Circulating Tumor DNA Predicts Outcome from First-, but not Second-line Treatment and Identifies Melanoma Patients Who May Benefit from Combination Immunotherapy

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Introduction

Metastatic melanoma is an aggressive type of skin cancer and responsible for most skin cancer–related deaths (1, 2). In the last decade, the emergence of targeted therapy and immune checkpoint inhibitor/s (ICIs) has significantly changed the clinical management and outcome of patients with melanoma (3, 4). Current treatment options for unresectable stage III and stage IV disease (5) include BRAF-targeted therapies for patients that have BRAF-mutant melanomas, and ICIs with anti–PD-1 alone or in combination with anti–CTLA-4. However, durable response is only seen in a minority of patients, and the optimal sequencing of therapies and the selection of the most effective first-line therapy, remain controversial (6, 7). The CheckMate 067 trial demonstrated that the combination ipilimumab and nivolumab resulted in superior long-term survival outcomes compared with either nivolumab or ipilimumab monotherapy, with a 5-year overall survival of 52% (8). However, in addition to being a more costly regimen (9), patients treated with combination therapy experienced more grade 3 or 4 treatment-related adverse events relative to those treated with nivolumab or ipilimumab alone (9). Thus, there is a need to better stratify patients who will require upfront combination therapy from those who may derive a similar benefit from anti–PD-1 monotherapy.

The potential clinical utility of ctDNA for melanoma management has been demonstrated in multiple studies. Elevated baseline ctDNA levels have been shown to significantly correlate with low overall response rate (ORR) and short progression-free survival (PFS) in...
patients with melanoma receiving targeted therapy (10–13). However, there is a paucity of studies evaluating the predictive value of baseline ctDNA in patients treated with immunotherapy. Low baseline ctDNA level has been previously associated with long PFS in patients with melanoma treated with ICI (14) and was found to correlate with tumor shrinkage on radiology (15). In contrast, Lee and colleagues showed that a decline in ctDNA during treatment, but not low baseline levels, predicted longer PFS and OS (16). Thus, more studies are needed to clarify and further refine the predictive value of ctDNA in patients treated with immunotherapies, particularly taking into consideration the line of therapy, prior treatment regimens and mutation status.

In this study, we analyzed a prospective cohort of patients with melanoma receiving systemic therapies, including a large proportion of BRAF wild-type (WT) cases. We compared the predictive value of pretreatment ctDNA levels to inform survival outcomes in patients with metastatic melanoma receiving first-line or second-line systemic ICI. Our observations were validated using published datasets from two independent cohort studies (16–19).

Materials and Methods

Discovery cohort
We analyzed a total of 125 baseline plasma samples collected prior to commencing systemic therapy from 110 patients with unresectable stage IV cutaneous melanoma enrolled in the study between 2013 and 2018 at Sir Charles Gairdner Hospital (SCGH) and Fiona Stanley Hospital (FSH) in Perth, Western Australia. Patients’ characteristics are presented in Fig. 1 and Table 1. A subset of 15 patients were considered as baseline for their first- and second-line therapy. This study received approval from the Human Research Ethics Committee of Edith Cowan University (Nos. 11543 and 18957) and Sir Charles Gairdner Hospital (No. 2013–246). Written consent was obtained from all patients under approved human research ethics committee protocols that complied with the Declaration of Helsinki. Patients were clinically monitored, and the median follow-up period was 95 weeks (range, 16–257 weeks).

Validation cohort
We pooled data from two independent cohorts of 128 unresectable stage III (n = 3) and stage IV melanoma (n = 125) patients recruited from the Melanoma Institute Australia (NSW) affiliated hospitals and Peter MacCallum Cancer Centre (Victoria, Australia), as described previously by Lee and colleagues (16–18) and Wong and colleagues (19). Additional details and patient characteristics are shown in Fig. 1 and Table 1. Further comparison between the discovery and validation cohorts is presented in Supplementary Table S1.

Translational Relevance

Low pretreatment plasma ctDNA level is predictive of longer progression-free survival in patients with melanoma receiving first-line immune checkpoint inhibitors, but its predictive value is lost in the second-line setting, particularly after treatment with BRAF + MEK inhibitors. Patients with treatment-naïve melanoma with high pretreatment ctDNA levels showed a trend toward better outcomes when treated with combination anti–CTLA-4/anti–PD-1 ICIs rather than anti–PD-1 alone. Quantification of ctDNA may be useful for the stratification of patients who will likely benefit from combination immunotherapy.

