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ORIGINAL RESEARCH

Identification of CD24 as a marker for tumorigenesis of melanoma

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Objective: Cutaneous melanoma (CM) is a common skin cancer. Surgery is still the primary treatment for CM, as melanoma is resistant to chemotherapy. In the recent years, it has been found that cancer stem-like cells (CSCs) are responsible for this drug resistance. CD24 is a widely used marker to isolate CSCs. In this study, we aimed to analyze the properties of $CD24^+$ and $CD24^-$ subpopulation of melanoma cells.

Materials and methods: We isolated $CD24^+$ cells CSCs using magnetic-activated cell sorting system. We extracted total RNA and carried out reverse transcription polymerase chain reaction analysis. We counted the cell colonies using soft agar assay and assessed the cell invasion using cell migration assay. We implanted $CD24^+$ or $CD24^-$ cells into the flank of non-obese diabetic severe combined immunodeficiency mice, and measured the tumor volumes every 5 days until the end of the experiment. We carried out immunohistochemical analysis to study the tissue sections.

Results: We demonstrated that the $CD24^+$ subpopulation has self-renewal properties in vitro and in vivo by using soft agar assay and xenograft tumor model. Furthermore, we confirmed that CD24 expression is accompanied by activation of Notch1 signaling pathway.

Conclusion: This study provides new knowledge on the role of CD24 in the tumorigenic ability of melanoma.

Keywords: melanoma, CD24, apoptosis, migration, therapy

Introduction

Cutaneous melanoma (CM), a malignant tumor of the pigment-producing melanocytes, is the most lethal form of skin cancer.[1] Surgery is still the primary treatment for CM.[1] Several adjuvant therapies have been used for CM, such as radiation therapy and immunotherapy.[2,3] However, the major obstacle to an effective treatment for melanoma is tumor heterogeneity.[4]

Melanoma contains multiple cell populations with various antigens.[5] In order to elucidate the roles of the distinct cancer cell subpopulations in melanoma, we isolated two subpopulations based on CD24 expression. In our previous study, we have demonstrated that the levels of CD24 mRNA and protein were higher in cancer tissues than that in normal tissues of the melanoma patients.[6] CD24, a 27-amino-acid single-chain protein that binds to the extracellular matrix, is a widely used cancer stem cell marker.[7] $CD24^+$ cancer cell colonies isolated from some cancers, such as nasopharyngeal carcinoma,[8] ovarian cancer,[9] and pancreatic cancer, showed increased self-renewal and enhanced chemoresistance.[10] However, other studies showed that lack of CD24 expression appears to be critical for the identification of the breast cancer stem-like cells (CSCs).[11,12]

In this study, we demonstrated that CD24 was markedly associated with the properties of melanoma cells. CD24 expression in these cells is accompanied by activation of Notch1

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signaling pathway. This study provides new knowledge on the role of CD24 in the tumorigenic ability of melanoma.

Materials and methods

Cell lines and culture

The melanoma cell lines, A375 and B16F10, were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cell lines were grown in Dulbecco's Modified Eagle's Medium (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin) and maintained in a humidified incubator with 5% CO_2 at 37°C.

Isolation of $CD24^+$ cells from A375 and B16F10 cell lines

Magnetic-activated cell sorting (FACS Vantage SE; Becton Dickinson, Franklin Lakes, NJ, USA) was carried out using CD24 microbeads, according to the manufacturer's protocol (STEMCELL Technologies, Vancouver, BC, Canada). Cells were labeled with anti-CD24 antibody cocktail and mixed with magnetic microbeads, and then separated by a magnet. Sorted $CD24^+$ and $CD24^-$ cells were cultured on a six-well plate and used in the following studies.

