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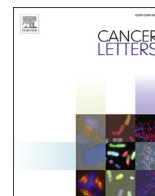
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Mini-review

Circulating tumour DNA (ctDNA) as a liquid biopsy for melanoma

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ABSTRACT

Circulating tumour DNA (ctDNA) has emerged as a promising blood-based biomarker for monitoring disease status of patients with advanced cancers. In melanoma, ctDNA has been shown to have clinical value as an alternative tumour source for the detection clinically targetable mutations for the assessment of response to therapy. This review provides a critical summary of the evidence that gives credence to the utility of ctDNA as a biomarker for monitoring of disease status in advanced melanoma and the steps required for its implementation into clinical settings.

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Introduction

Melanoma is the most aggressive form of skin cancer, representing only 10% of all skin cancers but responsible for more than 80% of skin cancer-related deaths. The advent of novel therapeutic approaches over the past several years has transformed the clinical management of metastatic melanoma [1]. The combination of BRAF and MEK inhibitors, commonly used to treat melanoma patients with BRAF mutations, can rapidly reduce disease burden and improve patient status [2–4]. Nevertheless, acquired resistance to these treatments, as a consequence of clonal evolution and selection, commonly develops within 12 months [5–7]. Antibody-mediated blockade of immune checkpoints, particularly the cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) and the programmed cell-death protein 1 (PD-1), have markedly improved patient outcome in the last 5 years [8–12]. There is evolving evidence that the use of immune checkpoint inhibitors can lead to durable responses and prolonged survival for a small proportion of patients. However, a significant number of patients are still incapable of achieving a meaningful benefit from immunotherapy. In addition, immune checkpoint inhibitors are costly and have the

potential to cause substantial toxicity [13–16]. Major efforts have been made to better identify factors that can guide patient selection for specific treatments, monitor disease evolution, and aid in clinical decision making in melanoma.

In recent years, much attention has been focused on utilising circulating cell-free DNA (cfDNA), specifically the tumour-derived cfDNA (ctDNA), as a biomarker of disease status in metastatic cancer patients [17–19]. cfDNA are short nucleic fragments (~166 bp) found in plasma, which are thought to be released as a result of cell apoptosis and/or necrosis [18,20]. In cancer patients, the high cell turnover rate of tumours results in the release of ctDNA. Numerous studies have shown that ctDNA carries genetic information from the entire tumour genome and can therefore provide insight into clonal heterogeneity and evolution of all solid cancers present at any one time [18,21]. Thus, the presence of ctDNA in the blood provides information via a minimally invasive 'liquid biopsy', eliminating the morbidity associated with serial sampling of tumours for monitoring patients with advanced solid cancers.

Various studies in breast, lung and colorectal cancers have demonstrated the potential application of ctDNA analysis at each stage of clinical management: early diagnosis [18,22], molecular profiling [21,23–25], prognostication [18,26,27], detection of residual disease [28,29], monitoring response and clonal evolution [30–34]. Lastly, the recent approvals by the Federal Drug Administration (FDA) and the European Medicines Agency (EMA) for detection of the tyrosine kinase resistant clone EGFR p.T790M

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mutation in plasma as a companion diagnostic for second-line treatment of metastatic EGFR-mutant non-small cell lung cancer (NSCLC) [35–37], and the inclusion of ctDNA analysis into multiple clinical trials (ClinicalTrials.gov), signals the recent integration of this form of biomarker into routine clinical oncology.

While several studies have shown the efficacy of utilising ctDNA for monitoring of BRAF mutant melanomas [38–45], it remains unutilised in clinical management of melanoma. Here, we review the existing evidence of the clinical validity of ctDNA as a biomarker for metastatic melanoma and critically appraise the challenges ahead for the implementation of this liquid biopsy into melanoma clinical management.

