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Annals of Oncology 30: 815–822, 2019
doi:10.1093/annonc/mdz075
Published online 12 March 2019

ORIGINAL ARTICLE

Pre-operative ctDNA predicts survival in high-risk stage III cutaneous melanoma patients

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Note: This study was previously presented, in part, at the Medical Oncology Group Australia Annual Scientific Meeting 2018.

Background: The outcomes of patients with stage III cutaneous melanoma who undergo complete surgical resection can be highly variable, and estimation of individual risk of disease recurrence and mortality remains imprecise. With recent demonstrations of effective adjuvant targeted and immune checkpoint inhibitor therapy, more precise stratification of patients for costly and potentially toxic adjuvant therapy is needed. We report the utility of pre-operative circulating tumour DNA (ctDNA) in patients with high-risk stage III melanoma.

Patients and methods: ctDNA was analysed in blood specimens that were collected pre-operatively from 174 patients with stage III melanoma undergoing complete lymph node (LN) dissection. Cox regression analyses were used to evaluate the prognostic significance of ctDNA for distant metastasis recurrence-free survival and melanoma-specific survival (MSS).

Results: The detection of ctDNA in the discovery and validation cohort was 34% and 33%, respectively, and was associated with larger nodal melanoma deposit, higher number of melanoma involved LNs, more advanced stage and high lactate dehydrogenase (LDH) levels. Detectable ctDNA was significantly associated with worse MSS in the discovery [hazard ratio (HR) 2.11 $P < 0.01$] and validation cohort (HR 2.29, $P = 0.04$) and remained significant in a multivariable analysis (HR 1.85, $P = 0.04$). ctDNA further sub-stratified patients with AJCC stage III substage, with increasing significance observed in more advanced stage melanoma.

Conclusion: Pre-operative ctDNA predicts MSS in high-risk stage III melanoma patients undergoing complete LN dissection, independent of stage III substage. This biomarker may have an important role in determining prognosis and stratifying patients for adjuvant treatment.

Key words: circulating tumour DNA, stage III, melanoma, survival, adjuvant therapy

Introduction

The presence of lymph node (LN) metastases in cutaneous melanoma patients is associated with increased risk of recurrence with 5-year survival rates ranging from 93% in surgically resected American Joint Committee on Cancer (AJCC 8th edition) stage IIIA disease to 32% for patients with resected stage IIID melanoma [1]. Despite these prognostic estimates, the calculation of individual risk of disease recurrence and mortality remains imprecise [2]. The need for accurate prognostic biomarkers is particularly relevant and timely with the recent demonstration that adjuvant mitogen-activated protein kinase (MAPK) and immune checkpoint inhibitors improve recurrence free survival (RFS) in resected stage III melanoma patients [3–5]. Indeed, a more precise stratification of AJCC

stage III patients for costly and potentially toxic adjuvant therapy will provide significant benefits.

There has been extensive research attempting to identify biomarkers that more accurately sub-stratify risk in stage III melanoma patients. Clinicopathological factors such as Breslow thickness and ulceration of the primary melanoma, older age, increased number of tumour-positive LNs, higher LN ratio (proportion of LN containing metastases in radical LN dissection specimens) and the presence of extranodal spread have been shown to be independent predictors of poorer survival in stage III patients [6–8]. The presence of *BRAF* and *NRAS* mutations and the absence of an immune-related transcriptome signature in melanoma tissue was also found to be associated with poor survival in this group [9]. Similarly, serum markers such as S100α and lactate dehydrogenase (LDH) were associated with poor prognosis, but S100α was an unreliable prognostic marker and LDH produced an unacceptably high false positive rate and has yet to be validated [10, 11].

The prognostic utility of circulating tumour DNA (ctDNA) has been demonstrated in stage IV melanoma patients, with undetectable or low ctDNA associated with longer progression-free survival in patients treated with MAPK inhibitors or immunotherapy [12, 13]. The mechanistic basis for the predictive and prognostic utility of ctDNA is not well understood, but presumably reflects its positive association with tumour burden and rate of tumour growth [14]. In this study, we sought to determine whether ctDNA measured within 1 month before surgery can further stratify patients with high-risk stage III melanoma (AJCC stage IIIB/C/D).

Methods and materials

Patients and treatment

Patients with confirmed LN metastasis amenable to curative LN dissection and managed at hospitals affiliated with Melanoma Institute Australia; Royal Prince Alfred Hospital, Mater hospital and Westmead Hospital; between October 2010 and July 2017 were included in the study. Written consent was obtained from all patients under approved Human Research Ethics Committee protocols from Royal Prince Alfred Hospital (Protocol X15-0454 and HREC/11/RPAH/444).