RNA isolation and reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from cells using the RNeasy Mini Kit (Biomed, Beijing, People's Republic of China). RT-PCR was carried out as previously described.[6]

Soft agar assay

This was performed using the method of Lin et al.[10] A six-well culture plate was coated with 2 mL of bottom agar mixture (0.6% agar). After the bottom layer solidified, 2 mL of top agar mixture (0.3% agar) containing 1×10^4 $CD24^+$ or $CD24^-$ cells was added, and the plate was incubated for 4 weeks. The plate was stained with 0.5% crystal violet, and the colonies were counted using a light microscope (Olympus CX31; Olympus, Tokyo, Japan).

Cell migration assay

Cell invasion was assessed using 24-well transwell chambers with a pore size of 8 µm (Corning Incorporated, Corning, NY, USA). Cells (1×10^5) were seeded in the serum-free media in the upper well and incubated for 48 h. Cells in the lower chamber were stained with Giemsa staining solution and counted by a microscope.

Western blot analysis

Proteins from cells were resolved by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a nitrocellulose membrane, and detected using the following antibodies: anti-Galectin-3 (1:200; sc-53127), anti-cleaved NICD1 (1:200; sc-32745), anti-JAG1 (1:100; sc-135955), anti-DLL4 (1:100; sc-18640), anti-Notch1 (1:200; sc-376403), anti-N-cadherin (1:200; sc-53488), and anti-E-cadherin (1:200; sc-71008) (Santa Cruz Biotechnology, Dallas, TX, USA). The bands were detected using an enhanced chemiluminescence system (Amersham Biosciences, Amersham, UK).

Xenograft tumor model

This study was approved by the Ethics Committee of China Medical University. The animal experiments were performed in accordance with the Care and Use of Laboratory Animals guideline of the Ethics Committee of China Medical University. $CD24^+$ or $CD24^-$ cells (3×10^7) were subcutaneously implanted into the flank of non-obese diabetic severe combined immunodeficiency mice (4- to 6-weeks old; Charles River, Wilmington, MA, USA). Ten mice were in each group. Every five days until the end of the experiment, one mouse from each group was randomly selected and anesthetized, photographed, and sacrificed. Tumors were excised, and then prepared for immunohistochemical analyses. Tumors were measured using calipers, and tumor volumes were calculated (tumor volume = length × width^2 × 0.52).

Immunohistochemistry

Tumor samples were formalin-fixed and embedded in paraffin. Paraffin sections were stained with the first antibody for CD24 (1:200; sc-7034) and Notch1 (1:200; sc-376403) (Santa Cruz Biotechnology) by incubating overnight at 4°C. Secondary staining with biotinylated secondary antibodies with horseradish peroxidase (HRP; Beyotime Biotechnology, Nanjing, People's Republic of China) was performed for 30 min at room temperature. Then, the sections were counterstained with hematoxylin (Beyotime).

Statistical analysis

Each experiment was performed in triplicate. Statistical analysis was performed using one-tailed Student's *t*-test (unilateral and unpaired). Survival rate was analyzed by the Kaplan–Meier method. Differences with a *P*-value less than 0.05 were considered statistically significant.

Results

Tumorigenic ability of $CD24^-$ and $CD24^+$ subpopulation of A375 and B16F10 cells in vitro and in vivo

In this study, $CD24^+$ cells were isolated from A375 and B16F10 cells by using a CSC-specific marker, CD24 (Figure 1). In addition, we found that *CD24* mRNA was significantly higher in

