Original Article

Hdc-expressing myeloid-derived suppressor cells promote basal-like transition and metastasis of breast cancer

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Abstract: Metastases are the greatest contributors to death from breast cancer. Here, we identified a distinct subpopulation of luminal breast cancer characterized by cytokeratin 14 (CK14) expression in secondary colonies rather than primary tumors. This entity possessed a poorer prognosis compared to their CK14⁻ counterparts. Immunohistochemical analysis showed that myeloid-derived suppressor cells (MDSCs) were recruited into the tumor microenvironment and exhibited a close spatial relationship with CK14⁺ cancer cells. We demonstrated that histidine decarboxylase (Hdc) is capable of labeling myeloid-biased hematopoietic stem cell/progenitor cell (HSC/HSPC) and immature myeloid cells infiltrating in tumor tissues. FACS data obtained from Hdc-CreER⁷²; eGFP; MMTV-PyVT female mice revealed an increased percentage of Hdc⁺ PMN-MDSCs in metastatic masses. Hdc⁺ PMN-MDSCs expressed high levels of canonical Wnts, including Wnt2, Wnt4, Wnt5a, and Wnt7b, to aberrantly activate Wnt/β-catenin signaling in CK14⁺ malignant cells. β-catenin translocated from the membrane into the cytoplasm and nucleus. Targeted ablation of Hdc⁺ PMN-MDSCs-derived Wnts through porcupine²flo/flo and iDTR transgenic models hampered the metastatic cascade, making Hdc⁺ immature myeloid cells an attractive candidate for tailored immunotherapies.

Keywords: Breast cancer, metastasis, histidine decarboxylase, Wnt/β-catenin, MDSCs

Introduction

Breast cancer (BC) is the second leading cause of female cancer-associated mortality, and more than 90% of the cases are caused by metastasis [1, 2]. To form metastatic lesions, malignant cells detach from the primary tumor, invade through the surrounding mesenchymal tissue, enter and survive in circulation system, and become circulating tumor cells (CTCs) (also known as collective invasion) [3, 4]. These stages serve as an important window for clinical tailored strategies, because metastasis may occur before onset of clinical symptoms. BC tissues are histologically complex, containing a variety of cell types. Considerable advances have been made in elucidating the cellular diversity through transcriptome-based analysis and subdivide BC into three main types: luminal (hormone receptor-positive), HER-2-overexpressing (hormone receptor-negative, HER2 positive), and triple-negative (hormone receptor and HER2 negative) BC.

Of note is the fact that the progression of BC is accompanied by an increase in cellular motility and matrix invasion. Cancer cells at the invasive front or in collective invasion exhibit different phenotypes compared to other tumor cells in animal models or clinical cases. Substantial evidence suggested that cytokeratin 14 (CK14), an essential protein for normal breast development, endows tumor cells with a conserved basal epithelial program. The migratory cells in the breast embryonic placode display CK14⁺ CK8⁺ SMA. Normal breast stem cells and cancer cells with enhanced invasiveness, rather than relatively indolent malignant cells, express...
CK14 [5-7]. Although normal tubular cells show negativity, the reacquisition of CK14 expression in luminal tumor cells facilitates their metastasis [5]. However, the underlying molecular mechanisms are largely unknown.

During the normal development, Wnt/β-catenin pathway contributes to the regulation of normal cellular behaviors, including stem cell pluripotency and differentiation. The aberrant activation of Wnt/β-catenin signaling leads to the breast dysplasia composed of CK14+ CK5+ CK8- αSMA basal-like cells, suggesting the possibility that the expression of CK14 may be regulated by Wnt/β-catenin pathway [8]. However, the exact cellular source of Wnts is unclear.