ctDNA as a predictor of response to therapy and prognosis in metastatic melanoma

The majority of studies on ctDNA as a biomarker of disease status in advanced melanoma have been applied to patients treated with BRAF inhibitors [38,39,42,44,46], via monitoring of the singular *BRAF*^{V600} mutation. Notably, these studies have shown that plasma ctDNA levels prior to commencement of BRAF inhibitor therapy correlated with response to therapy [38,39,42,44,46]. Moreover, baseline ctDNA levels were consistently found to be significantly associated with overall response rate (ORR) and progression free survival (PFS) (Table 1) [38–41,43]. In particular, the BREAK-2 study (a phase II trial), aimed at evaluating the safety and clinical activity of the BRAF-inhibitor dabrafenib, showed that high basal ctDNA levels correlated with lower overall response rate and lower PFS to targeted therapy [39]. These results were further confirmed in a large study (N = 836) that include the BREAK-3, BREAK-MB and METRIC clinical trials [41]. Overall, these studies provide sufficient evidence of the predictive value of ctDNA for response to targeted therapy in melanoma patients.

A small number of studies have evaluated the predictive value and dynamics of ctDNA in patients treated with immunotherapies. A study conducted by Lipson et al. [47] showed that the levels of ctDNA correlated with radiological outcomes in two melanoma cases treated with immunotherapy. Gray et al. [38] showed that baseline ctDNA levels predict response to immunotherapy in melanoma patients, and that low basal ctDNA levels were significantly associated with long term clinical benefit. More recently, Lee et al. [48] reported on a longitudinal assessment of ctDNA in metastatic melanoma patients treated with PD1 inhibitors. In this study, ctDNA levels at baseline and early during treatment provided an accurate prediction of tumour response, PFS and OS. Nevertheless, a prospective study of survival in a larger cohort of patients is urgently required to validate the predictive value of ctDNA in melanoma patients treated with immunotherapy.

ctDNA as an indicator of tumour burden to monitor response to treatment

Previous studies in melanoma have demonstrated a significant association between ctDNA levels and tumour burden. Levels of ctDNA were found to significantly correlate with clinical serological markers of disease burden, namely lactate dehydrogenase (LDH), S100 calcium-binding protein B (S100B) and melanoma inhibitory activity (MIA) [40]. However, ctDNA proved to be a more accurate measure due to its tumour specific origin and ability to expand over a dynamic range of 4–5 log scale units [40,42,49]. Conversely, LDH, which is used to stage metastatic melanoma, has a narrow dynamic range of less than 2 log units and is affected by non-specific inflammatory conditions. Thus, ctDNA appears to be a better blood-based biomarker for defining patient tumour status in the clinic.

The ability of ctDNA to accurately reflect tumour burden makes it viable for tracking treatment response in metastatic melanoma patients. Studies have consistently shown that ctDNA levels correlate with imaging scans showing a decrease in tumour burden, in response to targeted therapy [38,42]. For example, Tsao et al. [50] demonstrated that changes in ctDNA levels accurately reflected the contemporaneous disease status of six immunotherapy cases during disease course. Conversely, an increase in ctDNA levels during treatment is an accurate indication of disease progression [38,42]. Gray et al. [38] provided evidence that increasing ctDNA levels can precede radiological evidence of disease progression and acquired resistance to targeted therapy. Finally, a recent study by Wong et al. [45] highlighted ctDNA quantification as a suitable complementary modality to functional imaging for real-time monitoring of tumour burden. Overall, the significant correlation between plasma DNA and disease status suggests that ctDNA can be utilised in the clinic as a complement to imaging scans to aid in the assessment of melanoma tumour dynamics.

In the era of immunotherapy and durable responses, ctDNA surveillance in patients could also provide clinicians with important adjunct information. In particular, for patients who have achieved complete response but are otherwise still undergoing treatment, ctDNA levels can be used to determine if cessation of treatment is warranted. Conversely, for patients that have ceased treatment, continuous monitoring of ctDNA levels, concurrently or in parallel to imaging scans, can help identify emergence of recurrence. Thus, ctDNA could have clinical value in the surveillance of patients that are successfully treated.

Monitoring of clonal evolution and emergence of resistance to targeted therapy

Tumour heterogeneity poses a significant challenge in patients treated with targeted therapy. While the selective pressure of these targeted therapies on dominant clones can result in tumour regression, these therapies can also drive an increase in the population of resistant clones. As ctDNA is derived from apoptosis of any tumour cell, it is valuable for the identification of sub-clonal mutant cell populations in a tumour and/or mechanisms underlying resistance to targeted therapies.

