Activating IGFLR1 to Promote Melanoma Cell Apoptosis

Kehan Ren
College of Life Science and Technology, Huazhong University of Science and Technology, Wuhan, Hubei 430074, China

Keywords: Melanoma, IGFLR1, IFN-γ, IGFL1, IGFL3, CD4+ T Cell.

Abstract: Purpose: Melanoma is one of the most common types of cancer with a high incidence rate. Surgical resection therapy is difficult to be applied to patients with extensive metastasis. Previous studies have reported that IGFL3 promotes the activation of CD4+ T cell and secretion of IFN-γ through the transmembrane protein IGFLR1, and IFN-γ inhibits tumor growth by inducing apoptosis of cancer cells. Activating IGFLR1 will upregulate the release of IFN-γ and promote cancer cell apoptosis. This paper investigates the effect of activation of IGFLR1 using IGFL1 recombinant protein on anti-melanoma treatment in both *in vitro* and *in vivo* conditions. In this work, eight possible outcomes of the experiments are discussed respectively. Different results will indicate whether or not IGFLR1 activation has a therapeutic effect on the anti-cancer treatment and provide critical information for the future clinical trial of IGFLR1 activation therapy.

1 INTRODUCTION

Melanoma is the most dangerous type of skin cancer (World Cancer Report. World Health Organization. 2014). According to the American Cancer Society (ACS), it affects about 100 thousand people per year in the United States, and it has a long-term survival rate of less than 30% (American Cancer Society, 2021). Compared with other types of cancer cells, melanoma cell is more likely to spread to other parts of the body. Additionally, since some of the melanomas do not make melanin, people may miss prime therapy time.

Surgical resection is currently the primary treatment for malignant melanoma at the early stage, but it is not suitable for patients with extensive metastasis (Niederhuber, 2019). Immunotherapy is a treatment method that is widely applied to advanced cancer (Abbott, M., and Y. Ustoyev, 2019). It aims to stimulate or suppress certain type of the immune cells through certain target in order to help the body's immune system attack melanoma cells more effectively. Immunotherapy has revolutionized anti-melanoma therapy through anti-programmed cell death protein 1 (anti-PD-1) (Onitilo, 2019). However, there are still over 60% of the patients who do not respond or develop resistance to these treatments (Hugo, 2016). Therefore, a more suitable treatment for melanoma and new drug target on immune cells to enhance the anti-melanoma immmune response are still needed to be developed.

CD4+ T cell is an immune cell in the body's immune system that plays a vital role in fighting cancer (Luckheeram, 2012). CD4+ T cells would not kill the cancer cells directly. However, it can produce and secrete IFN-γ, which is a glycosylated protein (Ngai. 2007). IFN-γ inhibits tumor growth by inducing apoptosis and dormancy of tumor cells (Takeda, 2017). IFN-γ induces the expression of IRF1, a tumor suppressor gene (Yan Zhou, Crystal M. Weyman, 2008). It also participates in the IFN-γ/STAT1 pathway, which leads to the dormancy of tumor cells (Kortylewski, 2004).

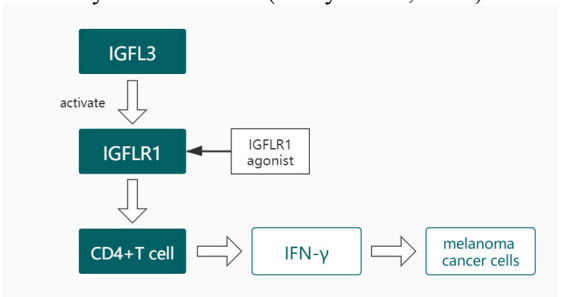

Figure 1: IGFLR1 Pathway. This figure shows the position and activities of IGFLR1.