Circulating Tumor DNA in Melanoma and Immunotherapy

Treatment response and disease progression assessment
Radiologic assessment of treatment response and disease progression was performed at 2- to 3-month intervals by CT and/or 18F-labeled fluorodeoxyglucose positron emission tomography (FDG-PET) scans. MRI of the brain was also used where indicated. PFS was defined as the time interval between the start of therapy and the date of first clinical or radiologic progression.

Plasma sample preparation and cell-free DNA extractions
Pretreatment blood samples were collected into EDTA vacutainer or Cell-Free DNA BCT (Streck) tubes. Isolated plasma was stored at −80°C until extraction. Plasma cell-free DNA (cfDNA) was isolated from 1–5 mL of plasma using QIAamp Circulating Nucleic Acid Kits (Qiagen), as per the manufacturer’s instructions. The recovered cfDNA was freezer-stored until ctDNA quantification.

tctDNA quantification
For the discovery cohort, the mutation target for ctDNA analysis was selected on the basis of the mutation reported in each patient’s molecular pathology result (BRAF mutant) or, if BRAF WT, obtained from next-generation sequencing of tissue biopsy using a custom melanoma panel, as described previously by Calapre and colleagues (20). Commercially available and/or customized probes were used to analyze ctDNA by droplet digital PCR (ddPCR). To cover all patients, we used a total of 22 different hotspot sequence variants in 10 different genes (Supplementary Tables S2 and S3). Droplets were generated using an Automatic Droplet generator QX200 AutoDG (Bio-Rad) and analyzed using the QuantaSoft analysis software version 1.7.4 (Bio-Rad). Amplifications were performed in 40-μL reactions using previously described cycling conditions (14). Quantification results were presented in copies of ctDNA per mL of plasma.

A cutoff of 20 copies/mL was used for comparison of our analyses with the results from different cohorts analyzed in three laboratories. This is the minimum ctDNA concentration that could be reliably detected given that ctDNA was isolated from 1–5 mL of plasma, eluted in 30–60 μL, an input of 5–8 μL in the ddPCR reaction and using different copies per mL of plasma (copies/mL) as threshold based on specific assays (Supplementary Table S2; refs. 16–19). We confirmed the suitability of this cut-off value through ROC curve analysis using the discovery cohort data for prediction of 6-month PFS (Supplementary Fig. S1). Patients with more than 20 copies/mL were defined as having a high ctDNA level, while patients ≤20 copies/mL were considered to have low ctDNA level.

Statistical analysis
A comparison of the patients’ characteristics between the discovery and the validation cohorts was performed using a χ2 or two-sided Fisher exact test, with the frequencies, percentages and the P values reported. Similarly, patient characteristics were compared by group using a χ2 or two-sided Fisher exact test, reporting their corresponding P values. A ROC curve was calculated to determine the best cut-off value to dichotomize ctDNA concentration to predict 6-month PFS. Multiple ctDNA cutoffs were calculated by averaging two consecutive ctDNA values. These values were then used to calculate survival HRs and 95% CIs for each ctDNA cutoff following a previously described analysis (21).

Median PFS was calculated using the Kaplan–Meier method, and survival curves statistical significance was determined using the log-rank and Gehan–Breslow–Wilcoxon test, when indicated, to stress the importance of early events. Univariate and multivariate Cox regression analyses were performed for PFS and OS comparisons in the discovery
cohort, the validation cohort and ICI monotherapy or combination cohort. All statistical analyses were performed using GraphPad Prism version 8 (GraphPad Software Inc.), SPSS version 25 (IBM) and R Studio (v.1.1.456). Results with $P < 0.05$ were considered statistically significant.

**Results**

**Patient characteristics**

The discovery cohort comprised 125 plasma samples. Of these, 66 were from patients with BRAF-mutant melanoma commencing first-line targeted therapy, and most patients received dabrafenib/trametinib (61/66, 92%; Fig. 1; Table 1). A total of 32 patients were treated with first-line ICI monotherapy or in combination (22 anti–PD-1 alone, 10 anti–CTLA-4 plus anti–PD-1 combination treatment) and 27 were treated with second-line ICI/s. Of the latter, 19 (70%) received a combination of BRAF/C6 MEK inhibitors as first-line treatment, while eight patients received ICI monotherapy as first line followed by combination ICI (Table 1; Supplementary Table S1). Overall, the discovery cohort included two BRAF-mutant patients in the first-line ICI group (2/32, 6%) and 21 in the second-line ICI group (21/27, 78%).