Recent studies have demonstrated the utility of ctDNA for the identification of mutations responsible for driving acquired resistance to BRAF and/or MEK inhibitors in melanoma [38,42]. The *NRAS* mutation, particularly at codon 61 (*p.Q61K/R*), became evident in the ctDNA of patients that developed acquired resistance to BRAF inhibitor treatments, even apparent prior to detection of radiological progression in some cases [38,42]. Mutations in the *NRAS* gene reactivate the mitogen-activated protein kinase (MAPK) pathway, which ensures the survival of melanoma tumours despite substantial BRAF inhibition [5,51]. In addition, recent reports have identified other potential mechanisms of resistance to targeted therapies either during treatment or at disease progression [42,45]. Overall, these results advocate for the implementation of serial ctDNA sampling to identify mutational mechanisms of drug resistance to allow for intervention or change of therapies prior to rapid escalation of tumour burden, which can be irreversible and fatal. Furthermore, ctDNA can also be used delineate those patients with intrinsic resistance, so BRAF inhibitors should be used in caution with these cases. Nevertheless, resistance to BRAF inhibition is not always driven by DNA mutations, and thus methodologies for an accurate and comprehensive assessment of tumour transcriptome are also needed.

It is important to note that for melanoma patients that harbour a BRAF mutation, targeted therapy remains the first-line treatment, particularly in Australia [52]. Given the high propensity of patients to

Table 1
Studies that shows utility of ctDNA as a biomarker of disease status in metastatic melanoma.

Author	Publication date	No. of patients	Stage	Mutations	Method	Analytical sensitivity	Treatment	Diagnostic sensitivity	Associated with or prognostic
Ascierto et al.	2014	91	IV	<i>BRAF</i> ^{V600E/K}	BEAMing	Not reported	Combination Dab + Tra	79–89%	Tumour burden, ORR & PFS
Bettegowda et al.	2014	20	IV	<i>BRAF</i>	BEAMing PCR-Ligation or Safe-SeqS	Not reported	–	85%	Not assessed
Lipson et al.	2014	12	IV	<i>BRAF</i> <i>ckIT</i> <i>NRAS</i> <i>TERT</i>	BEAMing & targeted reseq (TERT)	0.01%	Ipi/BMS-936559	50% (BRAF unbiased)	Not assessed
Sanmamed et al.	2015	20		<i>BRAF</i> ^{V600E}	BioRad ddPCR	0.005%	Vem and Dab	84%	Tumour burden, PFS & OS
Tsao et al.	2015	6	IV	<i>BRAF</i> ^{V600E/K} , <i>NRAS</i> ^{Q61H}	BioRad ddPCR	Not reported	Dabrafenib and MK3475	Not assessed	Not assessed
Gray et al.	2015	48	IV	<i>BRAF</i> ^{V600E/K} , <i>NRAS</i> ^{Q61K,R,L}	BioRad ddPCR	Not reported	Vem, Dab, Combi, Pembro, Nivo and Ipi	73%	ORR & PFS
Gonzales-Cao et al.	2015	22	IV	<i>BRAF</i> ^{V600E}	AS-PCR	0.005%	Vem and Dab	50%	PFS & OS
Santiago-Walker et al.	2015	836	IV	<i>BRAF</i> ^{V600E/K}	BEAMing	Not reported	Dabrafenib and/or Trametinib	88%	ORR, PFS & OS
Girotti et al.	2015	214	II, III and IV	<i>BRAF</i> ^{V600E/K} , <i>NRAS</i> ^{Q61R/K} , <i>PIK3CA</i> ^{E545K}	WES, Targeted re-sequencing	Not reported	Vem, Dab, Combi, Pembro, Nivo and Ipi	Not assessed	ORR & PFS
Chang et al.	2016	31	IV	<i>BRAF</i> ^{V600E/K} , <i>NRAS</i> ^{Q61K,R,L}	BioRad ddPCR	Not reported	–	80%	Not assessed
Knol et al.	2016	38	IIIc, IV	<i>BRAF</i> ^{V600}	Therascreen BRAF RGQ Kit (Qiagen)	Not reported	Combi, BRAF inhibitor	Not assessed	OS
Lee et al.	2017	76	IV	<i>BRAF</i> <i>NRAS</i> <i>KIT</i>	ddPCR	Not reported	Ipi, Pembro and Nivo	Not assessed	PFS & OS
Wong et al.	2017	52	IV	<i>BRAF</i> <i>NRAS</i> <i>TERT</i>	WES dPCR Targeted amplicon (TA) sequencing	0.1% 2%	MAPKi, Immunotherapy	Not assessed	Tumour burden, PFS

