRC, namely, *BCAT1* (branched-chain amino acid transaminase 1) and *IKZF1* (IKAROS family zinc finger 1) (Pedersen et al. 2015a, b; Symonds et al. 2016). We have also found that 83.3% of patients positive for ctDNA methylated in *BCAT1* and *IKZF1* at CRC diagnosis become ctDNA negative following surgery (Pedersen et al. 2015b). Detection of ctDNA based on these biomarkers might, therefore, be a useful non-invasive test for assessing adequacy of initial therapy if persistence of these markers after surgery reflects residual disease and subsequent risk of recurrence. This would inform ongoing management and prognosis by predicting risk for residual disease, recurrence and survival.

Here, we describe a prospective study in cases undergoing surgical resection for primary CRC to assess the relationship between presence of methylated ctDNA after surgery and presence of and risk for residual disease as well as recurrence-free and disease-specific survival.

Methods

This was a prospective observational study of a single cohort of cases with invasive CRC. ctDNA status was determined within 12 months after surgical therapy for the primary cancer by assaying blood for methylated *BCAT1* and *IKZF1*. ctDNA positivity was then related to presence of features indicating residual disease, or risk of such, as well as subsequent recurrence-free and disease-specific survival during follow-up.

Presenting adults, diagnosed with invasive CRC, were approached for the study (2011–2017). They were subsequently excluded if they did not undergo surgery, if blood was not collected within 12 months of surgery, or if the primary cancer was not adequately staged (Fig. 1).

The study was approved by the Southern Adelaide Clinical Human Research Ethics Committee (ethics #134.045). Written informed consent was obtained from all participants prior to study enrolment. The study is registered at Australian and New Zealand Clinical Trials Registry (#12611000318987).

Clinical assessments

Apart from clinical observation, the only intervention was collection of venous blood [18 mL collected into K_3EDTA Vacuette tubes (Greiner Bio-One, Frickenhausen, Germany)] during scheduled attendance at clinic, within the 12 months following surgery of the primary tumor. If more than one sample was collected in the 12-month period, the sample closest to the surgery date was used in the analysis. Samples were collected within 2.3 (median, IQR 1.6–7.3) months post-surgery. Most blood samples were collected following full treatment, with a minimum of 10 days since ceasing chemotherapy (93% of cases had a minimum of 4 weeks between chemotherapy and blood collection).

Clinical features of relevance to presentation (obstruction or perforation), demographics, tumor pathology and staging were ascertained by the study nurses from patient interviews and medical records, and recorded independently of knowledge of test results. Recurrence was recorded as determined by the multidisciplinary tumor group responsible

