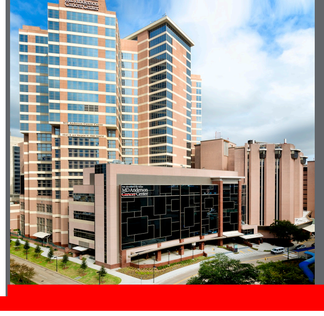

Identification of CD74 isoforms that complex with CD44 to initiate the oncogenic signaling pathway in melanoma

Kalli Majewski, Augustana College, Class of 2021

Dr. Yasunari Fukuda, Dr. Sung-Nam Cho, and Dr. Suhendan Ekmekcioglu

THE UNIVERSITY OF TEXAS MD Anderson Cancer Center

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Introduction

The protein Cluster of Differentiation 74 (CD74) is a type II transmembrane molecule that acts as a receptor for macrophage migration inhibitory factor (MIF) when it displays on the cell surface. The interaction between MIF and CD74 initiates oncogenic signaling in cancer to control the growth of a tumor. Our previous study showed this interaction was relevant in advanced melanoma[1] (Fig. 1). CD74 alone does not function to signal these pathways but does so with help of others including CD44, a non-kinase transmembrane glycoprotein, acting as a transducing co-receptor. Although it has been demonstrated that CD74 has four different isoforms (p31, p33, p41, and p43)[2], it is unclear which isoform makes this complex with CD44 to initiate the oncogenic signaling pathways in melanoma.

Objectives

Our group has previously shown that autocrine MIF-CD74 interactions in response to interferon-γ (IFN-γ) induced oncogenic pAKT pathway and tumor progression in cutaneous melanoma[3]. However, we have not shown whether the interaction between MIF and CD74/CD44 binding induces the oncogenic signaling.

In this proposal, we aim to identify

(1) if the binding of MIF/CD74/CD44 signaling is involved in oncogenic pAKT pathways
(2) which CD74 isoform makes the complex with CD44

in melanoma.

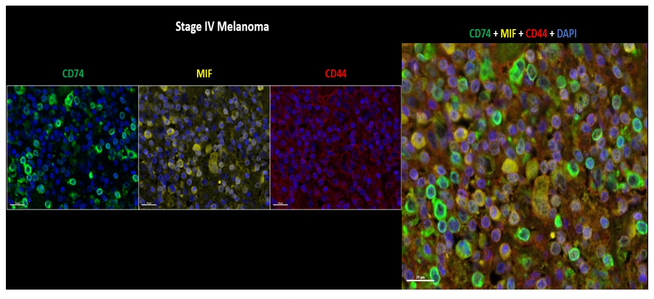

Fig. 1: Representative image of multiplex immunohistochemistry for CD74 (green), CD44 (red), and MIF (yellow) in patients with stage IV Melanoma. Unpublished.

Methods

Cell lines:
A375 (BRAF-mutant melanoma cell line),
SB2 (NRAS-mutant melanoma cell line)

Experimental overview

(1) Induction of the cell surface CD74 by recombinant human (rh) IFN-γ stimulation.

[Methods] We stimulated A375 and SB2 with different concentration of rhIFN-γ (0, 20, 100, and 500 IU/mL) and evaluated the CD74 expression by real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR), western blotting (WB), and flow cytometry.

(2) Activation of the AKT pathway by the binding of MIF to cell-surface CD74, induced by IFN-γ.