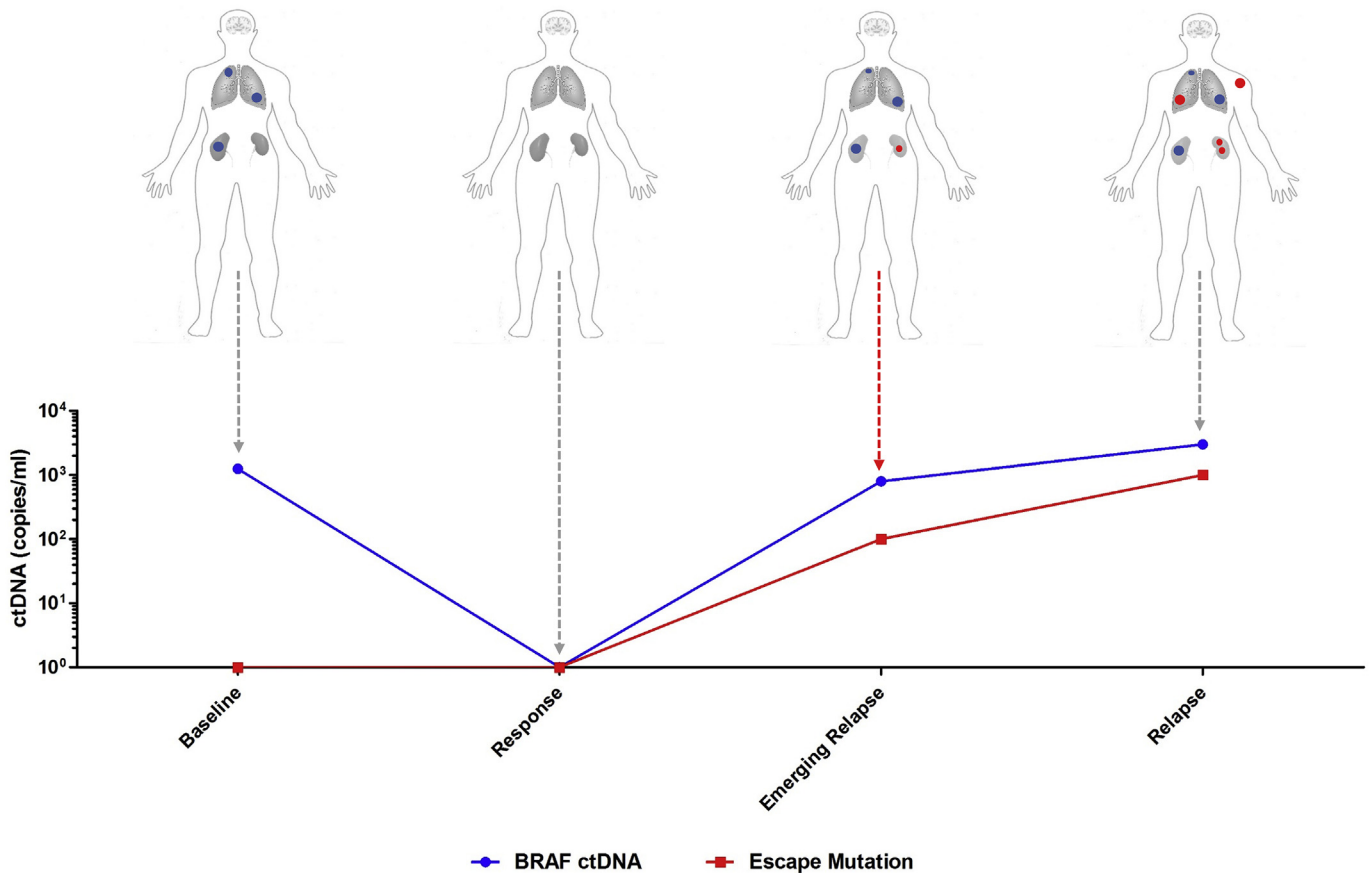


Fig. 1. ctDNA as an indicator of emerging resistance to targeted therapy. Serial ctDNA level measurement can be used to determine the exact point of emergence of acquired resistance to targeted therapy. At the point of emerging relapse, the tumour burden, and consequent ctDNA levels are low, therefore immediate change in treatment strategies may provide optimal clinical benefit in melanoma patients.

develop acquired resistance to BRAF and/or MEK inhibitors, it is crucial to determine the most appropriate time for switching therapy for improved overall survival. Tracking of *BRAF*^{V600} ctDNA levels and surveillance of potential mechanisms of acquired resistance, e.g. *NRAS* mutations, as patients undergo targeted treatment can help identify the period when resistance to targeted therapy emerges (Fig. 1). Modification of treatment at the onset of acquired resistance to targeted therapy, before rampant increase in tumour burden, potentially increases the response rate to subsequent second-line immunotherapy treatment. Previous studies have shown that low tumour burden is correlated with response to immunotherapy [10]. Thus, treatment modification prior to full disease escalation could give patients a better outcome. Nevertheless, ctDNA guided clinical trials are needed to determine the utility of ctDNA as a catalyst for a reactive treatment modification in melanoma.

ctDNA and the epigenetic landscape in melanoma

Aside from mutations, emerging research have shown that tumour-specific gene methylation patterns are detectable in ctDNA of cancer patients [53–59]. Methylation at the promoter region of tumour suppressor genes is considered a primary event in carcinogenesis, thus methylated cfDNA could be a promising biomarker for early detection of cancer.

Previous studies have demonstrated that detection of hypermethylation in the promoter region of genes in ctDNA such as *TFPI2*, *PTEN*, *RARB2*, *RASSF1A* and *MGMT* can significantly discriminate melanoma patients from healthy controls [60–62]. Thus,

methylation studies using cfDNA may be a viable non-invasive diagnostic biomarker for melanoma. Of note, hypermethylated *ESR1*, hypermethylated *AIM1* and hypomethylated *LINE-1* promoters in circulating DNA were found to be unfavourable prognostic markers of melanoma [63,64]. However, the utility of ctDNA for epigenetic studies in melanoma requires further evaluation, particularly in relation to the new treatment options, including early diagnosis, prediction of response, disease monitoring and/or identifying resistance mechanisms.