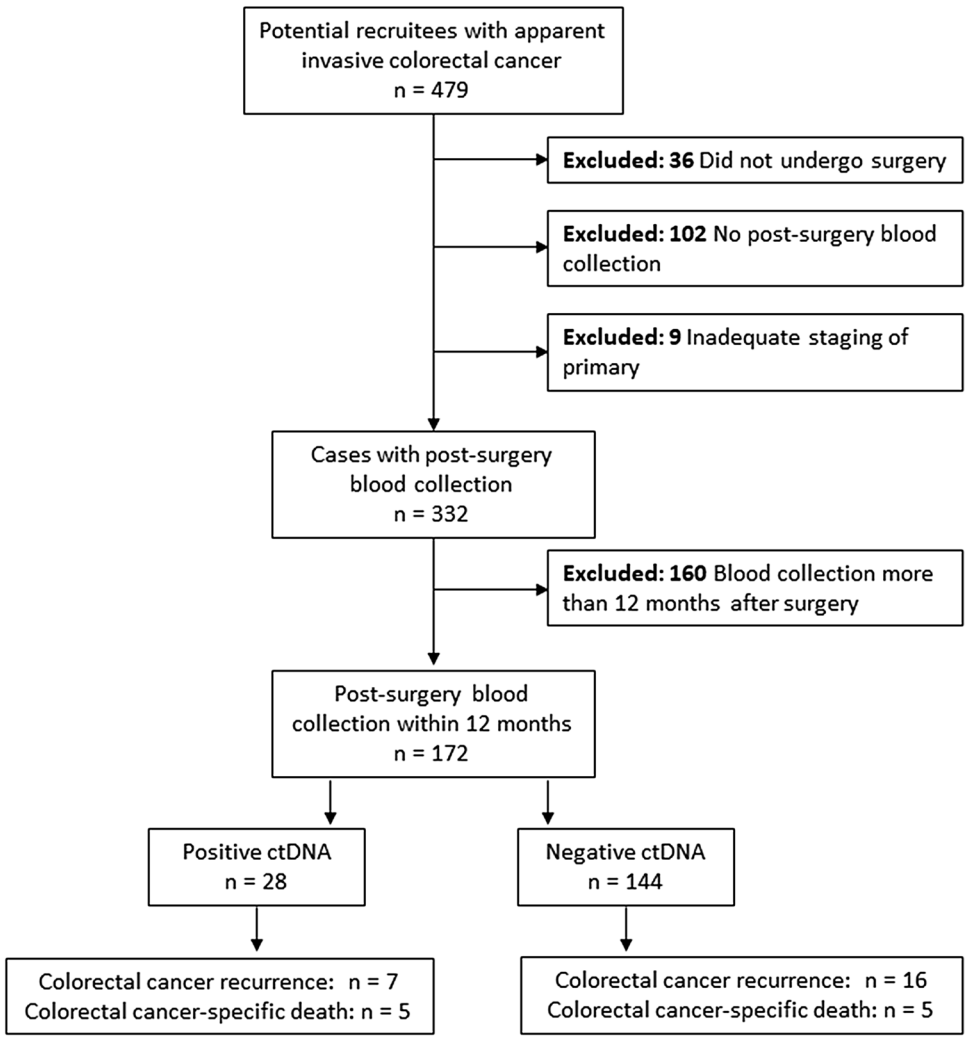

Fig. 1 Disposition of cases. Selection of 172 cases eligible for analysis from recruiting 479 patients with invasive colorectal cancer

for patient care based on radiological imaging including CT scan as previously described (Young et al. 2016). Extent of non-surgical therapy and whether or not this was completed as initially planned or suggested by the treating doctors was also recorded by the study nurses, without knowledge of the ctDNA result. Date and cause of death was ascertained from the South Australian Death Registry. Diagnosis and extent of disease [stage with AJCC and TNM (Edge et al. 2010)] were determined from colonoscopy, biopsy and imaging as well as clinicopathological findings at surgery. Distal tumors were those distal to the transverse colon, and if synchronous tumors were present, then stage and location was specified for the most advanced lesion. Histopathological features, as listed in Table 1, were determined by the responsible pathologist according to standard practice within the institution.

If ctDNA is detected in plasma after surgery then this may indicate minimal residual disease not suspected at surgery; consequently, assessment of cases for the likelihood of residual tumor took into account not only histopathological findings at the surgical margins (R1 or R2) (Wittekind et al. 2002) and distant metastases not removed at surgery (M1), but also involvement of the most proximal (apical) lymph node (reports with no mention of the apical lymph node were assumed to have no involvement), seven or more nodes involved (N2b), and peritoneal involvement (T4 extension) (Niedzwiecki et al. 2011; Quentmeier et al. 1990; Quah et al. 2008).

Methylation analysis

At least 3.9 mL plasma was assayed for methylated *BCAT1* and *IKZF1* DNA as previously described (Pedersen et al. 2015b). Briefly, cell-free DNA was extracted using a QIASymphony Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany) and bisulphite converted using the Epi-Tect Fast Bisulphite Conversion Kit (Qiagen). The resulting bisulphite-converted DNA was analysed as three replicates in a triplex real-time qPCR assay (*ACTB* control, methylated *BCAT1* and *IKZF1*). A sample was deemed ctDNA positive

Table 1 Characteristics of cases at diagnosis, categorized according to post-surgery ctDNA status