Insulin growth factor-like receptor 1 (IGFLR1) is a transmembrane protein, and it is encoded by the

Ren, K.
Activating IGFLR1 to Promote Melanoma Cell Apoptosis.
DOI: 10.5220/0011312600003443
In *Proceedings of the 4th International Conference on Biomedical Engineering and Bioinformatics (ICBEB 2022)*, pages 883-888
ISBN: 978-989-758-595-1

IGFLR1 gene located on chromosome 19 (Fagerberg, 2014). IGFLR1 is highly expressed in CD4+ T cells (Zhang, 2018). Insulin growth factor-like family member 1 (IGFL1) is known as the ligand of IGFLR1, and it is induced in inflammatory skin conditions (Song, 2020); (Lobito, 2011). IGFL3 is another ligand of IGFLR1, showing high-affinity interactions with IGFLR1 (Zhang, 2018). IGFLR1 pathway (See Figure 1) could be a potential drug therapy target. IGFL3 binds with IGFLR1, promoting CD4+ T cell activation and IFN-γ secretion (Zhang, 2018) . However, the effect of IGFL1 binding with IGFLR1 in immunotherapy has not be studied.

Therefore, in order to test the therapeutic effect of activating IGFLR1 in preclinical conditions, a comparative study should be designed. This paper investigates the effect of activating IGFLR1 using IGFL1 recombinant protein on anti-melanoma treatment in both *in vitro* and *in vivo* conditions. If IGFLR1 is activated in T cells through binding IGFL1 recombinant protein produced in e coli, then the function of T cell will be enhanced, and melanoma progression will be suppressed, because IGFL3 promotes CD4+ cell activation and IFN-γ release through IGFLR1, and IFN-γ plays an important role in killing cancer cells.

2 METHODS

2.1 Materials

This experiment will use a human melanoma cell line (A375), a murine melanoma cell line (B16-F1), human and murine CD4+ T cells. Cell line A375 and B16 are obtained from the China Center for Type Culture Collection. Human CD4+ T cells are isolated from healthy donor PBMCs, and murine CD4+ T cells are isolated from the murine spleen. For all the experiments, cells are randomly allocated to different experimental groups.

C57BL/6 mice will be used *in vivo* study. Four-week old C57BL/6 female and male mice are purchased from the Center of Medical Experimental Animals of Hubei Province (Wuhan, China). The mice are randomly assigned to the control group or the treated group. The mice are housed under specific pathogen-free conditions. Animals will be euthanized immediately if they display excessive discomfort.

IGFL1 recombinant protein is produced in e coli and IGFL3 is ordered from CUSABIO for both *in vitro* and *in vivo* experiments.

2.2 In Vitro Cell Culture

Co-culture system will be established for *in vitro* study. A375, B16 cells are cultured on RPMI-1640 supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin at 37 °C with 5% CO2 (Ye, 2017). The cells are passaged every 2 days using TrypLE. Isolated T cells are cultured in RPMI-1640 supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin and streptomycin and 2-mercaptoethanol (Zhang, 2018). T cells are primarily activated with anti-CD3 (UCHT1) and anti-CD28 (CD28.2) (Zhang, 2018). Melanoma cells are labeled with CFDA-SE. CFDA-SE-labeled melanoma cells and T cells are added into the 12-well plate, shaken evenly, and placed in a cell culture box for co-culture.

Each co-culture cells will be divided into six groups: (1) negative control: PBS; (2) positive control: 100ng/ml IGFL3 solution; (3) 10 ng/ml IGFL1 solution; (4) 50 ng/ml IGFL1 solution; (5) 100 ng/ml IGFL1 solution; and (6) 200 ng/ml IGFL1 solution. IGFL1 is given and incubated in the cell culture box for 2 hours. *in vitro* experiment will be repeated three times.

2.3 Flow Cytometry

The cell apoptosis will be measured through the Flow Cytometry system offered by ThermoFisher Scientific. The co-cultured cells will be collected and measured every 24 hours after treatment. The PI staining solution in the apoptosis PI staining kit is used for staining. The therapeutic effect of the *in vivo* experiment will be evaluated after one course of treatment (30 days), with three courses of treatment in total. Each experiment is repeated three times. Flow cytometry analysis showed that the ratio of CFDA-SE+/PI+ cells was the ratio of tumor cells killed after IGFL1 stimulated CD4+ T cells to exert an antitumor immune effect. Each experiment is repeated for five times.