Detectable DNA hypermethylation in the *RASSF1A* promoter in the serum of melanoma patients has been the most consistently reported epigenetic modification evident in melanoma ctDNA [60–62,65]. This has been associated not only with reduced overall survival [62] but is also predictive of worse response to biochemotherapy [63,65]. In addition, a recent report showed that levels of methylated *RASSF1A* ctDNA are significantly higher in *in situ* and metastatic melanoma patients ($n = 84$) than in healthy controls ($n = 68$), with the level of methylated *RASSF1A* cfDNA being more elevated in metastatic cases than in early stage melanoma [66]. *RASSF1A* methylation has also been reported to be detectable in variety of tumours such as breast [67,68], prostate [67], lung [68,69] and ovarian cancers [70–73]. Thus, the need for identification of ctDNA methylation markers that are specific to melanoma is necessary and useful for diagnostic purposes.

Notably, DNA methylation is not unique to tumour cells. Methylated DNA can be derived from non-tumour cells which can significantly affect the specificity of methylated ctDNA for diagnostic purposes. Studies clearly linking abnormal methylation

profiles to tumour derived circulating DNA are required to confirm the tumour specificity of these methylation profiles. Nevertheless, the preferential presence of methylated DNA in cancer patients may be an indicator of cancer related processes, and could still provide relevant diagnostic and prognostic information.

ctDNA analysis – challenges and new frontiers

ctDNA analysis and methodologies

Detection of ctDNA relies on the identification of tumour specific genetic alterations. However, tumour-specific mutation profiling can be limited by relatively low concentrations of ctDNA. Advances in technology have been aimed at better detecting low-frequency mutations. Current methods to detect mutations include PCR based methods such as BEAMing (beads, emulsion, amplification and magnetics) and droplet digital PCR (ddPCR), which are known to have limits of detection of 0.01 and 0.001% respectively [49,74–76].

