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Report

Isolation and Molecular Characterization of Circulating Melanoma Cells

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SUMMARY

Melanoma is an invasive malignancy with a high frequency of blood-borne metastases, but circulating tumor cells (CTCs) have not been readily isolated. We adapted microfluidic CTC capture to a tamoxifen-driven B-RAF/PTEN mouse melanoma model. CTCs were detected in all tumor-bearing mice and rapidly declined after B-RAF inhibitor treatment. CTCs were shed early from localized tumors, and a short course of B-RAF inhibition following surgical resection was sufficient to dramatically suppress distant metastases. The large number of CTCs in melanoma-bearing mice enabled a comparison of RNA-sequencing profiles with matched primary tumors. A mouse melanoma CTC-derived signature correlated with invasiveness and cellular motility in human melanoma. CTCs were detected in smaller numbers in patients with metastatic melanoma and declined with successful B-RAF-targeted therapy. Together, the capture and molecular characterization of CTCs provide insight into the hematogenous spread of melanoma.

INTRODUCTION

Recent advances in the treatment of metastatic melanoma have altered the outlook in this previously refractory cancer. In patients with metastatic B-RAF mutant melanoma, dramatic (albeit transient) responses to B-RAF-MAPK blockade require timely monitoring of drug response so that treatment options can be adjusted (Flaherty et al., 2012). The identification of effective treatments for metastatic disease suggests that these drugs may be applied even more successfully in patients in earlier stages of disease, such as stage II or III, where microscopic distant metastases may be eradicated by targeted therapies (Balch et al., 2009). However, identifying high-risk patients who should receive such postoperative "adjuvant" therapy is challenging using the current clinical staging, and would benefit from more reliable indicators of tumor invasiveness and recurrence risk. For patients with advanced melanoma, as well as those with localized disease, analysis of circulating tumor cells (CTCs) may therefore provide a novel biomarker to guide therapeutic decisions.

In addition to diagnostic applications, the detailed molecular characterization of melanoma cells circulating in the bloodstream may yield new insights into the process of melanoma metastasis (Liu et al., 2011; Ramsköld et al., 2012). However, there are significant challenges. CTCs are rare even in patients with advanced cancer. Moreover, melanomas do not express the classical epithelial cell surface marker EpCAM, which has formed the basis for most CTC isolation strategies (Yu et al., 2011). Some melanoma-specific cell surface epitopes have been proposed for CTC enrichment (Khoja et al., 2013), and the large size of tumor cells within primary melanomas has led to the application of filtering strategies to isolate melanoma CTCs (De Giorgi et al., 2010), although recent studies have suggested that melanoma CTCs may span a wide range of cell sizes (Ozkumur et al., 2013). Given the difficulty of isolating whole melanoma CTCs, RT-PCR-based measurements of blood-derived, melanoma-specific transcripts have also been employed. In patients with locally invasive tumors, positive PCR signals in such assays are associated with a poor prognosis and an increased risk of distant metastasis (Hoshimoto et al., 2012). Taken together, multiple approaches suggest the presence of melanoma cells in the bloodstream of patients in various stages of disease, but a robust cell-capture platform is essential for efficient detection and molecular characterization of these cells.