2.4 ELISA

IFN-γ expression level will be measured via sandwich ELISA. The sandwich is formed by adding the samples, then the second antibody, which has a measurable signal on it. Specific anti-murine IFN-γ antibodies and anti-human IFN-γ antibodies are precoated in the wells of the microplate. Samples and controls are then added into these wells and bind to the immobilized antibody. Then the second antibodies is added and bind to the samples. After

incubation, the intensity of signal is measured.

2.5 Western Blotting

Whole-cell lysates will be prepared with FLAG-IP lysis buffer (50 mM tris, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, and 10% glycerol), with protease inhibitor tablets. Protein concentration will then be determined with DC Protein Assay from Bio-Rad Laboratory. Proteins will be separated using SDS Page gel electrophoresis and wet-transferred to polyvinylidene difluoride membranes. Blots will be visualized with SuperSignal West Pico Chemiluminescent® substrate from Thermo Fisher Scientific. IGFL1 Antibody and IGFLR1 Antibody from ThermoFisher Scientific will be used for Western blotting.

2.6 Animal Model

C57BL/6 mice aged from 4 to 6 weeks will be used. B16 cells are injected subcutaneously to form a model of Subcutaneous melanoma. As a minimum of eight mice per group is required for having a statistical power, each group has ten mice. Each C57BL/6 mouse is injected with B16 cells subcutaneously at the left hind leg on day 0. The experimentalists are blinded from the expected outcome of the treatment.

Then the mice are randomly divided into three groups: (1) negative control: PBS; (2) positive control: 100ng/mL IGFL3 solution; (3) IGFL1 treatment, with the optimum blood concentration determined by the cell experiment. For the positive control group, there is no experimental data to confirm that IGFL3 would function *in vivo* experiments. However, IGFL3 is the possible molecule that may activate IGFLR1 *in vivo* experiments. *in vivo* experiment will be repeated three times.

2.7 In Vivo IGFL1 Delivery

IGFL1 will be given on day 8. Hydrodynamic tail vein injection will be performed. The growth of B16 tumors is monitored by measuring tumor size every other day. On day 30, all the mice will be euthanized. The IGFL1 level in the blood will be determined by western blot.

2.8 Histologic Evaluation and Immunohistochemistry

Tissue of mice in experimental groups is collected for histologic evaluation in order to determine tissue toxicity. Tumor tissue, lung, liver, spleen and stomach of mice are fixed by 4% paraformaldehyde and then embedded in paraffin and cut to ~4 μm thick sections by Thermo FINESSE 325. Organ sections are stained by H&E and slides are evaluated for tumor formation by a veterinary (Chen, 2018).

2.9 Statistical Analysis

The statistical significance of all numerical data gathered through ELISA, Western Blot, and Flow Cytometry will be analyzed using the student's T-Test on GraphPad Prism® at ($p < 0.05$).

3 RESULTS

Possible Results on Melanoma Cell Apoptosis (The overview of six possible results is also shown in Table 1.)

Table 1: Possible Results on Melanoma Cell Apoptosis.

Cell Lines	Result 1	Result 2	Result 3	Result 4	Result 5	Result 6
Killing of xenograft cells	+	-	+	-	-	-
Killing of Human A375 Cell Lines	+	+	-	-	+	-

Killing of Murine B16 Cell Lines	+	+	+	+	-	-

Note. "+" represents a significant increase in melanoma cell apoptosis. "-" represent not significantly different from negative control.

Possible Result 1: Applying IGFL1 promotes melanoma cell apoptosis in determined human and murine melanoma cell lines and the cell line from the *in vivo* animal models.

IGFL1 activates IGFLR1 in all *in vitro* CD4+ T cell samples, increasing the expression of IFN-γ. The apoptosis of melanoma cell samples is promoted significantly. The animal experiments display that IGFL1 activation of IGFLR1 has a therapeutic effect on melanoma.