Patients with clinically occult (i.e. detected by sentinel LN biopsy) or clinically and/or radiologically detected, pathologically confirmed stage IIIB/C/D melanoma according to the AJCC staging system 8th edition were eligible for this study [1]. To ensure melanoma ctDNA was being measured, an established gene mutation in *BRAF*, *NRAS* or *KIT* detected in tumour tissue using commercially available methods was required for inclusion in the study. ctDNA analysis was carried out on blood samples drawn within 1 month before surgery. Patients with in-transit metastases only were excluded from the analysis, but the presence of in-transit metastases concurrently with LN metastases was permitted. All patients were staged to exclude distant metastases with computerised tomography (CT) and/or fluorodeoxyglucose-positron emission tomography and magnetic resonance imaging or CT imaging of the brain.

In total 174 patients were eligible for the study; 119 patients recruited from multiple centres were in the discovery cohort (referral source B-L) and 55 patients recruited from a single and separate referral source (referral source A) were in the validation cohort (Figure 1).

Disease characteristics and response assessment

Patient demographics and clinicopathological features including AJCC stage, extranodal extension, number of tumour-involved LNs, serum LDH, clinical presentation (clinically/radiologically detected nodal metastasis or detected by sentinel LN biopsy) and maximum dimension of the largest melanoma deposit were included in the analysis. Following surgery, patients were reviewed routinely with a combination of clinical assessment and imaging. Recurrence was defined as distant metastatic disease and the date when new metastatic disease was identified was recorded. Follow-up duration was calculated from the date of lymph node dissection to the following three dates: date of death from melanoma, loss to follow-up or 31 March 2018. Primary outcome measure is melanoma-specific survival (MSS) with secondary outcome of distant metastasis recurrence-free survival (DM-RFS). Patients who died from non-melanoma causes were censored at their date of death, and only MSS is reported in this study.

Plasma collection, ctDNA extraction and quantification

Patient peripheral blood samples were collected prospectively at baseline using 10 ml EDTA vacutainer tubes and processed within 4 h of collection as previously described [13]. Droplet digital PCR was used to quantify DNA (supplementary Methods, available at *Annals of Oncology* online).

Statistical analysis

Patient and disease characteristics were summarised according to ctDNA detectability before surgery. Frequencies and percentages according to ctDNA detectability with their corresponding *P*-values were calculated using Fisher's exact test. DM-RFS and MSS were calculated using the Kaplan–Meier method, and the log-rank test was used for comparison of survival.

Multivariable Cox proportional hazard regression analyses for DM-RFS and MSS were carried out using the Wald test to assess the predictive ability of ctDNA detection categorised in two groups; detectable ($\geq$1 positive droplets) versus undetectable (no positive droplets). Other factors found to have a predetermined level of association with the clinical outcomes (*P*-value $\leq$0.15 from a univariable analysis) were included in a multivariable model. Model discrimination was assessed using the C-index, and calibration was assessed using calibration plots [15]. All analyses were carried out using statistical software Graphpad Prism (version 7.02) and R script 3.3.1. The reported *P*-values were considered significant if $<$0.05.

Results

Patient characteristics

One hundred and nineteen stage IIIB/C/D melanoma patients with somatic tumour-associated mutations and pre-operative plasma samples from multiple hospitals affiliated with Melanoma Institute Australia were identified. Patients from a single, independent referral source were further recruited as the validation cohort ($n = 55$) (Figure 1). ctDNA was detectable in 40/119 (34%) patients in the discovery cohort (median 38 copies/ml plasma, range 4–2275) and 18/55 (33%) patients in the validation cohort (median 17 copies/ml plasma, range 4–510). The cohorts were similar across all clinical and pathological characteristics (supplementary Table S1, available at *Annals of Oncology* online).

ctDNA biomarker status

Pre-operative blood samples were analysed in 119 patients in the discovery cohort to determine whether ctDNA detectability could be used as a prognostic biomarker in patients undergoing