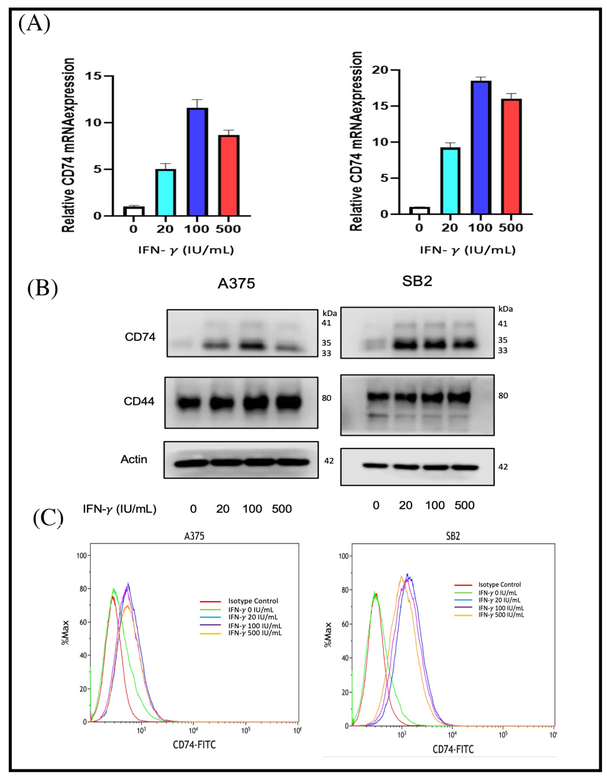

Fig. 2 Induction of CD74 by IFN-γ stimulation.
(A) qRT-PCR data showed that IFN-γ (20, 100, and 500 IU/mL) dramatically increased the mRNA CD74 expression in A375 and SB2. (B) WB analysis of change in CD44 and CD74 expression after IFN-γ stimulation (20, 100, and 500 IU/mL) in A375 and SB2. Actin was used as a loading control. (C) Flow cytometry analysis showed that IFN-γ stimulation (20, 100, and 500 IU/mL) increased the cell surface CD74expression in A375 and SB2.

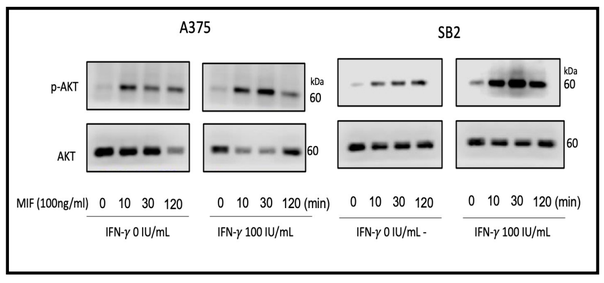

Fig. 3. Activation of AKT signaling after rhMIF stimulation.
We compared the p-AKT expression 0, 10, 30, and 120 min after 100 ng/mL rhMIF exposure under basal and IFN-γ stimulatory conditions in A375 and SB2. Total AKT was used as a loading control.

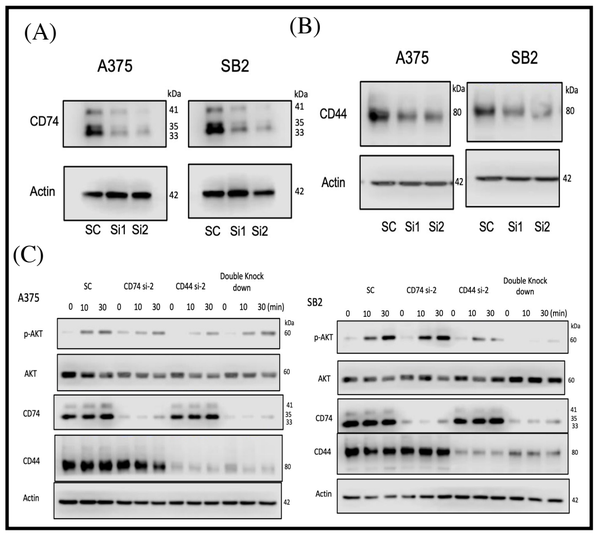

Fig. 4. Activation of AKT signaling after rhMIF stimulation after knocking down of CD74 and CD44
(A, B) Efficacies of two individual siRNAs in knocking down CD74 (A) and CD44 expression (B) were analyzed by WB in A375 and SB2 under 100 IU/mL IFN-γ stimulated conditions. SC siRNA was used as a control. (C) Under 100 IU/mL IFN-γ stimulated conditions, we compared the p-AKT expression 0, 10, and 30 min after 100 ng/mL rhMIF stimulation in A375 and SB2, transfected with scramble (SC) siRNA, CD74 si-2, CD44 si-2 and double (CD74 si-2 and CD44 si-2) by WB. Total AKT was used as a loading control.