Possible Result 2: Applying IGFL1 promotes melanoma cell apoptosis in determined human and murine melanoma cell lines, but not the cell line from the *in vivo* animal models.

IGFL1 activates IGFLR1 in all *in vitro* CD4+ T cell samples, increasing the expression of IFN-γ. The apoptosis of both human and murine melanoma cell samples is promoted significantly. However, IGFL1 does not successfully increase *in vivo* IFN-γ expression, or the animal experiments do not display a significant therapeutic effect of IGFL1 activation of IGFLR1 on melanoma.

Possible Result 3: Applying IGFL1 promotes melanoma cell apoptosis in the determined murine melanoma cell line *in vitro* and *in vivo* animal models, but not the human melanoma cell line.

IGFL1 activates IGFLR1 in murine CD4+ T cells, increasing the expression of IFN-γ. The apoptosis of B16 cell samples is promoted significantly. However, the IGFL1 does not successfully increase *in vitro* IFN-γ expression or the apoptosis of A375 cell samples. The animal experiments display that IGFL1 activation of IGFLR1 inhibits the growth of B16 tumors since this model uses the same melanoma cell line as *in vitro* murine cell samples.

Possible Result 4: Applying IGFL1 only promotes melanoma cell apoptosis in determined murine melanoma cell line.

IGFL1 activates IGFLR1 in murine CD4+ T cells. The apoptosis of B16 cell samples is promoted significantly. However, applying IGFL1 to the co-culture of human CD4+ T cells and A375 cells does not successfully increase *in vitro* IFN-γ expression or the apoptosis of A375 cell samples. Furthermore, IGFL1 does not successfully increase *in vivo* IFN-γ expression, or the animal experiments do not display a significant therapeutic effect of IGFL1 activation of IGFLR1 on melanoma.

Possible Result 5: Applying IGFL1 only promotes melanoma cell apoptosis in determined human melanoma cell line.

IGFL1 activates IGFLR1 in human CD4+ T cells. The apoptosis of A375 cell samples is promoted significantly. However, applying IGFL1 to the co-culture of murine CD4+ T cells and B16 cells does not successfully increase *in vitro* IFN-γ expression or the apoptosis of B16 cell samples. The animal experiment will not be successfully conducted in this scenario.

Possible Results 6: Applying IGFL1 does not promote melanoma cell apoptosis.

Applying IGFL1 does not successfully stimulate CD4+ T cells. The expression level of IFN-γ does not change significantly. Moreover, there is not a significant increase in the ratio of melanoma cell apoptosis.

Possible Result 7: Applying IGFL1 increases IFN-γ expression but does not promote melanoma cell apoptosis in any cell lines.

The IFN-γ level in the blood is upregulated. However, there is not a significant increase in the ratio of melanoma cell apoptosis after IGFL1 activation.

Possible Result 8: Applying IGFL1 promote melanoma cell apoptosis, but does not increases IFN- γ expression in any cell lines.

Melanoma cell apoptosis is prompted. However, there is not a significant increase in the IFN- γ level in the blood.

4 DISCUSSION

Previous studies report that activation of IGFLR1 will increase the secretion of IFN-γ in CD4+ T cells, which promotes cell apoptosis in many known cancer cell lines. In order to test the preclinical therapeutic effect of activating IGFLR1 in melanoma cell samples and animal models by using agonist, this study induces potential agonist IGFL1 to one well-studied melanoma cell line from humans and one from mice, as well as an *in vivo* animal melanoma model.

Possible Results 1 fully support the hypothesis. Compared with the negative group, the expression level of IFN-γ is upregulated and the melanoma cell apoptosis is promoted, which is consistent with the positive group. The result is consistent with previous studies investigating IGFLR1's effect on CD4+ T cells and IFN-γ's effect on cancer cells. It indicates that IGFL1 activating IGFLR1 therapy has potential value in anti-melanoma treatment. Further studies investigating the specific gene regulation mechanism of IGFLR1 should be done for a thorough understanding of its structures and functions. The relation between IGFLR1 and IGFL1 should also be investigated to investigate the more specific IGFLR1 pathway. Preclinical testing on more complex and representative animal models such as rabbits and monkeys should also be done before the transition to clinical testing of IGFLR1 activation therapy. In order to improve this therapeutic method, better delivery platforms like small molecule carriers or mechanisms involving controlled-release should be applied as well.