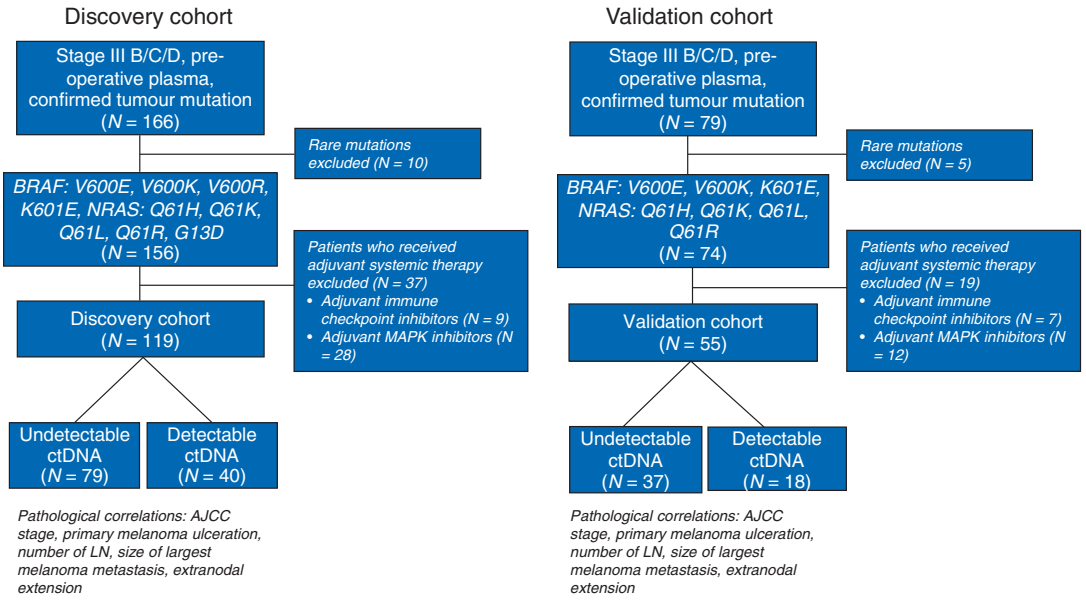

Figure 1. Flow diagram of patients included in the study. Patients referred from multiple centres affiliated with Melanoma Institute Australia were the discovery cohort and patients from a single and separate referral source (referral source A) were the validation cohort. Rare mutations; *BRAF* (*V600G, G469A, G469*, D594G, L597S*), *NRAS* (*G12D/E, G12R/P, G13C, G13S/N, G13S*), *KIT* (*L576P*), *KRAS* (*G12S, G12S/M, Q61R*), *MET* (*T992I*). MAPK, mitogen-activated protein kinase; ctDNA, circulating tumour DNA; ddPCR, droplet digital polymerase chain reaction.

complete surgical resection with AJCC stage III melanoma. ctDNA detectability was strongly associated with disease burden including size of largest melanoma deposit ($P < 0.01$), number of tumour-involved LNs ($P < 0.01$), method of detection ($P = 0.01$) and elevated LDH ($P < 0.01$). There was no association between ctDNA detection and AJCC stage or presence of extranodal extension (Table 1). These findings were consistent in the validation cohort except that ctDNA detectability was not associated with elevated LDH in the validation group ($P = 1.0$; supplementary Table S2, available at *Annals of Oncology* online).

Size of largest melanoma deposit showed the strongest association with ctDNA ($P < 0.01$). Furthermore, no patients with largest melanoma deposit <10 mm had a detectable ctDNA across both the discovery and validation cohorts (58/174 patients; supplementary Figure S1, available at *Annals of Oncology* online). However, there was no direct correlation between size of largest melanoma metastasis and ctDNA copies/ml plasma (Spearman rank; $R^2 = 0$, not significant) (supplementary Figure S2, available at *Annals of Oncology* online).

Detectable ctDNA associated with worse melanoma-specific and distant metastasis recurrence-free survival

Pre-operative blood samples were analysed to determine whether ctDNA can predict outcome in stage III patients undergoing curative surgical resection. At a median follow-up of 26 months in the discovery cohort, 62 (52%) patients had died from melanoma. Patients with detectable ctDNA had a median MSS of 17.6 months compared with 49.4 months in patients with undetectable ctDNA [log-rank test; HR 2.11 (95% CI 1.20–3.71), $P < 0.01$; Figure 2A]. In the separate validation cohort, 23/55 (42%) patients had died from melanoma at a median follow-up of 24.8 months. Patients with detectable ctDNA had a median MSS of 23.9 months compared with 92.1 months in patients with undetectable ctDNA [log-rank test; HR 2.29 (95% CI: 0.89–5.88), $P = 0.04$; Figure 2B].

Clinicopathological factors which were significant for MSS in a univariable analysis in the discovery cohort were LDH, stage and ctDNA. In a multiple Cox regression analyses, ctDNA and presence of extranodal extension were the only parameters which remained significant for MSS in both the discovery and validation cohort (Tables 2 and 3). Kaplan–Meier curves for MSS based on the Cox multivariable model including ctDNA detectability, stage, number of LNs, size of LNs and extranodal extension, demonstrated similar discrimination in both cohorts (Figure 2C). The C-index for MSS was 0.712 (discovery) and 0.705 (validation), with the prognostic index in the two cohorts centred on the mean (supplementary Figure S3, available at *Annals of Oncology* online).

At the time of analysis, 96/119 (81%) patients in the discovery cohort and 39/55 (71%) patients in the validation cohort had distant metastatic recurrence. Patients with a detectable ctDNA had a median DM-RFS of 6.2 months compared with 13.9 months in patients with undetectable ctDNA in the discovery cohort [log-rank test; HR 1.59 (95% CI: 1.0–2.52), $P = 0.027$; supplementary Figure S4, available at *Annals of Oncology* online]. Patients with detectable ctDNA had a median DM-RFS of 8.7 months compared with 14.5 months in patients with undetectable ctDNA in the validation cohort [log-rank test; HR 2.15 (95% CI: 1.04–4.47), $P = 0.014$, supplementary Figure S5, available at *Annals of Oncology* online]. In a multivariate analysis, no clinicopathological factors remained significant for DM-RFS in the discovery cohort (supplementary Table S3, available at *Annals of Oncology* online), but ctDNA and extranodal extension remained significant in the validation cohort (supplementary Table S4, available at *Annals of Oncology* online).