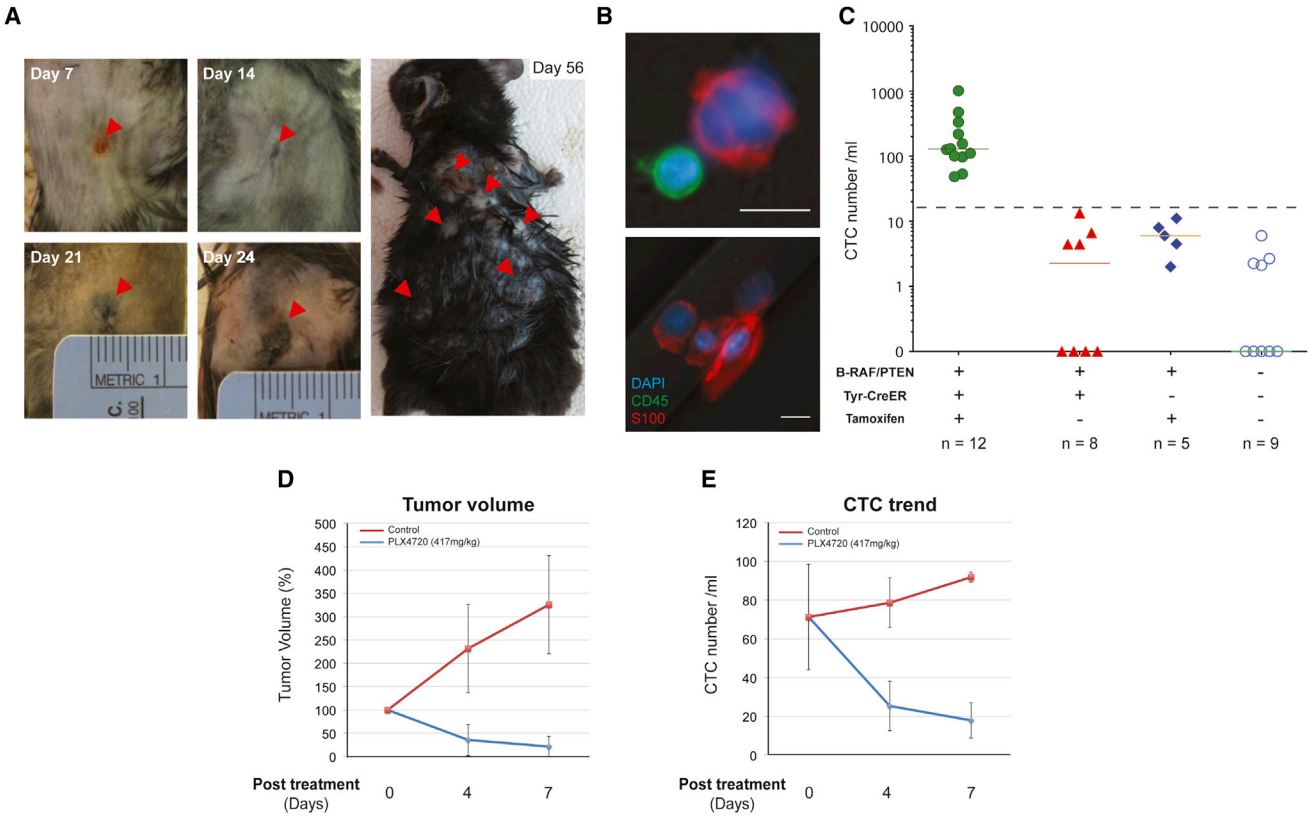

Figure 1. Identification of CTCs in the *B-raf*$^{CA/+}$/*Pten*$^{flox/flox}$ Mouse Melanoma Model

(A) Representative images of melanoma induced by focal tamoxifen injection. Arrowheads show tumor progression at the injection site from day 7 to day 24 (left) and cutaneous metastasis at day 56 after tumor induction (right).

(B) Upper: representative image of a mouse melanoma CTC adjacent to a leukocyte. Lower: a cluster of four CTCs. Blue, DAPI; green, CD45; red, S100. Scale bars, 10 μm.

(C) Quantification of CTCs from a cohort of tumor-bearing mice (green, n = 12) and control mice (red, genotype-matched tumor-free mice, n = 8; blue, Tyr-CreER− mice that received tamoxifen injection, n = 5; open circles, syngeneic C57BL/6 mice, n = 9). Solid lines, median CTC counts; dashed line, threshold of ≥14 S100$^+$ cells/ ml.

(D and E) Concordant changes in (D) tumor volume and (E) CTCs in tumor-bearing mice fed with control chow or PLX4720-containing chow (blue, PLX4720; red, control). Mean value, error bars represent SD.

See also Figure S1.

Here, we adapted a microfluidic platform, the ^{HB}CTC-Chip (Stott et al., 2010), to capture melanoma CTCs using panels of antibodies against melanoma-specific cell surface markers, followed by immunofluorescence (IF) staining for melanoma antigens and optimized on-chip imaging. We applied this ^{HB}CTC-Chip to a robust, inducible B-RAF-PTEN-driven mouse melanoma model (Dankort et al., 2009), in which we validated capture of bona fide melanoma CTCs and monitored their response to targeted therapy and the timing of CTC generation by localized lesions. The large number of CTCs generated in the mouse melanoma model, together with the consistent genetic background, allowed us to compare RNA sequencing (RNA-seq) profiles of matched primary tumors, metastatic lesions, and CTCs, thereby generating a melanoma CTC signature that identified high-risk subsets in human melanoma-derived specimens. Based on its validation in the mouse model, we applied the melanoma ^{HB}CTC-Chip to a pilot cohort of human samples.

RESULTS

Microfluidic Isolation of CTCs and Their Response to B-RAF-Targeted Therapy in a Mouse Melanoma Model

Given the considerable heterogeneity of human melanoma, we sought to adapt the ^{HB}CTC-Chip to capture cells from a genetically engineered mouse model of melanoma (Dankort et al., 2009). In this model, subcutaneous injection of tamoxifen at the flank of the animal leads to activation of oncogenic B-RAF^{V600E} coincidently with deletion of PTEN within melanocytes. Melanomas are formed at the injection site with 100% penetrance within 3 weeks, with distant cutaneous metastases appearing at 6–7 weeks (Figure 1A). After testing B-RAF/PTEN-driven mouse tumors for expression of multiple lineage-specific markers, we selected antibodies against the cell surface epitopes CSPG4 and MCAM for CTC capture, and antibody against the melanoma marker S100 for staining and imaging of captured CTCs (Figures S1A–S1D). We collected blood specimens from