Possible Results 2 partially support the hypothesis. In Possible Result 2, IGFL1 activation fails *in vivo* experiment. In mice experiment, compared with positive group, the ELISA result will indicate a low expression level of IFN-γ. And IGFL1 level is almost no higher than the negative group. The failure of the *in vivo* experiment is most likely to be caused by the unsuccessful delivery of IGFL1 *in vivo*: IGFL1 does not reach CD4+ T cells through vein injection or does not maintain in the body long enough for its functions. To improve the experiment, a highly efficient and dependable delivery method should be developed. The safety level of activating the IGFLR1 pathway to enhance antitumor immune responses should be improved before clinical trials. Furthermore, new agonists activating IGFLR1 could be developed in place of IGFL1.

Possible Results 3 and 4 both partially support the hypothesis. Both of the results indicate that IGFLR1 activation cannot promote the cell apoptosis in human melanoma cell lines. Compared with negative group, the ELISA result will indicate almost no difference in the expression level of IFN-γ. Only if the IGFLR1 activation promotes the cell apoptosis in human melanoma cell samples, the IGFL1 activating IGFLR1 treatment will potentially have therapeutic effects and should be carried on to clinical trial. Possible Results 3 and 4 indicate that the IGFLR1 activation is not qualified to be a universal treatment for melanoma either because the A375 cell line does not have an IFN-γ receptor or has a different type of IFN-γ mechanism. This will require future studies to re-evaluate the relationship between IFN-γ, IGFLR1, and general types of melanoma.

Possible Results 5 and 6 indicate potential errors in the experimental designs. Possible Results 5 partially support the hypothesis. The expression level of IFN-γ and cancer cell apoptosis rate in murine cell line does not change significantly. It indicates that IGFL1 cannot be applied to mice. Searching for new agonists will be required for future studies on animal cell lines. At the same time, IGFL1 activating IGFLR1 treatment should be applied to other types of human melanoma cell lines to investigate the therapeutic effect. Possible Results 6 contradicts the hypothesis. In result 6, IGFLR1 activation is unsuccessful in all cell samples. Compared with the negative group, the expression level of IFN-γ is not considerably changed. It indicates that IGFL1 does not activate IGFLR1, which means bioinformatic data deviates from the actual situation. This result may be caused by an inappropriate cell incubation method or IGFL1 concentration. In this case, changing experimental designs is required. This result may also be caused by IGFLR1 participating in multiple pathways. This will require further studies on IGFLR1 pathways and the development of new agonists aiming at IGFLR1.

The Possible Result 7 and 8 partially contradicts the hypothesis. In Result 7, The IFN-gamma level in the blood is upregulated, which is consistent with positive group. However, there is not a significant increase in the ratio of melanoma cell apoptosis after IGFL1 activation. This result indicates that CD4+ T cells is successfully activated, however, some oncogenic mutations may be present in the melanoma cell line, which means that future clinical melanoma therapies through activating IGFLR1 could not be applied for all kinds of melanoma types since each cell line can have such a mutation. However, this result is unlikely to happen on the well-studied melanoma cell lines since the IFN-γ pathways are relatively well studied in these cell lines. In Possible Result 8, melanoma cell apoptosis is prompted, which is consistent with positive group. However, there is not a significant increase in the IFN-gamma level in the blood. It indicates that an alternative IGFL1 pathway may be crucial for CD4+ T cell stimulation. Future studies should be focused on investigating the expression level of other cytokines to verify the effects of IGFL1 activation on CD4+ T cells.

