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Possible effects of treatments durations with Ruxolitinib on inhibiting jak phosphorylation of STAT1 in A549 lung cancer cells

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Abstract. This study explores the effects of different treatment durations of Ruxolitinib on inhibiting JAK phosphorylation of STAT1 in A549 lung cancer cells. The JAK/STAT signaling pathway is critical in lung cancer development and progression. By utilizing western blotting, a STAT luciferase reporter assay, and an MTT viability assay, the study evaluates the impact of Ruxolitinib treatment on STAT1 phosphorylation, STAT activation, and cell viability. Understanding how treatment duration influences JAK/STAT pathway inhibition provides valuable insights for developing targeted therapies in lung cancer. This research contributes to addressing the challenges of lung cancer treatment by exploring the potential of Ruxolitinib as a therapeutic agent.

Keywords: Jak/STAT protein family, Ruxolitinib, target gene.

1. Introduction

Globally, lung cancer stands as the leading cause of cancer-related fatalities in both men and women. Among men, it ranks as the most prevalent cancer, while among women, it ranks third, trailing behind breast and gastrointestinal cancers in frequency [1]. Various etiological factors could trigger lung cancer tumorigenesis, such as tobacco use, ethanol consumption, environmental pollution, and nutritional habits [1-2]. Considering the substantial morbidity and mortality from poor outcomes, more prophylactic strategies for lung cancer are imperative [3]. Lung cancer has two main types: nonsmall cell lung carcinoma and small cell lung carcinoma [2]. It is reported that long-term tobacco smoking is the cause of the vast majority (85%) of cases [4].

The STAT protein family are transcription factors that quickly activate target genes in response to specific signals from outside the cell, such as cytokines, growth factors and other molecules, by getting phosphorylated on tyrosine residues, mostly by JAKs [5-6]. Key cellular processes, including differentiation, proliferation, survival and functional activation, are influenced by these genes [7]. Studies that remove different STAT genes have shown that they are important for many processes, especially for making and working of blood and immune cells. They also affect breast development, milk production, growth after birth and maintaining balance in the body. JAKs are part of the tyrosine kinase (TYK) family. They share a fundamental structure comprising four structural domains containing seven similar regions known as JH1–7 [8]. Cytokines such as interferon-α, interferon-γ, and various ILs bind to receptors that have JAKs attached to them. These are enzymes that start the STAT pathway when

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the receptors are activated by cytokines. By stopping JAKs from working, the production of inflammatory cytokines is reduced because STAT proteins are not changed.

The JAK/STAT signaling pathway consists of three primary elements: the tyrosine kinase-associated receptor, JAK, and STAT. This pathway serves as a communication route for multiple cytokines and growth factors, including interleukin 2 to 7 (IL-2 to IL-7), granulocyte-macrophage colony stimulating factor (GM-CSF), growth hormone (GH), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and interferons (IFNs), enabling the transmission of signals within cells [2]. The tyrosine kinase-associated receptors are situated on the cell membrane and match with various cytokines and growth factors. These receptors lack kinase activity but possess a binding site for the tyrosine kinase JAK within their intracellular domain. Upon ligand binding, JAK catalyzes the phosphorylation of tyrosine residues on numerous target proteins. This phosphorylation triggers a signaling cascade, facilitating the transmission of signals from outside the cell to the interior.

Leonard et al. initially suggested that tumors might arise and progress due to the continuous activation of JAK/STAT signaling components. Activation of this signaling pathway can occur through multiple means, including the self-production or neighboring cell secretion of cytokines, interaction with receptors, mutations in JAK or upstream oncogenes that trigger JAKs, subsequently activating STAT members. Additionally, in uncommon instances, mutations in STAT genes themselves can lead to their activation. The ongoing activation of JAK/STAT signaling plays a significant role in the initiation, progression, metastasis, and resistance to treatments in lung cancer [9]. Although the mechanism of JAK/STAT signaling pathway in lung cancer metastasis is not fully elucidated, many studies have shown that reducing JAK/STAT activity can inhibit lung cancer metastasis, suggesting that JAK/STAT may be a therapeutic target for preventing lung cancer metastasis.

Ruxolitinib treats myelofibrosis and PV, which are blood cancers that affect the bone marrow. Myelofibrosis causes scar tissue to replace the bone marrow and reduce blood cell formation. PV causes the bone marrow to produce too many red blood cells. Ruxolitinib is for people who did not respond well to hydroxyurea. Ruxolitinib can also help people 12 years and older who have aGVHD. This is a problem that can happen after HSCT, when you get new bone marrow to replace the sick one. It usually shows up in the first few months after HSCT. Ruxolitinib is for those who did not get better with steroids. Ruxolitinib can also help people 12 years and older who have cGVHD. This is a problem that can happen after HSCT, when you get new bone marrow to replace the sick one. It usually shows up more than 3 months after HSCT. Ruxolitinib is for those who did not get better with 1 or 2 other treatments. Ruxolitinib belongs to a group of drugs called kinase inhibitors. It treats myelofibrosis and PV by stopping the signals that make cancer cells grow. This prevents cancer cells from spreading. It treats GVHD by stopping the signals of the cells that cause GVHD.

