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Clinical Utility of a Circulating Tumor Cell Assay in Merkel cell carcinoma

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Abstract

BACKGROUND—Quantitation of circulating tumor cells (CTCs) has utility in managing breast, colon and prostate carcinomas.

OBJECTIVE—Determine whether a commercially available CTC assay provides prognostic information in MCC and/or insight into treatment responses.

METHODS—We analyzed CTCs in 52 specimens from 34 MCC patients.

RESULTS—The presence of CTCs correlated with extent of disease at blood draw (p=0.004). Among 15 patients with regional nodal disease, CTC-negative patients had 80% disease-specific survival at 2 years after the test, versus 29% for CTC-positive patients (p=0.015). Among the entire cohort, those without CTCs had 72% MCC-specific survival while CTC-positive patients had 25% survival (n=34, median follow-up 19 months, p=0.0003). 57% of MCC patients had a cytokeratin "dot" visible in ≥20% of CTCs, a feature that was absent among CTCs from other carcinomas (zero of 13 cases).

LIMITATIONS—CTC assay was performed at variable times after diagnosis and heterogeneity in extent of disease affects interpretability of the data.

CONCLUSION—CTC detection in MCC is feasible and appears to add prognostic information, particularly in patients with regional nodal disease. It may also assist clinical management in certain situations, including differentiating metastatic MCC cells from those of other carcinomas.

Introduction

Merkel cell carcinoma (MCC) is an aggressive neuroendocrine skin cancer with a five-year disease-associated mortality of 30–40%[1,2]. Its reported incidence has tripled in the past 20 years to 1,600 cases/year in the US[3]. MCC commonly arises on sun-exposed skin of

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Caucasians who are older than 50. Etiologic factors include ultraviolet exposure, advanced age, immune suppression[4] and the associated Merkel cell polyomavirus (MCPyV)[5].

Although the levels of antibodies to the MCPyV T antigen oncoprotein in the serum can be used to track disease status[6], only about 50% of MCC patients produce such antibodies. Sentinel lymph node biopsy is the gold standard for detecting early occult metastases at diagnosis. Radiological imaging modalities (CT or PET-CT scans) are the major tools to determine extent of metastatic disease and response to therapy in sites not easily accessible by physical examination. These imaging studies are costly, expose the patient to clinically significant radiation and are prone to false positive and false negative results[7]. There is a need for less invasive and less costly biomarkers for prognosis and disease status monitoring.

Circulating tumor cells (CTCs) have been studied extensively in several cancers including prostate[8], colon[9], breast[10], ovarian[11], pancreatic[12] and neuroendocrine tumors such as small cell lung carcinoma[13]. CTCs have been proven to be useful prognostic markers in several carcinomas in which they correlate to disease progression and predict relapse[14,15]. In MCC, the presence of CTCs has been previously reported in 4 patients, typically as a single case [16,17,18]. None of these 3 reports focused on assessing the utility of CTCs. In this study we have analyzed 52 samples from 34 patients and correlated CTCs with outcomes to evaluate the clinical utility of CTCs in MCC.

Methods

Human subjects and clinical samples

This study was approved by the Fred Hutchinson Cancer Research Center IRB (Protocol #6585) and performed in accordance with Helsinki principles. All patients gave informed consent. Patients' blood samples (7.5 ml) were collected in CellSave Vacutainers™ containing EDTA and a cell stabilizing reagent (Veridex LLC™, Warren, NJ, USA).

CTC quantitation

Blood samples collected from MCC patients were maintained at room temperature and processed within 72h after collection. The CellSearch™ system (Veridex LLC™, Warren, NJ, USA) was used for isolation and counting of CTCs. The CellSearch Epithelial Cell Kit™ contains EpCAM(epithelial cell adhesion molecule)-specific antibodies conjugated to ferromagnetic particles to enrich epithelial cells. Isolated cells were fluorescently labeled with the nucleic acid dye 4′, 6-diamidino-2-phenylindole (DAPI) and monoclonal antibodies specific for leukocytes (CD45-allophycocyanin) and epithelial cells (cytokeratin 8, 18,19-phycoerythrin). To be defined as a CTC, an object must be round or oval, have a nucleus (DAPI-positive) contained within an epithelial cell (cytokeratin 8, 18, 19-positive), and lack expression of CD45. Identification and enumeration of putative CTCs were performed by the CellTracks Analyzer II™ and then subsequently verified by a trained operator [19]. Samples containing one or more CTCs per 7.5 ml blood were considered CTC-positive, whereas samples containing no CTCs were considered negative.