Immune cells, widely appreciated as one of the most important regulators of microenvironment, play a central role during BC tumorigenesis and progression. At the early stages, BC tissues can release cytokines/chemokines such as TGF-β, IFN-γ, and TNF-α, contributing to anticancer therapies [9]. When chronic injury and pathogens persist, the delicate balance will be ruined by non-resolving inflammation and serves as the initiating event of the sequential neoplastic progression [10]. BC cells can recruit myeloid-derived suppressor cells (MDSCs) into the tumor mass through mTOR signaling pathway or the secretion of granulocyte colony-stimulating factor (G-CSF) [11]. MDSCs, deriving from the hematopoietic stem cells/progenitor cells (HSC/HSPCs) in the bone marrow or spleen, can be subdivided into two major types: the granulocytic MDSCs (PMN-MDSCs, CD45+CD11b+Ly6Ghi) and monocytic MDSCs (M-MDSCs, CD45+CD11b-Ly6Glo) [12]. A recent study by our group suggested that Hdc can serve as a marker of the myeloid-biased HSC/HSPCs, which may differentiate into immature myeloid cells in the context of aging, injury, inflammation, and tumorigenesis [13]. Hdc-expressing PMN-MDSCs promotes the progression of colonic cancer in murine models by the recruitment of Foxp3+ regulatory T cells [14].

Considerable developments have been made to explore tailored strategies for inhibiting cancer-associated MDSCs over the last decade. However, several issues need to be addressed to fulfill these strategies. We have little definitive information about the exact entity of immune cells that promote the evolution of BC mainly due to the heterogeneity of this population. In this study, we investigated the role and regulatory mechanisms of CK14 expression in the advanced stages of BC on the basis of transgenic murine models and clinical archives. The results show that Hdc+ PMN-MDSCs specifically expanded in the setting of BC metastasis and expressed high levels of Wnts to abnormally activate Wnt/β-catenin signaling and enhance CK14 expression in adjacent tumor cells. Our data further unveil the interactions between Hdc+ PMN-MDSCs and invasive tumor cells and propose that the inhibition or depletion of these cells with targeted strategies might facilitate anti-BC therapies.

Materials and methods

Study cohort

This study included 229 patients with accurate pathologic diagnosis of luminal cancer, 112 of which were at early stages and 117 exhibited lymph node or distant organ metastasis. Achieves of primary and metastatic tissues were collected and evaluated. All patients gave written informed consent to participate in the study in accordance with the Helsinki Declaration, and this study was approved by the Medical Ethics Committee of The Fourth Affiliated Hospital of Nanchang University.

Mouse models

All animal experiment protocols have been approved by Nanchang University Institutional Animal Care and Use Committee. Hdc-CreERT2; eGFP mice were purchased from EMMA. MMTV-PyMT, porcupine flox/flox, and iDTR mice were obtained from the Jackson Laboratory. To explore the role of Hdc+ PMN-MDSCs-derived Wnts, Hdc-CreERT2; eGFP; MMTV-PyMT mice were crossed to porcupine flox/flox and iDTR repectively. All mice were backcrossed and maintained at C57BL/6 background and housed in the special pathogen-free animal facilities.

Histopathology and immunohistochemistry

Serial sections obtained from the formalin fixed and paraffin embedded blocks underwent H&E and immunohistochemical staining. The histopathologic evaluation of all sections was performed by two independent pathologists (X Xiong and H Pan), who were blinded regarding...
Hdc⁺ PMN-MDSCs and CK14⁺ breast cancer

details. Primary antibodies for CK14 (1:100; clone LL002, Novocastra, Leica; ab7800, Abcam), CD15 (1:150; clone Carb-1, Novocastra, Leica), anti-GFP (1:200; ab13970, Abcam), and β-catenin (1:200; ab32572, Abcam) were used in the slide stainer (Autostainer 360, Lab Vision, Thermo Fisher). A microscope (80i, Nikon) with the CCD camera (DS-Ri2, Nikon) was used to perform the image analysis. Counting of positive cells was conducted in non-overlapping fields using the 40 x objective. The average number of positive cells in each square centimeter was calculated for each specimen.

**In vivo treatment**

At 6-week-old, a tamoxifen (Harlan Laboratories) chow was used to delete porpupine in Hdc⁺ myeloid cells, resulting in a block of Wnts’ secretion (Figure 5A). To further exclude other influence factors, intraperitoneal diphtheria toxin (DT, Sigma) injections combined with a tamoxifen chow was applied to abolish Hdc⁺ myeloid cells in Hdc⁺CreERT2; eGFP; MMTV-PyMT; iDTR and Hdc⁺CreERT2; eGFP; MMTV-PyMT; porcupineflox/flox; iDTR mice (Figure 5A).