Characteristics	Number (%) $N=172$	Post-surgery ctDNA status		OR (95% CI)[1]	p[2]
		Negative ($n=144$, 84%)	Positive[3] ($n=28$, 16%)		
Age at diagnosis (years), mean (95% CI)[3]	–	66 (65–69)	63 (58–68)	1.0 (0.9–1)	0.101
Gender, male, no. (%)	105 (61)	88 (61)	17 (61)	1.0 (0.4–2.3)	0.969
Staging, no. (%)					
AJCC Stage III or IV	79 (46)	58 (40)	21 (75)	4.5 (1.8–11.1)	0.001
T stage, T3 or T4 (1 unstaged)	124 (73)	100/143 (70)	24 (86)	2.6 (0.8–7.9)	0.096
M stage, M1 (2 unstaged)	9 (5)	5/142 (4)	4 (86)	4.6 (1.1–18.2)	0.032
N stage, N1 or N2	72 (42)	54 (38)	18 (64)	3.0 (1.3–7)	0.011
Tumor deposits found (stage N1c)	11 (6)	4 (3)	7 (25)	11.7 (3.1–43.3)	<0.001
Location, distal[4], no. (%) (3 unknown)	95 (56)	76/142 (54)	19/27 (70)	2.1 (0.8–5.0)	0.111
Size, >40 mm, no. (%)	85 (49)	73 (51)	12 (43)	0.7 (0.3–1.7)	0.449
Histopathology, no. (%)					
Mucinous	22 (13)	18 (13)	4 (14)	1.2 (0.4–3.8)	0.796
Lymphatic invasion	41 (24)	26 (18)	15 (54)	5.2 (2.2–12.3)	<0.001
Perineural invasion	25 (15)	16 (11)	9 (32)	3.8 (1.5–9.8)	0.006
Venous invasion—extramural	15 (9)	9 (6)	6 (21)	4.1 (1.3–12.6)	0.014
Venous invasion—intramural	6 (3)	5 (3)	1 (4)	1.0 (0.1–9.2)	0.979
Poorly differentiated	33 (19)	26 (18)	7 (25)	1.5 (0.6–3.9)	0.396
Synchronous CRC, no. (%)	7 (4)	5 (3)	2 (7)	2.1 (0.4–11.6)	0.379
Perforation at presentation, no. (%)	5 (3)	2 (1)	3 (11)	8.5 (1.4–53.6)	0.022
Obstruction at presentation, no. (%)	14 (8)	11 (8)	3 (11)	1.5 (0.4–5.6)	0.588

[1]OR odds ratio determined by univariate logistic regression analysis; CI confidence interval

[2]p value, Wald test

[3]Positive for methylated *BCAT1* and/or *IKZF1*

[4]Tumors located at or beyond the splenic flexure were categorized as distal

if at least one PCR replicate was positive for either methylated *BCAT1* or *IKZF1*.

Statistical analyses

Logistic regression was used to assess the association between residual disease and post-surgery ctDNA. Multivariate analysis was performed with post-surgery ctDNA result as the outcome variable. A backward selection was performed considering the following characteristics: number of lymph nodes involved; T4 stage/peritoneal extension; less than 12 lymph nodes sampled; incomplete non-surgical treatment at time of venesection and adequacy of surgical resection (clear margins, absence of distant metastases or apical node involvement).

A Cox proportional hazards (PH) model was used for the association with CRC recurrence. Time to recurrence was measured from date of surgery to first positive radiological evidence of recurrence and was censored at last radiological follow-up. Time to CRC-related death was measured from date of surgery to time of death and was censored at last known follow-up. Multivariate analysis with CRC recurrence as the outcome event was performed with the following covariates: post-surgery ctDNA result; number of lymph nodes involved; T4 stage/peritoneal involvement; less than 12 lymph nodes sampled; incomplete non-surgical treatment at time of venesection and adequacy of surgical resection (clear margins, absence of distant metastases or apical node involvement).

A z test with a binomial distribution was used to estimate the power of the logistic regression analysis using an alpha of 5%, our observed ctDNA test positivity of 16%, a proportion comparison of 0.04–0.25 and an R-squared value of 0.1294 (based on the correlation between the three-feature composite for residual disease, namely, involved surgical margin or apical lymph node, or remaining metastasis, and incomplete non-surgical treatment at time of venesection). Based on these values, power was determined to be 83.2%. A Cox PH power analysis was performed using $n=138$, HR 3.81, standard deviation = 0.466 and a probability of recurrence = 0.17, estimating a power of 85.6%.