Table 1. Clinicopathological characteristics of the discovery patient cohort (N = 119) according to ctDNA

Characteristic	Total *N* = 119 (%)	Undetectable ctDNA *N*=79 (%)	Detectable ctDNA *N*=40 (%)	*P*-value[a]
Age in years, median (range)	64 (20–90)	61 (30–89)	68 (20–90)	
<65	63 (53)	46 (58)	17 (43)	0.1
≥65	56 (47)	33 (42)	23 (57)	
Sex				
Male	78 (66)	51 (67)	27 (68)	0.8
Female	41 (34)	28 (33)	13 (32)	
Primary site				
Upper/lower limb	44 (37)	26 (33)	18 (45)	0.07
Chest/abdomen/back	47 (39)	38 (48)	9 (23)	
Head/neck	15 (13)	10 (13)	5 (13)	
Occult[b]	11 (9)	4 (5)	7 (17)	
Unknown[b]	2 (2)	1 (1)	1 (2)	
Primary tumour (T stage)				
T1a–T2b	36 (30)	22 (28)	14 (35)	0.2
T3a–T4b	69 (58)	52 (66)	17 (42)	
Occult[b]	11 (9)	4 (5)	7 (18)	
Unknown[b]	3 (3)	1 (1)	2 (5)	
Primary tumour ulceration				
No	51 (43)	39 (50)	12 (30)	0.4
Yes	51 (43)	34 (42)	17 (43)	
N/A (occult primary)[b]	11 (9)	4 (5)	7 (17)	
Unknown[b]	6 (5)	2 (3)	4 (10)	
Mutation status				
BRAF	84 (71)	59 (74)	25 (63)	0.2
Non-*BRAF* (*NRAS/KIT*)	35 (29)	20 (26)	15 (37)	
Year of surgery				
2010–2013	66 (55)	46 (58)	20 (50)	0.4
2014–2017	53 (45)	33 (42)	20 (50)	
LN site of surgical resection				
Axilla	55 (46)	39 (49)	16 (40)	0.7
Groin	46 (39)	29 (37)	17 (43)	
Cervical	18 (15)	11 (14)	7 (17)	
AJCC stage III subgroup				
B	41 (34)	32 (40)	9 (23)	0.1
C	65 (55)	40 (51)	25 (62)	
D	13 (11)	7 (9)	6 (15)	
Number of tumour-involved LNs[c]				
1–2	63 (53)	49 (63)	14 (35)	<0.01
≥3	56 (47)	30 (37)	26 (65)	
Size of largest melanoma deposit[c]				
≤20 mm	65 (54)	52 (66)	13 (33)	<0.001
>20 mm	52 (44)	25 (32)	27 (67)	
Not reported[b]	2 (2)	2 (2)	0 (0)	
Extranodal extension				
No	56 (47)	38 (48)	18 (45)	0.8
Yes	59 (50)	38 (48)	21 (53)	
Not reported[b]	4 (3)	3 (4)	1 (2)	
Method of detection				
Sentinel node biopsy	36 (30)	30 (37)	6 (15)	0.01
Clinically/radiologically detected	83 (70)	49 (63)	34 (85)	
Lactate dehydrogenase				
≤ULN	94 (79)	69 (87)	25 (62)	<0.01
>ULN	10 (8)	3 (4)	7 (18)	
LDH not measured[b]	15 (13)	7 (12)	8 (20)	

[a]*P*-values were calculated using the Fisher's exact probability test.
[b]Not included when calculating *P*-value.
[c]Median values were used for these cut-offs—median number of LNs=2 (range 1–56), median size of largest LN=20 mm (0.5–120 mm).
ctDNA, circulating tumour DNA; LN, lymph node; LDH, lactate dehydrogenase; ULN, upper limit of normal.