Hypothesis: Since STAT3 is excessively activated in cancer cells, I predict that treatment with certain concentration and treatment duration with Ruxolitinib inhibits jak phosphorylation of STAT1 in A549 lung cancer cells. Measure STAT phosphorylation using western blots and STAT activation by a STAT luciferase reporter assay and cell killing by MTT assay and tumor size reduction in A549 xenograft mice.

2. Methods

2.1. Western blot

The cells were collected by spinning them down, then broken open with RIPA buffer. The cell debris was removed by spinning the lysates at 12000rpm for 10 minutes at 4°C. The amount of protein in the clear liquid was measured using the BCA Protein Assay Kit to make sure the samples had the same protein level. The proteins were separated by running them on a gel with SDS and then transferred to a PVDF membrane. The membrane was blocked with 4% non-fat milk in TBST for an hour at room temperature to prevent non-specific binding. Then, the membrane was washed with TBST three times for 5 minutes each and incubated with HRP-linked anti-rabbit secondary antibody for an hour at room temperature. After washing the membrane three more times with TBST for 5 minutes each, the bands

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were detected using the ECL system. The STAT1 protein expression was normalized to tubulin (as the loading control).

2.2. MTT assay

10,000 HCT116 lung cancer cells are mixed with 100 μL of media. Afterward, they're exposed to 10 μL of MTT reagent for approximately 3 hours. Then, a detergent solution is added to break down the cells and dissolve the colored crystals. The colorimetric detection measures the absorbance at 570 nm. The intensity of the color formed correlates directly with the quantity of viable cells present.

2.3. Xenograft tumor model

Around 2 million cells modified with lentivirus were injected just beneath the skin on both sides of the mice. Over the course of approximately every 3 to 4 days, the tumors' dimensions were gauged. Following a period of 30 days, the animals were euthanized, and the tumors were assessed in terms of their weight.

2.4. Treatment duration with Ruxolitinib

Divide the mice into eight groups and inject 1ml of 0.4mg/ml solution with Ruxilitinib into each group of mice two times a day for three days.

2.5. Statistics

The procedure will be replicated three times. All numerical data gathered from Western blot, luciferase, MTT assay, and the Xenograft tumor model will undergo statistical analysis for significance using the student's T-Test at a significance level of $p < 0.05$.

3. Result

Table 1. Possible results of experiment.

Combination of Possible Results (CR)	STAT1 phosphorylation decreased by Ruxolitinib	Increase in killing of A549 lung cancer cell by MTT	Decrease in Xenograft tumor size	Supporting Hypothesis?
CR1	+	+	+	Fully support
CR2	+	+	-	Partially
CR3	+	-	+	Partially
CR4	-	+	+	Partially
CR5	+	-	-	Partially
CR6	-	+	-	Partially
CR7	-	-	+	Partially
CR8	-	-	-	Non

"+" represents decrease of STAT1 phosphorylation, amount of cells and Xenograft tumor size."-" represents the opposite.

Combination of possible results 1(CR1): There was a significant decrease in the size of tumor of xenograft mouse, while the survival rate was relatively high in the MTT assay compared with transfection of cells with empty vector. The western blot analysis also showed that STAT phosphorylation was inhibited by Ruxolitinib.

Combination of possible results 2(CR2): There wasn't any decrease in the size of tumor of xenograft mouse, while the survival rate was relatively high in the MTT assay compared with transfection of cells with empty vector. The western blot analysis also showed that STAT phosphorylation was inhibited by Ruxolitinib.

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Possible results 3(CR3): There was a significant decrease in the size of tumor of xenograft mouse, while the survival rate was relatively low in the MTT assay compared with transfection of cells with empty vector. The western blot analysis also showed that STAT phosphorylation was inhibited by Ruxolitinib.

Possible results 4(CR4): There was a significant decrease in the size of tumor of xenograft mouse, and the survival rate was relatively high in the MTT assay compared with transfection of cells with empty vector. While the western blot analysis also showed that STAT phosphorylation wasn't inhibited by Ruxolitinib and STAT activation was caught by luciferase STAT.

Possible results 5(CR5): There wasn't a significant decrease in the size of tumor of xenograft mouse, and the survival rate was relatively low in the MTT assay compared with transfection of cells with empty vector. The western blot analysis also showed that STAT phosphorylation was inhibited by Ruxolitinib.

Possible results 6(CR6): There wasn't a significant decrease in the size of tumor of xenograft mouse, while the survival rate was relatively high in the MTT assay compared with transfection of cells with empty vector. The western blot analysis showed that STAT phosphorylation was inhibited by Ruxolitinib.