For both models an alpha level of 0.05 was used to determine statistical significance for covariates included in the final models and for assessing any potential issues with the Cox PH assumption in the survival analysis. All analyses were completed using Stata version 13.1.

Results

Study population

Figure 1 shows the disposition of 479 patients approached. Of 332 who provided a post-surgery blood sample, 172 (51.8%) did so within 12 months of surgery. Their clinical characteristics at diagnosis and nature of presentation are summarized in Table 1. The population studied included 61% males, and 56% of the tumors were located in the distal colon.

Post-surgery ctDNA status

Sixteen percent of patients ($n = 28$) returned a positive ctDNA result in the first sample collected within 12 months of surgery. Table 1 compares the clinical characteristics at diagnosis according to the post-surgery ctDNA result. A positive result was significantly more likely for later stage (AJCC or TNM), perforation at presentation, presence of lymphatic, perineural or extramural venous invasion, or for stage N1c (tumor deposits in adjacent mesentery).

Association between completeness of resection and ctDNA result

A comparison of surgical pathological features related to completeness of resection or risk of residual disease is shown according to post-surgery ctDNA status in Table 2. Specific features significantly associated with a positive result by univariate analysis were: increasing number of involved lymph nodes (OR 8.3 for seven or more), involved apical node (OR 15.4), remaining distant metastases (OR 17.2) or involved peritoneum (OR 3.8). We also compared different combinations of pathological features to post-surgery ctDNA status: The highest risk of a positive ctDNA test (OR 7.7) was observed if any of the following features of residual disease or risk for such were present, namely, involved margin or apical lymph node, or remaining metastasis. Significantly increased risk was also observed for obvious residual disease (involved margin or remaining metastasis) although numbers were small, 4/144 vs 4/28 ($p = 0.017$).

Multivariate modeling by logistic regression showed that a three-feature composite for residual disease or risk for such (namely, involved surgical margin or apical lymph node, or remaining metastasis) and incomplete non-surgical treatment at time of venesection were independent predictors of a positive blood result (Table 3). Cases with at least one of the three-feature composites present were 5.3 times (95% CI 1.5–18.4, $p = 0.008$) more likely to be ctDNA positive after surgery. Blood samples taken prior to completion of treatment (surgical resection of metastases and chemotherapy) were also more likely to return a positive ctDNA status [OR 3.4 (1.4–8.1), $p = 0.007$] compared to cases where the initial treatment had been completed.

Post-surgery ctDNA status and survival analysis

All cases were assessed for CRC-related mortality in November 2017. 10 died from CRC during this follow-up period. The number of CRC-specific deaths, while low in

Table 2 Characteristics of cases at surgery categorized according to post-surgery ctDNA status

Characteristics	Number (%) $N = 172$	Post-surgical ctDNA status		OR (95% CI)[1]	p[2]
		Negative ($n = 144$)	Positive ($n = 28$)		
A. Resection margins involved, no. (%)	4 (2)	3 (2)	1 (4)	1.7 (0.2–17.4)	0.637
B. Number of nodes involved by tumor, no. (%)					
0	112 (65)	100 (69)	12 (43)	Reference	0.004
1–3	41 (24)	34 (24)	7 (25)	1.7 (0.6–4.7)	
4–6	11 (6)	6 (4)	5 (18)	6.9 (1.8–26.2)	
7 or more	8 (5)	4 (3)	4 (14)	8.3 (1.8–37.7)	
C. Apical node involved, no. (%)	7 (4)	2 (1)	5 (18)	15.4 (2.8–84.3)	0.002
D. Distant metastasis remaining after surgery, no. (%)	4 (2)	1 (1)	3 (10)	17.2 (1.7-171.6)	0.016
E. T4 Stage/peritoneal involvement, no. (%)	32 (19)	21 (15)	11 (39)	3.8 (1.6–9.2)	0.003
Less than 12 nodes sampled, no. (%)	32 (19)	26 (18)	6 (21)	1.2 (0.5–3.4)	0.675
Incomplete non-surgical treatment at time of venesection	56 (33)	39 (27)	17 (61)	4.2 (1.8–9.7)	0.001
Any of A or D above, no. (%)	8 (5)	4 (3)	4 (14)	5.8 (1.4–24.9)	0.017
Any of A, C or D above, no. (%)	13 (8)	6 (4)	7 (25)	7.7 (2.3–25.0)	0.001
Any of A, C, D or E above, no. (%)	36 (21)	23 (16)	13 (46)	4.6 (1.9–10.8)	0.001