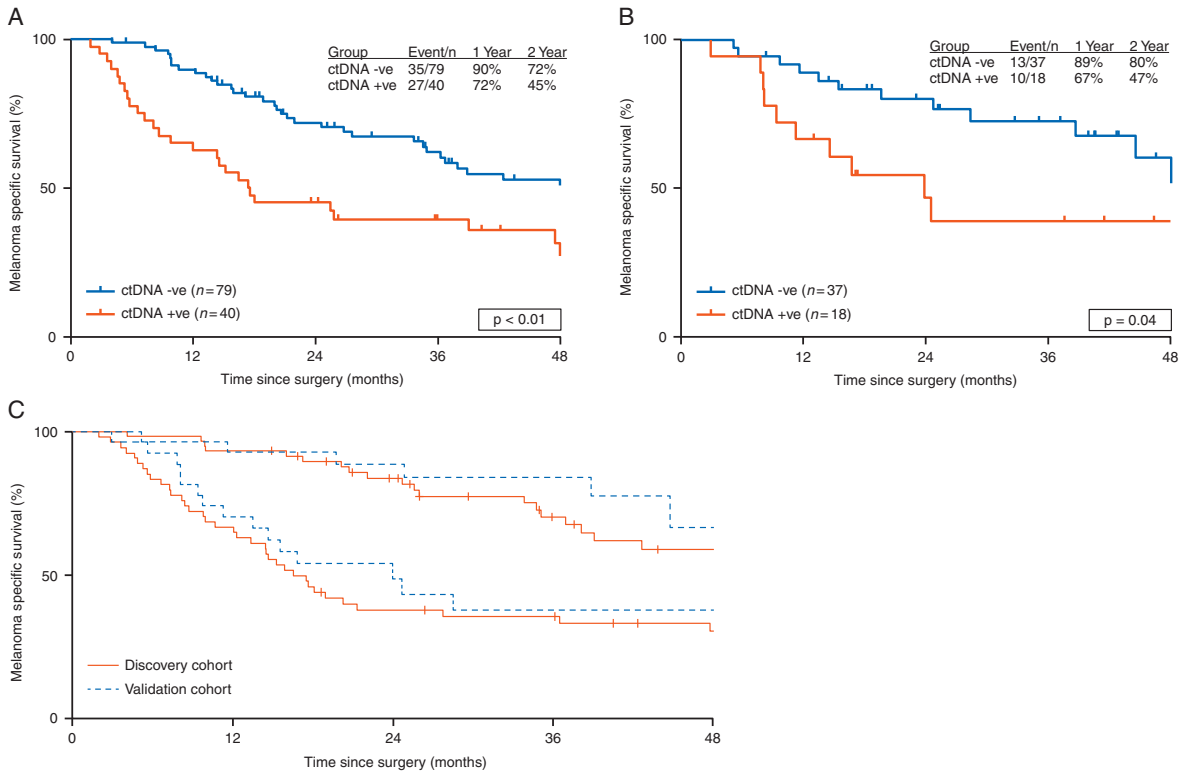

Figure 2. Kaplan–Meier estimates according to ctDNA profile groups of (A) MSS in the 119 patients in the discovery cohort (HR 2.1, $P < 0.01$) and (B) MSS in the 55 patients in the validation cohort (HR 2.3, $P = 0.04$). (C) Kaplan–Meier curves for MSS in the discovery and validation cohorts, based on Cox multivariate model including ctDNA detectability, stage, number of lymph node metastases, size of largest melanoma metastasis and extranodal extension.

ctDNA stratifies prognosis determined by AJCC stage III subgroup

The ability of ctDNA to further differentiate high- and low-risk patients within the AJCC stage III subgroups was analysed in all patients ($n = 174$). As expected, MSS decreased from AJCC stage IIIB to IIIC to IIID ($P = 0.02$, supplementary Figure S6, available at *Annals of Oncology* online). ctDNA was detectable in 16/64 (25%) stage IIIB patients and in 35/96 (36%) patients with IIIC disease. The 12-month MSS rates in stage IIIB patients with detectable and undetectable ctDNA were similar at 88% and 96%, respectively [HR 2.0 (95% CI 0.74–5.43), $P = 0.09$; supplementary Figure S7, available at *Annals of Oncology* online] whereas the 12-month MSS rates in stage IIIC patients with detectable and undetectable ctDNA was 57% and 85% [HR 1.7 (95% CI 0.95–3.11), $P = 0.05$; Figure 3A].

The MSS of AJCC stage IIIC patients who were stratified according to ctDNA detectability was compared with patients with stage IIIB melanoma. There was no difference in MSS between stage IIIC patients with undetectable ctDNA and all patients with stage IIIB disease [HR 1.4 (95% CI 0.80–2.35), $P = 0.2$]. However, the hazard ratio for MSS was 2.4 (95% CI 1.25–4.53, $P < 0.01$) for patients with stage IIIC and detectable ctDNA compared with stage IIIB patients (Figure 3A).

The difference in MSS was greatest in the 14 patients who had stage IIID disease, where ctDNA was detectable in 7 (50%) patients and undetectable in seven. All seven patients with detectable ctDNA have died, with four patients dying from melanoma within 6 months from date of surgery (2.9, 3.6, 4.0 and 4.9 months). Of the seven patients with undetectable ctDNA, four patients were still alive at 18.5, 53.7, 60 and 80.2 months follow-up and two patients died from melanoma at 8.4 and 21.2 months. One patient died after 5 months with cause of death unknown. The HR for death was 6.4 (95% CI 1.63–25.04, $P < 0.01$, Figure 3B) with a median OS for patients with detectable ctDNA at 4.9 months and unreached in patients with undetectable ctDNA.

Discussion

This study shows that detectable ctDNA before complete surgical resection in patients with AJCC stage IIIB/C/D (high-risk stage III) with a *BRAF*, *NRAS* or *KIT* mutant melanoma is an independent predictor of worse MSS in patients receiving no systemic adjuvant therapy. This has important implications with recent data demonstrating the efficacy of adjuvant therapy in stage III melanoma patients [3–5].