**Baseline ctDNA and PFS**

Consistent with previous studies, for patients treated with first-line targeted therapy, low plasma ctDNA level at baseline was predictive of longer PFS (median: 57 vs. 29 weeks; HR, 0.54; 95% CI, 0.30–0.98; $P = 0.025$; Supplementary Fig. S2).

We then evaluated the predictive value of baseline ctDNA levels in patients receiving first- or second-line ICI in the discovery cohort. No statistically significant differences between clinical patients’ characteristics were associated with ctDNA levels (Fisher exact test; Supplementary Table S4). However, patients with low ctDNA levels prior to first-line treatment initiation ($n = 18$) had a significantly longer PFS, with undefined median, compared with patients with high levels of ctDNA (median PFS 8 weeks; HR, 0.20; 95% CI, 0.07–0.53; $P < 0.0001$; Fig. 2A). The predictive value of ctDNA was significant across multiple cut-off values (Supplementary Fig. S3A). Multivariate Cox regression analysis controlling for age, sex, tumor stage, brain metastases, and BRAF status confirmed that low ctDNA level at baseline was an independent predictor of longer PFS (HR, 5.18; 95% CI, 1.88–14.31; $P = 0.001$; Supplementary Table S5).

Analysis of patients receiving ICI as second-line ($N = 27$) failed to demonstrate an association between low ctDNA and longer PFS (median PFS 31 vs. 26 weeks; HR, 1.05; 95% CI, 0.41–2.72; $P = 0.913$; Fig. 2B) using 20 copies/mL as cutoff or any other value (Supplementary Fig. S3B). Similar results were observed when removing five patients with intracranial disease only (Supplementary Fig. S3A). As 19 of 27 (70%) of patients receiving second-line ICIs, had BRAF inhibitors (with or without MEK inhibitors) as first-line treatment, we evaluated the PFS outcome of this subgroup of patients. Low baseline ctDNA in patients commencing ICI as second-line after failing therapy with BRAF/C6 MEK inhibitors was not a predictor of longer PFS (median: 30 vs. 3 weeks; HR, 0.59; 95% CI, 0.16–2.24; $P = 0.356$; Fig. 2C; Supplementary Fig. S3C), contrary to the first-line ICI setting. Nevertheless, this observation was derived from a small cohort (14 vs. 5) patients.

**Validation cohort**

Two independent melanoma patient cohorts receiving ICI/s in the first- or second-line setting were combined and used to validate our findings ($N = 128$). This validation cohort comprised 77 patients treated with first-line ICI (37 anti–PD-1 monotherapy, 40 anti–CTLA-4...
plus anti–PD-1 combination) and 51 patients treated with second-line ICIs. Of these 51 patients, 36 (71%) were treated with first-line BRAF/C6 MEK inhibitors (Supplementary Table S1), while 14 (27%) were treated with ipilimumab and 1 (2%) was treated with ipilimumab plus nivolumab as first-line treatment. The cohort included 35 (35/77, 45%) BRAF-mutant patients in the first-line ICI group and 38 (38/51, 75%) in the second-line ICI group. The validation cohort had a significantly higher number of BRAF-mutant patients treated with first-line ICI than the discovery cohort (35/77, 45% vs. 2/32, 6%, P < 0.0001, respectively; Supplementary Table S1). Similarly, there was a significantly higher number of patients treated with combination ICIs in the first-line setting than in the discovery cohort (P = 0.048). No other statistical difference in patient characteristics was found between the validation and the discovery cohorts.