Possible results 7(CR7): There was a significant decrease in the size of tumor of xenograft mouse, while the survival rate was relatively low in the MTT assay compared with transfection of cells with empty vector. The western blot analysis also showed that STAT phosphorylation wasn't inhibited by Ruxolitinib.

Possible results 8(CR8): There wasn't a significant decrease in the size of tumor of xenograft mouse, the survival rate was relatively low in the MTT assay either compared with transfection of cells with empty vector. The western blot analysis also showed that STAT phosphorylation wasn't inhibited by Ruxolitinib.

4. Discussion

Previous research has shown that Ruxilitinib can interfere with the phosphorylation process of cancer cells and alleviate cancer-related symptoms. The JAKs/STAT protein family plays a vital role in lung cancer development, and some therapies aim to stop the cancerization of cells by disrupting transcription or phosphorylation. Therefore, this research intends to demonstrate that Ruxilitinib treatment durations can reduce the number of lung cancer cells by affecting the phosphorylation of STAT1 protein.

Combination of result 1 (CR1) shows that Ruxilitinib influences phosphorylation of JAK/STAT proteins. The result of western blot and MTT assay experiments indicate that free STAT1 protein decrease and this cause a massive percentage of cancer cells died. In addition, the measurements of Xenograft tumor model shows that Ruxilitinib could effectively slow down the spread of cancer cells. All the evidences support hypothesis.

The combination of results 2 (CR2) shows that the transcription of JAKs/STAT proteins was affected for some reason, and there was an increase in the death of lung cancer cells as well. However, the tumor size of the xenograft did not show a significant decrease, which indicates that maybe another material or one of the products in the irregular process of transcription of JAKs/STAT proteins caused the death of cancer cells. Therefore, this result could not fully support the hypothesis. The result could only partially support the hypothesis.

The combination of results 3 (CR3) shows that the transcription of JAKs/STAT proteins was affected for some reason, and Ruxilitinib was involved in the reaction. However, it did not reduce the rate of cancer cells, which means Ruxilitinib may not play a decisive role in decreasing cancer cells. More experiments on other materials are needed. The result could only partially support the hypothesis.

The combination of results 4 (CR4) shows that injecting Ruxilitinib into mice can reduce the number of lung cancer cells and partially cure the cancer. However, the mechanism is not related to affecting the transcription or phosphorylation of JAKs/STAT proteins. Ruxilitinib can alleviate some symptoms of lung cancer, but there is no direct connection between the death of cells and the injections of Ruxilitinib. The result can only partially support the hypothesis.

The combination of results 5 (CR5) shows that Ruxilitinib could affect the transcription of JAKs/STAT proteins, but there was no significant change in the number of cancer cells and the xenograft

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tumor size. This indicates that Ruxilitinib could not alter the state of cancer cells or slow down the cancerization. The result could only partially support the hypothesis.

The combination of results 6 (CR6) shows that the JAKs/STAT protein was correctly translated, and the xenograft tumor size did not show any sign of decrease. However, A549 lung cancer cells were still killed in the MTT assay, which means Ruxilitinib may affect other biochemical processes and reduce the cancer cells. The result could only partially support the hypothesis.

The combination of results 7 (CR7) shows that Ruxilitinib had no impact on the transcription of JAKs/STAT proteins or the killing of lung cancer cells, but it had a positive effect on the xenograft tumor mice. This indicates that Ruxilitinib may indirectly trigger another biochemical process and thus show a positive outcome in tumor mice. The result could only partially support the hypothesis.

Combination of result 8(CR8) shows that Ruxilitinib couldn't have impact on A549 lung cancer cells as it does in other symptoms of cancers. The result could not support the hypothesis at all.

5. Conclusion

In conclusion, this article highlights the significance of investigating the effects of Ruxolitinib treatment duration on inhibiting JAK phosphorylation of STAT1 in A549 lung cancer cells. The JAK/STAT signaling pathway plays a crucial role in lung cancer development and progression. By inhibiting JAK phosphorylation, Ruxolitinib has the potential to disrupt aberrant signaling pathways that promote cancer cell growth and metastasis.

The study hypothesizes that specific concentrations and treatment durations of Ruxolitinib can effectively inhibit JAK phosphorylation of STAT1. Through various experimental assays, including western blotting, STAT luciferase reporter assay, and MTT assay, the researchers aim to elucidate the impact of Ruxolitinib treatment on STAT1 phosphorylation, STAT activation, and cell viability.

Understanding the relationship between Ruxolitinib treatment duration and JAK/STAT pathway inhibition is crucial for the development of targeted therapeutic approaches in lung cancer. Identifying the optimal treatment duration and concentration of Ruxolitinib can provide valuable insights into improving patient outcomes and potentially preventing lung cancer metastasis.

Moreover, this research contributes to the broader understanding of the JAK/STAT signaling pathway as a therapeutic target in lung cancer. By highlighting the potential of Ruxolitinib in inhibiting JAK phosphorylation of STAT1, this study opens new avenues for the development of targeted therapies and paves the way for further investigations in the field of lung cancer treatment.

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