Treatment with Increasing Amounts and for Various Durations of the Anticancer Agent Curcumol's Effect on Stimulation of the Clearance of HEP3b Xenograft Tumors and Decreasing of Jak3/Stat3 Signaling and Phosphorylation.

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Abstract

Human Hepatocellular Carcinoma is the second leading cause of cancer deaths, and it is undoubtedly easy to be infected with a destructive lifestyle. However, humans' immune systems will be effective for cancer treatment if there is no suppression of T cells brought by Jak3/Stat3 molecular activities. Fortunately, Curcumol has been proven effective for several cancer treatments and might also be helpful in HCC cancer treatment. This paper tends to prove that treatment with increasing amounts and for various durations of the anticancer agent Curcumol will stimulate the clearance of HEP3b xenograft tumors and decrease Jak3/Stat3 signaling and phosphorylation in the in vivo conditions. STAT3 phosphorylation and STAT3 mediated transcription will be measured by western blot analysis and STAT3 reporter assay. HEP3b killing will be measured by MTT assay. In the experiment, the Positive control is Taxol, and the negative control is PBS/DMSO. Each experiment will be repeated five times with different concentrations and various treatment duration of Curcumol extractives, while all the experiments will be done in vivo. .

Keywords: Curcumol, Indoleamine 2,3-dioxygenase (IDO), Human Hepatocellular Carcinoma (HCC)

I. Introduction

Cancer is a disease where cells in human bodies grow and reproduce uncontrollably. The cancer cells can invade and destroy surrounding healthy tissue, including organs. Cancer sometimes begins in one part of the body before spreading to other areas [5]. It is one of the most intractable and tricky diseases that human have ever seen. The difficulty in treating cancer is its variety. There are more than 100 different types of cancers in the world, and they are caused by different things, so it is extremely tough to prevent them. Moreover, different cancers respond to different treatments, and it can evade our immune system or suppress key elements of the usual immune response, which increases the difficulty of their treatment [11].

Human Hepatocellular Carcinoma (HCC) is the most common cancer of the human liver. It is derived from hepatocytes and is caused normally because of environmental and genetic factors. Liver cirrhosis, excessive alcohol consumption, and several virus infections will increase the risk of the development of HCC. Its high morbidity and death rate made the World Health Organization (WHO) consider HCC as the second leading cause of cancer deaths [1].

However, humans' immune systems have developed their own way to inhibit tumor cells: The T-cells. It can recognize the combination of the major histocompatibility complex (MHC) molecule and an antigenic fragment and is activated to multiply rapidly into an army of specialized T cells [2]. Moreover, T-cells can be suppressed by Indoleamine 2,3-dioxygenase (IDO)[3]. IFN-g binds to its specific receptors IFN-gR1 and IFN-gR2, which causes dimerization of the receptor molecule, phosphorylation of Jak3, and form IDO. The two Jak3 molecules create a channel to get a Stat3 homologous dimer. The Stat3 dimer then phosphorylated by Jak3, and the phosphorylated Stat3 dimer detach from the receptor. Stat3 dimer enters the nucleus and binds to the GAS regulatory sequence of the IFN-g-induced gene, which activates the expression of IDO [3]. IDO's immune suppression effects are caused by decreased tryptophan availability and the generation of tryptophan metabolites, and suppress T cells from proliferation, and survival [4]. As a result, the immune system cannot effectively attack tumor cells. It will negatively affect the treatment of cancer by extending the treatment cycle of cancer [3].

Several Chinese Medicines have been proven to be effective to anticancer treatments. Rhioxma Curcumae Aeruginosae, roots of Curcuma aerugionosa Roxb., contains volatile oil consists of Curzerenone, Epicurzerenone, Curzenene, Curdione, Curcumol, and

other chemicals [5]. Its volatile oil has been shown to be a potent anticancer substance. The active anticancer nature of Curcuma's volatile oil is brought by Curcumol, a bioactive sesquiterpenoid [5]. It is known to possess numerous pharmacological effects such as anticancer, antimicrobial, antifungal, antiviral, and anti-inflammatory. The medicines made with this anticancer agent have been used in the treatment of several cancers and have gained remarkable results. As a result, this paper tends to prove the possibility that Curcumol may decrease the HCC cancer cells and decline the Jak3/Stat3 signaling.

Hypothesis: I predict that treatment with increasing amounts and for various durations of the anticancer agent Curcumol will stimulate the clearance of HEP3b xenograft tumors and decrease Stat3 phosphorylation and reporter.