[1]OR odds ratio determined by univariate logistic regression analysis; CI confidence interval

[2]Wald test, p value

Table 3 Multivariate analysis of association between post-surgery ctDNA status and surgical findings

Factors	Odds ratio (95% CI)	*p*
Involved surgical margin or apical lymph node, or remaining metastasis		0.008
Nil present	Reference	
At least one present	5.3 (1.5–18.4)	
Incomplete non-surgical treatment at time of venesection		0.007
No	Reference	
Yes	3.4 (1.4–8.1)	

CI confidence interval

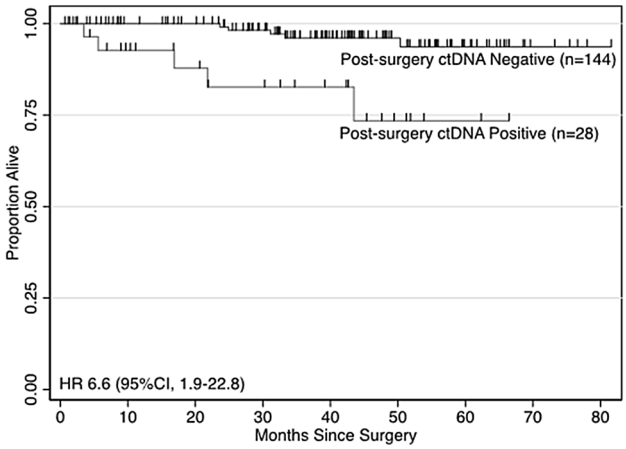

Fig. 2 CRC-specific survival. Kaplan–Meier estimates for time to disease-specific mortality among 172 patients with invasive CRC, by post-surgery *BCAT1*/*IKZF1* ctDNA status. The number of CRC-specific deaths was significantly greater following a positive ctDNA result within 12 months of surgical resection

rate, was significantly different between those with a positive ctDNA result after surgery compared to those with a negative result (18 vs 3%, respectively, $p = 0.011$). Survival curves for CRC-specific death are shown in Fig. 2. Multivariate analysis could not be performed using death as the outcome due to the limited number of events.

Cases considered to warrant surveillance with radiographic examination for recurrence ($n = 138$) were followed for a median 22.9 months (IQR 12.0–33.6). There was no difference in follow-up time between those with different ctDNA results [negative ctDNA 22.6 months (IQR 11.8–34.4); positive ctDNA 23.3 months (IQR 14.3–29.5), $p = 0.930$]. Recurrence was diagnosed in 23 (Table 4). It should be noted that only 61% of cases returning a positive ctDNA completed their initial course of treatment (surgery plus chemo and/or radiotherapy as indicated) compared to 87% of negatives (Table 4). This was due to either patients declining ongoing therapy, or due to comorbidities or complications precluding a full course of treatment.

Recurrence rates were not significantly different by a Fisher's exact test ($p = 0.066$); however, when follow-up time was considered (Fig. 3) it can be seen that there was a difference between post-surgical ctDNA results with rates of recurrence. The Cox Proportional Hazards multivariate model indicated that the ctDNA status determined within 12 months was the only significant predictor of recurrence when modeled with the features detailed in Table 2. Cases with a positive ctDNA post-surgery result had an increased

Table 4 Predictors of recurrence in 138 cases with radiological follow-up

Variable	No. of patients	No. recurrence	Disease-free survival ($n = 138$)		
			HR	95% CI	*p* value
Positive ctDNA (vs. negative ctDNA)	23	7	3.81	1.5–9.5	0.004
Number of lymph nodes involved					
0	90	9	Reference	–	0.019
1–3	31	7	2.46	0.9–6.6	
4–6	9	3	4.66	1.3–17.3	
7 or more	8	4	5.01	1.5–16.5	
T4 stage/peritoneal involvement	31	6	1.61	0.6–4.1	0.325
Less than 12 lymph nodes sampled	27	3	0.77	0.2–2.6	0.679
Incomplete non-surgical treatment at time of venesection	42	9	2.46	1.1–5.7	0.036
Any of A, C or D from Table 2	13	4	2.28	0.8–6.7	0.138

