Annals of Oncology Advance Access published July 25, 2014

review

Annals of Oncology 00: 1–7, 2014
doi:10.1093/annonc/mdu207

Circulating tumour cells as tumour biomarkers in melanoma: detection methods and clinical relevance

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Received 1 February 2014; revised 24 April 2014; accepted 5 May 2014

Circulating tumour cells (CTCs) are cells of solid tumour origin detectable in the peripheral blood. Their occurrence is considered a prerequisite step for establishing distant metastases. Metastatic melanoma was the first malignancy in which CTCs were detected and numerous studies have been published on CTC detection in melanoma at various stages of disease. In spite of this, there is no general consensus as to the clinical utility of CTCs in melanoma, largely due to conflicting results from heterogeneous studies and discrepancies in methods of detection between studies. In this review, we examine the possible clinical significance of CTCs in cutaneous, mucosal and ocular melanoma, focusing on detection methods and prognostic value of CTC detection.

Key words: circulating tumour cells, melanoma, review, RT-PCR, ISET, immunomagnetic enrichment

introduction

Circulating tumour cells (CTCs) are cells of solid tumour origin detectable in the peripheral blood. They are considered a prerequisite step in establishing distant metastases. CTC number has been shown to correlate with clinical outcome in several cancers including breast, prostate, colon and lung cancer [1–5]. In 2011, the US Food and Drug Administration approved the semi-automated and robust immunomagnetic enrichment and staining system; CellSearch™ (Veridex, Raritan, NJ), as an aid for monitoring metastatic breast, colorectal and prostatic carcinomas.

Metastatic melanoma was the first malignancy in which CTCs were detected. Smith et al. [6] reported the presence of melanoma cells in the peripheral blood of patients with metastatic cutaneous melanoma by identifying melanoma CTCs through mRNA transcript detection of specific markers. Several studies have been published on CTC detection in melanoma at various stages of disease. The studies reported vary considerably with respect to patient populations, timing of sampling, method of CTC detection, and assay quality control measures. There is no general consensus as to the clinical utility of CTCs in melanoma, largely due to conflicting results in studies using differing approaches, the heterogeneity of melanoma CTCs and the scarcity of CTC analyses within prospective clinical trials.

In this review, we examine the clinical significance of CTCs in cutaneous, mucosal and ocular melanoma, focusing on detection methods, prognostic, and predictive value of CTC detection.

materials and methods

We carried out a literature search using PubMed and ISI Web of Knowledge. The key words variably combined included 'circulating tumour cells', 'CTC', 'melanoma', 'circulating melanoma cells', 'CMC' and 'prognosis'. Only studies published in English from January 2000 till December 2013 in peer reviewed journals were considered. For cutaneous melanoma, only studies with >100 patients were reviewed for the assessment of CTC clinical significance.

methods of CTC detection in melanoma: an overview

Methods of isolation and detection exploit unique properties of CTCs including (i) expression of melanocyte-specific nucleic sequences (mRNA-based strategy), (ii) expression of melanocyte-specific proteins (protein-based strategy) and (iii) distinctive physical properties (size-based strategy) (Table 1). Normal healthy volunteer blood cohorts have been tested concurrently with patient samples in a number of studies. Healthy volunteer blood was either negative for melanocytic cells or melanocytic cell markers or detected below the threshold set in cancer patients.

Detection of CTCs by analysis of mRNA melanocyte-specific transcripts with reverse transcriptase-PCR (RT-PCR) has been explored by several investigators over the past 22 years. Different melanocytic markers have been employed with tyrosinase, Melan-A/Mart-1, gp100 and the MAGE proteins being most

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Table 1. Overview of CTC detection methods in melanoma

Strategy	Techniques	Markers	Ref.
mRNA-based strategy	RT-PCR	Tyrosinase, Melan-A/Mart-1, S100, MAGE-family, gp100, CD146, p97	[7–23]
Protein-based strategy	Immunomagnetic enrichment	Negative selection (HMB-45, Melan-A/Mart-1); positive selection (MCSP, CD146)	[24–27]
Sized-based strategy	ISET	HMB-45, Melan-A/Mart-1, S100	[28, 29]

commonly used [30, 31]. However, results from different laboratories have been contradictory [32]. Some of the discrepancies seen can be explained by differences in methodology and quality assurance, but insufficient sample size and heterogeneity of patients with respect to stage and treatment might have further contributed to conflicting results. Limitations of using PCR to detect CTCs include: (i) amplification of non-specific products, (ii) lack of thoroughly validated protocols for sample processing, RNA-preparation, cDNA synthesis and PCR conditions as well as (iii) lack of rigorous quality control measures on a per-sample basis. The lack of validated methods increases the possibility of variations in sensitivity, specificity and also the potential of non-specific amplification products due to protocol and primer design and carry-over contamination. New methods for CTC isolation have been devised to combat these limitations and aim to segregate and characterise intact tumour cells.