BEAMing and ddPCR technologies involve amplification of DNA templates within water-oil droplets followed by quantification. While the sensitivity of these technologies allow for detection of low frequency mutations, pre-identification of gene targets is required. In melanoma, the high frequency of patients that harbours either mutant *BRAF*^{V600} (~50%) or *NRAS*^{Q61} (~20%) enhances the efficacy of these highly sensitive methodologies for ctDNA analysis in the majority of cases. However, melanomas that are wild-type *BRAF* and/or *NRAS*, approximately 30% of patients overall, are more challenging to monitor.

Mutations in the promoter region of the telomerase reverse transcriptase (*TERT*) gene, particularly *TERT*^{C250T} and *TERT*^{C228T}, are present in 50–70% of melanomas [77] and are associated with rapid growing tumours and poor prognosis [78–80]. Detection of *TERT* mutations in plasma are therefore an alternative mutant ctDNA for assessment of melanoma status, as well as a relevant mutation for prognostication [45]. Nevertheless, the fact that these mutations are present in the promoter region provides a significant challenge. Stability of cfDNA is highly dependent on its association with the nucleosome core particle and linker histones, which preferentially protect it from nuclease cleavage [81]. Given the frequent absence of nucleosomes in *TERT* promoter regions, due to its high level of expression in melanomas [82], it is susceptible to enzymatic cleavage, suggesting that mutations in this region may be less represented in ctDNA. This hypothesis highlights the need for that further studies of ctDNA biology prior to its clinical implementation.

It is important to note that while *TERT* promoter mutations are common aberrations in melanoma, these tend to co-occur in tumours with *BRAF* and/or *NRAS* mutations [82,83]. Therefore, ctDNA identification in *TERT*, *BRAF* and *NRAS* wild-type melanomas would require interrogation of multiple additional loci using targeted sequencing of the plasma. Since cfDNA levels range from 1 to 10 ng/ml of plasma in most patients [18], this presents a significant challenge for any library preparation methodology required for sequencing. In addition, current sequencing technologies have error rates greater than the frequency abundance required for ctDNA analysis [84]. Improvements in library preparation, particularly addition of unique identifier barcodes and/or running multiple replicates [85], can increase the sensitivity of these methods. However, these types of analyses are currently restricted to highly specialised laboratories.

ctDNA in the cerebro-spinal fluid (CSF) for monitoring of brain metastasis

A major limitation of ctDNA analysis is its unsuitability as a biomarker for tracking evolution of tumours that metastasise to the

brain [25,86]. Patients with brain tumours often have low and/or undetectable ctDNA, suggesting that the blood-brain barrier may significantly impact the release of tumour DNA into the systemic circulation [25,45,86]. However, studies have shown that ctDNA kinetics can be derived from cerebro-spinal fluid (CSF), allowing assessment of tumour dynamics from metastatic tumours in the brain of patients with melanoma [86,87]. Given the highly invasive nature of repeated lumbar punctures, the clinical utility of this approach would need to be clearly vindicated.

ctDNA analysis as a viable marker of residual disease

The potential role of ctDNA in the clinic may extend beyond merely monitoring disease status of patients with advanced cancers. Plasma DNA analysis may also be exploited for detecting residual disease and/or early relapse in pre-metastatic stages. Recent prospective studies in breast and colorectal cancers have shown that detection of ctDNA in plasma after curative resection, either at post-surgical or serial time points, is significantly correlated with residual disease and eventual recurrence [29,88]. Analysis of ctDNA may elucidate the presence of residual tumour mass that was not eradicated by standard treatment, and thus identify patients at high risk of recurrence.