CI confidence interval

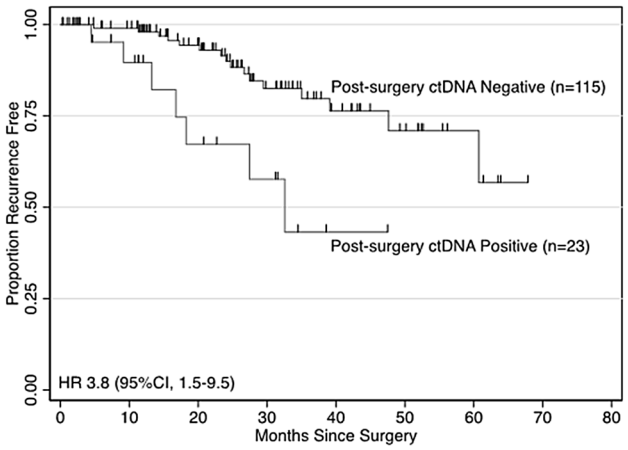

Fig. 3 Recurrence-free survival. Kaplan–Meier estimates for time to recurrence among 138 patients with invasive CRC, by post-surgery *BCAT1*/*IKZF1* ctDNA status. The number of recurrences was significantly greater following a positive ctDNA result within 12 months of surgical resection

risk of recurrence (HR 3.8, 95% CI 1.5–9.5, $p = 0.004$) at any time compared to subjects with a negative result. The Kaplan–Meier survival plot of the recurrence incidence is provided in Fig. 3.

Discussion

Using methylated *BCAT1* and *IKZF1* as two epigenetic markers of ctDNA, this prospective study identified a relationship between a positive post-surgery ctDNA results in patients operated on for primary CRC and the presence of and risk for residual disease, as well as survival. The demonstration of a relationship to recurrence-free survival is important as this points to the likelihood that a positive post-surgery ctDNA result in this context indicates residual disease and/or prognosis independent of already established parameters. This in turn might warrant a change in, or addition of, adjuvant therapy.

There is considerable interest in understanding the association between detection of ctDNA biomarkers and detection of minimal residual disease and disease recurrence, as well as predicting survival (Thierry et al. 2014; Tie et al. 2016). Patients are known to have significantly better progression-free and overall survival rates after successful surgical clearance (Quentmeier et al. 1990). Features known to inform completeness of surgical resection and risk of residual disease include known unresected metastases and involvement of the surgical margin as captured in the R sub-classification of the TNM system (Edge et al. 2010). In addition, residual disease and subsequent recurrence is more likely where there is apical node involvement, more than six lymph nodes involved, or peritoneal involvement (T4 extension) (Niedzwiecki et al. 2011; Wittekind et al. 2002; Quah et al. 2008).

We found in univariate analysis that a positive ctDNA status post-surgery was more likely if any of three markers of residual disease or risk of residual disease was present (OR 7.7); while the increasing number of involved lymph nodes (OR 8.3) and involved peritoneum (OR 3.8) was also associated with a positive result. Multivariate modeling determined the three-feature composite for residual disease (surgical margins or apical node involved, distant metastases) to be an independent predictor of post-surgery ctDNA status. Cases positive by ctDNA were 5.3 times more likely to either have or be at risk of residual disease. Currently available non-ctDNA risk predictors for residual disease are generally associated with a two–three-fold increase in risk (Niedzwiecki et al. 2011; Quah et al. 2008).

