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Aldehyde dehydrogenase isozymes: markers of cancer stem cells in human melanoma

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In recent years, the cancer stem cell (CSC) hypothesis has challenged conventional models of cancer initiation and growth. The hypothesis not only maintains that cancers consist of heterogeneous cell populations, but it also proposes that cancers harbor a unique population of cells that retain an increased capacity for self-renewal and differentiation. Dubbed CSCs may also possess a propensity for tumor initiation and propagation when transplanted into immunocompromised mice, thereby earning them two additional designations: cancer initiating cells and tumor initiating cells. It has been proposed that current cancer treatment modalities target non-CSCs, which comprise the bulk of the tumor. Following tumor debulking, CSCs may remain viable due to intrinsic survival mechanisms and chemoresistance [1,2].

In the 1990s, Dick described a subset of human leukemic cells that retained the capacity to self-renew and differentiate [3]. Following the identification of CSCs in acute myelogenous leukemia and other hematologic malignancies, numerous reports of CSCs in solid organ tumors surfaced. The gold standard assay to validate the presence of a candidate CSC subpopulation that fulfills the criteria of 'self-renewal' (serially transplantable) and 'differentiation' (generating heterogeneous lineages recapitulating an original tumor) is

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serial transplantation of tumor cells in an immunocompromised mouse model [1,2,4]. Thus far, these CSCs have been identified and characterized by specific cell-surface markers. In the context of human melanoma, cell-surface markers such as CD133 [5], ABCB5 [6], and CD271 [7] have been used to identify a phenotypically distinct CSC in the nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mouse model. Despite a large body of evidence supporting the presence of CSCs in other cancers and the identification of the aforementioned melanoma CSC surface markers, the existence of CSCs in human melanoma has remained controversial. In two seminal studies, Quintana *et al.* [8,9] used a highly immunocompromised NOD/SCID IL-2R γ^{null} (NSG) mouse model to demonstrate an increased frequency of tumorigenic cells, such that one in four human melanoma cells were tumorigenic. The NSG model is unique in that residual natural killer cell activity which characterizes the NOD/SCID mouse has been eliminated. Some have used these data to reject the existence of CSCs in human melanoma, while others consider that multiple CSC populations can coexist within a tumor. The studies also failed to identify a cell surface marker which reliably correlated with tumorigenic capacity, including previously described markers for CSCs in human melanoma in the NOD/SCID model (ABCB5, CD271, CD133). It should be noted, however, that the aforementioned studies did not examine high aldehyde dehydrogenase (ALDH), a key intracellular CSC marker thought to be less vulnerable to CSC isolation procedures. Furthermore, Quintana *et al.* did not conduct *in vivo* serial transplantation experiments, the gold standard of assays to validate the presence of a CSC population [8,9]. Since Quintana *et al.*'s first report in 2008 [8], four groups including ours have compared tumorigenesis of human melanoma cells in NOD/SCID mice and NSG mice [7,10–12]. Although these four independent groups have also identified increased tumorigenicity in the NSG mouse model, the magnitude of the increase described by Quintana *et al.* has not been validated. These discrepancies may be explained in part by variations in methodologies. Nonetheless, the sum of the evidence certainly favors the existence of a CSC population in human melanoma.

More recently, physiological and functional stem cell properties have been utilized to identify CSCs. Due to the conflicting data on the existence of CSCs in human melanoma, we decided to employ these properties to identify CSCs in human melanoma *in lieu* of cell-surface markers that could be susceptible to experimental conditions. Specifically, we studied ALDH, a detoxifying enzyme responsible for the oxidation of retinal to retinoic acid, which in turn regulates many genes associated with cell proliferation and differentiation. Indeed, increased ALDH activity has been reported in normal stem cells [13]. Moreover, the ALDH isozyme family has been identified as reliable intracellular markers for CSCs in other solid organ malignancies [14]. We investigated tumor initiating capacity of ALDH⁺ versus ALDH⁻ human melanoma cells via intradermal injections into NOD/SCID and NSG mice [12]. We demonstrated a higher frequency of tumor initiating cells in ALDH⁺ cells in both mouse models. These findings validated our hypothesis that ALDH activity can distinguish tumorigenic and nontumorigenic melanoma cells in both NOD/SCID and NSG mice.