5 CONCLUSION

As a newly identified potential drug target, IGFLR1 has not been thoroughly studied in melanoma treatment. This study explores the therapeutic effect of IGFLR1 activation in human and murine melanoma cell lines, as well as Xenograft murine models. The result of this study will indicate whether or not IGFLR1 activation has a therapeutic effect in preclinical conditions, preparing the basis for the transition to clinical trials. The feasibility of using IGFL1 as the agonist of IGFLR1 will also be tested. Future studies should focus on improving *in vivo* IGFL1 delivery methods, including active targeting delivery and small molecule carriers, as well as developing IGFLR1 agonists such as monoclonal antibodies (mAbs). And we could pay attention to potential IGFLR1 functions in different kinds of T cells. Further studies on IGFLR1 pathways may find new targets for enhancing T cell function in immunotherapy of melanoma.

REFERENCES

American Cancer Society. Facts & Figures 2021. American Cancer Society. Atlanta, Ga. 2021.

Abbott, M., and Y. Ustoyev. "Cancer and the Immune System: The History and Background of Immunotherapy." Seminars in Oncology Nursing 35.5 (2019): 150923.

Chen, Junwei et al. “Inhibition of cancer stem cell like cells by a synthetic retinoid.” Nature communications vol. 9,1 1406. 11 Apr. 2018.

Fagerberg, Linn et al. "Analysis of the Human Tissue-specific Expression by Genome-wide Integration of Transcriptomics and Antibody-based Proteomics." Molecular & Cellular Proteomics 13.2 (2014): 397-406.

Hugo, Willy, et al. "Genomic and transcriptomic features of response to anti-PD-1 therapy in metastatic melanoma." Cell 165.1 (2016): 35-44.

Kortylewski, M., et al. "Interferon-gamma-mediated growth regulation of melanoma cells: involvement of STAT1-dependent and STAT1-independent signals. " Journal of Investigative Dermatology 122. 2(2004):414-422.

Luckheeram, R. V., et al. "CD4+T Cells: Differentiation and Functions." Clinical & Developmental Immunology 2012.12(2012):925135.

Lobito, Adrian A., et al. "Murine insulin growth factor-like (IGFL) and human IGFL1 proteins are induced in inflammatory skin conditions and bind to a novel tumor necrosis factor receptor family member, IGFLR1." Journal of Biological Chemistry 286.21 (2011): 18969-18981.

Niederhuber, John E., et al. Abeloff's Clinical Oncology E-Book. Elsevier Health Sciences, 2019.

Ngai, P., et al. "Gamma interferon responses of CD4 and CD8 T-cell subsets are quantitatively different and independent of each other during pulmonary Mycobacterium bovis BCG infection. " Infection & Immunity 75.5(2007):2244.

Onitilo, A. A., and J. A. Wittig. "Principles of Immunotherapy in Melanoma." Surgical Clinics of North America 100.1(2019).

Pajcini, Kostandin V et al. “MAFB enhances oncogenic Notch signaling in T cell acute lymphoblastic leukemia.” Science signaling vol. 10,505 eaam6846. 14 Nov. 2017.

Song, Wenjing, et al. "IGFLR1 as a Novel Prognostic Biomarker in Clear Cell Renal Cell Cancer Correlating With Immune Infiltrates." Frontiers in molecular biosciences 7 (2020).

Takeda, K., et al. "IFN-γ is required for cytotoxic T cell-dependent cancer genome immunoediting." Nature Communications 8(2017):14607.

World Cancer Report. World Health Organization. 2014. pp. Chapter 5.14.

Yan Zhou, Crystal M. Weyman , et al. "IFN-gamma induces apoptosis in HL-60 cells through decreased Bcl-2 and increased Bak expression. " J Interferon Cytokine Res 28.2(2008):65-72.

Ye, Jun, et al. "Vitamin E-rich nanoemulsion enhances the antitumor efficacy of low-dose paclitaxel by driving Th1 immune response." Pharmaceutical research 34.6 (2017): 1244-1254.

Zhang, Lei, et al. "Lineage tracking reveals dynamic relationships of T cells in colorectal cancer." Nature 564.7735 (2018): 268-272.