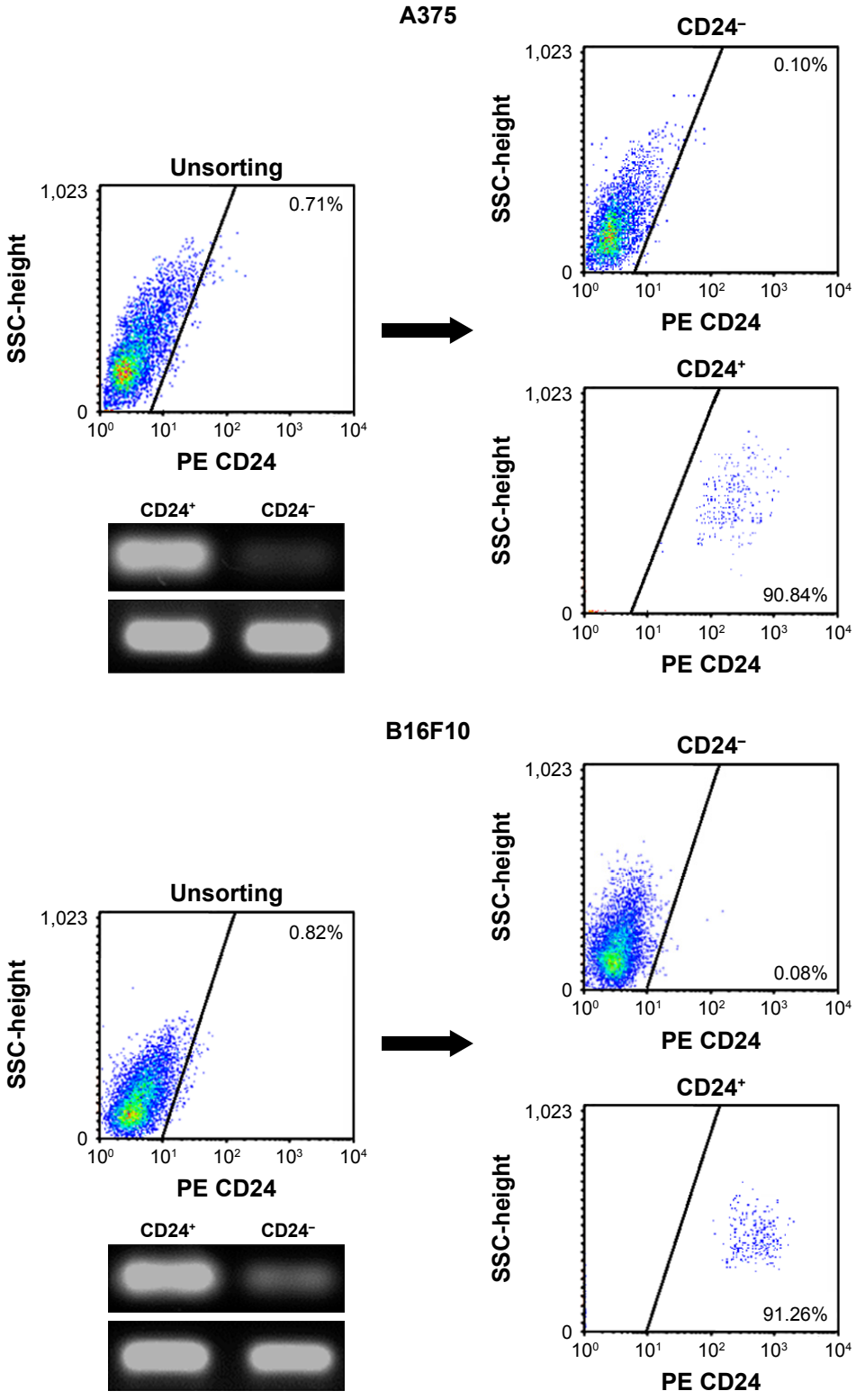

Figure 1 Identification of CD24+ and CD24− cells in the melanoma cell lines, A375 and B16F10.
Note: Analysis and sorting of CD24+ and CD24− cells were performed, and *CD24* mRNA expression was determined by RT-PCR.
Abbreviation: RT-PCR, reverse transcription polymerase chain reaction.