Immunomagnetic enrichment of rare CTCs followed by flow cytometry or immunohistochemistry for visualisation, quantification, and characterisation has been extensively used for epithelial cancer CTC detection. Melanoma CTC detection has been established, utilising two enrichment strategies: either negative enrichment by immunomagnetic CD45 depletion of the leukocyte fraction [24, 33] or positive selection of CTCs through antibody-coated magnetic beads against melanocyte-specific surface markers [25, 34, 35]. CTCs were subsequently identified by molecular and/or morphological analysis by means of flow cytometry or microscopy. Enrichment protocols and CTC detection differed in enrichment strategies and in markers used for detection. Published studies to date have been small and there is insufficient data on reproducibility of different methods. However, a specific kit for melanoma has recently been developed for use on the CellSearch platform. This melanoma CellSearch CTC kit uses CD146 (MelCAM) for CTC capture. Captured cells are then visualised using multi-marker fluorescent staining against the melanoma-associated chondroitin sulphate proteoglycan (MCSP) to detect melanoma CTCs. Co-staining with CD45 and CD34 is employed to differentiate CTCs from leukocytes and endothelial cells, respectively, and DAPI is used to visualise the nucleus. Stained cells are presented on the CellTracks Analyser for manual review and enumeration.

Both immunomagnetic-based enrichment techniques and RT-PCR detection of melanocytic markers are limited by the possible lack or loss of specific melanocytic antigens during tumour progression [36]. Size-based filtration devices could potentially overcome this limitation. The isolation by size of epithelial tumour cells (ISET) is a direct method for CTC identification, which has been applied in various epithelial cancers. Tumour cells are collected by filtration because of their large size in comparison with peripheral blood leukocytes and, after staining for specific markers, cells are identified by immunohistochemistry and/or molecular-genetic analyses. The method has been recently validated for melanoma [28, 37] and is being further investigated in large prospective clinical trials.

CTC in cutaneous AJCC (American Joint Committee on Cancer Care) stage I-III melanoma

Nine studies qualified for further analyses (Table 2). Three studies enrolled only stage III patients [10, 15, 16] and one study [12] focused on stage I–II patients. Except for Hishimoto et al. [16] multiple samples were drawn starting from the time of diagnosis of the considered disease stage or from a time point during follow-up. All the studies investigated presence of CTCs by detection of melanocytic transcripts with RT-PCR in either whole blood or peripheral blood mononuclear cells (PBMCs). All studies, bar Hoshimoto et al, [16], included tyrosinase as a melanocytic marker. Brownbridge et al. [8], Garbe et al. [9], Schimdt et al. [12] and Fusi et al. [15] used Melan-A/Mart-1 as an additional marker and Scoggins et al. [13] added MAGE3 and gp-100. Hoshimoto et al. [16] used a multi-marker assay which included Melan-A/Mart-1, MAGE3 and GalNAc-T. The sensitivity of the various assays differed; generally a ≥2 marker assay was more sensitive compared with detection of tyrosinase alone. Whole blood was preferable to PBMCs to avoid potential loss of melanoma cells after density gradient isolation or red blood cell lysis [17].

There was great variability in the rates of CTC detection in the different studies as reported in Table 2. Proebstle et al. [7] detected CTCs in 16% of patients with stage I–III melanoma whereas Brownbridge et al. [8] reported a detection rate of around 80% in a study with a similar patient population and disease stages. Schmidt et al. [12] found tyrosinase and/or Melan-A/Mart-1 transcripts in 142 of 236 (60%) patients with stage I and II cutaneous melanoma whereas Voit et al. [11] observed a lower detection rate (45%) in a cohort of patients of stage II and stage III melanoma in a study with more samples per patient, similar design but using only tyrosinase as a marker. In the other two studies with stage II and III melanoma [9, 13], the detection rate was lower (15%). In studies including only stage III patients, detection rates ranged from 14% [13] to 49% [9]. The highest detection rate of 80% reported by Brownbridge et al. [8] appears high raising doubts about the specificity of the assay while the detection rates of 10%–15% of other studies [7, 9, 13] suggest that the assay may not be sensitive enough to predict relapse in high-risk patients.