Patients with a detectable ctDNA before surgical resection had a median DM-RFS of only 6 months, indicating the presence of micrometastatic disease at time of surgical resection. Thus, if combination immunotherapy proves more effective than single agent PD1 inhibitors in the adjuvant setting (NCT03068455), ctDNA may identify patients requiring combination

Table 2. Univariate and multivariate analysis for MSS in patients in the discovery cohort (N = 119)

Variables	Univariable for MSS		Multivariable for MSS[a]	
	HR (95% CI)	*P*-value[b]	HR (95% CI)	*P*-value[b]
ctDNA detectable				
Yes versus no	2.12 (1.28–3.50)	0.003	1.85 (1.02–3.35)	0.042
Stage				
IIIC/IIID versus IIIB	2.13 (1.19–3.81)	0.011	2.10 (1.03–4.30)	0.042
Number of LN				
≥ 3 versus 1–2	1.45 (0.88–2.40)	0.147	0.85 (0.46–1.58)	0.618
Size of LN				
>20 mm versus ≤20 mm	1.51 (0.91–2.49)	0.110	1.12 (0.63–2.00)	0.708
Extranodal extension				
Yes versus no	1.61 (0.96–2.69)	0.070	1.72 (1.02–2.90)	0.042
Age ≥65				
≥65 versus <65	1.04 (0.63–1.72)	0.864		
Gender				
Male versus female	0.71 (0.42–1.20)	0.201		
Primary site				
Central versus periphery[c]	1.03 (0.59–1.81)	0.514		
Primary tumour (T stage)				
T3a–T4b versus T1a–T2b	1.53 (0.85–2.74)	0.152		
Primary ulceration				
Yes versus no	1.01 (0.59–1.75)	0.962		
Mutation (*BRAF*)				
Yes versus no	0.73 (0.43–1.23)	0.234		
LN resection site				
Cervical versus axilla	0.60 (0.25–1.45)	0.518		
Groin versus axilla	0.96 (0.57–1.62)			
Method of detection				
Clinically detectable versus SLN biopsy	1.08 (0.63–1.88)	0.777		
Lactate dehydrogenase[d]				
>ULN versus ≤ULN	2.80 (1.31–6.01)	0.008		

[a]Variables with a *P*-value ≤15% from the univariable cox regression are included in the multivariable model.
[b]Wald statistics, C-index = 0.71.
[c]Central: back, chest and abdomen; Periphery: upper and lower limbs.
[d]LDH was not included in the multivariable model given the high proportion of missing values ($n = 15$, 12%).
ctDNA, circulating tumour DNA; LN, lymph node; SLN, sentinel LN; ULN, upper limit of normal.

immunotherapy. We also suggest that ctDNA analysis should be incorporated into future clinical trials examining optimal duration of adjuvant immunotherapy (i.e. 6 versus 12 months). Recent data have confirmed the benefit of neoadjuvant immunotherapy in clinically detectable stage III melanoma [16] and based on stage IV data [13], we expect that ctDNA pre- and post-neoadjuvant therapy may be valuable in guiding the type of surgery and the need for post-operative adjuvant treatment. ctDNA detectability was associated with larger size of nodal melanoma deposit and clinical/radiological detectability, which is consistent with stage IV melanoma data that demonstrated a correlation between ctDNA mutation fraction and baseline disease volume [17]. The size of the largest tumour-involved LN did not correlate with MSS in our cohort, indicating that the prognostic significance of ctDNA detectability was not solely due to tumour volume.

An interesting finding is the ability for ctDNA to stratify outcome of patients within the AJCC substage. Despite no association between ctDNA detectability and AJCC stage III substage, these two pathological parameters independently predicted MSS in our cohort. Furthermore, the difference in MSS between patients with detectable and undetectable ctDNA was more pronounced in those with stage IIID compared with IIIC disease, indicating the importance of ctDNA with more advanced stage III subclass. We propose that ctDNA measurement before surgical resection of high-risk stage III melanoma may be an important addition to the current AJCC staging system for more accurate prediction of MSS. Larger prospective studies would help validate the prognostic value of including ctDNA analysis to the AJCC staging system, but this may not be possible as adjuvant melanoma therapy has now become the standard of care.

Table 3. Univariate and multivariate analysis for MSS in patients in the validation cohort (N = 55)