CD24+ cells than that in CD24− cells (Figure 1). The results of colony formation assays showed that CD24+ cells from both A375 and B16F10 cells could form more cell colonies than CD24− ones ($P<0.05$, Figure 2A). It indicated that the proliferation ratio of CD24+ cells is higher than CD24− ones. Migration of CD24− and CD24+ cells was detected using transwell assay. More CD24+ cells migrated to the lower membrane compared with CD24− ones ($P<0.05$, Figure 2B).

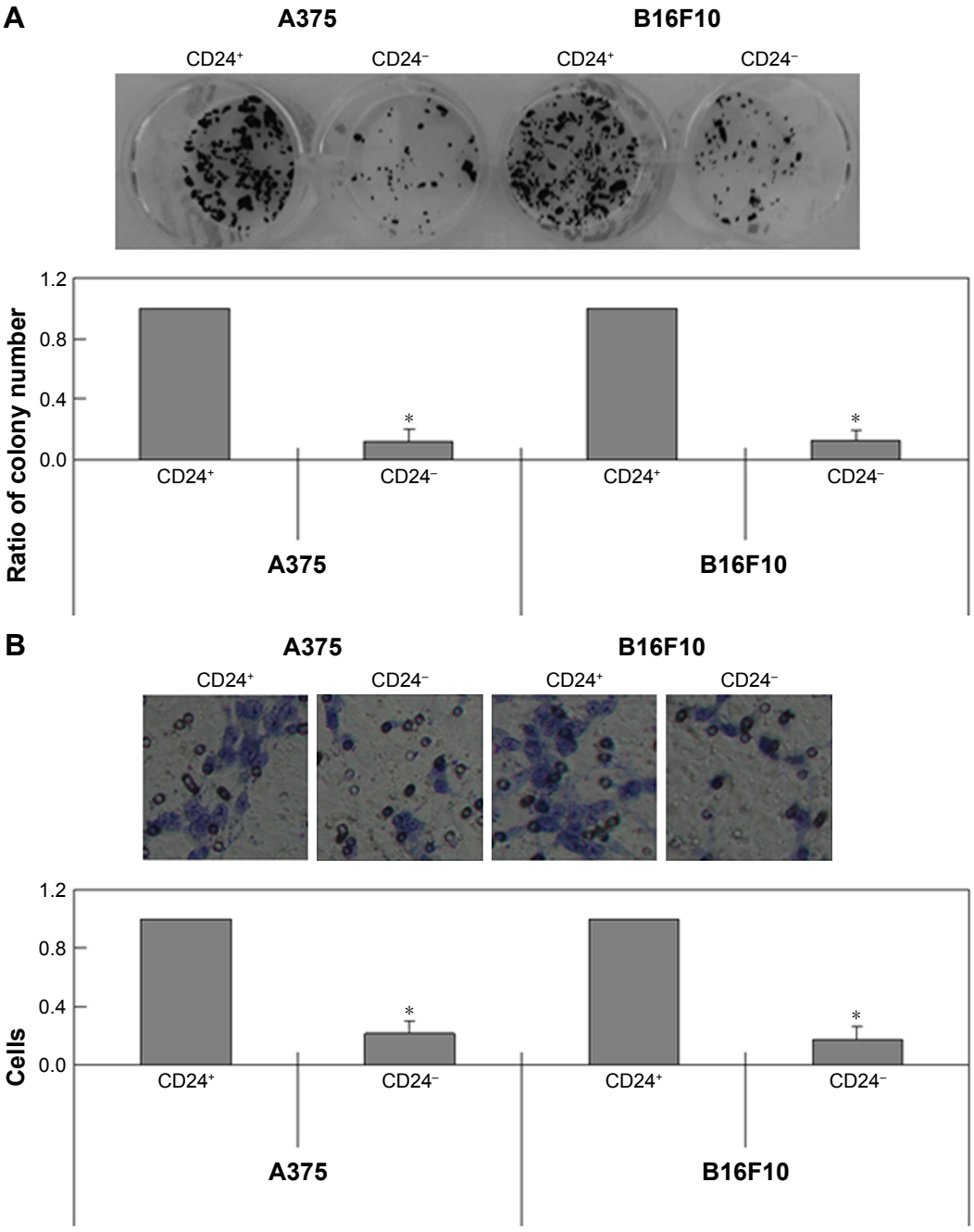

Figure 2 Tumorigenic properties of CD24+ and CD24− cells in vitro.
Notes: (**A**) The proliferation ratio of CD24+ and CD24− cells was determined by colony formation assay. (**B**) The migration of each cell line was determined by Transwell assay. *$P<0.05$.