Cytokeratin Staining Pattern Evaluation

CTC results (earliest positive draw) were analyzed in the 14 MCC patients with a positive result, and in 13 consecutive patients with positive CTCs in three other cancers (4 breast, 4 colon, 5 prostate). One expert technician, blinded as to diagnosis, counted and sorted CTCs into 2 categories according to their cytokeratin (CK) staining pattern: "typical dot" CK staining or "other" CK-staining pattern. In 2 patients whose CTC count exceeded 80 per 7.5 ml, 27 cells were analyzed in each case for staining pattern.

Statistical Analyses

Study analysis was performed using GraphPad Prism 5™ (GraphPad Software™) with values of $p<0.05$ considered significant. Differences in baseline characteristics between positive and negative CTC were analyzed with chi-squared and *t* tests. Association between presence of CTCs and extent of disease was assessed with Fisher's test for trend. A one-way ANOVA was used to analyze the relationship between CTC count and extent of disease. Overall survival was defined as the time between blood draw and either time of death or last follow-up. Median overall survival rates were calculated using Kaplan-Meier analysis, and differences between curves were analyzed by log-rank test. Differences between MCC and other cancers were analyzed by Fisher's exact test and *t*-test.

Results

Patients

Between June 2010 and March 2011, 34 patients seen in a multidisciplinary MCC clinic were recruited into the study and followed through the end of the study period (September 2012) with respect to outcome. Patients with no evidence of disease, localized, nodal or distant disease were all represented, as were both newly diagnosed and follow-up patients. This cohort thus represents a cross-section of patients typical for a tertiary care center. Clinical characteristics of the patients are shown in Table I. Median age at diagnosis was 68.5 years (range 41 to 90 years); 26 patients were men. Tumor site distribution was as follows: head or neck (12 patients), trunk (3), upper limb (5), lower limb (9), nodal disease with unknown primary (5). AJCC stage of MCC at diagnosis and stage at time of initial CTC assay are shown in Table 1. Median time from diagnosis to initial CTC count was 224 days.

52 blood samples from 34 patients were analyzed. CTCs were detected in 21/52 blood samples (40%). Median number of CTCs detected was 2 CTCs/7.5 ml (range: 1 to 711). 14 of 34 (41%) patients had CTCs detected in one or more blood draw.

There was no correlation between presence of CTCs at time of 1st blood draw and clinical characteristics of the tumor at initial diagnosis: stage, primary tumor size, sentinel lymph node status or lymphovascular invasion (*data not shown*). In contrast, CTC detection was strongly associated with extent of disease at the time of the assay. Specifically, correlation between CTC positivity at time of 1st CTC assay and extent of disease was as follows: 0 of 7 positive among patients with no clinical/radiographic evidence of disease, 1 of 6 positive among those with microscopic or local disease, 7 of 15 positive among those with regional disease, and 4 of 6 positive in patients with distant disease ($p=0.004$, Fisher's test for trend). Among 6 of 15 patients with regional disease on whom we were able to ascertain the number of involved nodes, there was no significant correlation between number of involved nodes and presence of CTCs.

Median time between initial CTC draw and last follow-up was 19 months. Presence of CTCs was strongly correlated with subsequent disease progression ($p=0.009$, Fisher's exact test) (Fig. 1). Thirteen patients had died and 21 were alive at the completion of the study. All deaths in this cohort were due to MCC, therefore overall survival and MCC-specific survival were identical in this study. Median overall survival time among all 34 patients had not been reached at 24 months of follow-up. Median survival time for patients with initial positive CTC was 10.5 months, while it had not yet been reached at 25.6 months for patients with negative CTC. As shown in Fig. 2 **panel A**, a statistically significant difference in overall survival was found between CTC-positive and CTC-negative groups of patients ($p=0.0003$). When grouped by extent of disease at time of CTC assay, the correlation between CTC

status and survival remained significant for patients with regional nodal disease (Fig. 2 **panel B**).