II. Methods

A. Materials

In the experiment, Jak3/STAT3 phosphorylation are measured by Western Blot. STAT3 mediated transcription is measured by a STAT3 reporter assay. HEP3b killing is measured by MTT assay. Stat3 phosphorylation's increasing in xenograft, Stat3 Reporter's increasing in xenograft, and HEP3b killing increased amount will be tested. Each experiment will be repeated for 5 times.

Different concentration of Curcumol extractive will be injected into animals with different treatment duration, which had been injected with HCC cancer cell lines ahead. Compared the size of tumor, Jak3/STAT3 phosphorylation, HEP3b killing, and Stat3 reporter increasing amount. The mice were housed under specific pathogen–free conditions. Animals will be euthanized immediately if they display excessive discomfort. Animals will be divided into different groups based on different treatment duration of Curcumol, different concentration of Curcumol, positive control, and negative control.

Each cell line will be divided into five groups: (1) negative control: PBS/DMSO; (2) positive control: Taxol; (3) 10 μM Curcumol extractive in 1 hours; (4) 10 μM Curcumol extractive in 9 hours; (5) 50 μM Curcumol extractive in 1 hours; (6) 50 μM Curcumol extractive in 9 hours. This experiment will only be done in the *in vivo* condition.

B. Western Blot Analysis

Protein was harvested and quantified at different time points after Curcumol was incubated with or without CEP at different concentrations. An amount of 50 μg of total protein per sample was separated by 10% or 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred by electro-blotting onto a nitrocellulose membrane. The membrane was blocked in 5% bovine serum albumin and then incubated with antibodies against STAT3 and phospho-STAT3 in 5% BSA overnight at 4 °C. The membrane was then washed and incubated with horseradish peroxidase-labeled secondary antibodies for 1 h at room temperature. Bands were visualized by use of a Western super-sensitive chemiluminescence detection system. Autoradiographs were quantitated by densitometry. β-actin was used as the internal control for protein normalization. Changes in size of tumor and Jak3/STAT3 phosphorylation will be measured[6].

C. STAT3 Reporter Assay

HEK-293 T cells were seeded in 96-well plates at a density of 5000 cells per well. After 24 h, the cells were transiently transfected with a mixture of 5 ng of pRL-CMV Renilla luciferase reporter, 50 ng of the firefly luciferase reporter, and 5pmol small RNA (siRNA or miRNA mimics). After 48 h, luciferase activity was measured using the dual-luciferase reporter assay system[7]. STAT3 mediated transcription is measured.

D. MTT Assay

Cells were plated at a density of 7000 per well of a 96-well plate and, 24 hours after plating, treated as the indicated concentrations. Twenty microliters MTT with a concentration of 5 mg/ml was added to each well for an additional 4 hours. The blue MTT formazan precipitate was then dissolved in 150 L of dimethyl sulfoxide per well with incubation for 120 minutes in a rotary platform at 37 C. Cell proliferation inhibition ratio was calculated according to the absorbance at a wavelength of 490 nm (A value) in each well by ELISA analyzer. Cell proliferation inhibition ratio (%) = (A value of control group A value of treated group)/A value of control group 100%[8]. HEP3b killing amount will be measured.

E. Animal Models

The mice will be divided into three groups of 6: negative control, positive control, and Curcumol treatment with different concentrations and time. HCC tumor cells will be injected into all 5 groups. Three weeks after administration, all mice were sacrificed. HEP3d killing, Jak3/STAT3 phosphorylation, and STAT3 mediated transcription will be measured.

F. Statistics:

All the experiment will be repeated for 5 times in different concentration of Curcumol and treatment time. The data were presented as mean ± standard deviations (S.D). Statistical analysis was evaluated by Student's *t*-test (between negative control and positive control) or one-way (between positive control and Curcumol treatments) ANOVA followed by Dunnett's *t*-test for multiple

comparisons using SPSS statistical program. A level of $P < 0.05$ was considered statistically significant [9].

III. Results

Possible Results on Curcumol's effect on HEP3b xenograft tumors, Stat3 phosphorylation, and Stat3 reporter (The overview of eight possible results is shown in Table 1):

Table I. Possible Results on Curcumol's effect on HEP3b xenograft tumors killing, Jak3/Stat3 phosphorylation, and Stat3 reporter in various treatment durations and different concentrations of Curcumol

Measured parameter	Result 1	Result 2	Result 3	Result 4	Result 5	Result 6	Result 7	Result 8
Tumor size decrease	+	+	+	+	-	-	-	-
Jak3/Stat3 phosphorylation decrease	+	-	+	-	+	-	+	-
Stat3 reporter decrease	+	-	-	+	+	+	-	-

Note. "+" represents changes occur in the experiments. "-" represent changes do not occur in the experiments, there is no significant difference from the negative control.