Multiple sampling and longer follow-up may raise the number of positive samples. Fusi et al. [15] showed that, in stage III

Table 2. Studies on CTC detection by RT-PCR evaluation of melanocytic markers in stage I–III cutaneous melanoma

Ref.	No. of patients		Stage I, II, III		Multiple sampling (from)	Detection rate (%)	Median follow-up (months)	Outcome	Prognostic significance
Proebstle *et al.* [7]	188	106	56	26	Yes (follow-up)	16.1% (30/188)	36	DFS	Yes (only stage III)
Brownbridge *et al.* [8]	262	91	86	85	Yes (follow-up)	80.5% (211/262)	NR	–	NE
Garbe *et al.* [9]	296	–	167	129	Yes (follow-up)	13.8% (41/296)	19	OS	No
Osella-Abate *et al.* [10]	110	–	–	110	Yes (surgery)	49.0% (54/110)	20	DFS	Yes[a]
Voit *et al.* [11]	111	–	78	33	Yes (follow-up)	45.1% (50/111)	75	DFS	Yes
Schmidt *et al.* [12]	236	157	79	–	Yes (surgery)	60% (142/236)	66	DFS OS	No
Scoggins *et al.* [13, 14]	817	353	266	207	Yes (surgery)	14.0% (115/817)	37	DFS DMFS OS	Yes[a] (only stage III)
Fusi *et al.* [15]	299	–	–	299	Yes (surgery)	36.5% (109/299)	43	DMFS	Yes[a]
Hoshimoto *et al.* [16]	320	–	–	320	No (only post-surgery)	33.8% (108/320)	51	DMFS DFS	Yes

[a]Only including follow-up samples. Baseline sample were not associated with prognosis.
NR, not reported; NE, not evaluated; DFS, disease-free survival; DMFS, distant metastases-free survival; OS, overall survival.

melanoma patients, the fraction of samples positive for ≥1 melanocytic maker was 5.7% at baseline but this subsequently rose to 36.5% during follow-up. A similar trend was observed by Osella-Abate et al. [10].

Hashimoto et al. [16] collected blood from patients with stage III melanoma at baseline only after complete lymphadenectomy. They employed a three-marker PCR to detect CTC. Around 35% of patients were positive for ≥1 marker whereas the detection rate dropped to 10% when CTC positivity was defined as detection of ≥2 markers. Only the later definition correlated with clinical outcomes.

What is clear from these studies is that detection rates are influenced by many factors including study design, sensitivity and specificity of the method, sampling time, sample per patient, markers used and numbers of markers analysed, definition of positivity etc. There is a trend to increased detection in multiple sampling and when more markers are used.

Taking this into account the prognostic significance of CTC detection in stage I–III melanoma was evaluated in eight of the nine studies (Table 2). Six studies found CTC detection to be correlated with early recurrence, shorter disease-free survival (DFS) or shorter overall survival (OS) [7, 10, 11, 13–16]. Specifically in the sub-population of patients with stage I and II disease only Voit et al. [11] demonstrate an association between CTC and outcome. However, the study by Voit et al. [11] had longer follow-up (6.3 years) and higher relapse rate (28% for stage II patients) compared with the other studies.

