Studies on the effect and mechanism of CD147 on melanoma stem cells

Yuan Jiang a, Renyi Liang b, Liqun Li c *, Jian Guan a *

a Department of Wound Healing and Plastic and Reconstruction Surgery, the Sixth Affiliated Hospital of Wenzhou Medical University, Lishui, Zhejiang Province, China
b Department of Tumor Radiotherapy and Chemotherapy, Lishui City People's Hospital, Lishui, Zhejiang Province, China
c Department of Plastic Surgery, the First Affiliated Hospital of Wenzhou Medical University, Wenzhou, Zhejiang Province, China

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Abstract
Background: Melanoma is the most aggressive form of skin cancer. Melanoma stem cells (MSCs) are one of the driving forces of melanoma invasion and metastasis. Therefore, it is of great significance to explore the mechanisms that maintain the stemness of MSCs. In this study, CD147-positive (CD147+) MSCs derived from A375 cell line were characterized.

Methods: Side population (SP) and non-SP cells were sorted from A375 cells. Quantitative real-time polymerase chain reaction and Western blot analysis were conducted to determine the expression of CD147 in SP and non-SP cells. Subsequently, CD147+ and CD147-negative (CD147−) cells were isolated from SP cells. Stem cell characteristics and metastatic potential of CD147+/- antigen-presenting cells were identified by sphere-forming, wound-healing, and transwell assays. Western blot analysis was performed to evaluate the protein levels of transforming growth factor-beta1 (TGFβ1) and neurogenic locus notch homolog protein 1 (Notch1) signaling pathway. Xenograft tumor experiments were conducted to investigate the tumorigenic capacity of CD147+ cells in vivo.

Results: CD147 was highly expressed in SP cells of A375 cell line. CD147+ cells have stronger abilities for sphere forming, migration, and invasion in vitro. The protein levels of TGFβ1, notch1, jagged1, and Hes1 were higher in CD147+ cells than in CD147− cells. Moreover, the CD147+ cells showed stronger tumorigenic and metastatic potential in vivo.

Conclusion: SP cells of A375 cell line expressed high levels of CD147, and CD147+ SP cells possessed much stronger stem-like characteristics and motility, which is linked to the activation of TGFβ1 and notch pathways.

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Introduction
Melanoma is a highly malignant tumor originating from melanocytes, and it commonly occurs in the skin. Melanoma is characterized by early metastasis, poor prognosis, and easy recurrence. For decades, both incidence and mortality of melanoma have increased by 3%-5% annually. It is reported that the 5-year survival rate of patients with early melanoma is as high as 99%, but the 5-year survival rate of patients with metastatic melanoma is less than 10%. Studies have found that a small subset of melanoma stem cells (MSCs) in melanoma, with stem cell-like features, is the key for melanoma genesis, metastasis, and recurrence. Therefore, exploring molecular mechanisms for regulating the biological behaviors of MSCs is extremely beneficial for developing effective targeted strategies for melanoma treatment.

CD147, a transmembrane glycoprotein belonging to the superfamily of immunoglobulins, has been demonstrated to play a significant role in the aggressive behavior of melanoma and serves as an independent prognostic biomarker for melanoma in a previous study. Furthermore, CD147 actively facilitates the initiation and metastasis of melanoma. Therefore, we hypothesized that CD147 could potentially function as a novel marker for MSCs. In this study, a side population (SP) of CD147-positive (CD147+) cells derived from the human melanoma cell line A375 was identified. Further, the role of CD147 was investigated in the maintenance of MSCs.

Materials and Methods

Cell culture
Human melanoma cell line A375 was cultured in H-Dulbecco’s Modified Eagle Medium (H-DMEM) (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin in a cell incubator with 5% CO₂ at 37°C. After centrifugation of collecting cells, these were resuspended in phosphate-buffered saline solution (PBS) with a density of 1×10⁶ cells/mL. A fluorescence-conjugated antibody against CD147 or immunoglobulin G (IgG; as a control) was used to stain the cells. CD147+ cells were sorted and analyzed using FACS Aria III cell sorter (BD Biosciences).

Quantitative real-time polymerase chain reaction (qRT-PCR)
Total RNAs were extracted from non-SP and SP cells using the miRNeasy mini kit (QIAGEN, Hilden, Germany). Synthesis of complementary DNA (cDNA) was performed using a first strand cDNA synthesis kit (Takara, Beijing, China). Next, qRT-PCR was conducted using a SYBR Green kit from Tiangen (Beijing, China). Primers used in this study are as follows: CD147: forward: 5’TTCATCTACGAGAAGCCGG-3’, reverse: 5’CAGGAAGAGTTCCTCTGCGG-3’; GAPDH: forward: 5’GGAGCGACATCCCTCCCCAA-3’, reverse: 5’GGCTGTGTCATACCTTCTCATGG-3’. Relative expression level of CD147 was calculated by using 2⁻ⁿᵈⁿ⁻ⁿ⁻ΔΔ method.