While we and others [10] provide strong evidence that ALDH⁺ human melanoma cells were enriched with tumorigenic cells, Prasmickaite *et al.* [15] were unable to corroborate these findings. The discrepancy is multifactorial and probably secondary to variations in tumor

cell samples, methodologies and data interpretation. For instance, cultured cells were used in Prasmickaite *et al.*'s report rather than patient tumors or xenografted patient tumors. Of note, neither Boonyaratanakornkit *et al.*'s report [10] nor Prasmickaite *et al.*'s report [15] analyzed the self-renewal capacity of ALDH⁺ cells by serially transplanting human melanoma cells into mice, an important criterion for CSCs. Therefore, to investigate whether ALDH⁺ cells and/or ALDH⁻ cells possess the CSC properties of self-renewal and differentiation, tumor cells derived from ALDH⁺ and ALDH⁻ tumors were serially transplanted *in vivo* [12]. Whereas secondary and tertiary tumors were observed from ALDH⁺ cells, ALDH⁻ cells failed to form palpable tumors during the 24-week observation period. This observation confirms the self-renewal capacity of ALDH⁺ cells. The ability of ALDH⁺ cells to differentiate was examined in both the *in vitro* and *in vivo* setting. *In vitro*, ALDH⁺ cells demonstrated the capacity to differentiate into ALDH⁻ cells, whereas ALDH⁻ cells retained the ALDH⁻ phenotype. *In vivo* studies validated these findings. Producing both ALDH⁺ and ALDH⁻ cells, tumors generated from ALDH⁺ cells re-established tumor heterogeneity. Conversely, tumors generated from ALDH⁻ cells were comprised entirely of ALDH⁻ cells. Collectively, our findings confirm the presence of a phenotypically distinct subpopulation of tumorigenic melanoma cells and validate the existence of a CSC population in human melanoma. To date, we are among three groups [6,7,12] who have examined the self-renewal and differentiation properties of CSCs in human melanoma. Whether these melanoma CSCs (ABCB5⁺, CD271⁺ or ALDH⁺) are distinct or overlapping populations remains under investigation.

The Aldefluor[®] assay was utilized in our study and others to identify CSCs measures ALDH enzymatic activity in living tissue specimens. By contrast, immunohistochemical staining quantifies ALDH expression in formalin-fixed tissue specimens and has been utilized to characterize the clinical significance of increased ALDH expression in solid-organ cancers. The availability of ALDH isozyme-specific antibodies has allowed for immunohistochemical analysis of tissues. Previously, it was thought that ALDH1A1 activity was responsible for Aldefluor assay positivity [16]. Therefore, initial studies investigating ALDH expression analyzed tumor specimens using ALDH1A1 specific antibodies for immunohistochemical staining. In many, but not all of these studies, ALDH1A1 expression correlated with prognosis [17]. Recently, however, Marcato *et al.* identified ALDH1A3 in Aldefluor-positive cells from human breast cancer, thereby providing evidence that the ALDH activity measured by the Aldefluor assay is not necessarily due to ALDH1A1 isozyme [18]. Furthermore, they demonstrated a positive correlation between prognostic factors and ALDH1A3, but not ALDH1A1, expression. These data underscore the need to understand the biological significance of each ALDH isoform in CSCs. We identified ALDH1A1 and ALDH1A3 as the predominant ALDH isozymes expressed in human melanoma tumors. Further biologic analysis of the ALDH⁺ melanoma cells revealed that these ALDH isozymes are vital to the function of these cells. Silencing ALDH1A3 by siRNA or shRNA led to cell cycle arrest, apoptosis, decreased cell viability *in vitro* and reduced tumorigenesis *in vivo*. We also noted decreased chemoresistance in ALDH⁺ cells following silencing of ALDH1A3 expression [12]. Collectively, these findings not only underscore the diagnostic, prognostic and therapeutic potential for ALDH isozymes in human melanoma, but they also open the door for future investigations. For example, the

diagnostic utility of ALDH isozyme immunohistochemical staining may be assessed by examining the differential expression of ALDH isozymes in benign nevi versus melanoma. Immunohistochemical staining with ALDH1A1 and ALDH1A3 may also provide valuable prognostic information. Finally, development of isozyme-specific enzyme inhibitors may broaden our understanding of ALDH isozyme physiology and pathophysiology, potentially aiding in the creation of targeted therapeutics selective for CSCs [19].

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Biographies

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