Possible Result 1: Hypothesis: Treatment with increasing amounts and for various durations of the anticancer agent Curcumol increased the HEP3b killing, decreased Jak3/Stat3 phosphorylation, and decreased Stat3 reporter.

Curcumol decreases the size of HCC tumor, stimulate the clearance of HEP3b xenograft tumors. The Jak3/Stat3 phosphorylation decreased, and the Stat 3 reporter decreased. The result showed that increasing amounts and for various durations of Curcumol extractive has therapeutic effect on HCC cancer.

Possible Result 2: Hypothesis: Treatment with increasing amounts and for various durations of the anticancer agent Curcumol increased the HEP3b killing, but it did not decrease Jak3/Stat3 phosphorylation and Stat3 reporter.

Curcumol decreases the size of HCC tumor, stimulate the clearance of HEP3b xenograft tumors. However, the Jak3/Stat3 phosphorylation and the Stat 3 reporter did not decrease. The result showed that increasing amounts and for various durations of Curcumol extractive has therapeutic effect on HCC cancer.

Possible Result 3: Hypothesis: Treatment with increasing amounts and for various durations of the anticancer agent Curcumol increased the HEP3b killing and decreased Jak3/Stat3 phosphorylation, but it did not decrease Stat3 reporter.

Curcumol decreases the size of HCC tumor, stimulate the clearance of HEP3b xenograft tumors. The Jak3/Stat3 phosphorylation decreased, but the Stat 3 reporter did not decrease. The result showed that increasing amounts and for various durations of Curcumol extractive has therapeutic effect on HCC cancer.

Possible Result 4: Hypothesis: Treatment with increasing amounts and for various durations of the anticancer agent Curcumol increased the HEP3b killing and decreased Stat3 reporter, but it did not decrease Jak3/Stat3 phosphorylation.

Curcumol decreases the size of HCC tumor, stimulate the clearance of HEP3b xenograft tumors, and decreased Stat 3 reporter. However, the Jak3/Stat3 phosphorylation did not decrease. The result showed that increasing amounts and for various durations of Curcumol extractive has therapeutic effect on HCC cancer.

Possible Result 5: Hypothesis: Treatment with increasing amounts and for various durations of the anticancer agent Curcumol did not increase the HEP3b killing, but it decreased Jak3/Stat3 phosphorylation, and decreased Stat3 reporter.

Curcumol did not decrease the size of HCC tumor or stimulate the clearance of HEP3b xenograft tumors. However, the Jak3/Stat3 phosphorylation still decreased, and the Stat 3 reporter also decreased. The result showed that increasing amounts and for various durations of Curcumol extractive has no therapeutic effect on HCC cancer.

Possible Result 6: Hypothesis: Treatment with increasing amounts and for various durations of the anticancer agent Curcumol did not increase the HEP3b killing and did not decrease Jak3/Stat3 phosphorylation, but it decreased Stat3 reporter

Curcumol did not decrease the size of HCC tumor, stimulate the clearance of HEP3b xenograft tumors or decrease Jak3/Stat3 phosphorylation, but it decreased the Stat 3 reporter. The result showed that increasing amounts and for various durations of Curcumol extractive has no therapeutic effect on HCC cancer.

Possible Result 7: Hypothesis: Treatment with

increasing amounts and for various durations of the anticancer agent Curcumol did not increase the HEP3b killing and decreased Stat3 reporter, but it decreased Jak3/Stat3 phosphorylation.

Curcumol did not decrease the size of HCC tumor and did not stimulate the clearance of HEP3b xenograft tumors. However, it decreased phosphorylation of Jak3/Stat3 and Stat3 reporter in short treatment duration and high concentration of Curcumol. The result shows that Curcumol has no therapeutic effect on HCC cancer.

Possible Result 8: Hypothesis: Treatment with increasing amounts and for various durations of the anticancer agent Curcumol did not increase the HEP3b killing, did not decrease Jak3/Stat3 phosphorylation, and Stat3 reporter.

Curcumol did not decrease the size of HCC tumor, did not stimulate the clearance of HEP3b xenograft tumors, did not decrease phosphorylation of Jak3/Stat3, and did not decrease Stat3 reporter in short treatment duration and high concentration of Curcumol. Nothing happened during and after the experiment. The result shows that Curcumol has no therapeutic effect on HCC cancer.