Sphere-forming assay
The CD147+ and CD147- cells were seeded in ultra-low attachment, 24-well plates at a density of 1000 cells/well. The sphere number was counted and the image was acquired after cells were cultured for 6 days. Sphere formation rate (%) was calculated as follows: (Number of formed spheres ÷ Number of seeds) × 100.

Wound-healing assay
CD147+ and CD147- cells were cultured in 6-well plates until they formed a complete monolayer. Then, a single wound in each well was scratched with tip of a micropipette. The scratch gap width was measured with an inverted microscope (ZEISS, Jena, Germany) after 0 and 24 h. A relative scratch width was calculated based on the distance migrated from the original scratch width.

Transwell-invasion assay
In order to evaluate invasion of CD147+ and CD147- cells, a transwell chamber (Corning, Acton, USA) precoated with Matrigel was used in a 24-well plate. Briefly, 200-µL cell suspension (5×10⁴ cells) was loaded into the upper chamber, and 600-µL DMEM containing growth factor was added in the lower chamber. Cells were cultivated at 37°C for 24 h. Subsequently, washing transwell inserts with PBS and removal of noninvasive cells in the upper chamber were done with cotton swabs. An axioskop optical microscope (ZEISS) was used to count invasion cells in the lower chamber after fixing them with methanol and staining with crystal violet.

Western blot analysis
For protein analysis, previously described techniques were used to prepare cell lysate for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Then,
the proteins were transferred to polyvinylidene fluoride (PVDF) membranes. After blocking, the membranes were incubated with primary antibodies, including anti-CD147 (ab108308, 1:1000; Abcam, Cambridge, UK), anti-transforming growth factor beta 1 (anti-TGFβ1, sc-7892, 1:100; SantaCruz, CA, USA), anti-neurogenic locus notch homolog protein 1 (notch1 signaling pathway) (ab280898, 1:1000; Abcam), anti-jagged1 (ab27198, 1:3000; Abcam), anti-Hes1 (ab71559, 1:2000; Abcam), and anti-GAPDH (ab59164, 1:2000; Abcam), followed by incubation with the second antibody. With an enhanced chemiluminescence (ECL) imaging system, protein bands were developed and photographed, and subsequently Image J was used to calculate protein gray value.

Tumorigenicity assay in vivo

Ten BALB/c nude mice (6-8 weeks) from Vital River Laboratory (Beijing, China) were randomly divided into CD147+ group and CD147- group (n = 5 per group). Subsequently, 100-µL CD147+ or CD147- cell suspension (approximately 5x10^5 cells) was injected subcutaneously into the right flank of each mouse.11 Tumor volume was measured every 4 days after the appearance of tumor by using the following formula: (length × width^2) ÷ 2.

After 24 days, tumors were removed, tumor weights were recorded, and animals were sacrificed.

Hematoxylin and eosin (H&E) staining

The lung tissues were excised after mice were euthanized. Next, according to the method described by Nakai et al., 4-µm paraffin sections of lung tissue were sliced for H&E staining to detect lung metastasis.17

Statistical analysis

Three replicates were performed for each experiment mentioned above. Using the GraphPad Prism 8.0 software, the data were analyzed and presented as mean ± SD, and unpaired Student’s t-test was used for comparison; P < 0.05 was considered statistically significant.
Figure 2  CD147+ cells possessed stem cell characteristics. (A) Sorting of CD147- and CD147+ cells from SP cells was done by flow cytometer. (B) Sphere formation assay of CD147- and CD147+ cells was performed. ***P < 0.001 vs. CD147- cells.

Figure 3  CD147+ cells demonstrated stronger migration and invasion abilities. (A) Wound-healing assay of CD147- and CD147+ cells in vitro. (B) Transwell invasion assay of CD147- and CD147+ cells in vitro. *P < 0.05, **P < 0.01 vs. CD147- cells.
CD147+ cells showed an increased number of invasion cells by transwell assay (Figure 3B). These results indicated that CD147+ cells exhibited robust migration and invasion abilities, compared with CD147- cells.

**CD147+ cells showed high expression levels of TGFβ1 and notch1 pathway**

Furthermore, Western blot analysis was employed to assess the expression levels of TGFβ1, notch1, jagged1, and Hes1 in both CD147+ and CD147- cells. As shown in Figure 4, the expression levels of all the proteins were elevated in CD147+ cells, compared to CD147- cells. The result established that CD147 activated both TGFβ1 and notch1 pathways.