Next, we determined the tumorigenic ability of CD24− and CD24+ cells in mouse models. As in the in vitro experiments, the tumor size in mice carrying CD24− cells was smaller than that in mice with CD24+ cells (Figure 3A). We observed fewer metastases in liver tissues in CD24− groups compared with CD24+ groups (Figure 3B). The survival rate of the CD24− tumor-bearing mice was increased up to 60% compared with CD24+ tumor-bearing mice ($P<0.05$, Figure 3D).

Changes of Notch1 signaling pathway in CD24− and CD24+ cells

The levels of Galectin-3, NICD1, JAG1, DLL4, Notch1, and N-cadherin were observed to be higher in CD24+ cells compared with CD24− cells using western blot (Figure 4). The expression level of E-cadherin was lower in CD24+ cells than that in CD24− ones (Figure 4). Furthermore, we found that increased CD24 was accompanied by high Notch1 expression in vivo (Figure 3C).

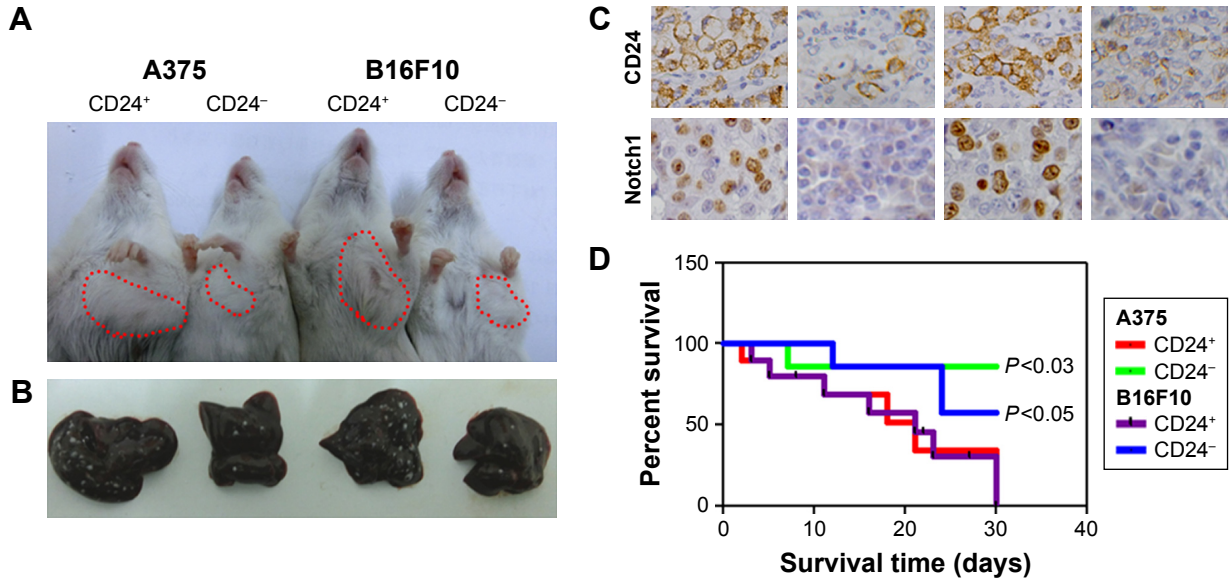

Figure 3 Tumorigenic properties of CD24⁺ and CD24⁻ cells in vivo.
Notes: (**A**) Macroscopic appearance of subcutaneous tumors in CD24⁺ and CD24⁻ groups. (**B**) Representative images of liver metastases from CD24⁺ and CD24⁻ groups. (**C**) Immunohistochemical staining of resected tumor tissues from each group using CD24 and Notch1 antibodies. (**D**) The survival rate of the mice was determined until 30 days after the CD24⁺ or CD24⁻ cells injection. Magnification ×200.