Variables[a]	Univariable for MSS		Multivariable for MSS[a]	
	HR (95% CI)	*P*-value[b]	HR (95% CI)	*P*-value[b]
ctDNA detectable				
Yes versus no	2.36 (1.01–5.49)	0.046	3.02 (1.15–7.93)	0.025
Stage				
IIIC/IIID versus IIIB	1.17 (0.50–2.74)	0.720	0.81 (0.31–2.12)	0.668
Number of LN				
≥3 versus 1–2	1.77 (0.76–4.09)	0.184	1.58 (0.65–3.82)	0.314
Size of largest LN deposit				
>20 mm versus ≤20 mm	1.14 (0.49–2.66)	0.758	0.64 (0.23–1.77)	0.388
Extranodal extension				
Yes versus no	2.32 (0.98–5.45)	0.054	2.93 (1.15–7.46)	0.024
Age				
≥65 versus <65	0.47 (0.20–1.12)	0.088		
Gender				
Male versus female	1.22 (0.51–2.92)	0.657		
Primary site				
Central versus periphery[c]	1.08 (0.41–2.82)	0.992		
Primary tumour (T stage)				
T3a–T4b versus T1a–T2b	0.75 (0.29–1.92)	0.940		
Primary ulceration				
Yes versus no				
Mutation status				
BRAF versus non-*BRAF*	0.57 (0.25–1.31)	0.183		
LN resection site				
Cervical versus axilla	0.63 (0.18–2.35)	0.397		
Groin versus axilla	0.54 (0.21–1.34)			
Method of detection				
Clinically detectably versus SLN biopsy	3.34 (0.98–11.43)	0.054		
LDH				
>ULN versus ≤ULN	3.01 (1.01–8.92)	0.047		

[a]Only variables selected from the discovery analysis are included in the validation regression.
[b]Wald statistics, C-index=0.71.
[c]Central: back, chest and abdomen; periphery: upper and lower limbs.
ctDNA, circulating tumour DNA; LN, lymph node; LDH, lactate dehydrogenase; ULN, upper limit of normal.

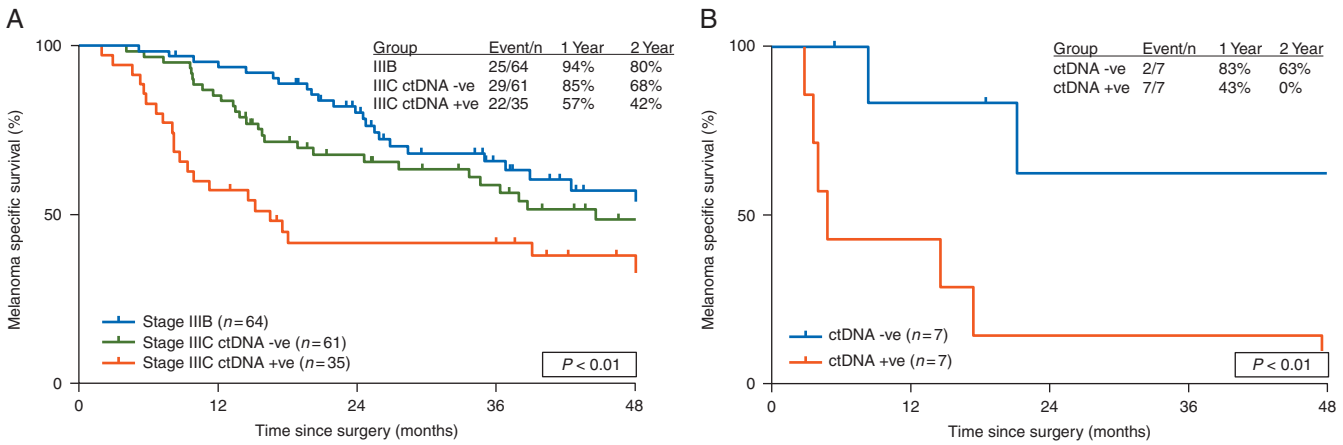

Figure 3. (A) Kaplan–Meier estimates for MSS ctDNA profile groups in patients with AJCC stage IIIC disease ($N = 96$) compared with all patients with stage IIIB melanoma ($N = 64$) in all patients. The Hazard ratio between detectable ctDNA and stage IIIC versus stage IIIB was 2.4 ($P < 0.01$). (B) Kaplan–Meier estimates for MSS patients with stage IIID disease only (HR 6.4, $P < 0.01$).

It is worth noting that the high-rate of recurrence in our patient cohorts was due to tumour tissue mutation testing not being routine for stage III melanoma patients, and only carried out at time of recurrence. Nevertheless, pre-operative ctDNA was predictive of DM-RFS in both cohorts. In addition, ctDNA analysis could only be carried out in patients with mutations affecting *BRAF*, *NRAS* or *KIT*. Although these mutations occur in ~80% of all cutaneous melanomas [18], ctDNA analysis in melanoma can be expanded with the availability of sensitive sequencing technologies, such as CAPP-seq, that simultaneously monitor many loci [19].

In this study, we did not analyse ctDNA after surgery, as a recent study found that post-operative ctDNA was only detectable in 12% of high-risk melanoma patients [20]. Nevertheless, ctDNA post-surgery was predictive of recurrence and survival in high-risk resected melanoma, and a direct comparison of the predictive value of pre- and post-operative ctDNA in stage III patients is warranted.

In summary, ctDNA measured before surgical resection predicts MSS in patients with high-risk stage III disease with a driver mutation. When used in combination with AJCC staging, detectability of ctDNA may provide more precise risk stratification, although further validation is required. Longer follow-up in larger cohorts of patients with prospective molecular testing, receiving adjuvant treatment with immunotherapy or targeted therapy, would provide more robust data regarding patient selection and RFS.