The patient population in which CTC detection may have the greatest clinical utility are stage III patients. The role of CTCs as a prognostic factor for relapse and a predictive factor for response to adjuvant therapy has been evaluated in three prospective clinical trials. Scoggins et al. [13, 14] assessed 820 patients who underwent sentinel node biopsy before subsequent lymphadenectomy in a multicentre study (Sunbelt Melanoma Trial) for presence of CTC in serial sampling starting from time of sentinel node biopsy. Two hundred and seven (25%) patients had a stage III melanoma. RT-PCR was carried out on PBMCs from 820 patients using four markers: tyrosinase, Melan-A/Mart-1, MAGE3 and gp-100. One hundred and fifteen (14%) had evidence of ≥1 RT-PCR marker at some point during follow-up. Stage III melanoma patients with a positive PCR test for >1 marker at any time point showed worse DFS ($P = 0.006$) and OS ($P = 0.0012$) compared with patients with only one positive marker. Stage III melanoma patients with a positive PCR test for tyrosinase showed significant worse DFS ($P = 0.0007$) compared with patients with no positive markers. The finding that >1 positive marker was significant for survival was of questionable clinical relevance as it applied to only seven stage III patients. Fusi et al. [15] evaluated the prognostic role of CTC by serially testing blood for tyrosinase and Melan-A/Mart-1 transcripts in a subset of patients enrolled in EORTC 18991 phase III trial, comparing pegylated interferon-α2b with observation. Of the 299 patients enrolled, 109 (36.5%) were positive for CTC, defined as ≥1 positive sample for tyrosinase or Melan-A/Mart-1. RT-PCR result (positive versus negative) at any given time point had no prognostic impact on subsequent distant metastasis-free survival whereas Cox time-dependent analysis evaluating prognostic significance of a sample positive for CTC at baseline or during follow-up indicated a significantly higher risk of developing distant metastasis in patients with a positive sample with a

hazard ratio (HR) of 2.23 [95% confidence interval (CI) 1.40–3.55; $P < 0.001$]. Adjuvant PEG-IFN-α2b had a positive impact on relapse-free survival in the whole population of stage III patients enrolled in the EORTC18991 study. RT-PCR results were however not predictive for treatment effect in multivariate analysis, possibly due to the relatively low number of patients enrolled in the substudy. Hashimoto et al. [16] evaluated stage III patients' DFS after complete lymphadenectomy before entering into a randomised adjuvant vaccine programme. Samples were drawn only once post radical surgery and CTCs were detected using a multi-marker RT-PCR. No correlation between presence of CTCs and established prognostic variables was found. The presence of ≥2 positive markers was significantly associated with shorter DFS survival with a HR of 2.13 (95% CI 1.20–3.76, $P = 0.009$) in a multivariate analysis. This is the only study to date to show an association between baseline CTC status and outcomes.

Taken together these data suggest that CTCs may be prognostic in patients with stage IIII melanoma. This cohort of patients therefore warrants further investigations with new detection platforms.

CTC in cutaneous AJCC (American Joint Committee on Cancer Care) stage IV melanoma

CTC detection in stage IV melanoma has been evaluated in pooled studies across all stages of disease that recruited small numbers of stage IV patients. Only three studies confined to stage IV disease met the criteria for further evaluation (Table 3).

Hoshimoto et al. [18] evaluated CTC detection by RT-PCR using the markers MART1, MAGE A3 and PAX3 in 244 patients recruited to the MMAIT-IV study. Patients underwent metastectomy followed by randomisation to either vaccine treatment or placebo. CTC detection was carried out before surgery and at 1 and 3 months after surgery. At least one marker was detected in 54.1% of all patients at baseline; 32% had one marker, 19.2% had two markers and 2.9% had three markers detectable. Detection of ≥1 marker at baseline was an adverse prognostic factor by multivariate analysis for both DFS (HR = 1.64, 95% CI 1.19–2.24, $P = 0.002$) and OS (HR = 1.53, 95% CI 1.05–2.24, $P = 0.028$). For the 214 patients who had serial CTC detection during follow-up post metastectomy; percentage CTC positivity at baseline, 1 and 3 months post-surgery did not differ significantly and CTC detection (i.e. detection of any marker) at any time point was associated with inferior DFS (HR = 1.91, 95% CI 1.11–3.30, $P = 0.020$) and OS (HR = 2.57, 95% CI 1.23–5.36, $P = 0.012$) by multivariate analysis at 1, 2 and 3 years. Treatment received (vaccine or vaccine plus BCG) had no impact on either CTC detection or clinical outcome.

Quaglino et al. [19] used tyrosinase RT-PCR for CTC detection in 200 stage IV patients; 149 on medical treatment and 51 post metastectomy. CTC evaluation was carried out at baseline (defined as before medical treatment or post metastectomy) and up to nine other time points. No CTCs were detected in the surgical patients at baseline and 54.9% of these remained negative throughout. 72.3% of the stage IV patients with measurable disease at study enrolment had detectable CTCs. All 149 patients with stage IV disease underwent chemotherapy (CT) or combination CT and immunotherapy (CT-I) and 34 of the 51 patients post metastectomy had adjuvant CT or combination CT-I. Three patient groups on medical treatment were defined during follow-up: patients who were negative throughout, positive throughout or had a succession of positive and negative samples post enrolment. Changes from positive to negative CTC detection were associated with response (assessed by WHO criteria) in patients with measurable disease. New metastases in all patients were associated with positive CTC detection at ≥1 time point, i.e. positive throughout or negative and then positive at any time point.