**Flow cytometry analysis**

Fresh tissues obtained from breast or lymph node were manually minced and incubated in DMEM with Collagenase A (Roche) and DNAse I (Roche) for 45 min at 37°C. Suspensions were filtered three times using a 70 μm nylon mesh to remove dead cell debris and enrich leucocytes. No more than 1 × 10⁶ cells were incubated with the antibody panel composed of CD45 (30-F11, eBioscience), CD11b (M1/70, eBioscience), and Ly6G (1A8, eBioscience). PMN-MDSCs were identified based on their phenotype: CD45⁺CD11b⁺Ly6Ghi. Hdc⁺ cells were characterized by their high level of GFP expression (GFP hi). CD45⁺CD11b⁺Ly6G⁺GFPlo cells harvested from eGFP wild type littermates were used to set the gate. Stained cells were fixed with Cytofix (BD Bioscience) for 30 min on ice and analyzed by LSR II flow cytometer (BD Bioscience).

**RNA-seq analysis**

Hdc⁺ PMN-MDSCs were sorted and lysed in ARCTURUS PicoPure RNA isolation kit according to manufacturer’s instruction (Life Technologies). Total RNA was isolated followed by cDNA amplification. Libraries were established using SMARTer Ultra Low Input RNA kit (Clontech Laboratories) and Nextera XT DNA Library Preparation Kit (Illumina). Sequencing was performed on Hiseq 2500 (Illumina).

**Gene microarray analysis**

Both Hdc⁺ and Hdc⁻ PMN-MDSCs were harvested from breast and metastatic lesions. Total mRNA was extracted using Rneasy Micro Kit (Qiagen) and labeled by 3' IVT Expression Kit (Affymetrix) before hybridized to the Affymetrix GeneChip mouse genome 430 2.0 array (Affymetrix). Arrays were performed using an Affymetrix Scanner 300-7G scanner with GCOS software. A significance cut-off of a Benjamini-Hochberg false discovery rate ≤ 0.05 was applied.

**Quantitative RT-PCR**

Total mRNA of sorted cells was isolated using Rneasy Micro Kit (Qiagen) and underwent reverse transcription using SuperScript III First-Strand Synthesis System (Life Technologies). PrimerQuest Tool (Integrated DNA Technologies) was used to design sequences of SYBR Green Primer (Integrated DNA Technologies). Quantitative PCR was performed with the StepOne Plus machine (Applied Biosystems). Relative gene expression was normalized to GAPDH.

**Statistical analysis**

Experimental results were replicated at least once, unless otherwise indicated. Sample sizes for each study were estimated on the basis of the expected differences and previous experience with the particular assay. All data are shown as the mean ± SEM. Kaplan-Meier survival was statistically analyzed by Log-rank test. Other statistical comparisons were evaluated with Student’s t test or one-way ANOVA. Significance levels were set at *P < 0.05; **P < 0.01; ***P < 0.001; n.s., not significant. N indicates biologic replicates. Data analyses were carried out using Prism 8 (Graphpad).

**Results**

**Metastatic lesions reacquired CK14 expression**

Normal mature luminal cells show negativity for CK14, while a high incidence of metastasis was
Hdc+ PMN-MDSCs and CK14+ breast cancer

MDSCs infiltration in metastatic lesions

MDSCs are a heterogeneous entity recruited from the bone marrow to the microenvironment and promote neoplastic progression. Consistent with previous studies, PMN-MDSCs, pinpointed by the combination of CD15+ and morphology, surrounded tumor cells at the invasive front or leading edge (Figure 2A) [17]. The number of PMN-MDSCs in metastatic tissues was significantly higher than that of non-metastatic tumors (Figure 2B). The spatial and temporal distribution of PMN-MDSCs in BC suggested their central role in the tumorigenic progression.