Acknowledgements

We thank the Melanoma biobank across Royal Prince Alfred Hospital, Melanoma Institute Australia and Westmead Hospitals, Sydney Vital Translational Cancer Research Centre, and the surgeons who contributed patients.

Funding

This work was supported by the National Health and Medical Research Council (grant numbers 1093017 and 1130423) and the CLEARbridge Foundation (no grant number applicable). HR, RAS and GVL are supported by NHMRC Fellowships (grant numbers 1104503, 1141295, 1119059).

Disclosure

The following authors: MSC, GVL, AMM, JFT and RFK sit on advisory boards for BMS, GSK, MSD, Roche, Amgen, Array, Novartis, Provectus and/or Pierre-Fabre. All remaining authors have declared no conflicts of interest.

References

1. Gershenwald JE, Scolyer RA, Hess KR et al. Melanoma staging: evidence-based changes in the American Joint Committee on Cancer eighth edition cancer staging manual. CA Cancer J Clin 2017; 67(6): 472–492.
2. Thompson JF, Scolyer RA, Kefford RF. Cutaneous melanoma in the era of molecular profiling. Lancet 2009; 374(9687): 362–365.
3. Long GV, Hauschild A, Santinami M et al. Adjuvant dabrafenib plus trametinib in stage III BRAF-mutated melanoma. N Engl J Med 2017; 377(19): 1813–1823.
4. Eggermont AM, Chiarion-Sileni V, Grob JJ et al. Prolonged survival in stage III melanoma with ipilimumab adjuvant therapy. N Engl J Med 2016; 375(19): 1845–1855.
5. Eggermont AMM, Blank CU, Mandala M et al. Adjuvant pembrolizumab versus placebo in resected stage III melanoma. N Engl J Med 2018; 378(19): 1789–1801.
6. Khosrotehrani K, van der Ploeg AP, Siskind V et al. Nomograms to predict recurrence and survival in stage IIIB and IIIC melanoma after therapeutic lymphadenectomy. Eur J Cancer 2014; 50(7): 1301–1309.
7. Spillane AJ, Cheung BL, Winstanley J, Thompson JF. Lymph node ratio provides prognostic information in addition to American Joint Committee on cancer N stage in patients with melanoma, even if quality of surgery is standardized. Ann Surg 2011; 253(1): 109–115.
8. Crookes TR, Scolyer RA, Lo S et al. Extranodal spread is associated with recurrence and poor survival in stage III cutaneous melanoma patients. Ann Surg Oncol 2017; 24(5): 1378–1385.
9. Mann GJ, Pupo GM, Campain AE et al. BRAF mutation, NRAS mutation, and the absence of an immune-related expressed gene profile predict poor outcome in patients with stage III melanoma. J Invest Dermatol 2013; 133(2): 509–517.
10. Mocellin S, Zavagno G, Nitti D. The prognostic value of serum S100B in patients with cutaneous melanoma: a meta-analysis. Int J Cancer 2008; 123(10): 2370–2376.
11. Majithia N, Velazquez Manana A, Yan Y et al. The prognostic role of pre-operative serum lactate dehydrogenase (LDH) in patients with resected advanced melanoma. J Clin Oncol 2017; 35(Suppl 15): e21054–e21054.
12. Santiago-Walker A, Gagnon R, Mazumdar J et al. Correlation of BRAF mutation status in circulating-free DNA and tumor and association with clinical outcome across four BRAFi and MEKi clinical trials. Clin Cancer Res 2016; 22: 567–574.
13. Lee JH, Long GV, Boyd S et al. Circulating tumour DNA predicts response to anti-PD1 antibodies in metastatic melanoma. Ann Oncol 2017; 28(5): 1130–1136.
14. Muhanna N, Di Grappa MA, Chan HHL et al. Cell-free DNA kinetics in a pre-clinical model of head and neck cancer. Sci Rep 2017; 7(1): 16723.
15. Royston P, Altman DG. External validation of a Cox prognostic model: principles and methods. BMC Med Res Methodol 2013; 13: 33.
16. Blank CU, Rozeman EA, Fanchi LF et al. Neoadjuvant versus adjuvant ipilimumab plus nivolumab in macroscopic stage III melanoma. Nat Med 2018; 24(11): 1655–1661.
17. Ascierto PA, Minor D, Ribas A et al. Phase II trial (BREAK-2) of the BRAF inhibitor dabrafenib (GSK2118436) in patients with metastatic melanoma. J Clin Oncol 2013; 31(26): 3205–3211.
18. Cancer Genome Atlas Network. Genomic classification of cutaneous melanoma. Cell 2015; 161: 1681–1696.
19. Newman AM, Bratman SV, To J et al. An ultrasensitive method for quantitating circulating tumor DNA with broad patient coverage. Nat Med 2014; 20(5): 548–554.
20. Lee RJ, Gremel G, Marshall A et al. Circulating tumor DNA predicts survival in patients with resected high-risk stage II/III melanoma. Ann Oncol 2018; 29(2): 490–496.