**CD147+ cells promoted tumor growth and metastasis in vivo**

In order to verify that CD147+ cells were more tumorigenic than CD147- cells, 5×10^2 CD147+ cells or CD147- cells were injected subcutaneously in nude mice. The results demonstrated that tumors in the CD147+ group were significantly larger and heavier compared to those in the CD147- group (Figure 5A). Moreover, a significant increase in the number of metastatic lung lesions was observed in CD147+ group (Figure 5B). These results indicated that CD147+ cells had a stronger tumorigenic and metastatic growth in vivo, which were consistent with in vitro results.

**Discussion**

Cancer stem cells (CSC) are a small group of cancerous cells capable of regeneration, thus causing metastases and tumor proliferation. Targeting CSC or CSC-like cells has become an important strategy of treating cancer. SP phenotype analysis, which does not depend on cell surface markers, is one of the most effective methods for sorting CSC. Herein, both SP and non-SP cells were sorted from A375 cells, and CD147 expression was verified as being high in SP cells. Later on, CD147+ and CD147- cells were isolated from AP cells, and CD147+ cells were demonstrated to have much stronger MSC potential than CD147- cells, both in vitro and in vivo.

CD147 is abundant in melanoma, and is closely associated with the proliferation, invasion, and metastasis of melanoma cells. The study conducted by Su et al. demonstrated that CD147 played a significant role in the modulation of melanoma tumor proliferation via glucose metabolic pathway. Reger et al. reported that CD147 facilitated the development of lymphatic vessels in in vitro and in vivo models of melanoma, suggesting that targeting CD147 holds potential for effectively inhibiting melanoma metastasis. Liu et al. found that CD147 affected MMP9 expression in a NFAT1-dependent manner, thereby facilitating the metastasis of melanoma. In addition, CD147 was confirmed to induce non-stem cancer cells convert into the stem-like features and may serve as a marker of breast and pancreatic CSCs. However, the role and function of CD147+ cells in MSCs remained unclear.

In the present study, SP cell subpopulation in A375 cells was isolated using flow cytometry, and it was ascertained that CD147 exhibited high expression in these SP cells. Further analysis by flow cytometry revealed that CD147+ SP cells constituted more than 90% of the population. Notably, CD147+ cells demonstrated robust stem cell properties through sphere formation experiments. Additionally, the migratory and invasive capabilities of CD147+ cells were confirmed as being much stronger through wound healing and transwell assays. The in vivo assay further confirmed that CD147+ cells had stronger tumorigenic and metastatic abilities.

![Image of Western blot analysis](Figure 4) CD147+ cells showed high expression levels of both TGFβ1 and notch1 signaling pathway. Representative Western blot bands and quantification of TGFβ1, notch1, jagged1, and Hes1 in CD147- and CD147+ cells. **P < 0.01, ***P < 0.001 vs. CD147- cells.
Figure 5  CD147+ cells promoted tumor growth and metastasis in vivo. (A) Representative tumor images were shown, and tumor volume and weight were measured. (B) H&E staining was performed to detect the lung metastasis of mice. **P < 0.01, ***P < 0.001 vs. CD147-group.

TGFβ is a versatile peptide cytokine capable of inducing epithelial mesenchymal transformation (EMT), thereby promoting tumor cell invasion and metastasis as well as conferring stem cell-like properties to tumor cells.30 In hepatocellular carcinoma and tongue squamous cell carcinoma, CD147 cells have been demonstrated to mediate the TGFβ1-induced process of EMT, and subsequent cell invasion.31,32 The notch pathway is crucial for regulating cell self-renewal and cell differentiation in CSCs.33 Studies have shown that notch1 signaling pathway enhances the stemness of melanoma and liver cancer cells.34,35 Herein, the results of Western blot analysis indicated that the expressions of TGFβ1, notch1, jagged1, and Hes1 were enhanced in CD147+ cells. These results suggested that both TGFβ1 and notch1 pathways were activated in CD147+ cells. However, additional investigations are required to provide definitive evidence to demonstrate whether this molecular mechanism is responsible for the maintenance of stemness of MSCs by CD147 cells.

Conclusion

This study unveiled that CD147 cells serve as a novel marker for the identification of MSCs, thereby presenting a potential therapeutic target for interventions in melanoma.

Competing Interests

The authors state that there was no conflict of interest to declare.

Ethical Approval

Ethical approval was obtained from the Ethics Committee of the Sixth Affiliated Hospital of Wenzhou Medical University.

Data Availability

The authors declare that all data supporting the findings of this study are available within the paper and any raw data can be obtained from the corresponding author upon request.

Author Contributions

Yuan Jiang, Renyi Liang, Liqun Li, and Jian Guan designed the study and carried out all experiments, supervised data collection, data analysis, and interpretation. All authors prepared the manuscript for publication and
CD147 enhanced the stem-like properties of melanoma stem cells

References