Hdc+ PMN-MDSCs were recruited during metastasis

Our previous study indicated that Hdc marks the myeloid-biased HSC/HSPC [13]. Hdc+ PMN-MDSCs are involved in the initial and advanced stages of colon cancer through the interactions with stem cells and other microenvironmental elements [14, 18]. The transgenic Hdc-CreER<sup>T2</sup>; eGFP; MM-TV-PyVT female mice showed breast tumors in situ by 5-weeks-old and developed invasive ductal carcinoma by 12 weeks, with the lung and lymph node metastases (Figure 3A, 3B). We examined CK14 and GFP expression in both primary and metastatic tumors. Consistent with IHC results obtained from clinical archives, CK14+ positivity was observed in up to 27.1 ± 1.3% of metastatic masses, which was higher than that of non-metastatic cases (P < 0.05) (Figure 3C, 3D). Our previous studies demonstrated that Hdc+ immune cells represented a subtype of PMN-MDSCs [14]. The serial sections stained with GFP antibody indicated the recruitment of Hdc+ PMN-MDSCs into metastatic colonies (1.41 ± 0.12 × 10<sup>4/cm<sup>2</sup></sup>) rather than primary tumors (0.22 ± 0.03 × 10<sup>4/cm<sup>2</sup></sup>) (Figure 3E). FACS data further supported the results of IHC and suggested that, in secondary lesions, up to 45.2 ± 3.6% of PMN-MDSCs expressed high levels of Hdc. By contrast, only 1.3 ± 0.2% of immune cells in primary sites were Hdc positive (P < 0.01) (Figure 3F).
Hdc+ PMN-MDSCs and CK14+ breast cancer

MDSCs-derived Wnts involved in BC tumorigenesis

Wnt/β-catenin, a conserved pathway, contributes to the regulation of normal cellular behaviors, including stem cell pluripotency and differentiation. Aberrant activation of Wnt/β-catenin signaling is well studied in many solid tumors. To gain more insight into the molecular mechanisms by which metastatic tumor cells overexpress CK14, we first performed IHC on tumor sections by β-catenin antibody and found the translocation of β-catenin from the membrane to the cytoplasm and nucleus, reflecting the status of Wnt/β-catenin signaling (Figure 4A).

Despite recent advances in our understanding of the important role of Wnts during the malignant transformation of mammalian glands, the cellular sources are largely unknown because of the complexity of microenvironment. Our previous study suggested that myeloid cell-derived Wnts exerted pivotal effects on the repairment of intestinal stem cells [19]. In colorectal cancer, the expression of Wnt2 and Wnt5a in infiltrating tumor-associated macrophages, a heterogenous entity derived from MB-HSC, was upregulated during the neoplastic progression from adenoma to adenocarcinoma [20]. RNA-seq analysis data revealed that Hdc+ PMN-MDSCs obtained from metastatic masses expressed higher levels of canonical Wnts, including Wnt2, Wnt4, Wnt5a, and Wnt7b, compared to non-metastatic counterparts (Figure 4B). These results were further confirmed by RT-PCR (Figure 4C), suggesting the involvement of Hdc+ PMN-MDSCs-associated Wnt/β-catenin signaling in the advanced stages of BC.

Attenuation of metastasis by Wnt inhibition

Given that Hdc+ PMN-MDSCs may serve as a major cellular source of Wnts in the metastatic stages of BC, we hypothesized that blocking of Hdc+ PMN-MDSCs-derived Wnts would inhibit the metastatic process. Thus, the Hdc-CreERT2; eGFP; MMTV-PyVT mice were crossed with porcupine<sup>lox/lox</sup> (Porcn) mice to eliminate Wnts in Hdc-expressing cells. At 6 weeks of age, the mice were induced to delete porcupine by a tamoxifen-containing diet (Figure 5A). FACS results indicated that the downregulation of porcupine did not influence the percentage of Hdc+ PMN-MDSCs (P > 0.05) (Figure 5B). However, the number of metastatic lesions decreased significantly in Porcn group (2.3 ± 0.3) compared to that of Hdc-CreERT2; eGFP; MMTV-PyVT mice (7.5 ± 0.6).
To further elucidate the central role of Hdc⁺ PMN-MDSCs, we employed the inducible diphtheria toxin receptor (iDTR) to deplete Hdc⁺-expressing myeloid cells in Hdc-CreER²; eGFP; MMTV-PyVT; DTR mice (Figure SA). At 10 weeks of age, the number of secondary colonies (1.9 ± 0.2) and Hdc⁺ PMN-MDSCs (4.8 ± 0.3%) in DTR⁺ mice decreased significantly compared to Hdc-CreER²; eGFP; MMTV-PyVT; porcinefloxflox; DTR mice to explore the possibility that Hdc⁺ PMN-MDSCs-derived Wnts serve as a main driver for the reacquisition of CK14 in tumor cells. Consistent with above-mentioned
Hdc+ PMN-MDSCs and CK14+ breast cancer