Multivariate analysis for CTC detection at baseline and outcome was significant; time to progression (TTP): HR = 1.45, 95% CI 1.00–2.12, $P = 0.046$ and OS: HR = 1.57, 95% CI 1.05–2.36, $P = 0.024$.

Patients with detectable CTCs during follow-up had inferior TTP (HR = 3.60, 95% CI 2.51–5.16, $P < 0.001$) and OS (HR = 4.83, 95% CI 3.49–6.68, $P < 0.001$) by multivariate analysis. CTC positivity at any time point was also associated with development of new metastatic sites in surgical patients; HR = 7.05, 95% CI 4.59–10.83, $P < 0.001$ and progression of existing lesions in stage IV patients; HR = 3.13, 95% CI 2.21–4.43, $P < 0.001$, OS: HR = 3.20, 95% CI 2.14–4.80, $P < 0.001$. Taken together these results indicate that tyrosinase may be useful in monitoring disease i.e. as an assessment of disease burden at any time point. This measure also appears to have independent prognostic significance.

Using the CellSearch platform, Khoja et al. [25] showed that detection of ≥1 CTC in 101 patients with metastatic or inoperable stage III disease was an adverse prognostic factor in both univariate and multivariate analyses. The range of CTC number/7.5 ml blood at baseline was 0–36 (mean 2, median 0), 40% had ≥1 CTC and the optimum CTC cut-off as defined in previous studies, was 2. Patients with <2 CTCs had significantly longer median OS than patients with ≥2 CTCs (7.2 versus 2.6 months, HR = 0.43, 95% CI 0.22–0.81, log-rank test $P = 0.009$). Forty-five patients had serial CTC enumeration during medical treatment.

Table 3. Studies on CTC detection in stage IV cutaneous melanoma

Ref.	No. of patients	Multiple sampling	Method	Markers	Outcome	Prognostic significance
Hashimoto *et al.* [18]	214	Yes	RT-PCR	Mart-1; Mage-3; Pax3	DFS, OS	Yes
Quaglino *et al.* [19]	200	Yes	RT-PCR	Tyrosinase	TTP, OS	Yes
Khoja *et al.* [25]	101	Yes	CellSearch	MelCAM; MCSP; CD45	OS	Yes[a]

[a]Prognostic significance for all CTC cut-off levels with greatest specificity and sensitivity at the CTC >2 cut-off level.

MCSP, melanoma-associated chondroitin sulphate proteoglycan; NE, not evaluated; DFS, disease-free survival; TTP, time to progression; OS, overall survival.

Changes in CTC counts reflected response as assessed by CT scan; those with increases in CTC count progressed and those with decreases in CTC count responded to treatment. The prognostic significance of CTC number recorded before and at any time point during treatment of all 45 patients confirmed a shorter OS for patients with ≥2 CTCs at any time point during treatment (median OS 7 versus 10 months, HR = 0.34, 95% CI 0.14–0.81, $P = 0.015$). Thus, CTCs as detected by the CellSearch platform may be prognostic and pharmacodynamic in metastatic melanoma. The patients recruited to this study were a heterogeneous group and further studies in clearly defined patient populations are needed to validate these preliminary findings.

CTC in mucosal melanoma

No published studies on CTC detection in mucosal melanoma were found. Our own data using the CellSearch and ISET platforms demonstrates that CTCs are detectable in this melanoma subtype [38]. CellSearch detected CTCs in 6 of 11 (55%) mucosal melanoma patients (range 1–125, mean 21 median 1). ISET detected CTCs in 10 of 12 (83%) mucosal melanoma patients (range 1–26, mean 4 median 2).

CTC in ocular melanoma

Ocular and cutaneous melanocytes have a similar embryological origin in the neural crest, but their malignant counterparts display different biological behaviours. Ocular melanoma metastasises by haematogenous routes because of the absence of draining lymphatics, whereas cutaneous melanomas often spread to regional lymph nodes first. [39]. Due to early haematogenous spread uveal melanoma is a good model to estimate the significance of CTC in predicting distant metastases and to better understand CTC biology. Ocular and cutaneous melanoma share similar melanocytic markers and the platforms for CTC detection optimised for cutaneous melanoma have been used in ocular melanoma.