Discussion

CD24 is a widely used biomarker for isolating CSCs.[13] Previous studies have shown that CD24⁺ cells have high capability to form tumors.[8–10] However, other studies showed that CD24 is not a cancer stem cell marker.[11,12,14] For example, Xu et al[14] found that both A549 and H560 CD24⁺ cells did not show enhanced tumor-forming ability than CD24⁻ cells.

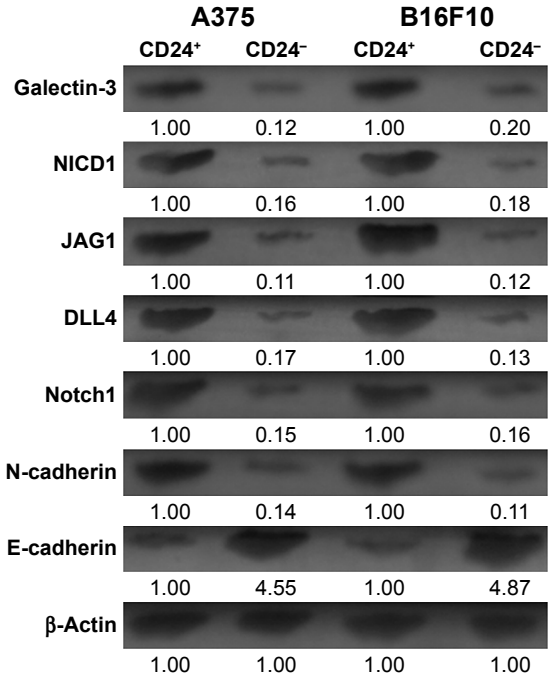

Figure 4 Western blot analysis of Notch1 signaling pathway.
Notes: Galectin-3, NICD1, JAG1, DLL4, Notch1, N-cadherin, and E-cadherin in cell lysates were determined.

In this study, using the soft agar assay, we demonstrated that the CD24⁺ subpopulation has self-renewal properties. We also confirmed the tumorigenic potential of the distinct population in mouse models.

The main finding of this study was that CD24 expression is accompanied by activation of Notch1 signaling pathway. Notch1 was found increased in many kinds of malignant tumors, such as melanoma, prostate cancer, and hepatocellular carcinoma.[15] Notch1 could promote the growth, survival, and metastasis of melanoma.[16] Previous studies showed that Notch1 controls another cancer stem cell marker (CD133) expression at transcriptional and posttranscriptional levels and regulates the survival and proliferation of glioma cells[17] and melanoma cells.[18] So et al[19] found that the CD44⁺/CD24$^{-/low}$ subpopulation of breast cancer cells showed elevated Notch1 signaling and increased cell proliferation compared to the CD44⁺/CD24^{high} subpopulation. In contrast, we found the activation of the Notch1 signaling pathway in CD24⁺ cells. Many previous studies showed that Notch1 pathway plays a crucial role in CSCs maintaining stemness.[20–22] Despite the opposite roles of Notch1 signaling in CD24 expression in breast cancer cells and melanoma cells, the results of both our study and So et al's study confirmed that Notch1 signaling maintains the tumorigenic ability of CSCs.

Conclusion

In conclusion, in our study, we found CD24⁺ population in melanoma cells has the characteristics of CSCs. CD24

expression may be related with activation of Notch1 signaling pathway.

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Disclosure

The authors report no conflicts of interest in this work.

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