Figure 4. Aberrant activation of Wnt/β-catenin pathway in metastatic cells. (A) The majority of metastatic cells, rather than primary tumor cells, showed cytoplasmic and nuclear immunopositivity for β-catenin. (B) RNA-seq results demonstrated that Wnt2, Wnt4, Wnt5a, and Wnt7b were upregulated in Hdc+ PMN-MDSCs cells obtained from metastatic tumors. (C) qRT-PCR further supported high levels of Hdc+ PMN-MDSCs-derived Wnts. Original magnification × 200 (left, A) and × 400 (right, A).

Discussion

Initiation of colony growth at the lymph nodes or secondary organs is the rate-limiting step during tumor progression, suggesting that cellular survival is a key feature of a successful metastatic cell. Here we have identified a subgroup of metastatic luminal BC with poor prognosis that reacquired CK14 expression. MDSCs were recruited into the metastatic masses and exhibited a close spatial relationship with CK14+ tumor cells. Hdc labeled the majority of the infiltrating PMN-MDSCs, which expressed high levels of canonical Wnts, including Wnt2, Wnt4, Wnt5a, and Wnt7b, to aberrantly activate Wnt/β-catenin signaling in metastatic cells. The ablation of Hdc+ PMN-MDSCs-derived Wnts in vivo by porcupine−/− or iDTR decreased the metastatic rate.

Breast luminal cancer is a heterogeneous disease deriving from the ductal epithelium. The growth of secondary lesions at distant sites is a key step for the majority of BC-related deaths. A previous study demonstrated that the genetic diversity was higher in the distant metastases compared to primary tumors [21]. These changes promote breast cancer cells to gain, at least in part, stem cell or progenitor-like characteristics and display phenotypic discordance. Basal-like triple negative breast cancers can express CK14, one of the normal myoepithelial/basal cells markers [22]. The discordance rate regarding hormone receptor ranges from 6.4-54% and 0-33% in HER2 group [23]. Consistent with previous studies, almost one-third of metastatic luminal BC in this study expressed CK14 in secondary lesions. Our data further extend the panel of discordance markers and suggest that CK14 may serve as a marker for a distinct entity of metastatic ductular cancers with poor prognosis.

However, the exact molecular mechanisms contributing to phenotypic discordance remain a subject of intense investigation mainly because of the complex bi-directional interactions between malignant cells and microenvironment factors in the oncogenic cascade. Myeloid-derived immune cells, including MDSCs, tumor-associated macrophages (TAMs), tumor-associated neutrophils (TANs), are essential for the tumorigenesis and progression of many solid tumors through direct or indirect effects [24-26]. BC cells can secret CXCL17 to enhance the...
expression of PDGF in MDSCs, resulting in the angiogenesis and metastasis in the lung [27]. High levels of MDSCs in patients with advanced breast cancer have been linked to poor prognosis [24]. In breast cancer tissues, γδ T-derived IL-17 has been demonstrated to induce G-CSF-dependent expansion of TANs, which sequentially inhibit CD8 T cells and facilitate the lymph node and lung metastasis [28]. However, considerable advances have elucidated the fact that MDSCs are also heterogenous.

More precise molecular mechanisms are urgently needed for developing tailored immune therapies. The expansion of PMN-MDSCs has been closely correlated with carcinogenesis in a murine model [29]. Our previous studies have demonstrated that Hdc serves as a promising marker for myeloid-biased HSCs/HSPCs and immature myeloid cells in tumor microenvironment [13, 14, 30]. In this study, Hdc+ PMN-MDSCs were recruited into metastatic lesions rather than primary site, contributing to the establishment of secondary colonies. The close spatial relationship between Hdc+ PMN-MDSCs and CK14+ malignant cells raised the possibility that these immature myeloid cells may play an important role in the reacquisition of basal-like phenotype through a paracrine pattern [13].