**Baseline ctDNA predictive value in the validation cohort**

Similar to the discovery cohort, patients with low baseline ctDNA levels prior to first-line ICI showed a significantly longer PFS than patients with high ctDNA levels (median PFS undefined vs. 42 weeks; HR, 0.42; 95% CI, 0.22–0.83; P = 0.006; Fig. 2D). The predictive value of ctDNA was significant across multiple cut-off values (Supplementary Fig. S3D). Multivariate Cox regression analysis controlling for age, sex, tumor stage, brain metastases, BRAF status, and LDH confirmed that low ctDNA level at baseline prior to first-line ICI, was an independent predictor for longer PFS (HR, 2.423; 95% CI, 1.17–5.02; P = 0.017; Supplementary Table S6). Similar to the discovery cohort, low ctDNA was not associated with longer PFS in the second-line setting in the validation cohort (median PFS 49 vs. 13 weeks, HR 0.61, 95% CI, 0.30–1.25, P = 0.143; Fig. 2E)

### Table 1. Clinical characteristics at baseline of the patients with melanoma included in the study.

<table>
<thead>
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<th>Variable</th>
<th>Discovery cohort</th>
<th>Validation cohort</th>
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<td></td>
<td>First-line ICI</td>
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Abbreviation: TT, targeted therapy.
Figure 2.
Kaplan–Meier curves for PFS of patients with melanoma treated with ICIs. Patients were stratified into those with low (green) or high (red) baseline ctDNA levels. Each graph denotes PFS outcomes in the discovery (A–C) or validation cohorts (D–F); for patients treated with ICI as first-line (A and D) or second-line treatment (B and E); or for BRAF-mutant patients receiving ICI after failing first-line targeted therapy (C and F). Log-rank P values, HR, and 95% CIs are indicated for each plot.

Overall, ctDNA was found to be predictive of PFS in patients treated with first-line ICI. However, patients with high ctDNA levels in the validation cohort showed longer PFS compared with patients with similarly high baseline ctDNA levels in the discovery cohort (8 vs. 42 weeks, red lines in Fig. 2A and D). It is important to note that in the discovery cohort, only 29% (4/14) of patients with high ctDNA levels at baseline were treated with combination anti–CTLA-4 plus anti–PD-1 therapy, compared with 58% (19/33) of patients in the validation cohort (Supplementary Tables S4 and S7).

To investigate whether the combination of ICIs is more effective in patients with high ctDNA, patients from both cohorts were combined and dichotomised according to whether they had high or low baseline ctDNA levels. Within these groups, survival outcomes were compared in patients who received single agent anti–PD-1 versus combination of ICIs. Comparison of patient characteristics revealed a larger proportion of BRAF-mutant patients with low ctDNA levels received combination therapy (P = 0.003; Supplementary Table S8).

Albeit not significant, a trend showing longer PFS was observed for patients with high baseline ctDNA treated with combination of ICIs when compared with those who received anti–PD-1 monotherapy (median: 42 vs. 7.5 weeks; HR, 1.79; 95% CI, 0.90–3.53; P = 0.081; Fig. 3A). Similarly, patients treated with combination therapy showed longer OS (median: 186 vs. 43 weeks; HR, 1.91; 95% CI, 0.87–4.21; P = 0.104; Fig. 3B). However, OS was significant when the Gehan–Breslow–Wilcoxon test was used (P = 0.028), reflecting the difference in events occurring at the beginning of the curve. Patients with low ctDNA levels showed no differences in PFS or OS when treated with combination of ICIs versus monotherapy (HR, 1.97; 95% CI, 0.87–4.47; P = 0.124; Fig. 3C and HR, 1.74; 95% CI, 0.63–4.79; P = 0.306; Fig. 3D, respectively). However, none of the groups reached median PFS or OS, and a limited number of events were recorded within the follow-up time.

Discussion

Studies investigating the ability of baseline ctDNA to predict treatment outcome in melanoma patients undergoing ICI are scarce, mainly including BRAF-mutant melanomas and do not differentiate between treatment lines (14, 16, 22). In this study, we showed that baseline ctDNA in patients with metastatic melanoma receiving first-line ICI is a strong predictor of clinical outcome, as shown in both the discovery and validation cohorts. In contrast, the predictive value of ctDNA is lost in second-line ICI, particularly in patients failing first-line targeted therapy. Overall, our results redefine the context of use of ctDNA as a predictive biomarker in patients with melanoma receiving immunotherapy, confining its utility to the first-line treatment setting.
Currently, there is a paucity of published data related to outcomes of patients who experience disease progression on BRAF inhibitor therapies and are treated with second-line ICI. A recent retrospective study demonstrated that while ICI first-line efficacy appears comparable with trial populations, ICI treatment of BRAF-mutant patients failing targeted therapy demonstrated a significantly lower response (23–25), and indeed, any drug therapy has lower efficacy in the second-line setting (26–28). Consistent with these findings, our results demonstrate the limitation of ctDNA as a predictive biomarker in the second-line setting. Mason and colleagues found that BRAF-mutant patients refractory to first-line targeted therapy have a high proportion of brain metastases (47%; ref. 23), a finding similar to that observed in the discovery and validation cohorts (37% and 58%, respectively). The brain is a common site of targeted therapy failure, often contributing to death (29). Given that brain metastases have been reported to shed less ctDNA into the circulation (18, 30), the large proportion of disease progression within the brain, may contribute to the limited predictive value of ctDNA in this setting. In line with this, removal of patients with only intracranial metastases from the analysis improved the ctDNA predictive value in the validation cohort. However, ctDNA still failed to predict PFS for patients who received prior treatment with targeted therapies.