Methods (continued)

[Methods] We pre-treated A375 and SB2 with 100 IU/mL rhIFN-γ (no IFN-γ stimulation for control) for 24 h to induce the cell-surface CD74 expression and sequentially stimulated by 100 ng/mL rhMIF. After 0, 10, 30, and 120 min exposures to rhMIF, we prepared the cell lysates to evaluate p-AKT expression by WB.

(3) Interaction between MIF and CD74/CD44 binding is involved in the AKT pathway.

[Methods] The change in p-AKT expression 0, 10, and 30 min after 100 ng/mL rhMIF exposure was evaluated by WB with knocking down of CD74 and CD44 (as single and double) by short interfering (si)RNA in A375 and SB2. Scramble (SC) siRNA was used as a control.

(4) Identification of the isoform of CD74 that complexes with CD44

[Methods] We will pre-treat A375 and SB2 with 100 IU/mL rhIFN-γ for 24 h to induce cell-surface CD74 expression and perform CD44/CD74 co-immunoprecipitation, followed by WB.

Results

(1) Induction of the cell surface CD74 by IFN-γ stimulation.

CD74 mRNA expression was dramatically upregulated in response to IFN-γ in A375 and SB2 by q-RT PCR (Fig. 2A). The upregulation of 3 isoforms of CD74 (p33, p35, and p41) under IFN-γ stimulatory conditions were found in A375 and SB2 by WB (Fig. 2B). On the contrary, the CD44 expression was not affected by IFN-γ (Fig. 2B). In addition, flow cytometry analysis revealed that the cell-surface CD74 was induced by IFN-γ, particularly in SB2 (Fig. 2C).

(2) Activation of the AKT pathway by the binding of MIF to cell-surface CD74, induced by IFN-γ.

In order to explore if binding of MIF to cell-surface CD74 initiates AKT signaling, we compared the p-AKT expression 0, 10, 30, and 120 min after rhMIF exposure under basal and IFN-γ stimulatory conditions. As shown in Fig. 3, the upregulation of p-AKT expression after rhMIF stimulation was more marked in A375 and SB2 pre-treated with rhIFN-γ than in those cultured in basal conditions, particularly clearer in SB2. These results indicate the interaction between MIF and the cell-surface CD74, induced by IFN-γ, activates oncogenic signaling pathway in melanoma.

(3) Interaction between MIF and CD74/CD44 binding is involved in the AKT pathway.

Results (continued)

In order to assess if MIF/CD74/CD44 signaling is involved in the activation of the AKT pathway, we silenced CD74 and/or CD44 by siRNA. We prepared siRNA reagents targeting 2 different sequences of CD74 and CD44 (si-1 and si-2, respectively). Efficacies of transfection were confirmed by WB (Fig. 4A, B) and CD74 si-2 and CD44 si-2 were used for the following experiment. Next, under 100 IU/mL rhIFN-γ stimulated conditions, we compared the p-AKT expression 0, 10, and 30 min after 100 ng/mL rhMIF stimulation in A375 and SB2 between cells transfected by scramble (SC) siRNA, CD74 si-2, CD44 si-2 (single), and CD74 si-2/CD44 si-2 (double). When compared, cells with SC siRNA, p-AKT expression was slightly decreased in cells with CD74 si-2 and was remarkably attenuated in cells with CD44 si-2 (Fig. 4C). Furthermore, AKT signaling was synergistically suppressed in SB2 with double knocking down of CD74 and CD44.

(4) Identification of the isoform of CD74 that complexes with CD44

Lastly, in order to identify the isoform of CD74 that complex with CD44, we will collect cell lysates after 100 IU/mL rhIFN-γ stimulation and perform CD44/CD74 co-immunoprecipitation, followed by WB.

Conclusions

The present study showed that the three isoforms of CD74 (p33, p35, and p41) was upregulated by IFN-γ, and the interaction between MIF and CD74/CD44 was associated with the activation of the AKT signaling pathway in melanoma.

We are currently studying to identify the isoforms of CD74 (p33, p35, and p41) that complexes with CD44. Given that CD74 also works as a chaperon for MHC-class II, it may be unpreferable to inhibit the functions of all isoforms of CD74.

In this regard, our result may not only provide a better understanding of the signaling pathways, but also help develop the therapeutic drug against the specific isoform of CD74 that is associated with the promotion of oncogenic signaling in melanoma.

References

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