Detection of residual disease will be particularly useful in high risk patients after surgical excision of the primary melanoma or after lymphadenectomy, given the highly aggressive nature of this cancer. Studies are needed to determine whether ctDNA positivity in melanoma patients post-surgery is indicative of recurrence. However, Bettegowda et al. [18] showed that early stage cancers often present with fewer than 10 copies per 5 ml of plasma. Given that ctDNA levels correspond significantly with tumour burden, the feasibility of ctDNA detection in stage I-II melanoma patients is unclear and requires evaluation. However, screening for ctDNA in stage III and IV melanoma patients after lymph node biopsy, lymphadenectomy or surgical resection of a metastasis could distinguish those patients with residual disease needing further adjuvant therapies.

ctDNA as a predictor of response to immunotherapy

As indicated above, recent clinical advances with immune checkpoint inhibitors, anti-CTLA4 anti-PD1–PDL1, have successful activity in treating melanoma [13,47]. Nevertheless, only a small cohort of patient respond to immunotherapy, which underscores the need for biomarkers that can be used to predict response to immune blockade inhibitors.

Mutations, genetic rearrangements, insertions and deletions have the capacity to encode novel, cancer specific neo-antigens. Activation of T-cells is initiated by the recognition of novel peptides presented as human leukocyte antigens (HLA). Thus, a cancer mutation can result in the formation of a novel antigenic peptide recognisable by the surveillance immune system. UV induced melanomas with a higher mutational burden, irrespective of whether these are driver mutations, may present a larger number of novel neoepitopes. As immune checkpoint inhibitors essentially unleash existing anti-tumour T cell responses, highly mutated tumours are more likely to be sensitive or more responsive to immune checkpoint blockade. Thus, high mutational load may be of clinical value for patients treated with immunotherapy.

In the last few years, studies have shown that tumour mutational load can be a relevant predictive biomarker for monitoring responses to immunotherapy, particularly for a PD-1 inhibitor in NSCLC [89,90], and for CTLA4 inhibition in metastatic melanoma [81]. Melanomas commonly have the highest mutational load of all human tumours [91], which might explain their high response