Studies of CTC estimation in ocular melanoma are limited. Of the eight reviewed, four employed a RT-PCR-based detection method, two immunomagnetic enrichment, one the ISET system and one used ISET and RT-PCR (Table 4). Seven studies were carried out in non-metastatic patients and five assessed the prognostic significance of CTC (including stage IV patients).

Callejo et al. [20] conducted a study in 30 non-metastatic uveal melanoma patients and evaluated CTC detection in blood samples drawn every 3 months. The majority of patients had ≥4 evaluable samples. The detection rate of tyrosinase and Melan-A/Mart-1 transcripts in peripheral blood increased by multiple sampling and CTC were identified in 29 of 30 patients. Their prognostic significance was not assessed because of short follow-up time. Boldin et al. [21] reported on detection of tyrosinase transcripts with RT-PCR in 41 patients with non-metastatic disease. PCR was carried out in patients before and after treatment of the primary tumour. Sixty-nine percent of the PCR samples with a positive result before therapy revealed a negative result after therapy. A positive PCR was significantly associated with poorer 5-year survival ($P = 0.023$). Pinzani et al. [28] came to similar conclusions in a similar study in which CTC were detected both by RT-PCR (tyrosinase transcripts) and by ISET. A significant correlation was observed between mRNA tyrosinase levels and DFS ($P < 0.05$) and OS ($P < 0.05$). Furthermore, the mRNA tyrosinase levels correlated to the number of CTC isolated with the ISET system. In a larger study, Schuster et al. [22] showed that the presence of tyrosinase or MelanA/Mart-1 transcripts was an independent prognostic factor in patients with high-risk primary uveal melanoma. Blood samples from 110 patients were collected at the time of the primary treatment and during follow-up. In multivariate analysis, patients with ≥1 positive sample for CTC had a significantly higher risk of developing distant metastases (HR of 7.3) and >22 times the risk of dying from melanoma. Detection of one CTC was significantly associated with TTP ($P < 0.001$) and disease-specific survival ($P < 0.001$). Similar results were obtained by the same group in a later study with a similar design, but in a cohort of patients with metastatic uveal melanoma [23]. Multivariate analyses revealed CTC detection as independent prognostic factors for DFS (HR 2.2) and OS (HR 4.0).

In a recent publication, Mazzini et al. [29] evaluated the prognostic significance of CTC detected by ISET in a cohort of 31 consecutive non-metastatic uveal melanoma patients. Patients with >10 CTCs/10 ml blood had significantly shorter DFS

Table 4. Studies on CTC detection in ocular melanoma

Ref.	No. of patients	Disease	Method	Markers	Outcome	Prognostic significance
Callejo *et al.* [20]	30	Non-metastatic	RT-PCR	Tyrosinase	–	NE
Boldin *et al.* [21]	41	Non-metastatic	RT-PCR	Tyrosinase Mart-1/MelanA	OS	Yes
Pinzani *et al.* [28]	41 (16)[a]	Non-metastatic	RT-PCR; ISET	Tyrosinase	PFS, OS	Yes
Schuster *et al.* [22]	110	Non-metastatic	RT-PCR	Tyrosinase Mart-1/MelanA	TTP, DSS	Yes
Ulmer *et al.* [27]	52	Non-metastatic	IM	MCSP	–	N.E.
Suesskind *et al.* [26]	81	Non-metastatic	IM	MCSP	–	No[b]
Schuster *et al.* [23]	68	Metastatic	RT-PCR	Tyrosinase Mart-1/MelanA	PFS, OS	Yes
Mazzini *et al.* [29]	31	Non-metastatic	ISET	Tyrosinase Mart-1/MelanA, S100	DFS, OS	Yes

[a]Patients evaluated also with ISET.
[b]No correlation with development of distant metastases.
IM, immunomagnetic-based enrichment; MCSP, melanoma-associated chondroitin sulphate proteoglycan; NE, not evaluated; PFS, progression-free survival; TTP, time to progression; DFS, disease-free survival; DSS, disease-specific survival; OS, overall survival.

($P = 0.012$) and OS ($P = 0.017$). CTC >10/10 ml blood were associated with known prognostic clinico-pathological characteristics in ocular melanoma (tumour basal diameter, tumour height, tumour–node–metastasis).