The current clinical approach to defining risk of recurrence for patients is based primarily on stage with refinements based on recognition of certain other clinicopathological features and genotype, but these only modestly indicate recurrence risk (Niedzwiecki et al. 2011; Quah et al. 2008). In this study, ctDNA status was the only significant predictor of recurrence when modeled with other post-surgery variables as patients with a positive result had a significantly increased risk of recurrence (HR 3.81) regardless of when status was ascertained in the 12 months after surgery. Only one other study has shown an independent association of methylated ctDNA with risk of recurrence, which was found with methylated *SST* in stage I–III preoperative blood (HR 2.60 95% CI 1.37–4.94) (Liu et al. 2016). In the multivariate analysis, the study mainly assessed diagnostic predictors of recurrence (e.g., stage), and not surgical considerations (e.g., involvement of the apical lymph node) as done in the current study. They also found that methylated *SEPT9*, commercially applied for CRC screening, was not significantly associated with recurrence (HR 0.77, 95% CI 0.42–1.71). Recommendations for surveillance of CRC cases after initial treatment vary around the world, but all advise more intensive surveillance for those showing features associated with a higher risk for recurrence. Our observations suggest that blood testing for methylated *BCAT1* and *IKZF1* ctDNA should be added to the list of features associated with a higher risk.

This observational study has some limitations as it was conducted in a usual-care moderate-sized clinical service where follow-up protocols were subject to variance according to practitioner protocols and risk for recurrence. Future highly structured prospective trials with defined blood sampling intervals are required to clarify the best time to determine the ctDNA status in terms of predicting (as opposed to diagnosing), especially relative to other treatments such as chemotherapy. Results from a recent study using mutation biomarkers of ctDNA demonstrated the difference in

timing of blood collection (Tie et al. 2016). In this study, when blood was collected upon diagnosis of recurrence, the personalized ctDNA mutation markers used were 85% sensitive for recurrence, but when plasma was collected in the immediate postoperative period, of those subsequently experiencing recurrence, a positive blood sample was seen in only 48% (Tie et al. 2016). However, this might simply be a result of differences in tumor burden with the lowest burden being in the period immediately following surgery. In the current study, the variable time to ctDNA testing after surgery was due to the goal of minimizing any spikes in ctDNA levels resulting from an effect of chemotherapy on the tumor (Diehl et al. 2008; Ng et al. 2017; Tie et al. 2015). ctDNA testing was typically performed once the initial treatment was finished, regardless of whether it was a complete course or not. Assessment of the methylated *BCAT1* and *IKZF1* ctDNA for longitudinal monitoring post-resection is underway.

Despite the relatively large number of patients in the study, there were a modest number of patients who were ctDNA positive after surgery and who later developed recurrence. This limited the subgroup analyses that could be done, and also makes the study susceptible to inherent biases, which could limit the extension of study findings to the population at large.

Epigenetic markers of ctDNA have received little attention, despite the fact that the crucial role of epigenetic alterations in the acquisition of malignant phenotype is well understood (Baylin and Jones 2011). Aberrant IKZF1 and BCAT1 expression has been shown to correlate with more aggressive cancer growth and progression, although their respective roles vary between cancers.

The DNA binding protein, IKZF1, regulates a small set of cell cycle-regulator genes, which restrict the G1-S transitioning of the cell cycle by recruiting the histone deacetylase complex and leads to chromatin changes in the promoter regions of these genes (Javierre et al. 2011; Malinge et al. 2013; Song et al. 2016). Aberrant methylation of the IKZF1 promoter has been reported for other types of cancers (Zhu et al. 2007), and in CRC this has been linked to loss of function as part of long-range epigenetic silencing region events (Javierre et al. 2011). This leads to deregulation of several of its targets, including notch, which plays a crucial role in the self-renewing process of colon crypt stem cells (Noah and Shroyer 2013; Riccio et al. 2008).

BCAT1 catalyzes the first step in the breakdown of branched-chain amino acids (BCAA) which are essential nutrients for growth and it has been demonstrated that when BCAT1 expression is blocked, the lifespan increases nearly 25% in nematodes (Mansfeld et al. 2015). Aberrant BCAT1 expression has been shown to sustain cancer proliferation in addition to migration and invasion, although the role is poorly understood as the function of BCAT1 appears to vary between cancer types (Ananieva and Wilkinson 2018). In CRC, dysfunctional BCAT1 expression has been associated with distant metastasis and a predictor of short disease-free survival (Yoshikawa et al. 2006). In glioblastomas, BCAT1 expression is linked to mutations in IDH1 or IDH2, which contribute to epigenetic silencing of BCAT1 via promoter methylation (Carrillo et al. 2012; Chaumeil et al. 2014). This leads to genome-wide alterations in histone and DNA methylation and likely mediates tumor development (Ward et al. 2010; Xu et al. 2011). The possibility of a BCAT1 and IDH1/IDH2 link in CRC has not been studied (Vogelstein et al. 2013).