Wnt/β-catenin signaling (also called the canonical Wnt pathway) is characterized by their crucial role in stem cell maintenance and differentiation. Aberrant activation of this pathway has been tightly associated with many malignant entities. Normal breast development could be hampered by the aberrant activation of Wnt/β-catenin in transgenic models, leading to ductular hyperplasia composed of undifferentiated basal-like cells (CK5/14+CK8 α-SMA) [8]. These immature cells seem to be capable of glv-
# Table 1. Primers for quantitative RT-PCR

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<th>Gene Symbol</th>
<th>Organism</th>
<th>Gene Name</th>
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<th>Reverse Primer (5'-3')</th>
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Figure 6. Schematic illustration of the interactions between metastatic cancer cells and Hdc⁺ PMN-MDSCs. Breast cancer cells detach from primary tumors, enter and survive in circulation, and proliferate in a foreign microenvironment. Hdc⁺ PMN-MDSCs, as an important component of tumor associated microenvironment, are recruited and expand in the colonies. High levels of Wnts derived from Hdc⁺ PMN-MDSCs can aberrantly activate Wnt/β-catenin signaling of cancerous cells with a paracrine pattern. β-catenin accumulates in the cytoplasm and nucleus, where it upregulates the expression of CK14. CK14 endows metastatic cells with a basal-like phenotype, which is characterized by enhanced abilities of proliferation and anti-apoptosis. Thus, infiltrating Hdc⁺ PMN-MDSCs contribute to the establishment of secondary tumors. Further studies are needed to elucidate the interactions between metastatic cells and Hdc⁺ myeloid-biased HSC/HSPCs.
ing rise to breast tumors. The membrane expression of β-catenin decreased with the sequential neoplastic progression from dysplasia to metastasis [31]. The cytoplasmic and nuclear β-catenin positivity was always observed in advanced luminal or basal-like cancers, which are characterized by CK14 expression [31]. Our immunohistochemical results that metastatic masses, rather than primary luminal cancers, showed CK14 positivity and translocation of β-catenin further supported this conclusion.

Despite considerable advances in the role of Wnts, relatively little is known of the cellular sources and regulatory mechanisms. The components of tumor environment can secret different Wnts in the setting of homeostasis and tumorigenesis. Epithelium-derived Wnts mainly contribute to organ development and stem cell expansion after injury [32]. Mesenchymal cells have been demonstrated to be an important source of Wnts for the maintenance of the stem cell or progenitor niche [33-35]. M2 macrophages can secret Wnt1 to promote the mucosal regeneration in inflammatory bowel diseases [36]. TAMs upregulate Wnt5b and Wnt7b levels to drive tumor metastasis and angiogenesis [19, 37]. We identified high levels of canonical Wnts in Hdc⁺ PMN-MDSCs, which may possess the same cellular source as TAMs and TANs. The block of Hdc⁺ PMN-MDSCs-restricted Wnts via porcupine<sup>flox/flox</sup> or DTR transgenic animal models significantly inhibited metastatic progression, reflecting its role in establishing secondary lesions.

In summary, we have identified and characterized Hdc⁺ PMN-MDSCs as a distinct subpopulation, which secrete higher levels of canonical Wnts to activate Wnt/β-catenin pathway in disseminated BC cells, leading to the reacquisition of a basal-like phenotype (Figure 6). Targeted ablation of Hdc⁺ PMN-MDSCs-derived Wnts attenuated the metastatic ability of cancer cells and thus hampered tumor progression. A previous study indicated that BC cells-derived cytokine could influence the differentiation of HSCs/HSPCs in the bone marrow [38]. There has been considerable interest in the crosstalk and regulatory loop between BC cells and Hdc⁺ MB HSCs/HSPCs. Given the more accurate definition of MDSCs subpopulation and pro-metastatic ability, the tailored immunotherapies based on targeting Hdc⁺ PMN-MDSCs might be of therapeutic benefit.

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**Disclosure of conflict of interest**

None.

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Hdc⁺ PMN-MDSCs and CK14⁺ breast cancer