The predictive significance of ctDNA levels prior to commencing targeted therapies has been previously demonstrated, with the absence of detectable ctDNA correlating with longer survival in large cohorts of patients with melanoma (10–13). Similarly, we found that baseline ctDNA level was a strong predictor of clinical outcome in melanoma patients in the first-line targeted therapy setting, showing that high ctDNA level is associated with poorer clinical outcomes.

The selection of first-line monotherapy over ICI combination is currently a complex decision and factors such as median tumor size, LDH levels, BRAF status, presence of brain metastases, and comorbidities must be carefully considered (8, 31, 32). We observed that in the validation cohort, more patients with high baseline ctDNA levels were treated with a combination of ICIs (19/33, 58%), while the patients in our discovery cohort were mainly treated with single-agent ICI (10/14, 71%). In addition, the validation cohort had a significantly higher proportion of patients treated with a combination of ipilimumab and pembrolizumab as first-line therapy instead of ipilimumab plus nivolumab (P = 0.006), as part of a clinical trial. Molecular and preclinical assessments of nivolumab

Figure 3.
Kaplan-Meier plots comparing survival of patients receiving first-line single-anti-PD-1 and ICI combination. Patients were separated on the basis of high (A and B, bold) and low (C and D, gray) baseline ctDNA levels, treated with anti-PD-1 alone (dashed line) or with combination anti-CTLA-4 plus anti-PD-1 (solid line). Graphs represent progression-free survival (PFS; A and C) and overall survival (OS; B and D). Log-rank P-values, HR, and 95% CIs are indicated for each plot. *, Represents Gehan-Breslow-Wilcoxon P values.
and pembrolizumab suggest that these drugs could be interchanged and differences seen in clinical trials are likely related to patient populations rather than be drug-dependent (33). Consequently, we showed that patients with high pretreatment ctDNA levels tend to benefit from the combination of anti–CTLA-4 and anti–PD-1 as first-line therapy. Although ctDNA level correlates significantly with tumor burden (19, 34), multivariate analyses in previous studies have shown ctDNA to be an independent predictor of survival (16, 34). In this context, our results suggest that more aggressive treatment will be particularly beneficial to those patients with high ctDNA levels.

Results from the Checkmate 067 study favors the use of ICI combination over anti–PD-1 monotherapy, showing that patients treated with a combination of ICIs had increased both response (58% vs. 45%) and 5-year overall survival rates (52% vs. 44%) compared with those treated with anti–PD-1 monotherapy. These differences were accentuated in patients with BRAF mutations, with an increased 5-year survival rate in the combination group (60% vs. 46%; ref. 8). In addition, it has been described that in patients with PD-L1–tumors, the combination of PD-1 and CTLA-4 blockade was more effective than was either agent alone (8, 35). On the basis of our results, elevated ctDNA may identify a group of patients with melanoma that could benefit from ICI combination treatment. However, our study was limited by the low number of patients with high ctDNA included in the survival analysis of single-agent anti–PD-1 and combination of ICIs. Further prospective clinical trials are needed to confirm our observations and validate the use of ctDNA as a predictive biomarker for the treatment of patients with melanoma.