CK staining pattern in CTCs

Images of 396 CTCs were evaluated for CK staining pattern (range: 1 to 128 per patient). Among 14 MCC patients, 8 had "typical-dot" CTCs (57%; Fig. 3). Among 13 patients with other cancers, only 4 had "typical-dot" CTCs (23%, NS). The percentage of "typical-dot" cells among all CTCs was significantly higher in MCC patients (median=35%) as opposed to other cancers (median=0%) (p=0.0024). In three patients with previously diagnosed MCC, the "typical-dot" pattern of the CTC was felt to indicate that a new, highly suspicious visceral or osseous lesion was likely to be MCC and hence management proceeded without biopsy of the new lesion.

Longitudinal studies

Eleven patients had blood samples drawn for CTC analyses at more than one time point (median draws per patient: 2.7, range: 2 to 7). In 7 patients, CTC levels correlated with tumor burden and accurately reflected responses to treatment. In 2 patients, CTC results could not be linked to a clearly defined disease burden (CTC counts were done during therapy and/or disease status assessment was not recorded at the time of blood draw). Finally, in 2 patients CTC counts were consistently negative, regardless of measurable disease. In managing our patients, in 6 cases the CTC test proved beneficial in clinical management in the following ways: 3 patients avoided a biopsy of a highly suspicious lesion in a difficult-to-biopsy location (as described above), 1 patient showed early and prolonged disease remission, and 2 patients showed insufficient response to treatment, suggesting a need for particularly close follow-up.

Case vignette (Fig. 4)

A 69-year-old man with no known immune suppression was diagnosed with a stage IIIB MCC in the left parotid lymph node basin with no known primary. A blood draw 2 weeks after the diagnostic parotidectomy showed no CTCs. The patient was treated with definitive fractionated radiation (60 Gy) to the left parotid and left neck. 3 months after the end of the treatment, a routine PET-CT showed a 4.5 cm mass in the posterior mediastinum. A blood draw 10 days later showed 13 CTCs/7.5ml. Importantly, several CTCs had a characteristic "dot-like" cytokeratin staining pattern. A single dose of ablative radiotherapy (6 Gy) was given to the mediastinal mass. CTC counts rapidly decreased to 1 CTC/7.5 ml by two weeks after radiation, and were negative 3 weeks later. In this case, the CTC results spared the patient an invasive biopsy of the mediastinal mass and provided reassuring data of therapeutic efficacy of palliative radiation.

Discussion

MCC is an aggressive cancer with a high tendency to develop metastases and a mortality rate that is three times higher than melanoma. The use of imaging remains controversial in this cancer because of its cost and lack of specificity and sensitivity[7,20]. There is a need to identify blood-based biomarkers to help track the disease and predict prognosis and response to treatment. CTCs are thought to be important mediators of tumor dissemination and metastatic disease progression[21]. We used a commercially available test that is FDA-approved for other cancers to identify CTCs in MCC. To our knowledge, this study is the first formal evaluation of CTCs in MCC, and the first exploration of the clinical utility of CTCs in the management of MCC patients. Among 52 samples from 34 patients, CTCs were found to reflect burden of disease and their presence showed a significant association with survival. In individual patients, serial CTC counts helped assess response to treatment and

finding the unique CK ‘dot-like’ staining pattern in CTCs was used to help guide clinical management in several individuals with MCC.

CTCs have been shown to aid in the therapeutic management of patients in a large number of different carcinoma types. In multivariate analyses carried out on breast, prostate and colorectal cancers, CTCs at baseline were an independent predictor of progression-free survival and overall survival[10,22,23]. Moreover, CTCs can act as a surrogate marker to determine response to treatment, and CTC changes during therapy can predict survival benefit from the treatment[18,24,25]. In MCC, publications have reported the presence of CTCs on peripheral blood smears in three patients with metastatic MCC to the bone marrow[16,17]. Furthermore, the presence of Merkel cell polyomavirus DNA in the peripheral blood of MCC patients (presumably indirectly reflecting the presence of CTCs) has been shown to predict poor survival[26]. In addition, CTC analysis using the CellSearch platform has been used to assess the response to treatment in one MCC patient[27].