IV. Discussion

Recent research showed that Curcumol can actively inhibit tumor cells and can help the treatments of several cancers. Curcumol has been proved to be able to block the cell cycle at both G2/M and G0/G1[5]. This paper tried to test if Curcumol will help the treatment of Human Hepatocellular Carcinoma (HCC) by stimulate the clearance of HEP3b xenograft tumors and decrease Jak3/Stat3 phosphorylation and Stat3 reporter to let T cells to proliferate. What is worth mentioning is that there is a potential rule: when Jak3/Stat3 phosphorylation decreases, Stat3 reporter must also be decreasing [3]. In the experiment, there are changes in treatment time and difference in concentration: (1) 10 μM Curcumol extractive in 1 hours; (2) 10 μM Curcumol extractive in 9 hours; (3) 50 μM Curcumol extractive in 1 hours; (4) 50 μM Curcumol extractive in 9 hours.

Curcumol has been proved to reduce the expression of phosphorylated Stat3 through Jak1, Jak2, and Src pathways [10]. As a result, normally when Curcumol extractive has been injected, the Stat3 phosphorylation and Stat3 reporter should decrease. In Possible Result 1,3,4,5,6,7. The declination have been discovered. Moreover, normally when the expression of phosphorylated Stat3 has been reduced, Indoleamine 2,3-dioxygenase (IDO) expression will also be reduced, and T-cells will proliferate because their suppression factor IDO was gone [3]. The proliferated T-cells will recognize the HCC cancerous cells and fight with these cells during the cancer treatment, and HEP3d killing should increase if there are no interrupting factors. Only Possible Result 1 meet two requirements. This result happened in different concentrations and various treatment time with Curcumol.

In possible result 2, Curcumol stimulate the clearance of HEP3b xenograft tumors and increase the HEP3b killing, but it does not decrease Jak3/Stat3 phosphorylation and Stat3 reporter. This result can be explained by the potentially existence of some other substances in animal model's bodies that react with Curcumol extractives and kill the tumor cells. These substances do not inhibit tumor cells by proliferate T-cells. They may use some other unknow method to heal HCC and further studies should be done. This new method may provide important information for the future clinical trial of Curcumol therapy.

In possible result 3 and 7, decreasing in Stat3 phosphorylation was discovered, but the Stat3 reporter did not change. This means that Stat3 molecules did phosphorylated, but it did not enter the nucleus due to some unknown reasons. In result 3, there is increasing in HEP3b killing, which means that Stat3 molecules enter the nucleus, but the killing of cancerous cells still increases. This might show that some interferences have been involved in the experiment and kills HEP3b cells. In result 7 the HEP3b killing did not change, which is possible since the IDO expression is still active. T- cells have still been suppressed so they cannot help to kill cancerous cells.

The Stat3 reporter decreased in possible result 4 and 6, which means that less Stat3 molecules enter the nucleus without been phosphorylated. In result 4, increasing in HEP3b killing was discovered, which means that the IDO expression might be suppressed by unphosphorylated Stat3 molecules in the experiments. In result 6, there is no discovered change in HEP3b killing, which possibly shows that unphosphorylated Stat3 molecules cannot affect IDO expression.

In possible result 5, only decreasing of Stat3 reporter and Stat3 phosphorylation were discovered during experiments. The HEP3b killing does not increase. There may be something that blocks the T-cells from attacking tumor cell, so the proliferation of tumor cells was not inhibited. Further research needs to be done to find what this substance is and reveal its characteristics.

There is nothing happened in possible result 8. There is no change in HEP3b killing, in Stat3 phosphorylation, nor Stat3 reporter. There may be some unknow reaction happened in animal's bodies, so Curcumol extractive has no effect on HCC tumor treatment. The issue may be most

likely to be caused by the cells or organ in animal's bodies did not absorb the Curcumol extractive. Maybe mice were not the best option for the *in vivo* experiments, so nothing happened. The animal model needs to give a second thoughts. Or the Curcumol extractive should be injected with some other chemical used as assistance. Some further studies should be done to figure this out.

All the experiments were done in various treatment durations and different concentrations, so this may also be an interference agent of the experiment results. Research showed that Curcumol might be most effective for blocking cell cycle and induces cell apoptosis with high concentration around 75 and 100 μg/mL, for 48 h after the treatment [11]. As a result, relatively high concentration and long treatment time may increase the success rate and accuracy rate of the results. Future studies should consider the change in Curcumol concentrations and treatment as important variants.

V. Conclusion

In summary, this experiment proved that different concentration and various durations of the anticancer agent Curcumol will stimulate the clearance of HEP3b xenograft tumors, decrease Jak3/Stat3 phosphorylation and Stat3 reporter. This experiment will provide new ideas for HCC cancer treatment. However, there still exists some potential problems in the experiment such as the potentially existing new method to heal HCC cancer, possibility that animal model's body don't absorb Curcumol extractive, the substance that may react with Curcumol and decrease tumor cells, and the obscure best concentration level and treatment time for Curcumol extractive. Hopefully there will be further research that could solve these problems and provide a new pathway for HCC cancer treatment.

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