Disclosure of Potential Conflicts of Interest

J.H. Lee reports other from Bristol Myers Squibb (travel support) and Bio-Rad (travel support) and personal fees from AstraZeneca (honoriaurum) outside the submitted work. M.A. Khattak reports grants from MSD Australia during the conduct of the study. T.M. Menzies reports personal fees and non-financial support from BMS; grants from Merck Sharp & Dohme and grants from Novartis outside the submitted work. S.J. Dawson reports other from Roche-Genentech (funding), other from CTx-CRC (research funding), and other from AstraZeneca (advisory board) outside the submitted work. M.S. Carlino reports personal fees from BMS (consultant advisor and honoraria), MSD (consultant advisor and honoraria), Novartis (consultant advisor and honoraria), Roche (consultant advisor), Pierre Fabre (consultant advisor), Eisai (consultant advisor and honoraria), Sanofi (consultant advisor), Merck (consultant advisor), Q biotics (consultant advisor), Ideaya (consultant advisor), Regeneron (consultant advisor), and Nektar (consultant advisor) outside the submitted work. A.M. Menzies reports personal fees from BMS (consultancy and honoraria), AstraZeneca (consultancy and honoraria), Sanofi (consultancy and honoraria), Merck (consultancy and honoraria), Q-88 (consultancy and honoraria), Skyline DX (consultancy and honoraria), and Sandoz (consultancy and honoraria) outside the submitted work. M. Millward reports personal fees from Aduro (consultant advisor), Amgen (consultant advisor), Bristol-Myers Squibb (consultant advisor), Highlight Therapeutics S.L. (consultant advisor), Mass-Array (consultant advisor), Merck (consultant advisor), MSD (consultant advisor), OncoSce Medical (consultant advisor), Pierre Fabre (consultant advisor), Roche (consultant advisor), Qbiotics (consultant advisor), Skyline DX (consultant advisor), and Sandoz (consultant advisor) outside the submitted work.

Acknowledgments

This work was supported by the National Health and Medical Research Council [NHMRC; APP1117991, to M.R. Ziman, M. Millward, B. Amanuel, H. Rizos, E.S. Gray, M.A. Khattak, APP1053792 and APP1107126, to S.Q. Wong, S. Sandha, S.J. Dawson, APP1128891, to H. Rizos, M.S. Carlino, A.M. Menzies; and APP1119059 and Program Grant, to R.A. Scolyer and G.V. Long]; the Cancer Council Western Australia (to M.A. Khattak, B. Amanuel, no grant number); the Perpetual Foundation (to M.R. Ziman, E.S. Gray, M. Millward, M.A. Khattak, B. Amanuel, no grant number); the Perpetual Foundation (to M.R. Ziman, E.S. Gray, M. Millward, M.A. Khattak, B. Amanuel, no grant number); the Merck Sharp & Dohme Investigator Studies Program (grant provided to M.A. Menzies, no grant number); the ECU Early Career Research (grant to J. H. Lee); the Spinknner Foundation (to M.R. Ziman, E.S. Gray, M. Millward, M.A. Khattak, B. Amanuel, no grant number); the Perpetual Foundation (to M.R. Ziman, E.S. Gray, M. Millward, M.A. Khattak, B. Amanuel, no grant number); the Cancer Institute NSW and Melanoma Institute Australia Health Translational Network (to A.C. McEvoy; no grant number); Senior Research fellowship from NHMRC (to H. Rizos; no grant number); mid-career fellowship from the Victorian Cancer Agency (to S.Q. Wong; no grant number); fellowship from Cancer Institute NSW and Melanoma Institute Australia (to A.M. Menzies; no grant number); G.V. Long is supported by the University of Sydney Medical Foundation. G. Marsavela is supported by a scholarship from the School of Medical and Health Sciences at Edith Cowan University. We would like to thank the patients with melanoma for their participation and support of the study. We also thank Aaron Beasley, Jamie Freeman, Paula van Miert, Mike Morici, Danielle Bartlett, and Pauline Zaenker for their help in the collection and processing of blood samples from patients and healthy controls. Furthermore, we extend our thanks to Dr. Tindaro Giardina from Pathwest for assistance with mutation profiling of tumors and Dr. Johnny Lo from Pathwest for assistance with mutation profiling of tumors and Dr. Johnny Lo for helping with part of the statistical analysis. Assistance from colleagues at Melanoma Institute Australia and the Ainsworth Foundation is also gratefully acknowledged.

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Received June 10, 2020, revised July 29, 2020; accepted September 9, 2020, published first October 16, 2020.
Circulating Tumor DNA in Melanoma and Immunotherapy

References


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