Two published studies [26, 27] used immunomagnetic enrichment to isolate CTCs from patients with primary uveal melanoma. Cells were positively selected from 50 ml blood for the expression of MCSP and visualised by immunocytologic staining. CTC detection was investigated before and after treatment of the primary tumour. CTC detection was associated with worse prognostic factors, but did not correlate with outcome.

In summary, all studies of CTC detection based on RT-PCR in ocular melanoma appear concordant in indicating a prognostic or predictive role of CTCs in this disease, whereas the study employing an immunomagnetic method failed to show any correlations with outcome. However, larger studies of emerging detection platforms are required to better define their significance in ocular melanoma.

conclusions

Detection of CTC by RT-PCR has been widely investigated in melanoma in the past 20 years. Taken as a whole, the studies reviewed here indicate a prognostic and possible pharmacodynamic value to CTC detection by RT-PCR albeit with several limitations as described above and before the advent of efficacious therapeutic agents. However, the real breakthrough in the CTC field emerged in the past 8 years with the development of robust technology to identify intact cells. The current gold standard for epithelial cancer is the semi-automated CellSearch system which allows segregation and phenotypic characterisation of CTCs. Its use in melanoma has been limited so far and its validation is currently ongoing in large prospective trials with new therapeutic agents.

In addition to enumeration and phenotypic characterisation of CTCs, isolation of intact CTCs allows genotypic characterisation of CTCs which could be relevant in monitoring tumour biology. While enumeration of CTCs or levels of mRNA transcripts could be of value in stratifying patients in clinical trials, an important clinical question is whether CTCs can be used as a 'liquid biopsy' to investigate tumour mutational profiles, clonal evolution under the pressure of current molecularly targeted therapies and mechanisms of drug resistance. All the studies described above were conducted before the advent of both targeted agents and effective immunotherapy in melanoma. Targeted therapy is usually associated with a rapid response rate, but the development of resistance is more or less universal. The identification of further novel mutations and a more accurate mutation profile of individual melanomas may lead to more effective therapy either with single agents or a combination of targeted therapies. Primary tumour is currently used for mutation profiling yet this does not reflect tumour evolution. Furthermore, a single tumour biopsy may not accurately reflect tumour sub-clones and in particular those with the greatest metastatic potential. CTCs may more accurately reflect the disease state within a patient and provide a more accurate assessment of therapeutic drug targeting and efficacy potentially predicting early resistance. Single CTC molecular profiling is possible and preliminary studies have shown discrepancies in BRAF and cKIT status between primary melanomas and CTCs [40]. The considerable challenge remaining is to determine the extent of CTC heterogeneity in melanoma patients; this challenge will be best addressed using marker independent enrichment technologies that can be readily deployed upstream of CTC isolation for molecular profiling.

A number of new platforms are under evaluation for CTC detection across a range of tumour types. Many of these address the limitations of established methodologies specifically by utilising multiple markers for CTC detection, allowing single cell or cell cluster isolation for downstream genomic applications and limiting cell loss during processing. The Amnis Imagestream platform combines flow cytometry with microscopy and imaging [41]. It can image 1000 cells per second acquiring up to 12 images per cell to detect simultaneous phenotypic and functional marker expression. Cells are selected for review by automated software analysis. The first-generation CTC chip developed at the Massachusetts General Hospital Centre for Engineering in Medicine and the Massachusetts General Cancer Centre used a microfluidic EpCAM coated plate to capture CTCs which were then stained and read using fluorescence microscopy. The capture and detection markers used were adaptable and CTC mutations could be assessed downstream [42]. This technique was successfully used to detect EGFR mutations in CTCs from non-small-cell lung cancer patients on treatment [43]. The third-generation CTC ichip is currently undergoing validation testing [44]. The use of one platform over another will depend on a number of factors including the sensitivity required; CTC detection in early-stage disease (where CTCs are rare), in contrast to stage IV disease and the clinical purpose of CTC detection; as a prognostic or pharmacodynamic or predictive biomarker.

CTC detection remains investigational in melanoma. There remain more questions than answers with respect to the optimal technologies for their detection and analysis, their biological significance and their clinical utility. Results from ongoing prospective studies in homogenous patient groups treated with new standards of care and using standardised protocols for CTC detection are awaited to more accurately define the role of CTCs as a novel biomarker.

disclosure

The authors have declared no conflicts of interest.

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