Our data show that CTC detection is feasible in MCC. This test did not reliably detect the presence of disease as 15 of 27 cases with disease at the time of blood draw had negative CTC results. However, CTCs were not detected in any patient without evidence of disease at the time of blood draw (n=7). More importantly, the presence of CTCs correlated with subsequent disease progression, and overall survival was significantly shorter in CTC-positive as compared to CTC-negative patients. Our study also demonstrates that in a majority of patients with serial blood draws, CTC counts correlated with tumor burden and response to treatment. Specifically, 2 patients had consistently negative CTCs despite measurable disease. In contrast, all 7 patients who had at least one positive CTC result had changes in counts that reflected tumor burden and response to treatment. Furthermore, in several cases, changes in CTC values occurred earlier than evaluable responses in imaging studies. Therefore, while this assay may fail to detect patients with measurable disease, for patients with a positive CTC result, serial monitoring of CTC counts may be appropriate to better inform clinicians of changes in extent of disease and therapeutic efficacy.

Our study also reveals that MCC CTCs often have a very specific cytokeratin staining pattern that is analogous to the classic MCC pattern seen on immunohistochemical staining of tissue. This finding could prove useful in differentiating CTCs derived from a MCC as compared to CTCs from other carcinomas. This would be particularly useful when imaging studies reveal a metastasis of uncertain origin. Although not a substitute for imaging nor pathological evaluation, a CTC study may provide earlier information on response to treatment and better discriminate between active disease and surgery- or radiation-associated inflammation.

There are several limitations to this study, including the fact that it is not a comprehensive longitudinal analysis. Its cross-sectional nature, with new and follow-up patients presenting with varying disease burdens, limits the interpretability of the data. Our patient population may not be representative of the general population of MCC patients because these patients all sought care at a tertiary center. Furthermore, because of the small number of subjects, the prognostic significance of CTCs can not be determined in a multivariate analysis. Specifically, these preliminary data are confounded by the link between extent of disease at blood draw and CTC count. In addition, the median 19-month follow-up period is sufficient to capture most recurrences but may not capture the majority of deaths from MCC. Indeed, patients with negative CTCs may have metastatic disease with a lower tumor burden than patients with positive CTCs, and therefore tend to recur and die later. These results need to be validated on a larger cohort in a prospective longitudinal study that includes a baseline CTC analysis at diagnosis.

This study demonstrates that the CTC assay can provide insight into prognosis, therapeutic efficacy and help improve follow-up care in MCC patients, particularly those with nodal disease at the time of evaluation. Further studies will be needed to determine the patient setting in which this assay will be most informative. As is the case for other cancers, the ability to detect CTCs may have an impact on the prognosis and treatment of patients with MCC by providing insight into the risk of the development of future metastases and disease progression, and a peripheral marker for treatment susceptibility and cancer surveillance.

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Abbreviations used

MCC	Merkel cell carcinoma
CTCs	circulating tumor cells
MCPyV	Merkel cell polyomavirus
CK	cytokeratin
NED	no evidence of disease
CT	computed tomography
PET	positron emission tomography
MRI	magnetic resonance imaging
XRT	radiation therapy
AJCC	American Joint Committee on Cancer