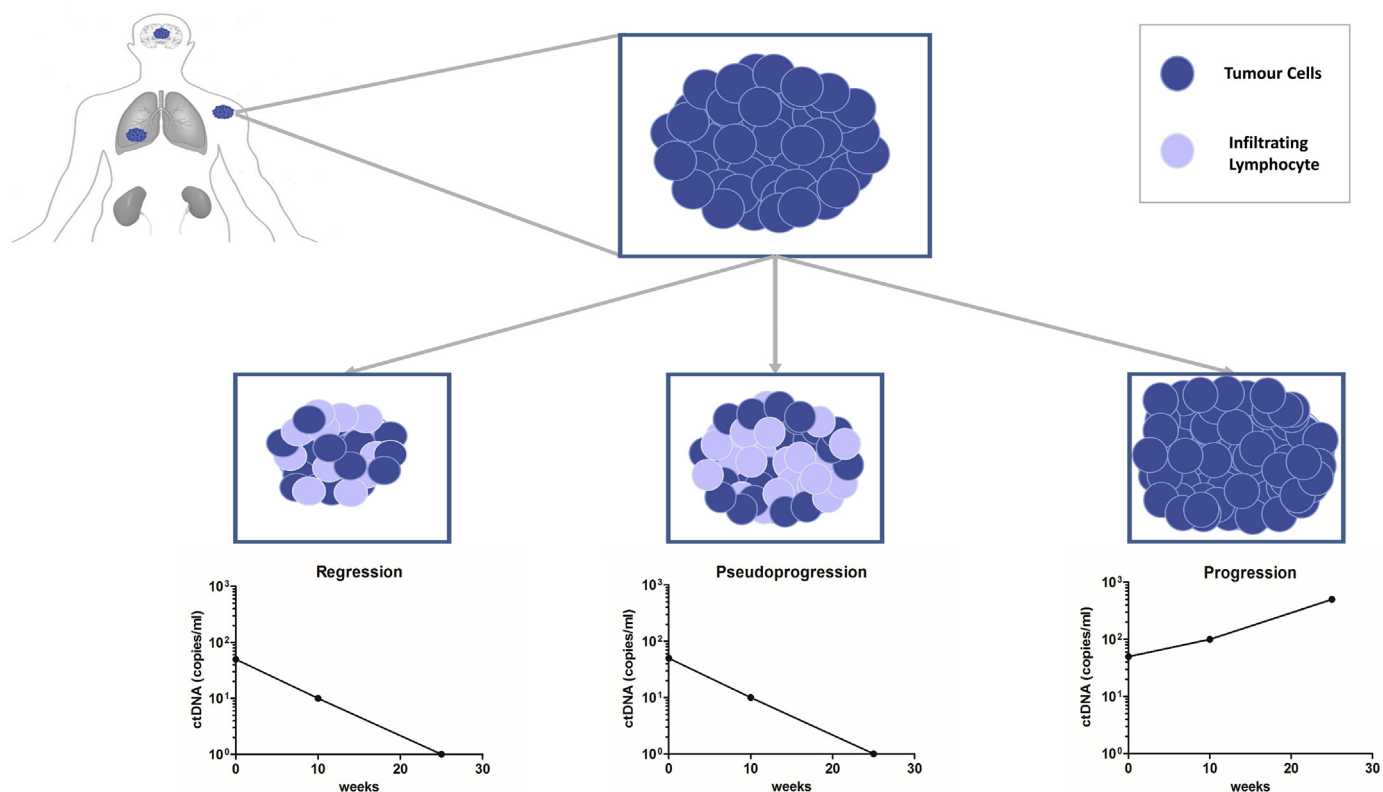


Fig. 2. Schematic representation of pseudoprogression in patients undergoing immunotherapy. ctDNA levels measurement can be used to distinguish between true progression (high) and pseudo-progression (low).

rates to anti-PD1 therapy (~30–40%) [92] and anti-CTLA4 monotherapy (15%) [12,13,81,93]. Next-generation sequencing of the tumour tissue is currently the gold standard for assessment of tumour mutational load. However, tissue biopsies can be highly invasive or the tumour may be inaccessible. Recently, a study showed that ctDNA can comprehensively capture spatial genomic heterogeneity of metastatic melanoma [45]. Therefore, ctDNA presents as an easily accessible and suitable surrogate tumour source for mutational load analysis to predict response to immunotherapy. Given the recent increase in sensitivity of next generation sequencing, it is feasible that ctDNA will be used in the future to estimate tumour mutational and neoepitope load, as a way predicting response to immune checkpoint inhibiting therapies.

While immunotherapy provides significant clinical benefit in patients that respond to treatment, delayed tumour regression and pseudo-progression, which is characterised by infiltration of lymphocytes into the tumour giving the appearance of an enlarged tumour in radiological scans, significantly complicate evaluation of patient response. As ctDNA levels correspond closely with tumour burden, ctDNA levels provide a measure that allows distinction between regressing, progressing and pseudo-progressing tumours (Fig. 2). While it is apparent that ctDNA monitoring provides reliable and highly specific information regarding disease status of melanoma patients, further analysis of the kinetics of response in patients undergoing immunotherapy is needed to confirm its utility as a companion to radiological assessments.

Conclusion

Overall, it is clear that ctDNA has significant clinical value both as a source of genetic material for mutational analyses and as a prognosticator of response to therapy. Its non-invasive nature,

accessibility and ability to accurately reflect disease burden also makes it a particularly reliable biomarker for the surveillance of melanoma patients during treatment course. In addition, emerging studies in epigenetics and residual disease suggest that ctDNA analysis could be a suitable diagnostic and prognostic marker of melanoma. As the goal of therapy in metastatic melanoma shifts from disease stabilisation to longitudinal complete response, ctDNA can therefore be exploited to provide a real-time assessment of patient status and, in cases of emerging relapse, be a catalyst for a reactive approach to treatment modification based on the biological changes observed. Nevertheless, clinical trials that looks at patient outcome as a result of ctDNA-guided clinical decisions are required before ctDNA can be successfully established as a melanoma-specific biomarker in clinical practice.

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Conflicts of interest statement

The authors declare no conflicts of interest.

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