There is also growing evidence of a link between aberrant BCAT1 expression and drug resistance. Elevated BCAT1 expression has been linked to relapsed tamoxifen-resistant breast cancer (Thewes et al. 2017), bevacizumab resistance in glioblastoma (Cho et al. 2016), and cisplatin resistance in prostate cancer and hepatocellular carcinoma, by enabling cancer cells to transition through the S-G2/M phase (Zheng et al. 2016; Zhu et al. 2017). Taken together, these studies suggest BCAT1 might serve as a prognostic marker and inform therapeutic strategy in the treatment of cancer.

Unlike mutations, epigenetic modifications linked to tumorigenesis are potentially reversible, and hence, provide an avenue for drug intervention. There are, however, very few studies investigating the therapeutic effect of epigenetic reversion. Gabapentin, a leucine analogue sold under the brand name Neurontin, has been shown to reduce the proliferation rate with almost 60% in glioma, by inhibiting BCAT1 (Tonjes et al. 2013). Hypermethylated FBP2 promoter in gastric cancer is associated with poor clinical outcome, and the hypermethylation in this gene has been shown to be reversible with the demethylating agent decitabine (5-aza-2′-deoxycytidine)(Choi et al. 2017).

An obvious question arising from this study is when might ctDNA biomarkers be informative for therapy. One might argue that testing after initial surgery is, in any case, the most logical if it is a sensitive marker for residual disease (Diehl et al. 2008; Ng et al. 2017; Tie et al. 2015, 2016), as this is more likely to influence decisions about treatment and surveillance protocols, than a result prior to surgery. It has already been shown that postoperative CEA is more informative than preoperative CEA (Konishi et al. 2018), although CEA testing is not ideal for detecting metastatic disease. Certainly, our previous studies show that testing for methylated *BCAT1*/*IKZF1* ctDNA is much more sensitive than CEA for recurrence, with a sensitivity of 68% compared to 32%, respectively (Young et al. 2016).

The decision to treat or not to treat a stage II colorectal cancer patient with adjuvant chemotherapy remains a debated issue with a clear need to identify better predictors of benefit from such treatment (Wirtzfeld et al. 2009). Similar to the promise shown with genetic mutation ctDNA

markers (so-called "liquid-biopsy"), and based on emerging evidence that epigenetic alterations appear to be more frequent and homogeneous than the somatic mutation frequency reported for specific mutations in known hot-spot genes (Markowitz and Bertagnolli 2009; Vogelstein et al. 2013), a further obvious question is whether epigenetic ctDNA markers might inform neo-adjuvant therapy at diagnosis (Tie et al. 2015). We are currently undertaking a long-term study to test methylated *BCAT1*/*IKZF1* ctDNA as a prognostic indicator when assayed at diagnosis, and their relationship to other prognostic indicators.

A recent review by the American Society of Clinical Oncology and College of American Pathologists highlighted the lack of evidence of clinical validity of ctDNA assays in detection of residual disease (Merker et al. 2018). We have shown with the current study that detection of ctDNA by assaying for methylated *BCAT1*/*IKZF1* in blood is informative with respect to completeness of surgical resection, risk of residual disease and recurrence-free survival. This has implications for decisions regarding adjuvant therapies in cases where these ctDNA markers are detected after initial therapy. Our findings now warrant validation in larger studies to define the degree of risk with greater confidence and to determine if monitoring epigenetic ctDNA status, together with adjustment to adjuvant therapy and surveillance strategies, leads to survival benefit.

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Compliance with ethical standards

Conflict of interest GPY is a paid consultant of Clinical Genomics. SKP and DHM are paid employees of Clinical Genomics. All other authors have no conflicts of interest to declare.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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