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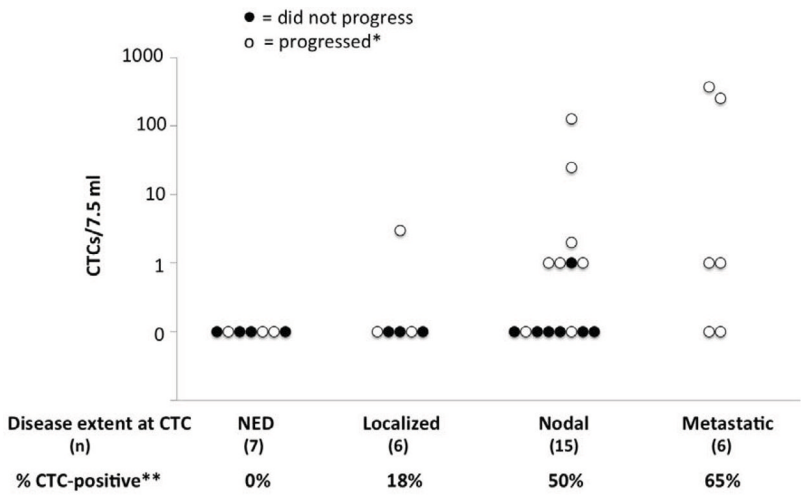

Fig. 1.

Correlation of CTCs with extent of disease and progression. The percentage of patients in each category of disease burden at time of CTC assay is indicated. Fisher's test for trend was significant for increased CTC positivity with advancing disease (**p=0.004). NED = no evidence of disease; Localized = primary tumor still in place or cutaneous recurrence at the primary site; Nodal = disease presenting or recurring in a lymph node; Metastatic = disease at a distant site. Each case is represented as an open or closed circle to indicate whether that patient did or did not progress (respectively) during follow-up. The presence of CTC was strongly correlated with subsequent disease progression (*median follow-up=582 days, p=0.009).

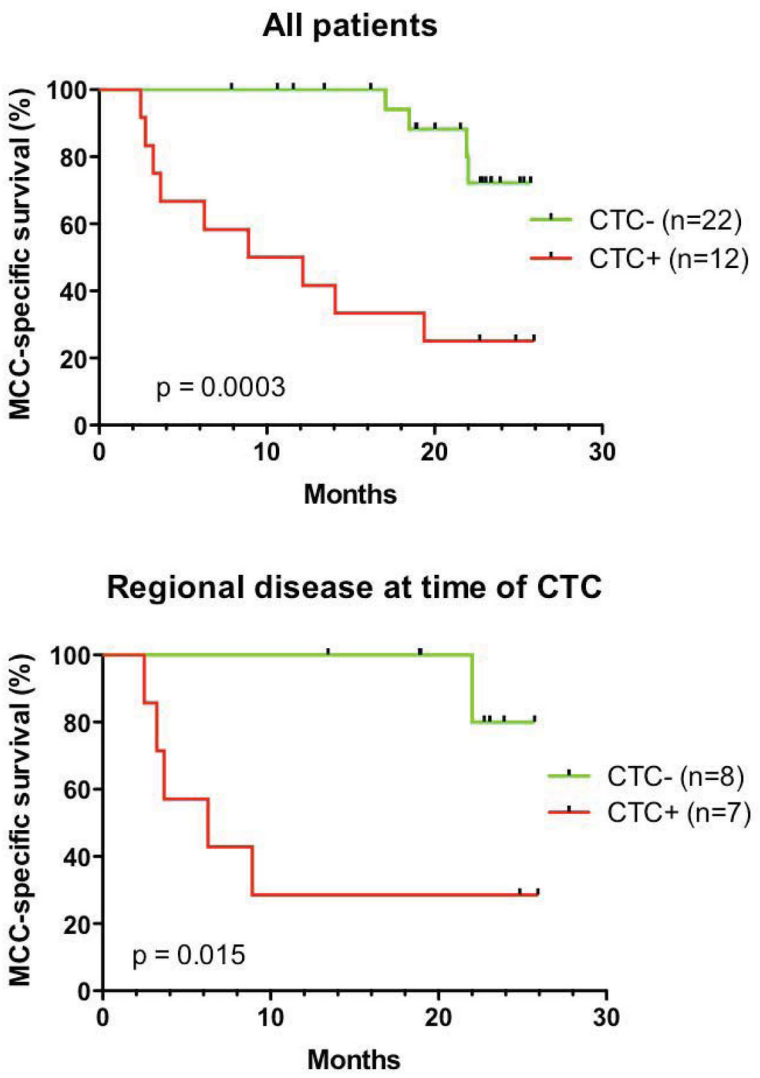

Fig. 2.

CTC status is associated with outcome in MCC patients. Among 34 patients, blood was collected at varying time points (11 to 3719 days) after initial MCC diagnosis. Among all patients, CTC status was strongly correlated with survival (panel A, p=0.0003). While the number of patients was small in other groups, CTC status was significantly associated with survival in patients who had regional nodal disease at the time of CTC assay (panel B, p=0.015).

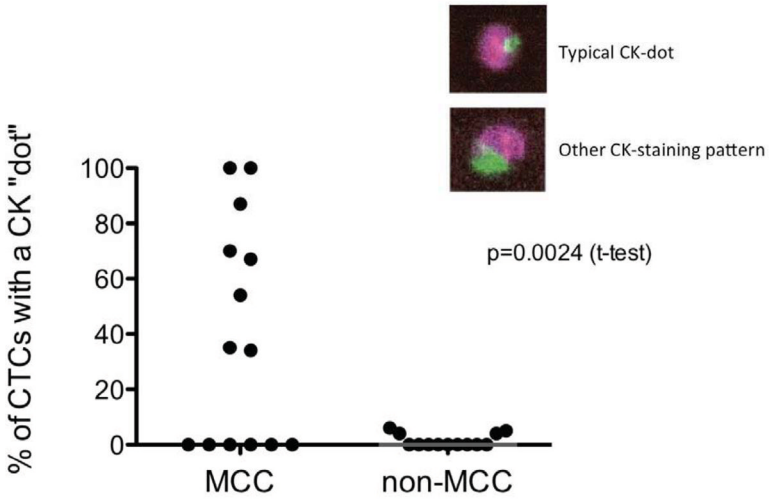

Fig 3.

"Dot-like" cytokeratin staining in CTCs from Merkel cell carcinoma. **Left**: 8 of 14 MCCs showed the frequent presence of dot-like CK in CTCs. **Right**: Of 13 non-MCC carcinomas (breast, prostate, colon), none contained a significant fraction of CTCs with dot-like CK.

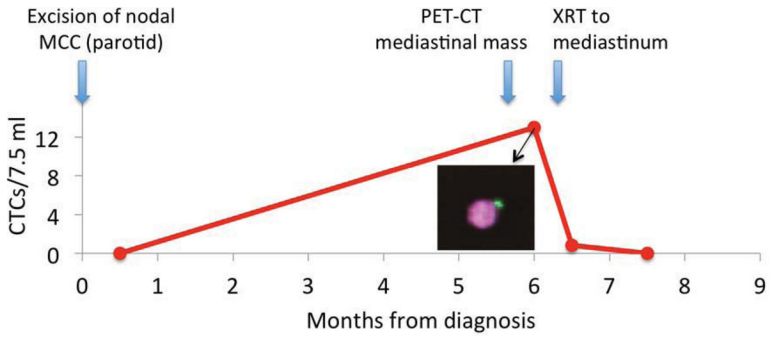

Fig. 4.

MCC case vignette illustrating the clinical utility of CTCs (See text for details). Typical "dot-like" cytokeratin (inset) in a CTC indicates that a mediastinal mass is MCC, sparing the patient a visceral biopsy.

Table I

Clinical characteristics of MCC patients who underwent a CTC study

	Number of patients	%
All patients	**34**	
Gender		
Male	**26**	**76%**
Female	**8**	**24%**
Median age at diagnosis: 68.5 years (range: 41–90)		
Stage at diagnosis (AJCC 7th edition)		
Stage I (≤2 cm primary)	**14**	**41%**
Stage II (>2 cm primary)	**5**	**15%**
Stage III (nodal)	**15**	**44%**
Stage IV (distant)	**0**	**0%**
Median primary tumor diameter: 1.6 cm (range: 0.3–6)		
Pathologic nodal evaluation at diagnosis (n=19)		
negative	**12**	**63%**
positive	**7**	**37%**
Median time from diagnosis to 1st CTC assay: 224 days (range: 11–3719)		
Extent of disease at 1st CTC sample		
No evidence of disease	**7**	**20%**
Local disease	**6**	**18%**
Nodal disease	**15**	**44%**
Distant disease	**6**	**18%**